The R563Q Mutation of the Beta Subunit of the Epithelial Sodium Channel: Prevalence and Effect

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MBBCh (Wits)

Thesis presented for the Degree of

Doctor of Philosophy

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January 2009

Declaration

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

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Erika Sherad Wilshire Jones	Date

Dedication

This thesis is dedicated to my father, Michael Jones, who inspired his daughters to achieve academically; my mother, Mandy Jones, who ensured we were always able to and my husband, Blaise Witney, who has supported me every step of the way through my thesis.

Acknowledgements

This work would not have been possible without assistance from many people to whom I am indebted.

I am very grateful to Professor Brian Rayner who introduced me to this topic and made it possible for me to do the thesis. He exposed me to the clinical aspects of the sodium channel, and salt sensitive hypertension. His patience and academic input has been inspiring. He encouraged me to be involved with the patients in the Hypertension Clinic to get a better idea of the care required for resistant hypertension. He also exposed me to the Hypertension Society and encouraged me to participate in conferences and academic meetings. He has overseen my project from beginning to end, making sure I had access to the people and information required.

Dr Tricia Owen, who exposed me to completely new techniques in the laboratory, encouraging me through all the trials of lab work, has supervised and encouraged me through all stages of this thesis. She made the process seem simple and ensured that I understood the reasoning behind the methods. She has given of her time and expertise with unfailing generosity. During the write-up of this thesis her encouragement has enabled me to take on the next chapter and plod through the seemingly endless volumes of work.

I am indebted to Professor Peter Meissner for his advice and guidance in the writing of this thesis and his encouragement during the research stages. His input to the compilation was invaluable. Dr Ike Okpechi has given generous advice as he worked

though his own thesis. I am grateful to Mauretta Makaleni, Donette Baines, Julie Kholevsohn and Helen Barrett for their clinical expertise that was generously supplied for many aspects of this thesis. I had assistance from many experienced people from the Inherited Metabolic Disease Laboratory: Surita Meldau, Felicity Leisegang, Lisa Lambson and Debbie Dawson. Di Sklar gave assistance in creating the family tree images. The statisticians who have advised and overseen the statistics for this thesis were essential. I am hugely indebted to Dr van der Merwe, Dr Isaacs and Dr Badri for their assistance.

I am grateful to Genevieve Jones whose experience in the academic arena enabled her to give generous time and advice to an inexperienced younger sister. Dr Michael Jones, my father, has given generously of his time by proof reading my thesis and making comments and corrections throughout. Mandy Jones, my mother, has given unstinting emotional support and encouragement and has been available for telephonic consultations at all times of the day. Anna Jones consistently gave practical advice on how to get through extended periods in front of the computer and through the thesis itself. Blaise Witney, my husband, I would like to thank you for the daily encouragement and drive required to get through the thesis.

Dr Stephen Tucker, from the Oxford Centre for Gene Function, gave advice and assistance with the physiological methods used in Chapter 9. Dr Tucker and Dr Lijun Shang made it possible to determine the sodium channel conductance in sodium channels with the R563Q mutation. The DNA from Johannesburg was made available by Professors Angela Woodiwiss and Gavin Norton. Their donation of 800 samples of DNA with data from the subjects made it possible to decrease the costs and collecting

time for this thesis. Professor Himla Soodyall from the SAIMR and her team (Carina Schlebusch, Heeran Makkan and Thijessen Naidoo) made it possible to travel into the Northern Cape to collect data from the Khomani San.

Dr Mike Pascoe organised funding from Wyeth Pharmaceuticals which enabled me to do this thesis. The funds, while making the research for the thesis possible, also enable me to attend courses and conferences. Mike also gave unceasing encouragement to this project. I was awarded a research grant from the UCT research council which helped to extend this thesis.

ABSTRACT

The R563Q Mutation of the Beta Subunit of the Epithelial Sodium Channel: Prevalence and Effect

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January 2009

Hypertension is a major worldwide predictor of morbidity and mortality. The search for genes that contribute to blood pressure is ongoing. The epithelial sodium channel genes were implicated when the beta subunit (SCNN1B, gene ID 6338) was found to have a mutation that caused Liddle's syndrome. The R563Q mutation in the beta subunit has been associated with hypertension and pre-eclampsia in the Xhosa and Coloured people in Cape Town.

The thesis consists of a cross-sectional analysis of the prevalence of the R563Q mutation in multiple ethnic groups in South Africa and a longitudinal functional assessment in response to saline infusion. The objectives were to determine the prevalence of the R563Q mutation and association with hypertension, and if it persists within families; to speculate as to the origins of the mutation; to determine if there were any relevant clinical differences between comparable patients with essential hypertension; to determine if the mutation predicted a difference in response to acute sodium loading and if a physiological difference is observed in sodium channel activity when expressed in oocytes.

A high frequency of hypertensives in Johannesburg and Cape Town were found to be heterozygous and the mutation associated with hypertension, including within families. In the Khoisan the R563Q mutation was found at a high frequency (19%) in a random sample, suggesting the mutation originated from this population.

The saline challenge illustrated the *in vivo* effects of the mutation. The results suggest that the sodium channel is innately overactive in heterozygous subjects and that counter-regulatory mechanisms are in place to compensate for changes in renal sodium handling. However, preliminary *in vitro* testing in oocytes did not show a difference in sodium channel activity.

Conclusion: This thesis has shown that the R563Q mutation is found in multiple ethnic groups in South Africa, in which it associates with hypertension; and possibly originated from the Khoisan. *In vivo* effects are described. The results are important because hypertension resulting from the R563Q mutation is a common and treatable cause of hypertension. It is recommended that hypertension units in South Africa screen for the mutation and alter treatment appropriately. A further recommendation is that a sodium channel inhibitor, such as amiloride, in an appropriate form, is registered in South Africa for the treatment of hypertension.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abbreviations

(Q)TDT – (Quantitative) Transmission Disequilibrium Testing
ACE – Angiotensin Converting Enzyme
ACTH – Adrenocorticotropic hormone
AGT – Angiotensin
ANP – Atrial Natriuretic Peptide
ARR – Aldosterone Renal Ratio
ATP – Adenosine Triphosphate
BMI – Body Mass Index
BP – Blood Pressure
cAMP – cyclic Adenosine Monophosphate
CTR – Cardiothoracic Ratio
DBP – Diastolic Blood Pressure
DNA – Deoxyribonucleic Acid
ECG – Electrocardiogram
EHT - Essential Hypertension
ENaC – Epithelial Sodium Channel
FENa – Fractional Excretion of Sodium
GFR – Glomerular Filtration Rate
GSH – Groote Schuur Hospital
GTP – Guanine Triphosphate
HWE – Hardy Weinberg Equilibrium
IQR – Interquartile range
LD – Linkage Disequilibrium
LRHT – Low Renin Hypertension
MAP – Mean Arterial Pressure
N – Number of channels
Na/K ATPase – Sodium-Potassium ATPase
Nedd – Neuronal Precursor Cell Expressed Developmentally Downregulated
PCR – Polymerase Chain Reaction
PKA – Protein kinase A
PKC – Protein kinase C
Po – Open probability
RAAS – Renin Angiotensin Aldosterone System
RFLP – Restriction Fragment Length Polymorphism
RNA – Ribonucleic Acid
S1A – Syntaxin 1A
SBP – Systolic Blood Pressure
SD – Standard Deviation
SGK1 – Serum Glucocorticoid Induced Kinase 1
SNP – Single Nucleotide Polymorphism

PART 1

INTRODUCTION TO THE THESIS

An African Home

In the traditional lifestyle in Africa sodium is a scarce resource that led to evolutionary pressures to conserve sodium.

CHAPTER 1

Introduction

Hypertension: An Epidemiological Nightmare

Hypertension is a common disorder affecting approximately a quarter of the world's adult population and is projected to increase to up to 30% by 2025. The prevalence of hypertension in 2000 ranged from 5.2% in rural India to 45.1% in Spain. While these figures could represent the differences between the more natural rural lifestyle compared with the industrialised lifestyle of established market economies, it could also be a reflection of the difference in discovery and reporting of diseases. The overall rate of hypertension was age related, ranging from 12.7% in the 20-29 year age group to 59.5% in those older than 70 years for men and 7.4% to 70% in women [Kearney et al., 2005].

South Africa is similarly affected by hypertension. In 1996, According to *Health Stats SA*, the deaths directly attributed to hypertension were 1.3% for males and 2.8% for females, of the total registered deaths in South Africa. This excluded deaths due to myocardial infarction or coronary vascular events. In 1998 the prevalence of hypertension using the World Health Organisation guidelines of a blood pressure (BP) greater than or equal to 140/90 was approximately 21% in South Africa [Steyn et al., 2001]. This became the recommended cut off defining hypertension for South Africa in 2006 [Seedat et al., 2006]. Hypertension is an increasing epidemiological problem facing rural South Africa. Thorogood et al. [2007] found hypertension in 42% of a group of adults studied in Agincourt (a rural South African town) and also showed increased total cholesterol level in 25.6% of subjects.

Hypertensive heart disease, ischaemic heart disease (IHD), stroke and kidney disease were all in the list of top 20 causes of death in 2000 for South Africa [Bradshaw et al., 2003]. Hypertension is a major risk factor for all of these diseases. The estimated years of life lost as a result of these four causes of death was 823 560, indicating the huge impact that hypertension has on the population. Hypertension, itself, was estimated to account for 9% of deaths in 2000 in South Africa [Norman et al., 2007].

Steyn et al. [2005], as part of the global INTERHEART study, assessed the risk factors that were associated with acute myocardial infarct in Sub-Saharan Africa. Black Africans presenting with first time myocardial infarct were significantly younger than the European or Coloured African groups, and hypertension was the dominant risk factor..

South African Population Groups

South Africa is a multiethnic developing country previously divided by racist policies. In 1994 South Africa underwent the first true democratic vote and for the first time the South African population demographics were properly documented by a national census in 2001, [Lehohla, 2003] which detailed the numbers of the various ethnic groups in different geographical areas. Unfortunately this census did not document the Khoisan groups. The Khoisan are few in number and often individuals have a mixed ancestry which may explain why, coupled with a pale skin colour, they may have been classified as "Coloured". The Khoisan people generally speak Afrikaans as their home language. Their native 'click' languages are spoken by only a few of the elder people. This thesis will use the descriptions for ethnic groups and geographical areas as described in the 2001 census and according to the suggestions made by Soodyall

[1993]. For the purposes of this thesis people of mixed ancestry (including a mixture of Khoisan, Caucasoid and Negroid, amongst others) will be called Coloured. In referencing, subjects will be referred to as they are in the reference. The Khoisan groups are made up of many smaller groups which inhabit different areas in Southern Africa. The Khoisan group from the Northern Cape identify themselves as Khomani San which was what they were termed for the purposes of this thesis.

South Africa is divided into nine provinces: Eastern Cape, Free State, Gauteng, KwaZulu Natal, Limpopo, Mpumalanga, Northern Cape, North West Province and the Western Cape (Figure 1.1). The Black African subjects in South Africa make up 79% of the population. The Coloured subjects amount to 8.9%; White subjects 9.6% and the Indian/ Asian subjects 2.5% [Lehohla, 2003]. The Black African subjects in each province are, similarly, higher than any of the other population groups except in the Northern and Western Cape where the Coloured group is the highest. (These are the areas where the Khoisan people traditionally lived.)

The groups according to home language in South Africa are: IsiZulu (23.8%), IsiXhosa (17.6%), Afrikaans (13.3%), Sepedi (9.4%), English (8.2%), Setswana (8.2%), Xitsonga (4.4%), SiSwati (2.7%), Tshivenda (2.3%), IsiNdebele (1.6%) and other languages (0.5%). The home languages spoken are a reflection of the population groups in each province with Black African languages being the dominant languages in most provinces, excluding the Northern and Western Cape (Afrikaans). The most common language spoken in each province is: Eastern Cape, IsiXhosa; Free State, SeSotho; Gauteng, IsiZulu (but mixed with Afrikaans, English, Sepedi and SeSotho); KwaZulu Natal, IsiZulu; Limpopo, Sepedi (and Xitsonga); Mpumalanga, SiSwati

(and IsiZulu); Northern Cape, Afrikaans (and Setswana) North West Province, Setswana and the Western Cape, Afrikaans (IsiXhosa and English).

Figure 1.1. Map of South Africa showing provinces and major cities

Soodyall [1993] classified the groups in South Africa as Bantu-speaking Negroid, Khoisan, Caucasoid and Hybrid ("Coloured") peoples. She showed that the Coloured people were a mixture of Khoisan, Negroid, Caucasoid and Malay ancestry (amongst others from imported slave labour) with a difference in frequency of genetic admixture depending on the geographical position of the individual. The Bantu-speaking Negroid groups in South Africa consist of the Nguni (Zulu, Swazi and Xhosa), Tsonga, Sotho/ Tswana (Sotho, Pedi, and Tswana), Venda and the Lemba.

For the purposes of this thesis, when grouped together, these ethnic groups will be called Black African.

This thesis presents the results from subjects originating in Johannesburg, Cape Town and the Northern Cape. The subjects from Johannesburg and Cape Town are from referral Hypertension Units attached to various teaching hospitals. The major cities in South Africa are Pretoria (Gauteng), Johannesburg (Gauteng), Cape Town (Western Cape), Durban (KwaZulu Natal), Port Elizabeth (Eastern Cape) and Bloemfontein (Free State). These major cities contain the tertiary referral units in South Africa. The population groups in Pretoria and Johannesburg are very similar (of the Black African groups – SeSotho and IsiZulu) and include the major ethnic groups represented in Durban and Bloemfontein. The IsiXhosa is the major Black African group in Port Elizabeth and Cape Town. Thus, sampling the population groups in Cape Town and Johannesburg gives a good representation of the population groups in the major cities in South Africa.

Furthermore, in order to obtain a sample of resistant hypertensives tertiary care referral units are required. The remainder of the South African cities do not contain referral units, so sampling in provinces like Limpopo and the North West is not logistically feasible. However, this thesis has samples from the Caucasian groups (English and Afrikaans), Coloured, IsiXhosa, IsiZulu and SeSotho which represent about 70% of the people in South Africa. (98.4% of the Coloured people speak either English or Afrikaans.) Thus, subjects studied in this thesis are a reasonable representation of the people living in South Africa.

A Racial Difference in Hypertension

Hypertension itself has a different disease pattern in the Black Africans when compared with those of European ancestry. Apart from the difference in risk for IHD and the difference in age, Black African hypertensives appear to be "salt sensitive", responding better to diuretics rather than angiotensin converting enzyme (ACE) inhibitors. Black African hypertensive patients were thought to have an increased propensity to absorb sodium, which was why diuretics are the mainstay of treatment. The difference in origin of hypertension between the different races is ascribed to genetics and has resulted in a concerted effort to determine which genes are responsible for hypertension. The prevalence of mutations in the epithelial sodium channel (ENaC) gene (e.g. T594M) is higher in the Black African population in London, but has not been reported in many of the Caucasian populations screened [Hannila-Handelberg et al., 2005; Persu et al., 1998; Dong et al., 2001; Ambrosius et al., 1999]. The prevalence of sodium channel mutations in Black African hypertensives could account for the response to the diuretic amiloride [Saha et al., 2005].

Amiloride is a potassium sparing diuretic that inhibits the ENaC [Rang et al., 1999]. The effect of the inhibition of the sodium channel is to decrease sodium and increase potassium reabsorption from the cortical collecting duct lumen in the kidney. A further advantage of amiloride is that it promotes the excretion of uric acid (which has been shown to have an independent effect on BP [Sundstrom et al., 2005] and coronary heart disease [Franse et al., 2000]) and has a duration of action of about 24 hours making it possible to use once a day. Furthermore, amiloride is well tolerated, apart from occasional hyperkalaemia.

A Genetic and Environmental Disorder

Over approximately the last 50 years the causes of hypertension have been investigated and questioned by many clinical and research teams. The association with micronutrient intake has been studied and sodium has been found more commonly to be associated with hypertension than any of the others [MacGregor, 1985; He and MacGregor, 2002]. Chloride has also been associated with hypertension [Whitescarver et al., 1984] whereas potassium has been found to be protective [Geleijnse et al., 2003].

Male gender is a risk factor in younger subjects but this changes as the individuals get older [Kearney et al., 2005], with females having a higher prevalence over 70 years of age. Age is one of the greatest risk factors for hypertension with the residual lifetime risk being 90% in middle-aged and elderly individuals [Vasan et al., 2002]. Regular exercise has been shown to decrease the risk of developing hypertension [Hayashi et al., 1999; Tanasescu et al., 2002] whereas excessive alcohol intake [Nanchahal et al., 2000] and cigarette smoking [Bowman et al., 2007] increase the risk. Obesity is also a known risk factor for hypertension, especially abdominal obesity [Okosun et al., 1999], and weight loss has been shown to reduce the risk for hypertension [Huang et al., 1998]. A healthy diet rich in fresh low fat produce has also been shown to improve blood pressure [Appel et al., 1997; Moore et al., 1999].

While the environmental factors are generally accepted, the clinically relevant genes associated with essential hypertension (EHT) remain to be elucidated except for rare cases of Mendelian forms of hypertension. The Mendelian forms of hypertension have highlighted potential candidate genes that may be implicated in common essential

hypertension. Many single nucleotide polymorphisms (SNPs) have been described to date but few occur in sufficient frequency in the general population and/or exert a clinically relevant effect on BP to be meaningfully relevant in the pathogenesis and treatment of EHT. Furthermore, some of these SNPs have had conflicting associations with hypertension in different geographical regions.

Development of the Thesis

The R563Q mutation of the beta subunit of the ENaC (β-ENaC or SCNN1B, GeneID 6338) has been found in many hypertensive patients attending the Groote Schuur Hospital (GSH) Hypertension Clinic and has previously been associated with hypertension [Rayner et al., 2003] and pre-eclampsia [Dhanjal et al., 2006]. This thesis aimed to detail further the association with hypertension, show that the mutation causes a change in function of the sodium channel, determine the possible origins of the mutation in the South African populations and if hypertensive patients with this mutation differ clinically from comparable patients without the mutation.

This thesis is divided into four parts. The first part introduces the thesis and critically reviews the relevant literature. In the literature discussion, the progression of hypertension as a disorder of individuals to the present day thinking of hypertension as a genetic conundrum is presented. The debate that was initiated in the 1960s is yet to be resolved and this thesis adds to the debate. Some rare Mendelian forms of hypertension are discussed because they introduce various candidate genes that are implicated in the origins of hypertension. The interaction between hypertension and genetics is then discussed in more detail.

Salt sensitivity is discussed as it is a major intermediary phenotype associated with hypertension and is of particular importance in the relationship between the sodium channel and hypertension. The renin angiotensin system is one of the major control systems for BP and salt sensitivity so this system is discussed, including some of the genetic findings that have been associated with hypertension. The sodium channel (the final regulator of sodium balance and the major area of interest in this thesis) is then discussed. This channel has been implicated in Mendelian forms of hypertension, EHT and genetic protection from hypertension. The β-subunit of the sodium channel is of particular relevance to this thesis.

The hypothesis, aims and methods are then presented. Parts two and three are the experimental sections of this thesis. Part two introduces the R563Q mutation as a common mutation in South Africa with a strong association with hypertension. This section discusses the prevalence in different population groups and tries to determine if one of these population groups might have been the original group. The association with hypertension is shown to be strong within families and in case-control analyses. The mutation is shown to exist in multiple ethnic groups in South Africa. Not all of the population groups show an association with hypertension, increasing the interest of the discussion. This section demonstrates that the Khoisan could be the population group of origin of the R563Q mutation in the South African groups.

Part three aims to show that the mutation alters the activity of the sodium channel from a physical and physiological perspective. This section aims to delineate the effects of the mutation in treated hypertensive patients with the mutation comparing their phenotype with treated hypertensives without the mutation. This is followed by

an analysis and discussion of the effects of acutely loading individuals with intravenous sodium. Sodium loading was used as a means to compare the renal and cardiovascular response of individuals with and without the R563Q mutation. This section also describes the effects of the R563Q mutation on sodium current in *Xenopus* oocytes, a well known means of illustrating a physiological alteration in sodium conductance. Further, in part three, a few interesting cases are presented, to illustrate observations that have been made in hypertensive patients with the R563Q mutation. Although case studies do not prove a point, they are useful in presenting observations from which further analyses and trials may be undertaken. They are thus important to the progression of research.

Part four brings together the whole thesis, presenting the conclusions and discussing areas for further research. Recommendations based on the findings are presented. In order to ensure certain terminology is understood in relevance to this thesis appendices have been compiled. Relevant documents are presented in the appendices.

CHAPTER 2

From an Observation to the Beta Subunit of the Sodium Channel

Hypertension: A History of the Developments of an 'Inherited Disorder'

Morgagni [1761] undertook more than 700 human dissections. Amongst his findings were the descriptions of a father and son, who both died of apoplexy (or stroke). Morgagni had previously noted the association between a rise in BP (or hypertension) and apoplexy. He suggested that the rise in BP could be of familial origin and was the first person to suggest hypertension was an inherited disorder.

It was about 150 years later before there was an attempt to delineate the genetic or familial origins of hypertension. Weitz [1923] studied the association of cardiovascular disease and hypertension over three generations. He showed that if there was a death in the parent from a cardiovascular disorder, there was hypertension in the next two generations which appeared to be independent of other factors. He also examined the association with BP and hypertension between monozygotic and dizygotic twins [Weitz 1954], and found that there was greater concordance for BP between monozygotic twins.

Ayman [1934] showed that hypertension had strong familial associations and proposed that heredity was the most important factor for the development of hypertension. Allan [1933] tried to determine if hypertension was a dominant or recessive trait using statistical modelling, but found there were insufficient data to

determine the mode of inheritance. Platt [1947] also tried to delineate the nature of the inheritance of hypertension, and suggested that EHT was a simple Mendelian dominant disorder with a distinct division between normotension and hypertension.

Pickering [1952] agreed that there was a strong familial association but argued that there was insufficient evidence to show the nature of inheritance, and alluded to the possibility of failure of homeostatic mechanisms within the kidney. Guyton [1990] was the first to describe this failed mechanism in 1966 and published a summary of his findings in 1990. He called the mechanism that controlled BP the *Infinite Gain Mechanism* of the kidney. Using a mathematical formula Guyton showed that only if there was failure in kidney homeostasis, would there be a long term disruption in BP control resulting in a rise in BP and hypertension. This was supported by clinical observations. In contrast, if there was normal renal function but abnormal cardiac or vascular function BP would change acutely, but would return to normal through the infinite gain mechanism of salt and water retention by the kidney.

The assumption was that abnormal kidney function would cause a resetting of BP homeostasis and could cause hypertension. Thus, in the early studies of EHT scientists were correct to exclude patients with chronic pyelonephritis (a known form of kidney disease caused specifically by environmental factors).

Pickering [1955a] proposed that hypertension was the extreme of the normal distribution of BP (the general population displays a Gaussian curve) which showed continuous variation. As there was no evidence for two populations, he argued against having an arbitrary division between normotension and hypertension. He believed that

hypertension had a multifactorial genetic and environmental basis with each individual trait having a small effect on BP. He suggested that the Mendelian dominance was seen due to an artefact by the artificial and arbitrary division between the normal BP and the hypertension. Rather, hypertension should be defined as deviance from the norm in each age group. He developed a complex formula involving the standard deviation (SD) for the age, which would then give the clinician a score that expressed the degree of hypertension or hypotension [Pickering, 1955b]. This method was never adopted due to the obvious clinical impracticalities.

In contrast, Platt [1964] showed that hypertension was not just an extreme of the normal distribution of BP. This was not the case in the normal population or in the families of known hypertensives across all age groups. Platt [1963], using twin studies, proposed a single gene theory: when inherited in the heterozygous form there would be a moderate rise in BP and when inherited in the homozygous form there would be a marked rise in BP causing severe hypertension. Platt suggested that the mutation could have created a survival advantage in the original population, who developed the mutation.

Platt's single gene theory has not been widely accepted. However, Liddle [1963] described a family who presented with a syndrome that was characterised by severe hypertension, hypokalaemic alkalosis and suppressed plasma aldosterone levels. They responded to triamterene – an inhibitor if renal ion transport in the ENaC, but not to an aldosterone antagonist. The BP response to triamterene was most effective when combined with a low sodium diet. The family tree demonstrated an autosomal dominant pattern of inheritance, and it was named Liddle's syndrome.

Shimkets et al. [1994] defined the mutation in the ENaC of this family. (The ENaC is found in the distal convoluted tubule and cortical collecting duct and is the final and rate limiting step in sodium reabsorption.) It was a mutation, R566X, which causes truncation of the carboxyterminal of the β subunit of the ENaC and increases activity of the β-ENaC resulting in sodium retention, hypokalaemia and hypertension. This mutation does not fully support Platt's single gene theory as it is only described in the *heterozygous* form with no homozygous cases reported, perhaps because of the extreme rarity of the mutation. However, in the heterozygous form the phenotypic presentation is extremely variable.

The biggest difficulty for the proponents of the single gene theory is the extreme rarity with which these single gene defects present and thus cannot account for the common condition of EHT.

Mendelian Forms of hypertension

The Mendelian forms of hypertension are rare and influence sodium retention or reabsorption. However, these forms of hypertension do bring to our attention candidate genes that could be responsible, or at least in part responsible, for a major portion of EHT. Searching for single nucleotide polymorphisms (SNPs) in these candidate genes may prove to be fruitful when trying to determine the genetic causes of hypertension. Figure 2.1 illustrates the interaction of multiple pathways that control BP and highlights known Mendelian forms of hypertension which will be discussed further.

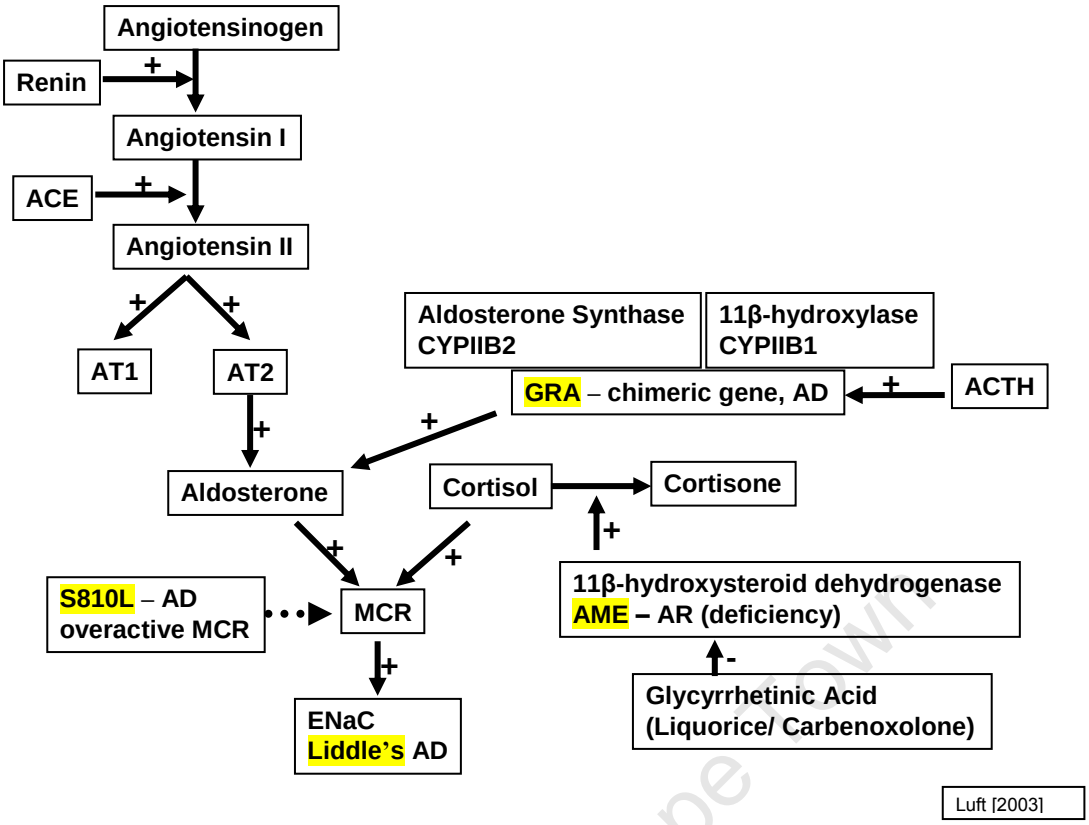

Figure 2.1. The renin angiotensin aldosterone system and the positions where Mendelian forms of hypertension occur. (ACE, angiotensin converting enzyme; AT1, angiotensin receptor 1; AD, autosomal dominant; AR, autosomal recessive; GRA, glucocorticoid remedial aldosteronism; ACTH, adrenocorticotropic hormone; MCR, mineralocorticoid receptor; AME, apparent mineralocorticoid excess; +, stimulates; - inhibits. Image created from information by Luft [2003])

Glucocorticoid Remedial Aldosteronism

Glucocorticoid Remedial Aldosteronism (GRA) is an autosomal dominant form of hypertension that presents with signs and symptoms similar to primary aldosteronism. The defect is a chimeric gene combining the aldosterone synthase gene and the 11β hydroxylase gene and has been described in 18 families [Pascoe et al., 1992]. A later study found a GRA-causing mutation in three subjects, one of whom was normotensive [Fardella et al., 2000]. (The production of the chimeric gene is shown in Figure 2.2.) These two genes are found on the same chromosome (8q) and unequal crossing over occurs with the aldosterone synthase gene gaining an

adrenocorticotropic (ACTH) sensitive promoter. Suppression of steroidogenesis with oral corticosteroids treats the hypertension as ACTH production is suppressed.

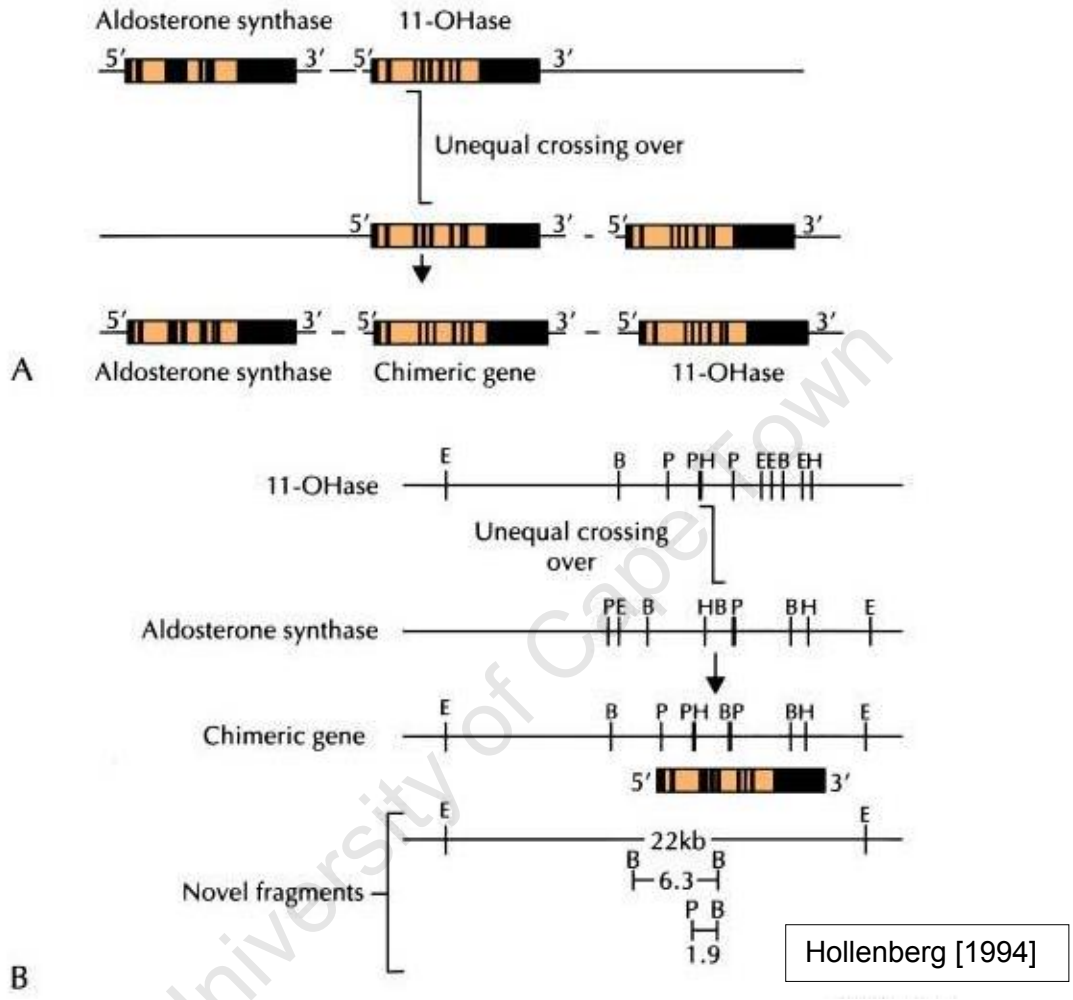

Figure 2.2. The production of the chimeric gene causing GRA. Image taken from *The Atlas of Heart Diseases, Hypertension: Mechanisms and Therapy*, Hollenberg [1994], p1.9

Apparent Mineralocorticoid Excess

Apparent mineralocorticoid excess (AME) is an autosomal recessive disorder which results from mutations in the 11 β-hydroxysteroid dehydrogenase gene, decreasing its function [Stewart et al., 1996]. Fewer than 100 cases have been reported [Palermo et al., 2004]. This enzyme converts cortisol to inactive cortisone. As cortisol and

aldosterone both stimulate the mineralocorticoid receptor and cortisol is present in higher concentrations, this enzyme is essential to prevent cortisol acting as a potent mineralocorticoid. Thus a deficiency of function of this enzyme will lead to constant stimulation of the receptor. This inherited disorder is mimicked by excess intake of liquorice which contains glycyrrhetinic acid which inhibits the enzyme.

Mineralocorticoid Receptor

The S810L mutation of the mineralocorticoid receptor [Geller et al., 2000] has been described in a family with early onset hypertension. The mutation changes the structure of the receptor making it innately active and sensitive to stimulation by mineralocorticoids that do not have the 21-hydroxyl group, especially progesterone. (Aldosterone has the 21-hydroxyl group.)

Liddle's Syndrome

Liddle's syndrome (as discussed above) is an autosomal dominant form of hypertension that is associated with hypokalaemia, hypoaldosteronism and metabolic alkalosis. It is the result of a defect in one of the sodium channel subunit genes. About 30 families have been described with this disorder [Rossier and Schild, 2008]. This syndrome will be discussed in detail later.

Gordon's Syndrome

Pseudohypoaldosteronism Type II (or Gordon's syndrome) is due to mutations in the WNK (with no lysine) kinase genes [Wilson et al., 2001]. These kinases alter the function of the sodium chloride cotransporter and mutations can increase its activity.

The genetic defect in this disorder has not been completely described but families have been described who exhibit clear autosomal dominant inheritance [Luft, 2003].

Autosomal Dominant Hypertension with Brachydactyly

Three families have been described with an autosomal dominant hypertensive disorder associated with shortened phalanges and metacarpals [Toka et al., 1998; Luft 1998]. The genetic defect has not been described but has been linked to chromosome 12p [Gong et al., 2003]. Neurovascular compression of the posterior inferior cerebellar artery has been found in some of these patients and has been suggested as the cause of the hypertension.

Congenital Adrenal Hyperplasia

Congenital adrenal hyperplasia (CAH) is a disorder resulting from a deficiency of the enzymes involved in cortisol synthesis, such as 11 β-hydroxylase [White et al., 1991], 17-α-hydroxylase [Yanase et al., 1989] or, more commonly, 21 hydroxylase [Morel et al., 1989]. This is a more common disorder with frequencies between 1 in 5000 and 1 in 15 000 births in western communities [Fauci et al., 1998]. The enzymes convert steroid precursors into cortisol and, if dysfunctional, cause a build up in the steroid precursors. (See Figure 2.3 for defects causing cortisol deficiency and CAH.) These deficiencies are a result of either autosomal dominant or recessive mutations. Mutations that cause this syndrome result in a protein with little or no enzymatic activity. The lack of cortisol stimulates the production of corticotrophin in the anterior pituitary which stimulates the adrenal gland. The build up of precursors is the likely cause of the hypertension. 11-β-hydroxylase deficiency also causes androgen excess but in 17-α-hydroxylase deficiency there tends to be an androgen deficiency.

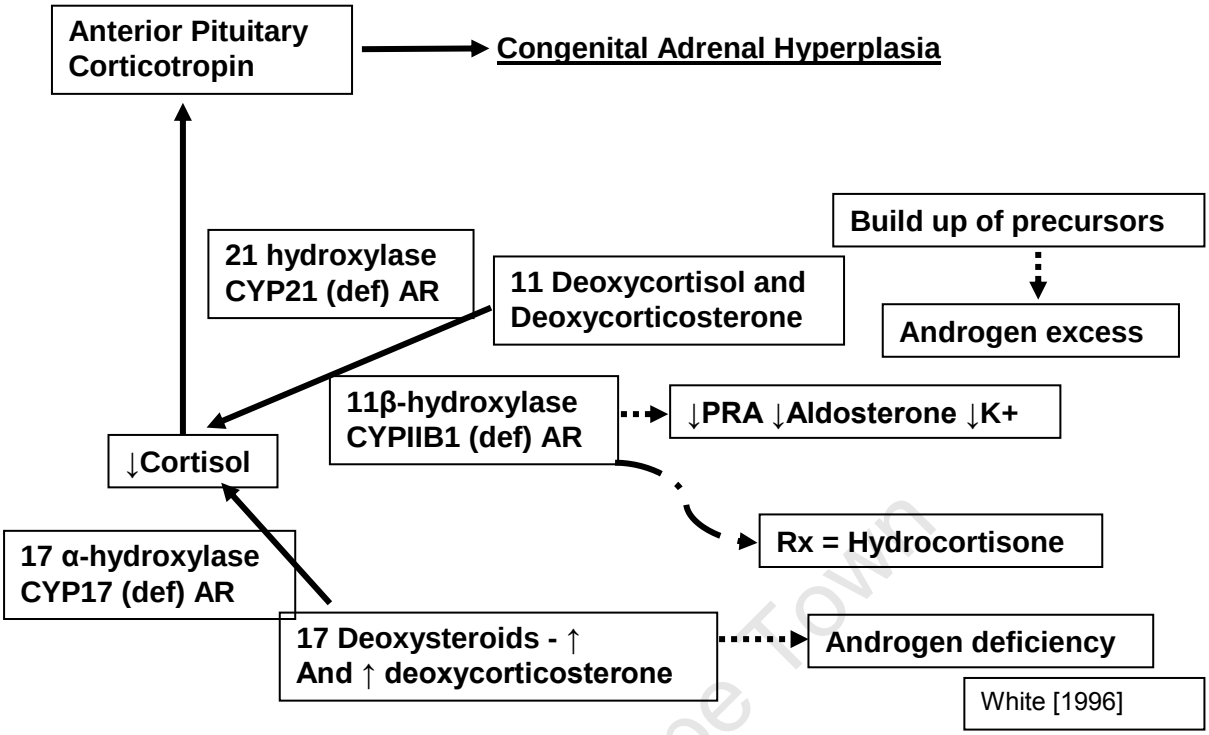

Figure 2.3. Defects causing CAH (AR, autosomal recessive. Image created with information from White [1996])

The above disorders illustrate the multiple possibilities for Mendelian forms of hypertension. These disorders are rare and often present with severe hypertension, but EHT is a common disorder that presents with a range of BP levels. More common SNPs in these genes with less dominant functional effects may form a reasonable approach to understanding the genetics of EHT.

Hypertension – A Geneticist's Nightmare

BP is a continuous variable resulting in an ongoing debate about the definition of hypertension. Platt and Pickering started off the debate in the 1960s and there have been differences of opinions ever since. Until recently, hypertension was defined as a BP ≥160/95mmHg in South Africa [Steyn et al., 2001], but the defining level has

been decreased to ≥140/90mmHg; in accordance with the European and American Guidelines [Lemogoum et al., 2003].

Determining the presence of hypertension in an individual is further complicated by the presence of a phenomenon called *white coat hypertension*. This is defined as the presence of a raised BP in a clinical setting but normal BP during the normal daily activities. It can only be diagnosed with home or ambulatory BP monitoring but is a risk factor for developing EHT [Ugajin et al., 2005].

The difficulty of determining the phenotype is highlighted when trying to associate genetic polymorphisms with hypertension. Many mutations described are associated with a minor difference in BP of questionable clinical application. Detecting these small changes can be difficult due to the natural variability of BP within an individual, the effect of BP measurement on BP (i.e. white coat phenomenon), and the poor reproducibility of single BP measurements. This problem is further compounded when comparisons are made between subjects.

There is clearly a strong inherited component to hypertension but the genetic causes remain elusive. This has lead to many different techniques employed by geneticists to determine the genotypes associated with hypertension.

One of the methods employed is genome-wide linkage analysis [Gong et al., 2003] which uses microarrays and chip-based technology to analyse up to 500,000 SNPs in a single sample. With the Human Genome Project it is now possible to pinpoint the potential candidate genes but in some cases associations have been found in areas of

the genome where there are no known genes. However the potential for false positive results is high due to the sheer number of associations.

Another method employed by geneticists to determine if there is an association with hypertension and a gene is the candidate gene approach. This method looks at specific genes (such as the β-ENaC) and determines if there are different haplotypes of the gene that are associated with hypertension. Once the presence of an associated haplotype is determined then it is possible to sequence the gene to define the mutation. Further association studies can then increase the information on the mutation.

However, these methods often fail to show a pure association with the phenotype being assessed. In which case, it is possible to determine if the mutation is associated with a difference in an intermediate phenotype that is known to result in the pathology that is being studied. For hypertension, one such intermediate phenotype is salt sensitivity.

Salt Sensitivity

Salt sensitivity is defined as a more than expected rise in BP after sodium chloride loading and a more than expected decrease with sodium restriction. The kidney is a finely tuned organ that filters large volumes of sodium every day (about 25560mEq/day); 99.4% (25410mEq/day) of which is reabsorbed [Guyton and Hall 1996]. Sodium reabsorption in each segment is dependent on the amount delivered to that segment (glomerulotubular balance). A defect in any of the channels responsible for sodium reabsorption is thus likely to have a major effect on sodium balance.

Examples of defects in sodium reabsorption are Bartter's, Gitelman's [Feehally et al., 2007] and Liddle's syndromes.

Bartter's syndrome is the result of an inactivating mutation in one of the genes encoding the major transport proteins in the thick ascending limb of the Loop of Henle. The major transport proteins are the sodium-potassium-chloride cotransporter, renal outer medullary potassium channel, the chloride channel and the Barrtin accessory protein to the chloride channel. Defects in these proteins result in sodium and chloride wasting associated with severe hypotension and hypokalaemia and often result in early death. Gitelman's syndrome is an autosomal recessive disorder and is less severe, presenting in early adulthood. It is due to a defect in the sodium-chloride cotransporter in the distal tubule. The syndrome is characterised by hypokalaemic metabolic alkalosis and hypotension with hypocalcuria and hypomagnesaemia. These disorders represent extreme situations resulting from mutations that completely disrupt the function of channels responsible for sodium reabsorption. It is possible that different genetic coding of these same channels will have a smaller effect and create situations like salt sensitivity. The kidney reabsorbs 99.4% of the sodium load thus, even mild to moderate perturbations in function can result in severe clinical syndromes. Milder defects in reabsorbtive function in these channels may be protective against hypertension in the face of high sodium diet; conversely, defects resulting in hyperfunction may favour survival in the face of sodium scarcity or cause salt-sensitive hypertension in environments of sodium excess.

Salt sensitivity is known to be more common in Black African people [Luft et al., 1991]. The original 'hunter-gatherer' people lived in an environment where sodium

was an extremely scarce commodity and had a total daily intake of about 0.3g to 3g a day [Carvalho et al., 1989]. Salt can be lost in the sweat (about 3-5g/day) and the stool (about 175mg sodium) [Guyton and Hall, 1996]. The loss of fluid and sodium in sweat would also tend to be larger in the heat of Africa compared with the losses observed in the cold European climate. Thus hunter-gatherers were in a precarious sodium balance. The ability to retain sodium by the kidneys was critical to survival and thus the ability to conserve sodium may have played a crucial role in genetic selection. This may be a reason why salt sensitivity is associated with population groups originating in Africa.

The descendants of these African people are now being exposed to the lifestyle that westerners have been living over many centuries. This includes a diet rich in sodium. Increased dietary sodium intake was been found to be associated with the risk of cardiovascular disease and mortality [He et al., 1999]. Two studies were initiated in an attempt to prevent hypertension with dietary sodium reduction. The interventions were education and counselling for periods of up to 48 months. In a 15 year follow up of those who had had the interventional advice the cardiovascular events were significantly lower than those who had had standard dietary advice [Cook et al., 2007].

Luft et al. [1991] showed that salt sensitivity developed as people got older and was due to a delay in sodium excretion. Falkner and Kushner [1990] studied normotensive young blacks and whites and also found that blacks were more salt sensitive than whites. They found that within these normotensive subjects those that were classified as salt sensitive had significantly higher BP than those that were salt resistant.

Salt sensitivity has been poorly defined, but is characterised by an increase in BP with higher sodium intake and a decrease in BP with sodium restriction. Sullivan et al. [1987] were one of the first teams to define salt sensitivity. They assessed subjects on their normal diet with added high (200mEq) and low (10mEq) sodium intake. They defined salt sensitivity as an increase in mean arterial pressure (MAP) by 5% on chronic dietary sodium loading. Salt sensitivity was present in 39% of the hypertensive subjects and 22% of the normotensive subjects. BP changes (outside of the normal range of 75-160mmHg) normally result in an alteration of sodium and water excretion which controls the BP [Guyton and Hall, 1996]. This pressure natriuresis and diuresis response may be defective in salt sensitivity.

Since then many attempts have been made to try and define salt sensitivity. Overlack et al. [1993] defined salt sensitivity as an increase in MAP by 5mmHg on a high sodium diet when compared with a low sodium diet. A difference of less than 5mmHg was defined as salt resistant and those in whom their BP decreased were labelled counter-regulators. They tested for salt sensitivity in a group of normotensive non-obese subjects and found that subjects that were older, female, had a lower body weight or a family history of hypertension were more likely to be salt sensitive.

Weinberger et al. [2001] defined salt sensitivity in the acute setting. Their definition of salt sensitivity was a decrease in BP of 10mmHg or more when given furosemide after a saline challenge of 2L 0.9% saline. A decrease in BP of less than 6mmHg was defined as salt resistant. This definition was applied to 708 subjects and their medical history and cause of death was followed up almost 30 years later. Salt sensitivity was found to be associated with mortality in both normotensive and hypertensive subjects.

Another team [Gill et al., 1991] described salt sensitivity as an increase in MAP by 8mmHg, between a week of a low (9mmol/day) sodium diet and a week of a high (249mmol/d) sodium diet. This dietary analysis was an in-hospital study to ensure strict compliance. They found that the salt sensitive group had a significantly higher urinary 3,4-dihydroxyphenylalanine (dopa) excretion which had a marked increase when the sodium was increased initially.

Although there are many definitions that are used for salt sensitivity, an indirect indicator is low plasma renin activity, plasma aldosterone and urinary aldosterone excretion that are suppressed by negative feedback in response to innate sodium reabsorption by the kidney

Racial differences in sodium homeostasis were well described by Ambrosius et al. [1999]. The Black group that was studied in America had lower urinary aldosterone-potassium ratios, increased sodium retention, decreased aldosterone excretion and increased potassium excretion, suggesting an underlying predisposition to salt sensitivity in African Americans. Furthermore, these results indirectly suggest that overactivity of the ENaC may be the underlying difference between Black African and Caucasian populations. Pratt et al. [1989] showed that even African American children under 12 years had lower aldosterone levels suggesting this may be a genetic difference. Furthermore, they [Pratt et al., 1993] showed the urinary aldosterone excretion in Black children did not increase with time and was persistently about 35% lower than in White children. These differences in aldosterone and renin levels are also described in South Africa. Rayner et al. [2001] showed that Black African

normotensives had significantly lower renin and aldosterone levels than the Caucasian normotensives, despite a similar sodium intake.

There is contrasting evidence for a racial difference in ENaC activity. Pratt et al. [2002] found that Blacks excreted less potassium and had higher serum potassium levels, and amiloride was shown to be a more effective antihypertensive in White than in Black individuals, indicating that Blacks have intrinsically lower ENaC activity. In contrast, Baker et al. [2001] showed that the ENaC was upregulated in Black people in London. Using a technique where they assessed nasal potential difference (which had been used to show a higher sodium channel activity in patients with Liddle's disease [Baker et al., 1998b]) they found that the maximum nasal potential difference was higher in Black subjects. Black hypertensives and normotensives did not have a difference in nasal potential difference.

Low renin, low aldosterone hypertension (LRHT) is a well described clinical entity. Pratt et al. [1999] showed that it is caused by sodium retention by the kidney rather than the result of increased mineralocorticoid activity. Fisher et al. [2002] demonstrated a familial association for low renin levels, possibly implicating genetic factors. As the RAAS is the major system controlling sodium homeostasis and BP [Guyton, 1990] it warrants further discussion.

Renin Angiotensin Aldosterone Syndrome

Renin is secreted by the juxtaglomerular cells of the kidney in response to a drop in BP, decreased glomerular filtration rate (GFR) and increased sympathetic activity.

Renin converts angiotensinogen (AGT) to angiotensin I which in turn is converted to angiotensin II, by ACE (Figure 2.4).

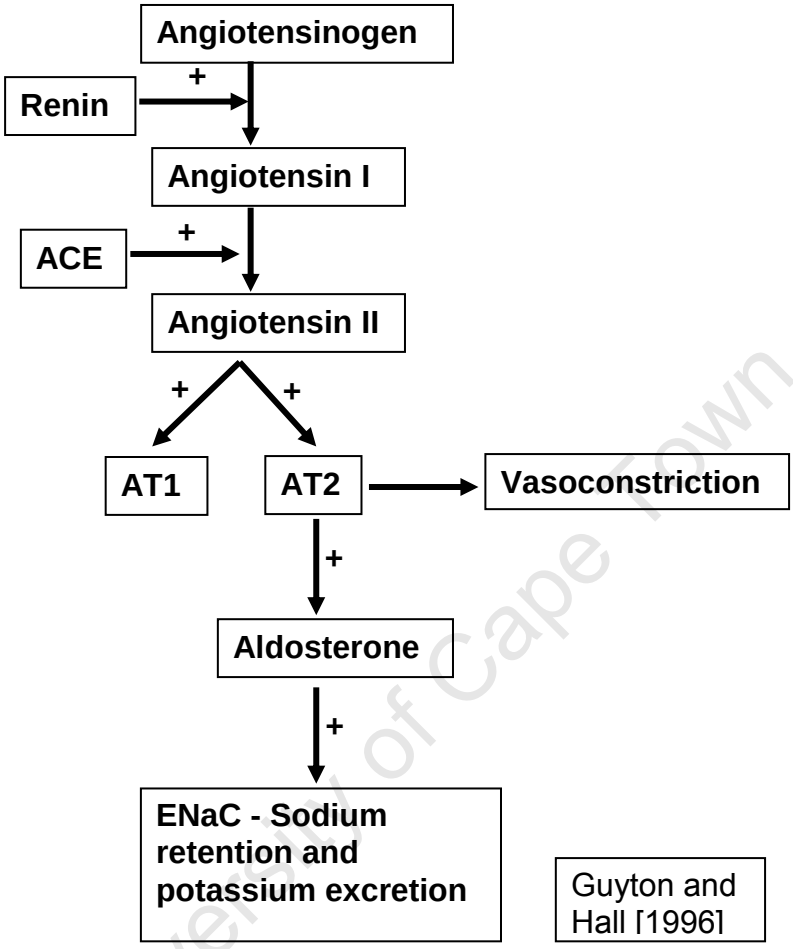

Figure 2.4. The renin angiotensin aldosterone system and its ability to control BP. (ACE- Angiotensin converting enzyme, AT1 and AT2 – Angiotensin receptors 1 and 2, ENaC- epithelial sodium channel)

Angiotensin II decreases renal blood flow, increases tubular reabsorption of sodium and water and stimulates the production of aldosterone. Angiotensin II is a potent vasoconstrictor, stimulates thirst, and via its receptors in the brain, stimulates sympathetic output and antidiuretic hormone secretion [Guyton and Hall, 1996]. Aldosterone stimulates sodium retention through stimulation of the mineralocorticoid receptor and the ENaC. All these effects act in concert to restore BP and sodium

balance. Thus the entire RAAS is a fertile source for the investigation of candidate genes for hypertension, particularly of the salt sensitive type.

Aldosterone is produced from the adrenal glands in response to angiotensin II or a decrease in potassium but is inhibited by a rise in osmolarity (increased sodium). It acts on the principal cells in the kidneys to increase sodium retention and increase potassium excretion. It stimulates and increases the apical sodium channels and the basal sodium-potassium adenosine triphosphatase (Na/K-ATPase) pump. Sodium absorption is increased, along with water, increasing the extracellular fluid volume and BP.

The activity of aldosterone on the ENaC is the final and rate limiting step in sodium reabsorption. However the aldosterone stimulus on BP is not as potent as angiotensin II [Guyton and Hall, 1996] which may account for the directed search for genetic polymorphisms in the renin and AGT genes. The genes that code for the proteins in the RAAS are candidate genes for hypertension. There have been many studies searching for polymorphisms in these genes to determine an association with hypertension.

Hasimu et al. [2003a] screened the renin gene for polymorphisms in Japan and then assessed for phenotypic differences. A missense mutation (G1051A) in the coding region of the renin gene was found and was associated with EHT. The GG genotype had significantly higher plasma renin activity. The same group [Hasimu et al., 2003b] also found a variable number of tandem repeat (VNTR) polymorphism in intron 7, 18bp upstream of exon 8, but this was not associated with hypertension. Niu et al.

[1999] found no evidence that the renin gene (or any other gene in the RAAS) influenced the pathogenesis of hypertension in the Chinese.

Nagy et al. [1999] showed that there was significant linkage between the renin gene and higher systolic BP (SBP) in the Hungarian population, using twin studies. They also showed that the β- and γ-ENaC genes were linked to increased SBP. Zhu et al. [2003] showed that a number of polymorphisms in the renin, AGT and angiotensin 2 receptor genes were linked to hypertension using family based studies and analysing the data using transmission disequilibrium testing (TDT) analysis. (For a description of the TDT analysis, see Appendix I.) These findings were particularly evident in the African American population that they studied.

The AGT gene is well researched as a candidate gene. This gene was found to have a high frequency of the M235T variant in many different populations. The T allele has been significantly associated with hypertension but Jeunemaitre et al. [1997b] showed that a SNP (G-6A) in the pretranscription region of the gene was in complete linkage disequilibrium (LD) with the M235T variant and when associated with the T allele was significantly associated with hypertension. However, Brand et al. [1998] observed that there needs to be a large number of families in a study for a significant linkage of hypertension to an AGT candidate gene. Tiago et al. [2002] found a significant relationship of the M235T AGT polymorphism with the influence of body size on SBP in South Africans.

ACE mutations were found that involved insertions and deletions. A Spanish study [Poch et al., 2001] found that an insertion mutation was associated with salt

sensitivity and a higher incidence of hypertension. Another study [Kosachunhanun et al., 2003] in the USA found that the ACE DD genotype, when co-expressed with the AGT 235 TT genotype, was associated with non-modulating salt response and thus a higher risk of hypertension. This study also found that subjects with the AGT 235 TT genotype had significantly lower plasma aldosterone levels and a higher SBP.

Other angiotensin gene polymorphisms have been described that are associated with EHT. Two studies [Caulfield et al., 1994; Atwood et al., 1997], in different populations confirmed the association of AGT gene GT dinucleotide repeat polymorphism with hypertension. Neither of these studies found a significant association with the M235T variant.

The angiotensin receptors have been contentious with regard to their influence on BP. The effects of the two receptors (AT_1R and AT_2R) on BP were characterised by Munzenmeier and Greene [1996]. They found that stimulation of AT_1R causes vasoconstriction and angiogenesis and stimulation of AT_2R inhibits this response. The AT1R gene is found on the X chromosome. Jones et al. [2003] found that the AT_1R11166CC genotype was found more frequently in hypertensives than in normotensives, and that, independent of BP, it was associated with cardiovascular risk. In the same study two polymorphisms were found in the AT_2R gene; AT_2R1675A>G and AT_2R3123C>A. The 1675G polymorphism was found to be protective against the effects of hypertension and if the 1675A genotype was co-expressed with the 3123A genotype there was a significant risk of hypertension and its complications. Jin et al. [2003] screened a Japanese population for polymorphisms of the AT_2R gene and discovered a polymorphism (C4599A in exon 3) that was

significantly associated with hypertension in women, particularly post-menopausal women.

These studies illustrate the difficulties of determining the effects of differing genotypes on multifactorial disorders. Associating a single mutation with a disease, independent of the remainder of the genetic make-up, excludes the additive effects of multiple polymorphisms.

Epithelial Sodium Channel

The ENaC is the final step in sodium reabsorption and thus of sodium balance. It is highly selective for sodium over potassium (>100x) and is inhibited by amiloride [Zhou and Bubien, 2001]. Mutations in the genes encoding the sodium channel subunits have been found to cause Liddle's disease (autosomal dominant hypertension with potassium wasting and hypoaldosteronism). Finding this disorder in the rate-limiting step for sodium reabsorption has made the ENaC genes attractive candidate genes for EHT.

The sodium channel is a ligand gated (i.e. opens or closes in response to a chemical messenger) transmembranous channel. The sodium channel is traditionally thought to be a tetramer made up of 2α, 1β and 1γ subunit as seen in Figure 2.5. However, recent evidence suggests that the channel is made up of equal numbers of each subunit: 1α:1β:1γ [Staruschenko et al., 2005a] being the most likely but two or three of each subunit also being a possibility. This team used a fluorescence based approach to illustrate the relative subunit stoichiometry and subunit titration experiments to show that the channels were more likely to be found with equal numbers of each subunit.

They also illustrated that the channels are preferentially found in the heteromer form as opposed to the homomer.

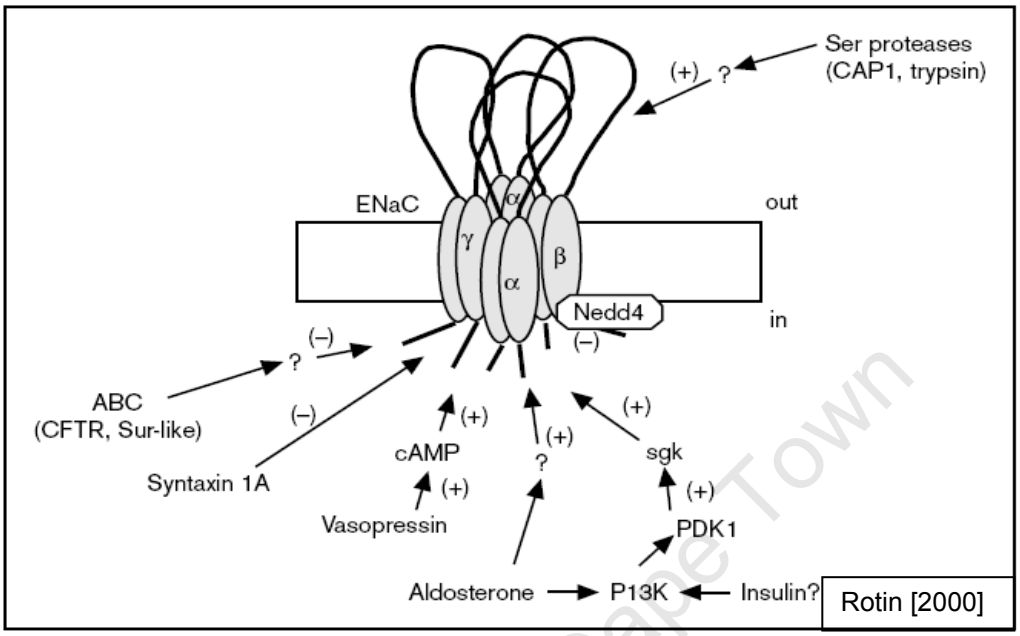

Figure 2.5. The ENaC is a transmembranous tetramer, composed of two α, one β and one γ subunit, influenced by many proteins. (Image taken from Rotin [2000]) ENaC, epithelial sodium channel; PDK1, 3-phosphoinositide-dependent protein; PI3K, PI3 kinase; CAP1, channel activating protease 1; CFTR, cystic fibrosis transmembrane conductance receptor; sgk, serum glucocorticoid induced kinase

These findings were supported by Jasti et al. [2007] who crystallised the acid-sensing-ion channel (ASIC). This channel has a similar amino acid sequence to the ENaC and is thought to have a similar evolutionary history. This team used crystallography and electrophysiological studies to determine the architecture and molecular properties of the ASIC. They found that the ASIC was expressed as a trimer but were unable to crystallise the intracytoplasmic region of the ASIC which influenced the ability to determine the function. This strongly suggests that the ENaC is also expressed as a trimer.

The ENaC was found in the apical plasma membrane of the principal cells in the collecting duct by Loffing et al. [2000] and has a high affinity for sodium [Babini et al., 2003]. Loffing et al. [2000] showed that dietary intake of sodium influenced the availability of the subunits and expression at the cell membrane. On a diet rich in sodium, mice had no detectable renal cell α-ENaC. The β and γ subunits were present in the cytoplasm but not at the apical surface. When the mice were on a low sodium diet all three subunits were found in the plasma membrane and in the subapical cytoplasm. The insertion of β- and γ-ENaC into the membrane coincided with detectable levels of α-ENaC. This regulatory feedback was replicated by Babini et al. [2003]. They found that upon exposure to high sodium concentrations the ENaC became inactive. Other factors were found to influence ENaC activity: activity is increased by aldosterone and vasopressin and inhibited by amiloride [Zhou and Bubien, 2001].

Upregulation

Insulin and aldosterone increase the number of sodium channels at the cell surface [Blazer-Yost et al., 1998]. The individual hormones stimulate the sodium current but together they act synergistically with the rate of decline of current being much slower when acting together. This effect was shown to be phosphatidylinositol 3 kinase (PI3 kinase) dependant [Blazer-Yost et al., 2003], suggesting a transcellular signalling pathway for ENaC insertion into the apical membrane.

Serum insulin acts on receptors at the basolateral membrane which then increases the sodium channel activity. Blazer-Yost et al. [2004] confirmed this pathway as a mechanism of insulin control of ENaC activity by showing that insulin increased PI3

kinase, which in turn resulted in phosphorylation of the ENaC with phosphatidylinositol 3,4,5-trisphosphate (PIP_3) causing insertion of the ENaC (from apical pools) into the apical membrane. Tong et al. [2004] showed that the PIP_3 and phosphatidylinositol 3,4-bisphosphate (PIP_2) had a direct stimulatory effect on the sodium channel by increasing open probability (Po) and confirmed that PI3 kinase mediated this pathway.

Tallini and Stoner [2002] also showed that insulin increased the open probability (Po) which increased the sodium channel conductance. Serum and glucocorticoid induced kinase 1 (SGK1) also increased sodium current. SGK1 was phosphorylated by PIP_3 and had an additive effect with aldosterone but abolished the insulin response [Arteaga and Canessa, 2005].

PIP_3 was shown [Pochynyuk et al., 2005] to interact with the γ-ENaC at the conserved positive amino acids just distal to the second transmembranous domain. This then regulates the signalling cascades of the γ-ENaC. The resulting alteration in channel function is thought to be the result of a difference in channel gating as Po is affected the most.

Aldosterone stimulates the sodium channel genes to increase production of the subunits that make up the channel [Zhou and Bubien, 2001]. The subunits are made in the endoplasmic reticulum. In the endoplasmic reticulum the subunits form an immature channel which enters the cytoplasm on acquisition of oligosaccharides before insertion into the cell membrane. This maturation process is the rate limiting step in the production of active channels [Valentijn et al., 1998].

Zhou and Bubien [2001] showed that aldosterone also activates the channel directly and that this process is adenosine triphosphate (ATP) dependent. This could also be how insulin and protein kinases A (PKA) and C (PKC) alter function with phosphorylation of the channel [Shimkets et al., 1998]. Cyclic adenosine monophosphate (cAMP) could assist in this process by increasing translocation of the channels into the cell membrane [Snyder, 2000].

Staruschenko et al. [2005b] showed that conserved threonines and serines within the cytoplasmic carboxy termini of the β-ENaC and γ-ENaC are required for interaction with epsin, dynamin and Nedd 4-2. The state of phosphorylation of the serine and threonine residues determined the interaction with these proteins which in turn influenced the channel activity. (For a description of Nedd 4, see glossary.)

Aldosterone also stimulates the production of protein 14-3-3β. This group of intracellular proteins binds to phosphorylated motifs on their target protein to control function. In the cortical colleting duct cells this protein binds to phosphorylated Nedd 4-2 inhibiting its interaction with ENaC, thereby decreasing ENaC internalisation [Liang et al., 2006].

Butterworth et al. [2005a] showed that the sodium channel is found in a subapical pool. This explained their earlier findings that activation of PKA results in insertion of channels (from a subapical storage pool of channels) into the membrane and a cAMP-induced increase in current [Butterworth et al., 2001]. This current could be G-protein dependant. Using B lymphocytes, Bubien et al. [1994] found that the ENaC is regulated by cAMP and guanidine triphosphate- (GTP)-binding proteins, which act on G proteins.

Hill et al. [2007] showed that the sodium channel inserts into the membrane with the aid of lipid domains (rafts). Many different proteins also use these lipid rafts to aid in their function, e.g. syntaxin 1A (S1A) which acts as a transporter for the sodium channel. As the lipid rafts are made up of cholesterol this makes the channels dependant on cholesterol. The interaction of lipid rafts and the sodium channel was found to be important for ENaC delivery to the apical membrane, but not for retrieval. This paper also showed that aldosterone did not alter the interaction between the ENaC and these lipid rafts suggesting that this means of delivery into the cell membrane is an independent pathway for increasing sodium channel activity.

Downregulation

The intracellular domains of the ENaC are available to interact with intracellular proteins to internalise the channels. The carboxy termini of the subunits each have a PY motif which has been found to be important in downregulation of the channels [Schild et al., 1996]. The PY motif is a proline-rich sequence which has a tyrosine within the motif. In the human β-ENaC this motif starts at amino acid 613 and ends with the tyrosine in position 618. The motif is TPPPxY, where T stands for threonine, P for proline, Y for tyrosine and x for any amino acid. Schild et al. [1996] showed that changing any P, the T or the Y within the motif increased the sodium channel activity to a level similar to that of the R566X mutation.

Nedd 4-2 is an ubiquitin-protein ligase that is involved in the ubiquitination of ENaC. Nedd 4-2 has multiple WW (tryptophan rich) domains that interact with the PY motif on the ENaC carboxy terminal allowing internalisation of the channel. (The evidence for this interaction was supported when Fouladkou et al. [2004] found a Nedd 4-2

variant (P355L) which decreased ENaC inhibition.) Once ubiquitinated, the channel is degraded by proteosomes or lysosomes [Staub et al., 1997]. WW domain containing protein 2 (WWP2) is another ubiquitin protein ligase (E3 protein) which has been found to internalise the ENaC, but not to the same degree as Nedd 4-2 [McDonald et al., 2002].

Nedd 4-2 is phosphorylated by SGK1 with the aid of 14-3-3 proteins, which inhibits the interaction between Nedd 4-2 and the ENaC [Bhalla et al., 2005]. In this way SGK1 increases ENaC activity by decreasing internalisation of the channel, increasing the number at the cell surface. SGK1 also increases the Po (chance of the channel being open) of the channel and may increase the synthesis of the channels [Alvarez de la Rosa et al., 2004].

Another mechanism of down regulating the transmembranous ENaC is by clathrin-mediated endocytosis. This is a process whereby channels are internalised into vesicles by the contractile protein clathrin. Shimkets et al. [1997] showed that the ENaC underwent endocytosis using clathrin which interacted with the β and γ subunits of the channel. Truncation of the carboxy termini of the β and γ subunits increased the half life of the channels, as did inhibition of clathrin. Linkage of clathrin lattices to the ENaC was found to be dependant on epsin [Wang et al., 2006], via an ubiquitin interaction motif in epsin. (The epsin family of proteins are important for protein-protein interactions, and interact with clathrin to allow clathrin-coated vesicle budding [Chen et al., 1998].)

Soluble N-ethyl-maleimide-sensitive factor attachment protein receptors (SNARE's) allow fusion of vesicles and membranes to transfer channels into the membrane. S1A is a target (t) membrane SNARE that interacts with the ENaC. S1A was found to increase the closed time of the channel within the membrane [Condliffe et al., 2004] and to decrease channel insertion into the membrane [Berdiev et al., 2004]. Accessory proteins stabilise the SNARE complex regulating vesicle insertion. Complexin binds to assembled SNARE complexes that consist of syntaxin and a vesicle-associated (v) SNARE and was found to inhibit ENaC activity by decreasing the number of channels (N) in the cell membrane [Butterworth et al., 2005b]

This indicates that S1A has more than one binding site onto the sodium channel. The variant S518K of the β-ENaC in the transmembrane domain leaves the channel in the open state which is insensitive to the initial action of S1A. However, after about an hour S1A decreases channel activity as a result of decrease in number of channels (N) at the cell surface [Condliffe et al., 2003]. The position where S1A, SGK, epsin and the protein kinases interact has not been determined. Any intracellular protein that interacts directly with the β-ENaC could potentially be influenced by the R563Q mutation. Alteration in the binding motif for any one of these, or other unknown proteins, could diminish the protein-protein interaction.

Internalisation of the sodium channel is described above but this does not encompass degradation of the channel. These pathways remove the channel from the apical membrane into intracellular vesicles. This then undergoes degradation or recycling. One method of degradation is the lysosomal pathway and another is the proteosomal pathway. The proteosomal pathway involves breaking down proteins by destroying

peptide bonds (proteolysis). This pathway involves tagging by ubiquitin and is catalysed by ubiquitin ligases (Nedd 4-2 is an ubiquitin ligase). Nedd 4-2 is a protein that is highly important for sodium channel control and so there is strong evidence that the proteosomal pathway is important for degradation of the channel. Malik et al. [2001] showed this but found no evidence suggesting the lysosomal pathway is involved in the degradation of the channel as others have [Staub et al., 1997].

In vivo Physical and Cellular Differences

Baker et al. [1998b] showed that a family with Liddles's syndrome had increased nasal ENaC activity suggesting increased renal ENaC activity. They [Baker et al., 1999] later found no difference in nasal potential difference in White normotensives and hypertensives, suggesting that White hypertensives are unlikely to have an increase in ENaC activity. This was not supported by Pratt et al. [2002], who found that Black American subjects with hypertension did not respond as well as the Whites to amiloride.

Zhou and Bubien [2001] showed that the ENaC is found in B lymphocytes. This team [Bubien et al., 2001] also found that lymphocyte ENaC activity accurately reflects renal ENaC activity. Carter et al. [2001] suggested that the B lymphocytes can be used to identify individuals who will respond to amiloride.

Genetics of the ENaC

The ENaC is the rate limiting step in sodium reabsorption, which prompted the need to clone the ENaC gene in the search for candidate genes for hypertension. The α-subunit gene is found on the short arm of chromosome 12 and the β and γ-subunit

genes are found close together on the short arm of chromosome 16. The β and γ subunit genes are found at 16p13-p12, within a 400kb fragment [Shimkets et al., 1994]. Voilley et al. [1995] reported the cloning and sequencing of the β- and γ-subunits. The genetic sequences for the two genes were highly similar, suggesting a common ancestor, possibly by gene duplication. Saxena et al. [1998] reported the exon-intron organisation of the β-ENaC. The last exon (13) contains the sequence for the second transmembrane domain and the PY motif.

Linkage analysis of chromosome 16p12 revealed linkage of the β- and γ-ENaC genes and SBP variation within families [Wong et al., 1999]. Pseudohypoaldosteronism Type 1 (PHA1) is an autosomal recessive [Pujo et al., 2007] disorder that presents with sodium wasting and hypotension. It is a disorder that presents in neonates and is incompatible with a prolonged life. The genetic defect is a mutation in the ENaC and prevents the sodium channel from reabsorbing sodium due to lack of response to aldosterone. Mutations are found from exons 2 to 13. Mutations of the mineralocorticoid receptors can also cause PHA1.

Liddle's Syndrome is a hypertensive disorder that results from mutations in the last exon (13) of the gene. Mutations that affect the intracytoplasmic tail of the channel are implicated in Liddle's. To cause the full Liddle's syndrome, disruption of the PY motif is required. This proline rich motif is the binding area for the intracytoplasmic ubiquitin protein ligase Nedd4-2. Nedd4-2 binds to the ENaC to internalise the channel, so decreasing the activity of the channel [McDonald et al., 2002].

PHA1 and Liddle's disease are two extreme disorders associated with genetic defects in the sodium channel genes. These two disorders indicate the level of importance of

the genes and many research teams have been analysing the effects of mutations within the genes encoding the three subunits.

The α-ENaC

The α-subunit of the sodium channel has, as yet, not been described with a mutation that causes full blown Liddle's syndrome, despite having a PY motif. This subunit also is the only subunit that can create a current for sodium when expressed alone in *Xenopus* oocytes. The gene encoding the α-subunit is found on chromosome 12p13.1 and chromosome 12p was found to have linkage for EHT [Gong et al., 2003]. Truncation of the α-ENaC (removing the PY motif) has been found to increase the sodium current *in vitro* [Schild et al., 1996], but truncating mutations have not been described *in vivo*.

A few point mutations of the α-ENaC have been described. The T663A missense mutation has been found more frequently in Caucasian subjects and has been found to be protective against hypertension [Ambrosius et al., 1999]. It did not have an effect on sodium current and did not show any association with the parameters that alter sodium balance. They found two other mutations (C618F and A334T) which did not differ between hypertensives and normotensives but were significantly more frequent in Black African subjects. Tong at al. [2006] described the effects of these three mutations. In Chinese hamster ovary cells the sodium current was assessed using patch clamping. They found that C618F and A663T increased and A334T didn't alter the current. This was achieved by an increase in number of active channels at the cell surface.

The β-ENaC

Shimkets et al. [1994] described four other kindreds with Liddle's syndrome (other than the original family). One of these families had the same mutation as that found in the original family, both families were in England. This mutation has been found in other countries: Sweden [Melander et al., 1998] and Japan [Kyuma et al., 2001]. All these mutations disrupted the PY motif. They were either mutations that introduced a stop codon, resulting in truncation of the carboxy terminal; or caused a frameshift of the gene, altering the encoded proteins of the carboxy terminal of the ENaC. (A frameshift mutation is the result of an insertion or deletion of one or more nucleotides resulting in a different amino acid sequence.)

Multiple point mutations of the β-ENaC have been described in association with hypertension. Many of these mutations cause an amino acid substitution. The T594M mutation has been found more frequently in individuals with hypertension [Baker et al., 1998a; Dong et al., 2001] who found that there was increasing frequency with increasing BP. Baker et al. [2002] found that amiloride alone controlled BP in those with this variant. There is no physiological evidence for over activity of the sodium channel with this mutation [Persu et al., 1998] and the association with hypertension was not replicated by Nkeh et al. [2003] or Ambrosius et al. [1999]. They did however find that the T594M mutation occurs more frequently in Black African individuals than in Caucasians. There was no significant difference in allele frequency between cases and controls in a South African study by Pegoraro et al. [2004] of pre-eclamptics and eclamptics compared with normotensive pregnant women. The expected two dimensional configuration of the sodium channel is shown in Figure 2.6.

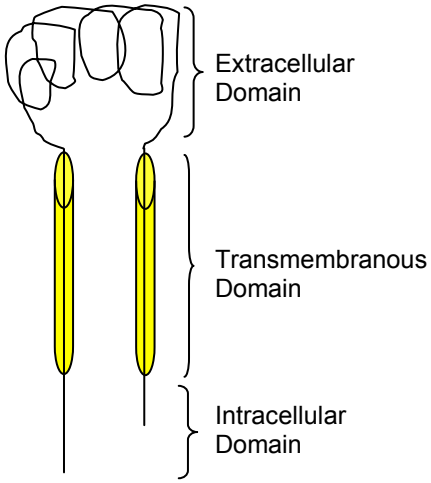

Figure 2.6. A 2D representation of the structure of the β-subunit of the ENaC.

Possible reasons for the T594M variant not to be an activating mutation are that it is found in a region of the sodium channel β-subunit gene that is poorly conserved across species and it is not near the vital PY motif. A further reason is that methionine and threonine are both non-charged amino acids.

The G442V mutation of the β-ENaC in exon 8 was also found more frequently in African individuals and is more frequent than the T594M variant [Persu et al., 1998]. There was no difference in allele frequency in three BP categories in a population screened by Dong et al. [2001]. The G442V variant also did not alter the sodium current when expressed in *Xenopus* oocytes [Ambrosius et al., 1999] but subjects with the G442V mutation had biochemical changes suggesting higher intrinsic activity of the ENaC.

Persu et al. [1998] screened the β-ENaC gene and found seven polymorphisms; five were found in exon 13. Melander et al. [1998] also sequenced the gene and found one point mutation in exon 13: βGly587Ser. This mutation was found in an individual who had early onset of hypertension and a positive family history of hypertension.

The mutations found by Persu et al. [1998] were more common in African Americans and some (G589S and R624C) altered sodium sensitivity when expressed in *Xenopus* oocytes, especially the amiloride-sensitive sodium uptake. R597S had a suppressed renin level and G589S tended toward hypokalaemia.

Hannila-Handelberg et al. [2005] also found one of the variants that Persu et al. [1998] found (G589S), when he screened the β-ENaC gene for mutations in Finland. However, this mutation was not significantly more frequent in hypertensives than in the controls and there was no difference in clinical characteristics or biochemical parameters. There was a non-significant blunted renin response to a captopril challenge and an increased excretion of potassium in relation to plasma renin and aldosterone. This mutation did not affect sodium current when expressed in *Xenopus* oocytes either. This team also found a variant in the intron, 17 nucleotides before the 13^{th} exon, which did not affect the ribonucleic acid (RNA) sequence. It was however more frequently found in hypertensives than in normotensives.

Mutations that disrupt the PY motif are more likely to cause differences in the biochemical parameters of the subjects and to alter the sodium current significantly when expressed in *Xenopus* oocytes. Shimkets et al. [1994] described four mutations that interfered with the PY motif. Two mutations introduced premature stop codons: R564X and Q589X, which removed the PY motif. Two other mutations resulted in a frameshift: a deletion of a cytosine in the six cytosine sequence from 593-595 resulting in altered protein sequence until the new stop codon at 673 and an insertion of cytosine at T-592 altering the encoded proteins till the new stop codon at 605. This kindred is described in more detail by Findling et al. [1997].

The R566X mutation was the mutation found in the kindred who originally presented to Dr Liddle, who described the syndrome. This mutation was expressed in *Xenopus* oocytes by Schild et al. [1995] and was found to increase sodium current significantly. The same team [Firsov et al., 1996], showed that the means of increasing the current was by increasing the N at the cell surface and by increasing the Po of the channel. Jeunemaitre et al. [1997a] described the deletion of 32 nucleotides starting at 579 which then introduced a new stop codon at position 582. Inoue et al. [1998b] described a cytosine insertion at Arg-597 also causing a frameshift and loss of the last 34 amino acids of the carboxy terminal.

All of these mutations were found in individuals or kindreds who had presented with full blown Liddle's syndrome and they all removed the complete PY motif. Schild et al. [1996] tried to determine the exact impact of the carboxy terminal by sequentially deleting portions of the carboxy tail. They found that deletions of the terminal amino acids, up to the last 10, increased sodium current *in vitro*. The increase in current was attenuated as the number of amino acids deleted decreased.

Subsequently it has been found that point mutations of the PY motif interfere with sodium current and can cause full blown Liddle's disease. The P616L missense mutation has been described as a *de novo* mutation in China [Yamashita et al., 2001], and in an unrelated family, also in China [Gao et al., 2001]. This mutation had been previously described in the USA [Hanssen et al., 1995b] and in Japan [Uehara et al., 1998] as a *de novo* mutation. Hanssen et al. [1995b] described the physiological characteristics of this mutation and found that the mutation caused a significant 8.8-

fold increase in sodium current and it altered the biochemical parameters of the individuals. (Some of the described mutations are shown in Figure 2.7.)

	ATC	GTC	TGG	CTG	CTC	TCG	AAT	CTG	GGT	GGC	CAG	TTT	GGC	
511	I	V	W	L	L	S	N	L	G	G	Q	F	G	525
	TTC	TGG	ATG	GGG	GGC	TCT	GTG	CTG	TGC	CTC	ATC	GAG	TTT	
526	F	W	M	G	G	S	V	L	C	L	I	E	F	539
	GGG	GAG	ATC	ATC	ATC	GAC	TTT	GTG	TGG	ATC	ACC	ATC	ATC	
540	G	E	I	I	I	D	F	V	W	I	T	I	I	552
	AAG	CTG	GTG	GCC	TTG	GCC	AAG	AGC	CTA	CGG	CAG	CGG	CGA	
553	K	L	V	A	L	A	K	S	L	R	Q	R	R	566
	GCC	CAA	GCC	AGC	TAC	GCT	GGC	CCA	CCG	CCC	ACC	GTG	GCC	
567	A	Q	A	S	Y	A	G	P	P	P	T	V	A	579
	GAG	CTG	GTG	GAG	GCC	CAC	ACC	AAC	TTT	GGC	TTC	CAG	CCT	
580	E	L	V	E	A	H	T	N	F	G	F	Q	P	592
	GAC	ACG	GCC	CCC	CGC	AGC	CCC	AAC	ACT	GGG	CCC	TAC	CCC	
593	D	T	A	P	R	S	P	N	T	G	P	Y	P	603
	AGT	GAG	CAG	GCC	CTG	CCC	ATC	CCA	GGC	ACC	CCG	CCC	CCC	
604	S	E	Q	A	L	P	I	P	G	T	P	P	P	616
	AAC	TAT	GAC	TCC	CTG	CGT	CTG	CAG	CCG	CTG	GAC	GTC	ATC	
617	N	Y	D	S	L	R	L	Q	P	L	D	V	I	629
	GAG	TCT	GAC	AGT	GAG	GGT	GAT	GCC	ATC	TAA				
630	E	S	D	S	E	G	D	A	I	STOP				639

Figure 2.7. Exon 13 of the β-ENaC gene. The amino acid numbers are shown on the sides. Yellow shading shows the transmembranous domain, green shading shows the PY motif, some published mutations that have been shown to cause to Liddle's syndrome are shaded in purple and non-Liddle's-causing mutations are shaded in blue. The arginine at position 563 is shaded in dark blue. Poorly conserved amino acids are shown in red writing.

Other point mutations of the PY motif have been found to cause Liddle's disease. P616R was described by Furuhashi et al. [2005] as a de novo mutation in a family who presented with Liddle's disease. Another proline in the motif has been implicated. P615S was found in a family who presented with Liddle's in Japan [Inoue et al., 1998a]. The substitution of tyrosine to histidine (Y618H) in the motif was also found to cause Liddle's [Tamura et al., 1996].

The R563Q mutation of the β-ENaC

The R563Q mutation of the β-ENaC is a SNP of the β-ENaC gene. The mutation is found in the exon of the carboxy-terminal cytoplasmic tail of the β-ENaC (Figure 2.8). It is a transition of guanine to adenine (CGG to CAG) in codon 563, resulting in a missense mutation from arginine to glutamine. The mutation was found to be associated with low renin, low aldosterone hypertensives in Black African and Coloured groups [Rayner et al., 2003].

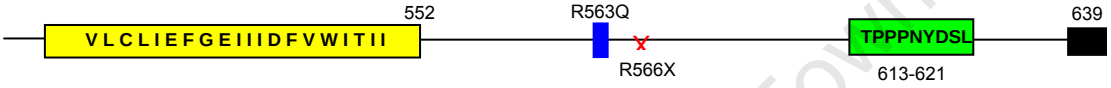

Figure 2.8. Exon 13 of the β-ENaC showing the close proximity of the R563Q mutation to the original Liddle's mutation (R566X), the transmembranous domain (yellow) and the PY motif (green), with the stop codon at position 639.

This polymorphism was found by sequencing of the carboxy terminal domains of the β- and γ-ENaC genes. Polymerase chain reaction (PCR) with restriction digest confirmed the presence of this mutation in a heterozygous form in 18 unrelated individuals. Fourteen of these individuals were tested for plasma renin and aldosterone levels. Nine had low renin and aldosterone levels and two had low aldosterone without a fully suppressed renin but one of these patients had renal impairment. Two patients had unprovoked hypokalaemia, one of them was severely hypokalaemic (2.1mmol/l). Eleven had severe hypertension with target organ damage.

Recently the R563Q mutation has been shown to be associated with pre-eclampsia by Dhanjal et al. [2006]. A racial difference in frequency of the mutation was evident; there was a higher incidence in Black African people than in Coloureds. Renin levels

were lower in individuals with the mutation, but there was no difference in plasma aldosterone and potassium levels. All subjects that have been found to have the mutation were heterozygous for the R563Q mutation.

The γ-ENaC

The γ-ENaC has also been implicated in Liddle's syndrome. Truncations of the carboxyterminal of the γ-ENaC have been found to cause full blown Liddle's syndrome. Yamashita et al. [2001] found a *de novo* truncating mutation (W576X) of the γ-ENaC in a patient who presented with Liddle's syndrome. This mutation truncates the carboxy terminal before the PY motif which starts at position 624 on the γ-ENaC gene. Another Liddle's mutation was found by Hansson et al. [1995a]. This mutation, K574X, is also a truncating mutation of the γ-ENaC and was found to alter the activity of the channel when expressed in oocytes.

Schild et al. [1996] also tested sequential deletions of the γ-ENaC. They found that there was significant gain in function if the PY motif was deleted. The last 21 amino acids, when deleted, did not alter the sodium current and there was not the sequential increase in current with increase in number of amino acids deleted as with the β-ENaC.

No point mutations found in the γ-ENaC have yet been found to cause full blown Liddle's disease. However, a few have been described in association with hypertension. Two variants (A531L and C582R) were found in one patient with diabetic nephropathy [Melander et al., 1998]. Neither was found in any of the control subjects and no functional analyses were implemented. They also detected a common mutation in codon 650: CTC to CTG. It did not alter the amino acid sequence and was

not found to associate with BP differences. An extra proline was also found, in one patient, after codon 594 due to the insertion of 3bp (CCT). 594insP was also found by Persu et al. [1999] but did not alter the sodium current in *Xenopus* oocytes. Neither did another mutation (R631H). Both these mutations were only found in one patient each.

Persu et al. [1999] found four other common mutations: three missense mutations (T387C, T474C and C549T) and a point mutation in the complementary DNA (C1990G). None of these mutations was more frequent in hypertensives but they were more commonly found in African Americans. Hannila-Handelberg et al. [2005] also found a common mutation in the γ-ENaC: V546I. This mutation did not alter sodium current in *Xenopus* oocytes or change the renin response and was not significantly more frequent in hypertensives. Ambrosius at al. [1999] also found three nucleotide variants which did not alter the amino acid sequence and were silent mutations. Poch et al. [2000] described a C to G transversion in codon 649 which did not associate with hypertension or salt sensitivity.

Summary

The ENaC is the rate limiting step in sodium reabsorption and is implicated in salt sensitive hypertension of genetic origin. It has been found to cause autosomal dominant forms of hypertension along with only a few other Mendelian forms of hypertension. There have been many mutations described, some of which have been associated with hypertension. Most of these mutations have been found in only a few individuals, but a few have been described at higher frequencies: T594M and G442V.

The R563Q mutation has been associated with hypertension and pre-eclampsia but further data are lacking. This study was designed to gather more information on this mutation from an *in vivo* physiological perspective and to study the prevalence in the South African multiethnic populations.

HYPOTHESIS AND AIMS

Hypothesis

The R563Q mutation of the ENaC is an activating variant found in the multiethnic populations in South Africa causing salt sensitivity and hypertension. The mutation originated in Southern Africa and has become a common variant as a result of scarce sodium supplies in traditional diets.

Aims

1. To demonstrate that the R563Q mutation associates with hypertension in family analyses by studying index cases and their families.
2. To investigate the geographical representation of the mutation across South Africa and its association with hypertension by collecting samples representing the Black African and Coloured groups living in Johannesburg, Cape Town and the Northern Cape.
3. To investigate the origin of the mutation. Specifically, to determine if the mutation in the South African Black African and Coloured groups might have originated in the Khoisan, who lived a hunter-gatherer lifestyle with scarce sodium supplies and a diet rich in potassium.
4. To document the clinical characteristics of the index patients heterozygous for the R563Q mutation compared with age and sex matched controls attending the Hypertension Clinic at GSH.
5. To examine the functional phenotype of subjects heterozygous for the R563Q mutation. Specifically, to investigate these subjects for impaired salt excretion and a greater BP response to an acute saline load compared with age matched normal controls.

6. To determine the effect of the R563Q mutation on sodium current when expressed in the *Xenopus laevis* oocytes compared with wild-type sodium channels

This study was approved by the Research Ethics Committee of the University of Cape Town. Reference Number: 269/2006 (see Appendix III)

CHAPTER 3

General Methods, Procedures and Equipment

This chapter describes the procedures and methods applied that were common to many sections of this thesis. Further details are described in each chapter, where relevant.

SUBJECTS

All subjects were counselled and gave signed informed consent for all clinical procedures and genetic testing. Subjects were selected for each section of the thesis as described in the methods for each section.

Consent forms are available in Appendix III.

CLINICAL METHODS

Blood pressure (BP) was measured in the sitting position after five minutes rest with a calibrated Accouson® mercury sphygmomanometer or a calibrated Omron® automatic sphygmomanometer (validated auscultatory BP monitors). The cuff size used was suitable for the arm circumference.

The mean of two stable readings was used. Hypertension was defined by a SBP ≥140 and/or diastolic BP (DBP)≥90 mmHg, or the use of antihypertensive medication. The MAP was calculated for each patient as one third of the pulse pressure plus DBP.

MAP = (SBP-DBP)/3 + DBP

BLOOD MEASUREMENTS

Renin, aldosterone, sodium, potassium and creatinine were measured by the National Health Laboratory Services (NHLS) at GSH. This is a South African National Accreditation System (SANAS) accredited laboratory and all the tests performed are accredited. The equipment used is calibrated regularly and standards and external quality control samples are run according to SANAS requirements.

Renin: 5ml blood was collected in an EDTA tube from subjects sitting for 10 minutes and kept at room temperature until delivery to the laboratory, within four hours. Renin was measured using RENIN III Generation® (a commercially available kit from CIS bio international, France). The renin level was determined by an immunoradiometric technique that used a pair of antirenin monoclonal antibodies which recognise the active and inactive forms of renin.

The serum was incubated in a polystyrene test tube with an insoluble monoclonal antibody on the wall of the tube that recognised both the inactive and the active form renin. A second monoclonal antibody labelled with 125-iodine was added to the tube which binds only the active renin. The solution was incubated at room temperature for three hours. The solution was washed to remove free reagent. Radioactivity was measured with a gamma counter for two minutes and compared with a standard serum sample. The technique and kit are internationally recognised as an accurate means of determining the active renin in human plasma.

Aldosterone: 5ml of blood was collected in a serum separator tube after the patient had been seated for ten minutes. Aldosterone was measured using radiolabelled

antibodies that bind to plasma aldosterone from a commercially available kit (Coat-a count Aldosterone Radioimmunologie®, Behring). This kit is an internationally accepted means of determining plasma aldosterone levels and the technique used was in accordance with the manufacturer's instructions.

Antibodies to aldosterone are coated onto test tubes. The sample was placed into the test tube. Radiolabelled (I^{125}) aldosterone was added to each tube which was then vortexed and incubated at room temperature for 18 hours. All residual fluid was removed and a gamma counter recorded the amount of radiation over one minute.

Sodium, potassium and creatinine: 5ml of blood was collected in a serum separator tube that allows the blood to clot. The tube was then spun and the serum undergoes analysis with an ion-specific electrode. This method uses an ion-exchange membrane to determine the difference in electromotive force between the sample and a reference solution. The activity across the membrane is directly proportional to the concentration of the electrolyte. This is a standard and accredited means of determining plasma electrolyte concentrations.

CALCULATIONS

Glomerular filtration rate (GFR) is an indicator of renal function as it is an estimate of the flow rate of filtered fluid through the kidney. The estimated GFR is calculated using the equation:

$GFR = 32788 \times (P_{Cr})^{-1.154} \times (age)^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if Black African})$

A GFR of less than 60ml/min/1.73m^2 is an indicator of chronic kidney disease (CKD). ($P_{Cr,}$ plasma creatinine)

Urinary sodium-creatinine ratio (Na/Cr) was calculated from the measured urinary sodium and creatinine levels. It is an indicator of sodium intake.

Fractional excretion of sodium (FENa) was calculated from the measured plasma and urinary sodium and creatinine levels.

$FENa = (UNa^{+}/PNa^{+})*(PCr/UCr)*100$

(U, urine; P, plasma; Na^{+}, sodium; Cr, creatinine)

Total urinary sodium-potassium ratio (UNa/K) was calculated as a ratio of the measured urinary sodium and potassium levels.

Total potassium excretion (TUK) was calculated using the equation:

$$\frac{UK^{+}*\text{Volume excreted}}{1000}$$

[U, urine; K^{+}, potassium (mmol/l); Volume (ml)]

MOLECULAR METHODS

The laboratory is maintained according to SANAS. Equipment is calibrated regularly and space is divided to ensure accuracy of results without the risk of contamination. Products used are stored according to the manufacturer's recommendations.

DNA Extraction: DNA from the Western Cape was extracted from whole blood collected in ethylene diamine tetra-acetic acid (EDTA) tubes. A minimum of 200µl whole blood was required. A commercially available extraction kit (Invisorb Spin Blood Mini Kit®) was used to extract the DNA.

200µl Lysis buffer A was added to the blood with 20µl Proteinase K, vortexed and incubated at 56^{o}C for 10 minutes. A receiver tube was then prepared with a spin filter. 400µl Binding Buffer B6 was added and the solution vortexed. The sample was loaded onto the Spin Filter and incubated for one minute then centrifuged at 12,000 rpm for two minutes. The receiver tube with the filtrate was discarded and the spin filter was placed into a new 2ml receiver tube.

500µl Wash Buffer I was added and the sample was centrifuged at 12 000 rpm for one minute. The filtrate was discarded and the spin filter returned to the 2ml receiver tube. 800µl Wash Buffer II was added and the sample was centrifuged at 12 000 rpm for one minute. The filtrate was discarded and the spin filter returned to the receiver tube. The washing step was repeated and the filtrate discarded. The spin filter was placed back into the 2ml receiver tube and finally centrifuged for four minutes at 13 000 rpm, completely removing the ethanol. The spin filter was placed into a new 1.5ml receiver tube and 200µl of Elution Buffer D at 56^{o}C was added to the centre of the spin filter then incubated for one minute. Then the product was centrifuged for one minute at 10 000 rpm. This step was repeated to improve the yield of genomic DNA. DNA was stored at -20^{0}C until further analysis.

The Khomani San DNA was extracted in Johannesburg at the South African Institute for Medical Research by C. Schlebusch. DNA was extracted from EDTA-blood or buccal swabs. Extracted DNA in both cases was dissolved in TE buffer (10mM Tris and 1mM EDTA, pH 8.0.) DNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Coleman Technologies Inc., LabVIEW®) and diluted to 50ng/ul using double distilled water. DNA was extracted using the salting-out method [Miller

et al., 1988]. The PureGene® Genomic DNA Purification Kit (Gentra Systems) was used for extraction of DNA from buccal swabs, according to the manufacturer's instructions.

DNA from a group of Black Africans from Johannesburg was donated by Professors Woodiwiss and Norton from the University of the Witwatersrand. The DNA was collected as part of another study [Nkeh et al., 2003] involving hypertensive patients attending a referral hypertension clinic in Johannesburg and matched controls. Consent was given for DNA analysis for hypertensive studies.

Polymerase Chain Reaction

Determination of the R563Q mutation in exon 13 of the ENaC: Exon 13 of the ENaC underwent amplification by PCR. Primers were designed using readily available software, Oligo®. Primer pairs were checked for length, compatible melting temperatures and possible primer dimers. The primer pair used for the analysis is shown in Figure 3.1.

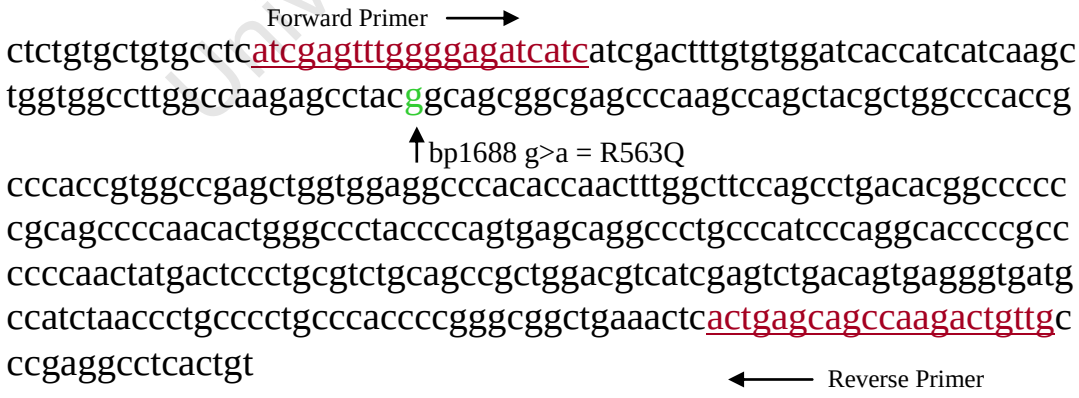

Figure 3.1. Primer positions on the β-ENaC

The PCR reagent contained: (for each 40µl PCR reaction)

- Premixed master mix consisting of:

  2.4µl $MgCl_2$ 5mM with a final concentration of 1.5mM $MgCl_2$

  1.6µl dNTP 5mM with a final concentration of 0.2mM dNTP

  8µl 5x Green GoTaq® (Promega) Flexi Buffer -pH8.5 with a final concentration of 1x buffer

  26.8µl Nuclease free distilled water

- 0.4µl Forward Primer 50pmol/l (5' atcgagtttggggagatcatc 3') in 1x TE buffer with a final concentration of 0.5µM
- 0.4µl Reverse Primer 50pmol/l (5' caacagtcttggctgctcagt 3') in 1x TE buffer with a final concentration of 0.5µM
- 0.2µl GoTaq® Flexi DNA polymerase 5U/µl with a final concentration of 1U

(TE buffer protects the nucleotides from degradation. $MgCl_2$ magnesium chloride, dNTP, deoxyribonucleotide triphosphate consisting of equal concentrations of dATP, dTTP, dGTP and dCTP)

The β-ENaC gene was amplified by adding 1µl DNA from each specimen to the PCR reagent. The DNA was denatured at 98^{o}C for three minutes, then underwent 35 cycles of 94^{o}C for 30s, 56.5^{o}C for 30s then 72^{o}C for 50s. After the cycles, the reaction was completed with eight minutes at 72^{o}C. The product was stored at 4^{o}C until restriction enzyme digestion.

The presence of PCR product was checked with a 2% agarose gel run for 20 minutes at 120V and 250mA. The agarose gel was made up using a commercially available agarose (LE, analytical grade, Promega) mixed in 1x Tris-acetate-EDTA [TAE -

40mM Tris, 20mM acetic acid, 1mM EDTA, ph 8.3 (Bio-Rad)] buffer to make up a 2% gel solution. The agarose was dissolved and 5µl of 10µg/ml ethidium bromide was added before being set. After electrophoresis the gel was viewed under ultraviolet light to detect the presence of PCR product (Figure 3.2).

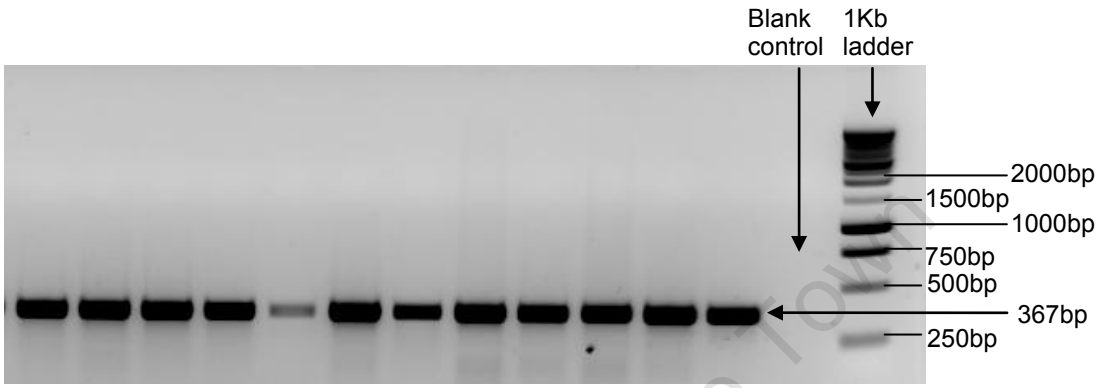

Figure 3.2. Gel confirming PCR product of the carboxy terminal of the β-ENaC. The presence of PCR product is confirmed by the dark bands. A control (blank) before the 1Kb ladder confirmed that there were no contaminants in the reagents or primers.

Restriction Enzyme Digestion: The R563Q mutation creates a *Sfc*1 restriction enzyme recognition site (CTACGG > CTACAG). The PCR product was digested for 16 hours at 25°C, 1 hour at 30°C and 1 hour at 37°C with *Sfc*1 restriction enzyme (New England Biolabs). Each 30µl PCR product was mixed with 1.8µl digestion buffer 4, 0.35µl albumin and 0.85µl *Sfc*1 restriction enzyme. (Buffer 4 contains 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol at a pH of 7.9 at 25°C.)

Restriction enzyme digestion involves detecting differences in DNA sequences by using restriction enzymes that recognise specific DNA sequences (usually about 4-6bp in length). These enzymes are produced by bacteria and cleave the DNA at

specific recognition sites. Restriction fragment length polymorphisms (RFLP) are the product of restriction enzyme digestion.

The *Sfc*1 restriction enzyme is produced by *Streptococcus faecium* and recognises these DNA sequences (see Figure 3.3 for positions in exon 13 of the β-ENaC):

5'…C^TRYAG…3'

3'…GAYRT^C…5'

Where: T= Thymine
A= Adenine
C= Cytosine
G= Guanine
R= either purine
Y= either pyramidine

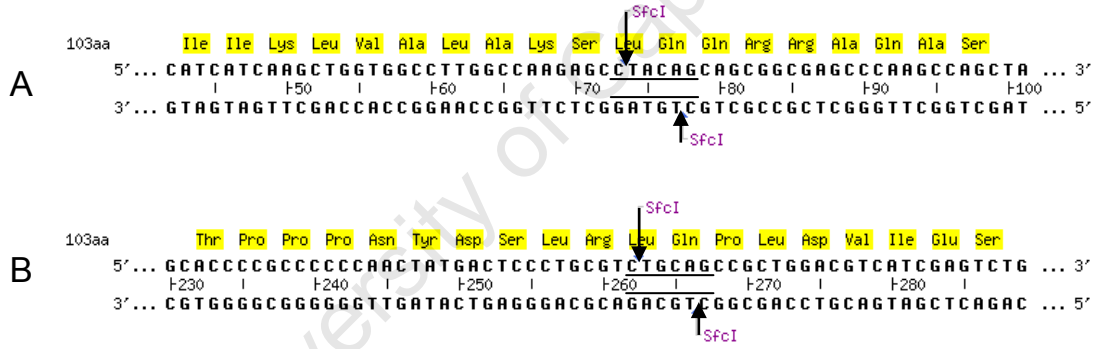

Figure 3.3. Restriction Enzyme Recognition Sites in exon 13. *A* shows the recognition site (underlined) created by the R563Q mutation and *B* shows the normal recognition site that is present towards the end of exon 13. The position where *Sfc*1 cleaves the DNA is shown by arrows.

The post digest product was run on a 2% agarose gel for 40 minutes at 120mV to detect the presence of the mutation. The normal β-ENaC cuts into two bands, consisting of 264 and 103bp bands; and the mutated gene cuts into bands of 189, 103 and 75bp (Figure 3.3).

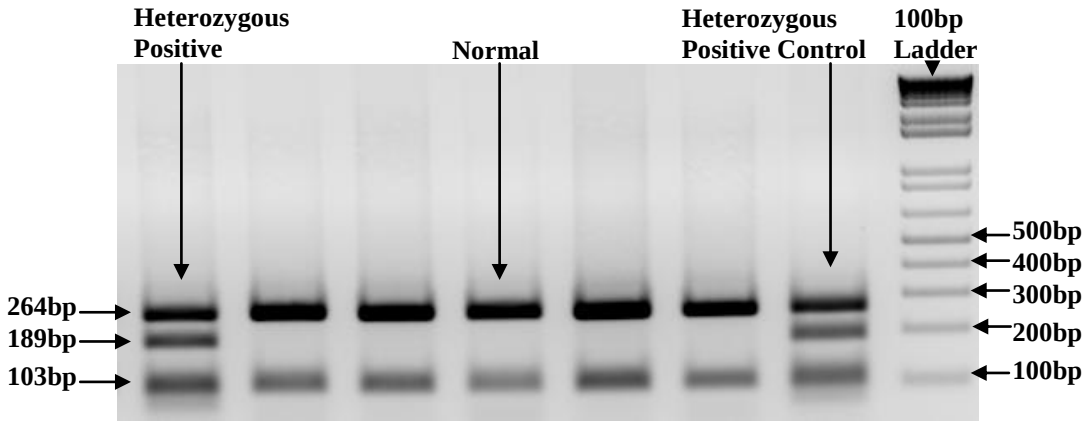

Figure 3.4. Restriction digest gel after the PCR product was digested with *Sfc*1 restriction enzyme. The normal sodium channel cuts into bands of 103 and 264bp and the allele with the mutation cuts into bands of 189, 103 and 75bp (not shown) long.

Sequencing: The PCR product was cleaned of dNTP's, buffers and primers before preparing for sequencing.

Purification: A commercially available kit (QIAquick® PCR purification Kit - Qiagen) was used for purification of the DNA, according to the manufacturer's instructions. 175µl binding buffer was added to 30µl PCR product and mixed, added to a spin column then centrifuged at 13 000rpm for one minute. The solution was discarded and the wash step repeated. To remove all fluid the column was centrifuged at 13 000rpm for one minute before being placed in an eppendorf tube and 30µl elution buffer was added to the column. The column was left standing for 60 seconds then centrifuged at 13 000rpm for one minute. The cleaned PCR product was stored at -20^{o}C until further analysis

Sequencing: A commercially available kit *(ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit*®, PE Biosystems) was used for sequencing of the purified DNA. The PCR product came to 200-500bp which requires a 3ng template.

The solution included 1µl BigDye®, 0.6µl sequencing buffer and 6.4µl sterile water, 1µl reverse (4R) primer and 1µl PCR product. The solution was mixed and placed in the thermocycler for 15s at 96^{o}C; 30 cycles of 15s at 96^{o}C, 15s at 50^{o}C, 4 minutes at 60^{o}C; then completed with 10 minutes at 60^{o}C.

The product was sent to the Central DNA Sequencer at the University of Stellenbosch where it was analysed on an ABI sequencer to determine the sequence. (The sequencer is used for determining the sequence for diagnostic purposes and thus complies with NHLS accreditation standards.) The sequences were read manually using Chromas lite® and confirmed with Bioedit®. Figures 3.5 and 3.6 show the normal and heterozygous sequences, respectively.

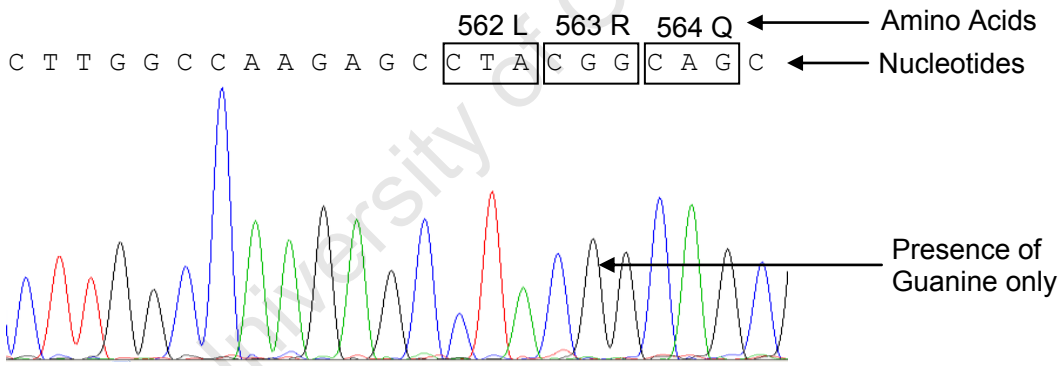

Figure 3.5. Normal Sequence

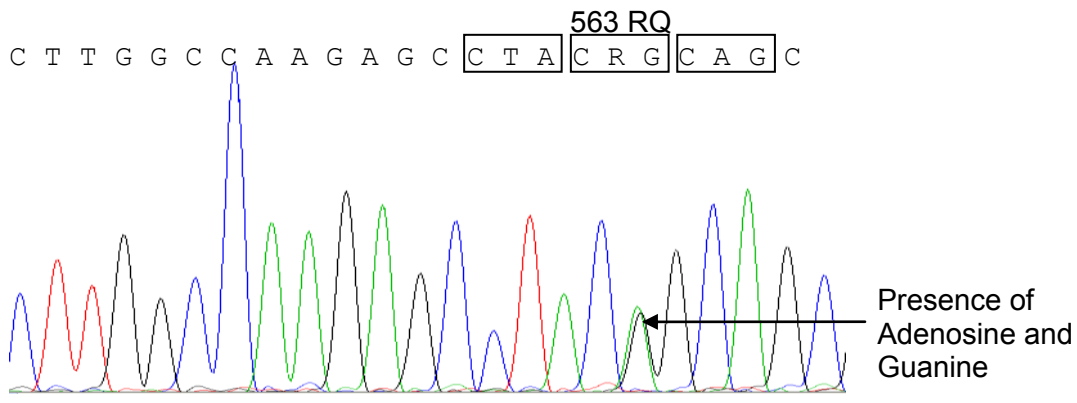

Figure 3.6. Sequence from a heterozygous patient

All samples that were homozygous for R563Q were checked with a second set of primers to exclude mutations or deletions that may have occurred in the location of the initial primers. Such a mutation could cause only one allele to amplify resulting in a false positive homozygous mutation rather than a heterozygous mutation. The alternative forward primer was 5'-GTC TGG CTG CTC TCG AAT CTG-3' and the reverse primer 5'-GTG AGT CCT CGG GCA ACA GT-3' (Figure 3.7).

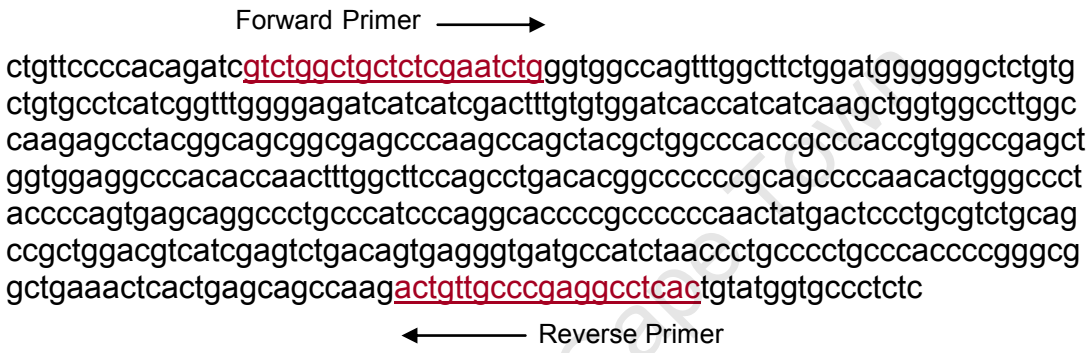

Figure 3.7. Alternative primer positions

The PCR using the alternate primers were amplified under the same conditions. However, the PCR product was larger (445bp), and after restriction enzyme digestion the normal alleles were 118bp and 327bp long and the mutant alleles were digested into fragments 118bp, 189bp and 138bp long.

STATISTICS

Results were analysed using SPSS® statistical software (SPSS® version 11.0, Chicago, USA). Categorical variables were analysed using Chi squared (X^2) analysis and, where the numbers analysed were less than five, Fisher's Exact Test. Continuous variables were analysed using Student's t test. Where the distribution deviated significantly from the normal distribution, as assessed by a significant Schapiro-

Wilk's test, data were log transformed before analysis. P values of less than 0.05 were considered significant. (Specific sections requiring further statistical analyses are discussed in the relevant section.)

MISSING DATA

There are missing variables in each section. This was due to inability to collect the data, inability to analyse the sample or lost samples. All results were included in the analysis and no results were excluded on the basis of inability to explain the result. Further information is given in each section as to the reasons why data were missing.

PART 2

A HIGHLY PREVALENT MUTATION

ASSOCIATED WITH HYPERTENSION

Hypertension:

A disorder with a familial association

CHAPTER 4

The R563Q Mutation is Associated with Hypertension in Kindreds

INTRODUCTION

Studies involving close relations have long been used to show linkage of genetic polymorphisms with phenotypes. Twin studies were the initial form of analysis. Wietz [1954] compared the difference between dizygotic and monozygotic twins as comparisons if there was an association between hypertension and inheritance. Platt [1963] used the fact that twins exhibited almost identical phenotypes to support his single gene hypothesis.

Family studies have progressed from twin studies to studies involving siblings [Allan, 1933; Platt, 1947] through to studies of parents and one or two children [Spielman, 1993] and large families [Abecasis et al., 2000b]. These studies often involve complicated statistical analyses [Luft, 2001; Abecasis et al. 2000a].

The aim of the present study was to investigate the phenotype of the R563Q mutation within affected kindreds, which provide a more uniform genetic and environmental background against which the phenotypic effects of the mutation can be assessed.

METHODS

Subjects were counselled and gave signed informed consent to join the study, including for DNA analysis. (See appendix III for consent forms.)

Individuals heterozygous for the R563Q mutation attending the Groote Schuur Hypertension Clinic and one heterozygous normotensive case (who was identified in the previous study [Rayner et al., 2003]) and their kindred were approached to participate in the study. Forty five index cases and 93 family members agreed. BP was measured as described in Chapter 3. Blood was then drawn for potassium (K^+), renin and aldosterone and DNA analysis. Antihypertensive medication was not withdrawn prior to BP measurement or blood sampling. DNA extraction, PCR amplification of the β-ENaC gene and restriction enzyme digestion for detection of the R563Q mutation are described in Chapter 3.

The variables were summarised as medians with interquartile ranges (IQR) due to extremes in the R563Q heterozygous group. Quantitative transmission disequilibrium testing (QTDT) [Abecasis et al., 2000a; Abecasis et al., 2000b] was used to estimate heritability and to test for association between transformed numerical variables and R563Q mutation status. Observations within families are more similar (correlated) than between families and QTDT takes the *degree* of relatedness into account in the analysis. The QTDT analysis assesses subjects within families, protecting the results from population stratification. The QTDT is unable to determine the mode of inheritance and does not estimate separate between and within family effects but rather calculates a single overall effect size, using all available information. Quantile normalisation [Pilia et al., 2006] was used to transform all to a normal distribution, before analysing them, because the distributions of some of the variables were highly skewed. The tests were adjusted for sex, race and age. (For further discussion of the QTDT analysis, see Appendix I.) Mixed-effects models were used to analyse measurements that did not undergo QTDT analysis, in order to control for the fact that

observations within families will be more similar than between families. This was used for variables that could be influenced by the presence of hypertension or antihypertensives (renin, aldosterone, potassium and creatinine). Numerical variables were log transformed to symmetry, where necessary.

RESULTS

Overall, 138 individuals (45 index cases and 93 family members) were studied, of whom 89 were heterozygous for the R563Q mutation and 49 normal. The demographics of the heterozygous and normal subjects are shown in Table 4.1. All patients and subjects were from either Coloured or Xhosa origins, and 49% (67) were male. No evidence of population stratification was found in the groups when they were assessed by the QTDT analysis.

Table 4.1. Demographics of the R563Q heterozygous and normal subjects

	R563Q heterozygous				R563Q normal	
	Index cases (45)		Relatives (44)		Relatives (49)	
	Number	Percentage	Number	Percentage	Number	Percentage
Male	19	42	24	55	24	49
Xhosa	14	31	8	18	9	18
Coloured	31	69	36	82	40	82
Hypertensive	44	98	27	61	17	35

Forty-four index cases had hypertension and one was a normotensive control identified in a previous study [Rayner et al., 2003]. Twenty two index cases had at least one relative willing to participate in the study. Of the relatives, 74 were first degree relatives (brother, sister, son, daughter, mother or father), 11 were nephew or niece, two half brothers, two half sisters and four were grandchildren, in relation to the index cases.

The main characteristics of the individuals heterozygous or normal for the R563Q mutation are summarised in Table 4.2. The p-values in Table 4.2 are for a test of association with mutation status, after adjusting for race, age and kindred-membership (except for age, which was only adjusted for kindred-membership). There was a significant association between age and R563Q mutation status ($p<0.001$), confirming the necessity of adjusting for age in all models. The subjects heterozygous for the R563Q mutation were older than the normal group, with a median difference of nine years.

Table 4.2. Characteristics of the R563Q heterozygous and normal groups

	R563Q heterozygous (91)			R563Q normal (47)			
	n	Median	IQR	n	Median	IQR	p-value*
Age, years	89	43	35, 57	46	34	26, 44	
SBP, mmHg	**89**	**150**	**135, 175**	**47**	**132**	**123, 149**	**0.003**
DBP, mmHg	**89**	**92**	**80, 103**	**47**	**84**	**73, 91**	**0.010**
MAP, mmHg	**89**	**113**	**99, 129**	**47**	**101**	**90, 110**	**0.005**
Potassium, mmol l^{-1}	84	4.2	3.8, 4.4	45	4.2	4.0, 4.4	0.224

*P values are for tests of association with R563Q mutation status, after adjusting for race, age and kindred membership, using QTDT. IQR, interquartile range

Of the R563Q heterozygous subjects, 71 (80%) had hypertension, while 17 (35%) of the normal subjects were hypertensive, $p<0.0005$. Mean SBP, DBP and MAP ($p=0.005$) were significantly higher in the heterozygous subjects (Table 4.2). This difference was present despite antihypertensive treatment not being removed prior to examination. More heterozygous subjects, compared with the normal group, were receiving antihypertensive therapy (65/89 (73%) vs. 14/49 (29%) respectively). Of the 71 heterozygous subjects with hypertension only 65 knew they were hypertensive before the study. Of these 65 only 10 (15%) had controlled BP (<140/90mmHg). Of the 17 hypertensive normal subjects 2 (12%) had controlled BP. There is no

significant difference between heterozygous and normal subjects with controlled BP. X^2 analysis of the relatives, excluding the index cases, to decrease ascertainment bias, revealed a significant association of the R563Q mutation with the presence of hypertension (p=0.008).

Six heterozygous subjects had hypokalaemia. Two displayed the full Liddle's syndrome phenotype during pregnancy. (These will be discussed in Chapter 10 as case studies.) Three patients had hypertension and hypokalaemia associated with the use of diuretics, and another was normotensive with unprovoked hypokalaemia (K^+ 3.3mmol/l). A R563Q normal family member had mild unprovoked hypokalaemia (3.4mmol/L) and a normal BP. However, the observed difference in the mean serum potassium between the heterozygous and normal groups was not significant (p=0.225).

A pedigree is shown in Figure 4.1 which demonstrates the autosomal dominant pattern of inheritance. All positive subjects in this study were heterozygous for the R563Q mutation. The pedigree also demonstrates the difficulty of associating a SNP with a common phenotype like hypertension in a single pedigree. In this pedigree there were no heterozygous normotensive subjects but in the entire group there were 18 normotensive individuals heterozygous for the mutation suggesting incomplete penetrance or added environmental factors, as some of these normotensive subjects were over 40 years old. Index cases were selected from the Hypertension Clinic, because this is where the mutation was originally identified. Patients attending the clinic have a severe uncontrolled form of hypertension making it unethical to stop their antihypertensive medication. Thus, blood was taken while the hypertensive

subjects were on medication. Renin and aldosterone levels did not undergo the QTDT analysis because the results would be affected by the presence of antihypertensive medication, invalidating the results in 60% of the subjects. As the renin and aldosterone levels were measured they were analysed to determine if a trend was observed, and the results were adjusted for age and family relationship.

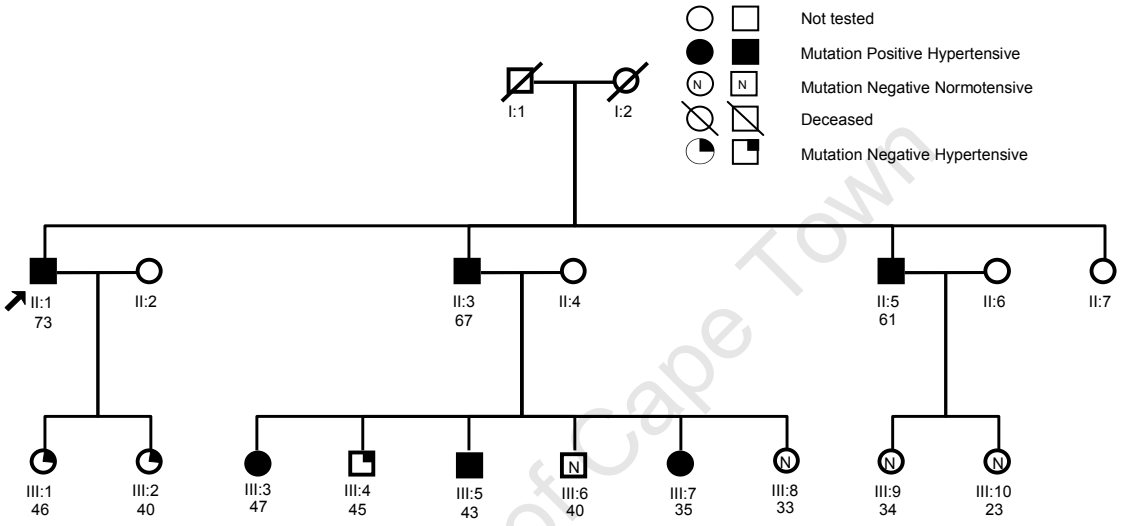

Figure 4.1. A pedigree showing the distribution of the mutation across two generations. (The arrow points to the index case. Subject ages are shown in years below identities.)

The observed differences in plasma renin (p=0.935) and aldosterone (p=0.326) levels between the two groups were not significant (Table 4.3). Suppressed aldosterone (<225 pmol/L) was present in 47% of R563Q-heterozygous subjects and in 56% of R563Q-normal subjects. Suppressed renin (<20 mU/L) was seen in 43 (48%) of R563Q-heterozygous and in 26 (52%) of R563Q-normal subjects. The serum creatinine levels were not significantly different between the heterozygous and normal groups (p=0.786). In the subjects who were not on treatment there was no difference between any of the parameters assessed (Table 4.4). Basic statistical methods were used for this analysis as there were very few related subjects.

Table 4.3. Renin, aldosterone and creatinine levels in the family analysis

	R563Q heterozygous			R563Q normal			
	n	Median	IQR	n	Median	IQR	p-value
Creatinine, μmol l^{-1}	88	76	67, 93	46	78	68, 87	0.786
Aldosterone, pmol l^{-1}	85	222	139, 390	47	206	142, 303	0.326
Renin, mmol l^{-1}	85	18	9, 33	47	18	12, 25	0.935

Table 4.4. Means for untreated subjects

	Heterozygous (24)	Normal (35)	
	Mean ±SD / Median (IQR)*	Mean ±SD / Median (IQR)*	P value
Age, y	35.4±13.1	30.8±13.5	0.197
SBP, mmHg	133±26	122±18	0.077
DBP, mmHg	80 (73;87)	80 (69;87)	0.121**
MAP, mmHg	96 (87;105)	94 (84;102)	0.092**
Renin, mmol l^{-1}	22.5 (11.5;27.6)	18 (12;22.8)	0.884**
Aldosterone, pmol l^{-1}	189 (125.3;301.3)	193 (144.5;292)	0.617**
Potassium, mmol l^{-1}	4.3±0.4	4.3±0.4	0.806
Creatinine, μmol l^{-1}	75.2±13.6	76.2±18.2	0.868

*Medians with IQR are displayed for log transformed variables; SD, standard deviation. ** P values for log transformed measurements

P values were calculated for the family analysis, excluding the index cases without families and excluding founders of the families (Table 4.5). This decreased the numbers analysed but also decreased the risk of bias toward associating the mutation with hypertension caused by selecting the families of hypertensive patients with the mutation. After excluding founders, only the SBP remained significant and after excluding index cases none of the variables were significantly different between the heterozygous and normal subjects.

Table 4.5. P values calculated with QTDT after excluding founders and index cases

	Original p-value	Excluding founders	Excluding index cases
SBP, mmHg	**0.003**	**0.041**	0.064
DBP, mmHg	**0.01**	0.064	0.175
MAP, mmHg	**0.005**	0.053	0.110
Serum Potassium, mmol l^{-1}	0.224	0.426	0.262

DISCUSSION

This family analysis examined the prevalence of hypertension and levels of BP in R563Q heterozygous and normal family members of index cases, and supports the suggestion that the R563Q mutation is a dominantly transmitted cause of hypertension in the Cape Town population forming the basis of this study. Table 4.1 gives the demographics of the groups. In the full group of 138 subjects the R563Q mutation was strongly associated with hypertension: 71 (80%) of the heterozygous subjects had hypertension, while 17 (35%) normal subjects were hypertensive, $p<0.0001$.

Further support for the dominant nature of the R563Q mutation was demonstrated in the clinically significant differences in SBP, DBP and MAP (Table 4.2). The differences in the medians for the heterozygous and normal groups (after adjusting for the effects of race, age and kindred membership) were 21mmHg, 8mmHg, and 14mmHg for SBP, DBP and MAP respectively. This was despite the difference between the groups in those on antihypertensive medication. 73% of heterozygous patients were on antihypertensive treatment compared with 29% in the normal group. If those on antihypertensives had been able to stop their medication it is expected that their BP would increase, increasing the differences between the heterozygous and normal groups.

The presence of antihypertensive medication also confounds the biochemical results. Most of the hypertensive subjects in this study are patients attending the Hypertension Clinic at GSH who have resistant hypertension requiring multiple medications to control the BP. The majority of the patients on antihypertensives would be taking an ACE inhibitor or a thiazide diuretic as part of their treatment. Both these drugs affect

the biochemical parameters that control BP. ACE inhibitors interrupt the renin angiotensin system and would tend to increase the renin level and decrease the aldosterone level. ACE inhibitors tend to cause hyperkalaemia whereas thiazide diuretics tend to decrease the potassium levels.

The subjects that had not been on treatment for hypertension were analysed as unrelated individuals. In this analysis there was no statistical difference between the groups with and without the mutation. The trends observed were as expected. Those with the mutation had higher BP and a lower aldosterone level, but the heterozygous group had a median age of nine years greater than the normal group.

The analyses could have been adjusted for the use of antihypertensive medication, as the results are confounded by these medications. However, if the SBP and DBP had been adjusted for the use of antihypertensive medication it would be tantamount to adjusting for the presence of hypertension, as the majority of the hypertensive subjects were on treatment. This would essentially remove the difference in BP. A further limitation of this analysis is that index cases are from the Hypertension Clinic, i.e. they are all hypertensive. For this reason the index cases were excluded from the analysis to exclude ascertainment bias. In the family based analysis the numbers were small so the association with a difference in BP was not significant, but the association with hypertension was significant when assessing the relatives by X^2 analysis.

The BPs in the family analysis support the hypothesis that the R563Q substitution is an activating ENaC mutation. However, lower renin and aldosterone levels in the

heterozygotes compared with the normal group were not found, as previously described [Rayner et al., 2003]. This is likely to be due to a combination of factors. Antihypertensive therapy was not withdrawn prior to testing, and renin and aldosterone may increase due to renal and vascular changes secondary to longstanding hypertension [Williams and Hollenberg, 1991]. Rayner et al. [2001] previously reported low renin and aldosterone in the normotensive population groups from which these families were drawn. This suggests that other genetic and environmental factors are present in these population groups, which also lead to suppression of renin and aldosterone. The serum creatinine level was not different between the heterozygous and normal groups, unlike the results in the Hypertension Clinic patient comparison (see Chapter 7). This is probably because there were fewer subjects involved in the family study with hypertension, which causes CKD.

Two of the index cases presented with the full Liddle's phenotype; they developed hypertension and severe persistent hypokalaemia during pregnancy. They are discussed further in Chapter 10. Only one relative of the hypokalaemic patients was available for study and she did not carry the mutation and was normotensive. The presence of hypokalaemia in only a minority of the heterozygous subjects is consistent with the low penetrance of hypokalaemia reported in kindreds with other Liddle's syndrome mutations [Findling et al., 1997], and suggests that other genetic or environmental factors are critical in determining the extent to which the full Liddle's syndrome phenotype is expressed.

CONCLUSION

The R563Q β-ENaC mutation associates with hypertension within kindreds. This finding strengthens the case for a causative role for the R563Q mutation in hypertension because the genetic background is more homogeneous within kindreds than in the general population.

CHAPTER 5

The Prevalence of the R563Q Mutation in South Africa

INTRODUCTION

As previously discussed, the R563Q mutation has been found to be common amongst people of Coloured or Xhosa descent (but not in people of European descent) with resistant hypertension in the Cape Town area [Rayner et al., 2003]. It has not been detected in other studies which included subjects of African descent [Dong et al.; 2001; Ambrosius et al., 1999] and it is possible that these findings are isolated to Southern Africa. The purpose of this chapter was, firstly, to determine the prevalence of the R563Q mutation in the ethnic groups living in the Johannesburg area compared with Cape Town and, secondly, to determine if the previously reported association with hypertension remains in other population groups.

METHODS

Cape Town

Records since 2002 of patients attending the referral Hypertension Clinic at Groote Schuur Hospital were examined for hypertension, gender, age, ethnic group and screening of the R563Q mutation. Normotensive controls from previous studies [Rayner et al., 2003] were included in the analysis but relatives were excluded because they would distort the prevalence of the mutation in the South African population.

Johannesburg

In Johannesburg patients are not routinely screened for the presence of the R563Q mutation but DNA has been obtained for research purposes from other hypertension studies. DNA from hypertensives referred to the tertiary hypertension clinics in Johannesburg and controls without a family history of hypertension were accessed from a data base at the University of the Witwatersrand, Department of Physiology, which is in the care of Professor Woodiwiss and Professor Norton. The subjects were defined as hypertensive if their clinical and daytime ambulatory DBP was greater than 89mmHg or their SBP was greater than 139mmHg. The control subjects had a mean DBP of less than 90mmHg after five minutes in the seated position. Controls were required to have been urban residents for at least two years and not to have a family history of hypertension. Secondary hypertension was excluded as described by Nkeh et al. [2003]. (At the time of sample collection hypertension was defined as a BP ≥160/95.)

All subjects gave consent for DNA analysis for research purposes. A history was taken and records were kept on subject age, gender and the presence of smoking and alcohol intake. Their height and weight were recorded, from which their body mass index (BMI) was calculated. The samples had been collected over many years and the DNA was stored at -20^{o}C until being prepared for PCR. Statistical analysis has been described (Chapter 3). Power was calculated using a freely available online calculator: Statistical Calculators.

RESULTS

Cape Town

1382 unrelated subjects were analysed in Cape Town. 1177 were hypertensive and 205 normotensive. 753 were of Coloured, 407 of Black African and 177 of Caucasian origin, with no significant difference between the groups with and without the R563Q mutation in the Coloured and Black African groups. Forty five subjects were not allocated into an ethnic group. 523 were male and 208 subjects had no record of gender. Forty nine subjects were heterozygous for the mutation; one of these subjects was normotensive. No homozygous subjects were found. 4.2% of the Coloured and Black African subjects were heterozygous for the mutation (p=0.002 for association of the mutation with hypertension). Table 5.1 summarises the data analysis.

Table 5.1. Cape Town subjects (and % of total) assessed for the R563Q mutation

		Heterozygous		Normal		P value*
		Number	Percentage	Number	Percentage	
Ethnicity	Coloured	28	2	725	52.5	
	Caucasian	0	0	177	12.8	
	Black African	21	1.5	386	27.9	
	Unknown			45	3.3	
Gender	Male	22	1.6	501	36.3	
	Female	27	2	623	45.1	
	Unknown			208	15.1	
Disorder	**Hypertensive**	**48**	**3.5**	**1129**	**81.7**	**0.002**
	Normotensive	**1**	**0.07**	**204**	**14.7**	

*P value is for comparison between hypertensives and normotensives.

The mean age of the individuals with the mutation was 54.1±14.9 years and those without 52.8±13.9 years. (Caucasian subjects were not included.) 21/307 (6.8%) Black African subjects and 28/648 (4.3%) Coloured subjects with hypertension had the mutation. The hypertensive group heterozygous for the mutation had lower renin and aldosterone levels but these did not reach statistical significance (Table 5.2). Figure 5.1 shows the ranges of age for the groups with and without the mutation. One

normotensive Coloured subject was heterozygous for the R563Q mutation and none of the Black African normotensives had the mutation.

Table 5.2. Renin and aldosterone levels in Cape Town hypertensives

	Heterozygous		Normal		
	Number	Median (IQR)	Number	Median (IQR)	P value*
Aldosterone, pmol l^{-1}	46	220 (132;354)	230	291 (202;431)	0.100
Renin, mmol l^{-1}	46	24 (10;37)	233	18 (8;54)	0.173

*P values for log transformed measurements

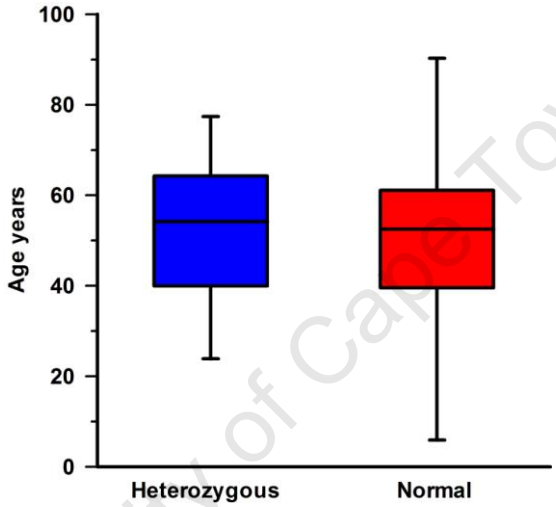

Figure 5.1. Mean ± standard deviation (SD) with extremes of age in the groups heterozygous and normal from Cape Town

Johannesburg

The DNA was available from three different ethnic groups: Nguni, Sotho and Venda. The subjects from Johannesburg were represented by 519 (66%) individuals of Nguni ethnicity, of which 333 (64%) were hypertensive, and 261 (34%) Sotho individuals, of which 184 (70%) were hypertensive. None of the Venda individuals tested had the mutation. There were very few people from this ethnic group (17 in total, 10 of whom were hypertensive) so they were excluded from the combined prevalence analyses. There were 527 (66%) subjects with hypertension and 270 (34%) normotensives in

total. In the entire group 7.4% of the hypertensives were heterozygous for the R563Q mutation and 2.6% of the normotensives (p=0.006). The hypertensive group was over represented by females (p<0.0005) but there were fewer smokers and alcohol drinkers (Figure 5.2). The hypertensives had significantly higher BP, BMI and weight but were shorter. The difference in age was not statistically significant (Table 5.3).

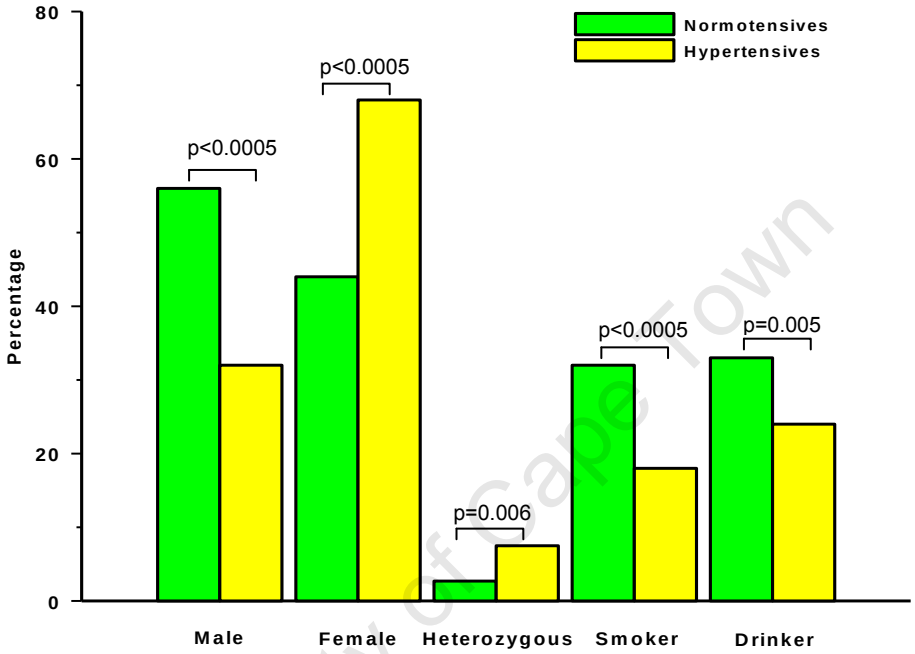

Figure 5.2. Percentages in the hypertensives and normotensives from Johannesburg

Table 5.3. Differences between hypertensives and normotensives from Johannesburg

	Hypertensives (527) Mean ±SD	Normotensives (270) Mean ±SD	P value
Age, y	53.6±10.3	52.3±9.7	0.088
Height, cm	**159.7±8.5**	**164.1±8.5**	**<0.005**
Weight, kg	**75.3±16.2**	**71.3±16.6**	**0.001**
BMI, kg m^{-2}	**29.7±6.9**	**26.7±6.9**	**<0.005**
SBP, mmHg	**150.9±13.4**	**122.9±12.4**	**<0.005**
DBP, mmHg	**93.2±9.1**	**76.4±8.8**	**<0.005**

Table 5.4 summarises the results of the DNA analyses. There were 333 hypertensive Nguni of which 28 (8.4%) were heterozygous for the R563Q mutation. 186 were normotensive of which 5 (2.7%) were heterozygous for the R563Q mutation. In the

Sotho population there were 184 hypertensives with 11 (6.0%) heterozygotes and of the 77 normotensives 2 (2.6%) were heterozygous for the R563Q mutation.

Table 5.4. Summary of Johannesburg DNA results

	Analysed		R563Q Heterozygous	
	Group	Number	Number	Percentage
Hypertensives	**Total**	**527**	**39**	**7.4**
	Nguni	**333**	**28****	**8.4**
	Sotho	184	11*	6.0
	Venda	10	0	0
Normotensives	Total	270	7	2.6
	Nguni	186	5**	2.7
	Sotho	77	2*	2.6
	Venda	7	0	0

Footnote: The frequency of the R563Q mutation was different between the hypertensive and normotensive groups, *p=0.242 for the Sotho group, **p=0.01 for the Nguni group and p=0.006 for the combined results, excluding the Venda.

Table 5.5 summarises the parameters of each of the ethnic groups divided into hypertensives and normotensives and into those with and without the mutation. This table highlights the differences between hypertensives and normotensives. The hypertensives have higher BP but the age and BMI are similar.

Table 5.5. Summary of Nguni and Sotho physical characteristics

	Nguni				Sotho			
	Hypertensive		Normotensive		Hypertensive		Normotensive	
R563Q	Hetero	Normal	Hetero	Normal	Hetero	Normal	Hetero	Normal
Number	28	305	5	181	11	173	2	75
Age, y	54±12	53±10	55±6	52±8	57±6	54±10	56±5	52±10
Height, cm	159±7	160±9	158±11	164±8	159±9	160±8	172±6	164±9
Weight, kg	77±16	75±16	76±18	73±17	73±18	76±17	69±5	67±15
BSA, m^2	1.79±0.17	1.76±0.18	1.77±0.17	1.78±0.18	1.75±0.2	1.79±0.18	1.81±0.1	1.72±0.18
BMI, kg m^{-2}	31±7	30±7	31±10	27±7	29±7	30±7	23±0	25±6
SBP, mmHg	151±14	152±13	129±17	123±12	155±17	150±14	131±9	122±14
DBP, mmHg	95±10	93±9	73±10	77±8	96±12	93±9	84±1	76±11

Footnote: Means ± SD. Within the hypertensive and normotensive groups the difference between the means of those with and without the mutation was not significant for any of the variables. BSA, body surface area; BMI, body mass index; Hetero, heterozygous

Table 5.6 shows the differences between the groups with and without the mutation. There was no significant difference in mean age or BMI between the groups with and without the R563Q mutation. Overall those with the mutation had significantly higher BP (p=0.003 for SBP and p=0.004 for DBP). The presence of hypertension was not significantly different between the Sotho heterozygous and normal groups despite the T test for blood pressures being adequately powered (observed power for SBP 0.996, and for DBP 0.990).

Table 5.6. Differences between combined Nguni and Sotho groups with and without the mutation.

	Heterozygous (46)		Normal (734)		P value
	Mean	SD	Mean	SD	
Age, y	55	10	53	10	0.296
SBP, mmHg	149	17	141	19	**0.003**
DBP, mmHg	92	13	87	12	**0.004**
BMI, kg m^{-2}	30	7	29	7	0.175

The normotensive subjects with the mutation tended to have higher SBP than those without the mutation (Table 5.7) but this difference was not significant (p=0.161). The DBP was not different between the groups. The groups with and without the mutation were represented by similar percentages of smokers, drinkers, males and females (Figure 5.5).

Table 5.7. BP in the normotensive Nguni and Sotho subjects from Johannesburg

	R563Q	N	Mean	SD
SBP	Heterozygous	7	129	14
	Normal	256	123	12
DBP	Heterozygous	7	76	10
	Normal	256	76	9

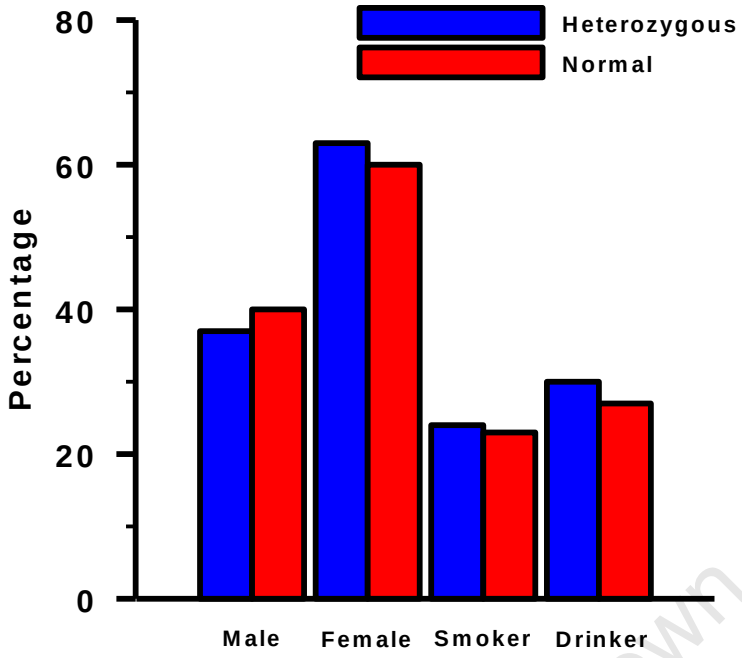

Figure 5.3. Percentage of males, females, smokers and those that drink alcohol in the Johannesburg subjects

Combined Results for Johannesburg and Cape Town

The Sotho and Nguni groups both belong to the Black African group (speaking a Bantu language) inhabiting South Africa. The Xhosa, which is the Black African group in Cape Town, also belong to the Nguni tribe but have lived separately from the Zulu (the major Nguni group in Johannesburg) for many centuries. The Coloured subjects from Cape Town are known as the Cape Coloured and have a mixture of Khoisan, Southeast Asian, western European and African Black genes [Rousseau et al., 2003]. Both the Coloured and Black African subjects analysed here have genetic input from the Bantu groups that migrated into southern Africa.

The samples in this thesis were collected from people in these groups attending two of the major referral hypertension clinics in South Africa. The control subjects were age matched normotensives. The Cape Town data set does not have records of the presence of smoking and alcohol intake, nor are there records of height, weight and

body surface area. The mean age of the Cape Town and Johannesburg subjects was very close; 52.8±13.9y and 53.1±10.1y respectively. The data sets were represented by similar frequencies of males, females and R563Q heterozygous individuals (Figure 5.6).

Figure 5.4. Frequency of males and subjects with the R563Q mutation and mean age of subjects in Johannesburg and Cape Town

In total, 2179 unrelated subjects were analysed for the mutation. 1704 (78%) were hypertensive and 475 were normotensive. 753 (35%) were of Coloured, 1204 (56%) of Black African origin and 177 (8%) were Caucasians. Forty five did not have an allocated ethnic group. The gender and race were not significantly different between the R563Q heterozygous and normal groups. In the combined group of Coloured, Nguni and Sotho people there were 95 (4.9% of the total) subjects who had the mutation; 87 (5.7%) of the hypertensives and 8 (1.7%) of the normotensives (p=0.0002 for association with hypertension) (Figure 5.5). The difference in mean age

between those with the mutation and those without was not significant, 54.4± 12.6 years vs. 52.9±12.5 years respectively.

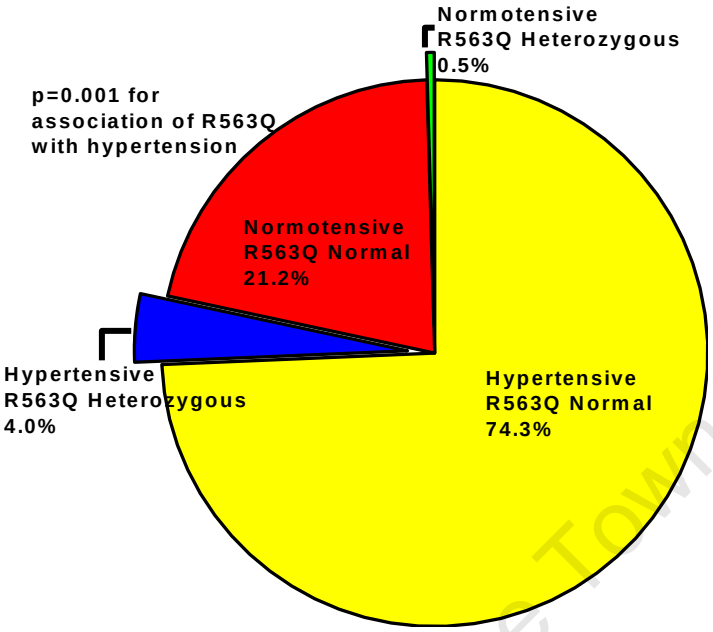

Figure 5.5. Percentages of subjects analysed in South Africa

DISCUSSION

The R563Q mutation of the β-ENaC has previously been found in subjects in the Cape Town area but has not been determined in other areas in South Africa. These results show that the R563Q mutation is prevalent and strongly associated with hypertension in three of the major ethnic groups in South Africa.

The frequency of the R563Q mutation in the GSH Hypertension Clinic referral clinic was found to be 6.8% in the Xhosa patients and 4.3% in the Coloured patients. It was not found in Caucasian hypertensive subjects and only one (0.5%) normotensive subject had the mutation. The R563Q mutation was found in 7.4% of the hypertensive group studied in Johannesburg and only in 2.6% of ethnically matched normotensive

controls. This difference was statistically significant as was the association with higher BP in the entire group.

In the search for candidate genes for hypertension many studies have found that mutations in the ENaC genes do not have a substantial role in EHT, [Munroe et al., 1998; Chang and Fujita, 1996]. There are a few studies that have found mutations at high frequencies, but their association with hypertension has been queried. The G442V mutation of the β-ENaC is found in exon 8 of the gene and was not associated with hypertension [Ambrosius et al., 1999]. It was associated with an increase in potassium excretion and a decrease in aldosterone excretion and aldosterone-potassium ratio suggesting an increase in ENaC activity. However, when expressed in *Xenopus* oocytes there was no difference in sodium current. The frequency of the G442V mutation in the Black African subjects of this study was 8.3%, but only 0.2% in the Caucasian subjects. This is in keeping with other reports of sodium channel mutations being found more commonly in Black Africans than in Caucasians [Persu et al., 1998]. A mutation in the α-ENaC (T663A) that was more common in a Caucasian population [Ambrosius et al., 1999], with a significant difference between the hypertensives and normotensives, was found to be protective against hypertension.

The T594M mutation appears to be the only other mutation of the sodium channel that has data published for South Africa and if the association of the T594M mutation with hypertension is spurious then there are no published mutations found at high frequencies definitively associated with hypertension in the ENaC genes of South Africans. The R563Q mutation has a strong association with hypertension in this study and is found at high frequencies in Johannesburg. In the data for this thesis there

was no difference in BMI (which has been shown to have an affect on the interaction between genotype and blood pressure in the same population groups [Tiago et al., 2002]) between those with and without the R563Q mutation (Table 5.4) but there was a consistent association of the R563Q mutation with higher BP.

The R563Q mutation is significantly associated with hypertension and BP differences. In subjects from Johannesburg there was an average difference of 8/5 mmHg between the heterozygous and normal individuals (p=0.006 for SBP and p=0.005 for DBP). This sample (780 Nguni and Sotho) represents two of the major Black African groups living in South Africa [Lehohla, 2001]. When the ethnic groups were separated, the presence of the mutation in the Sotho group did not significantly associate with hypertension. This could be due to the smaller numbers. However, the numbers were adequately powered. There was half the number of Sotho's sampled, with almost two thirds having hypertension.

In the combined Johannesburg and Cape Town sample analysed the association between the R563Q mutation and hypertension was still present (p=0.001). There was no difference between the R563Q heterozygous and normal groups with respect to smoking, alcohol intake, age, gender or BMI so they were not used as covariates in the analysis. This does not exclude the possibility that environmental factors contribute to the influence this mutation has on channel activity. It is thought that the R563Q mutation increases sodium channel activity and would thus be influenced by sodium intake of the individual. There were no data reflecting sodium intake for this sample (e.g. spot urinary sodium-creatinine ratio which is a reliable and easy indicator of sodium intake and is shown to have a direct correlation with BP in infants

[Pomeranz et al., 2002]). Thus, it was not possible to determine if the sodium intake was different between those with the mutation who were hypertensive or normotensive.

The R563Q mutation has not been found in a group of Black African people that has been analysed in London [Hartmann, 2007]. The Nguni and Sotho groups are Bantu-speaking people who originated from Central Africa. (This is also likely to be the place of origin of the individuals sampled in London.) The Nguni and Sotho come from the south-eastern Bantu migration into southern Africa, see Figure 5.6. At the time of this immigration southern Africa was populated by a hunter-gatherer population known today as the Khoisan who lived in the extreme and harsh environment of the Kalahari Desert but had migrated east and south, inhabiting most of southern Africa [Pereira et al., 2001; 2002; Soodyall et al., 1992].

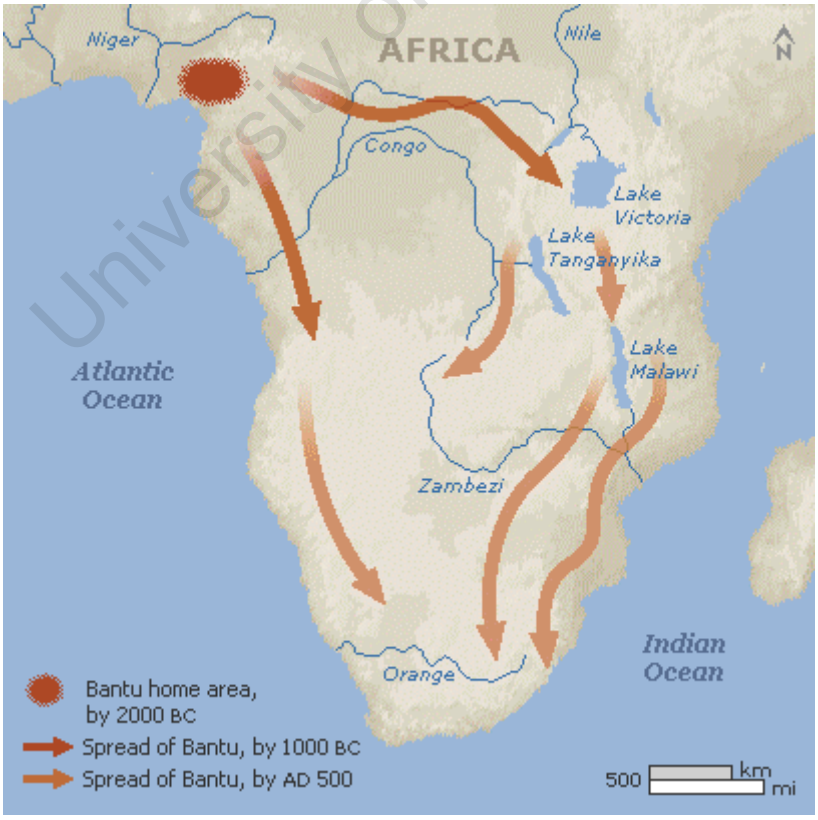

Figure 5.6. Bantu Migration [Encarta, 2008]

The Nguni in South Africa mostly consist of the Xhosa and Zulu tribes, who have a very high genetic correlation [Soodyall, 1993]. The Xhosa are the major group populating the Eastern and Western Cape whereas the Zulu traditionally lived in KwaZulu Natal. The Sotho inhabited the mountainous area Lesotho which lies inland and between the Eastern and Western Cape [Lehohla, 2001]. These groups have all been living in southern Africa for 2000 to 3000 years and the geographical boundaries have all but disappeared resulting in extensive genetic admixture between them [Salas et al., 2002; Soodyall, 1993].

The prevalence estimates in this chapter are not a true reflection of the prevalence within South Africans. These samples were selected according to blood pressures and thus show prevalences in these groups rather than the general population. Further studies are required to determine population prevalences.

CONCLUSION

The above data show that there is a strong association of the R563Q mutation with hypertension in areas other than the Western Cape. This association is independent of the patient's BMI and age. The mutation has a high prevalence in the hypertensive Black African and Coloured people of South Africa suggesting it may be the commonest clinically relevant cause of EHT described to date.

CHAPTER 6

The Khoisan: An Investigation into the Possible Origin of the R563Q Mutation in South Africans

INTRODUCTION

South Africa is a country with people of diverse origins. The aboriginal groups to southern Africa are the Khoikhoi and the San. These two groups became known together as the Khoisan and are found in the highlighted are in Figure 6.1. The Khoisan are thought to be direct descendents of the original *Homo sapiens* who inhabited Africa, as they have been found to have the oldest clades in mitochondrial DNA and the Y chromosome [Chen et al., 2000; Semino et al., 2002; Underhill et al., 2000].

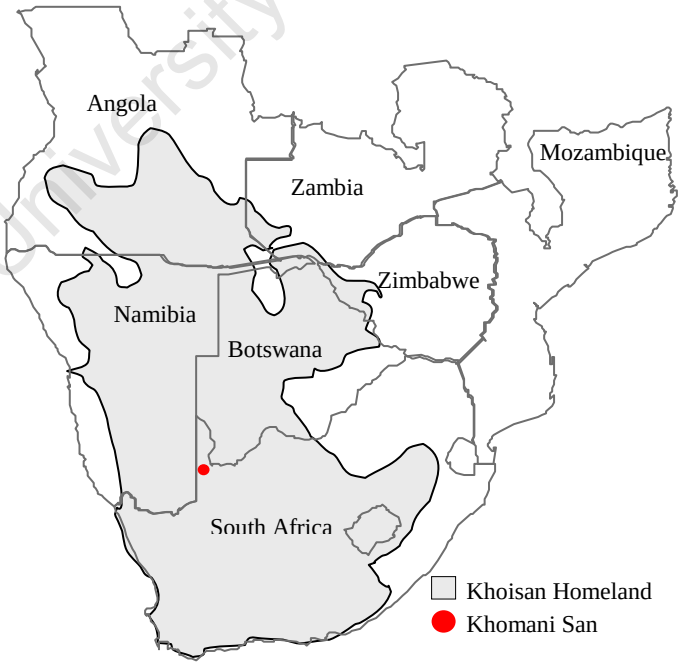

Figure 6.1. The Khoisan groups inhabit south western sub-Saharan Africa, including the Kalahari Desert. [Encyclopaedia Britannica, 2004]

The hunter-gatherer existence of the San included a diet rich in potassium and very low in sodium. The Khoikhoi were a pastoral group who have mostly been either incorporated into the San or the Black African groups that migrated into southern Africa. The Black African groups are the major groups that currently inhabit most of sub-Saharan Africa. These groups originated in central Africa at the confluence of the Niger and Benue rivers, and all speak Bantu languages. The groups migrated throughout sub-Saharan Africa, mingling with the aboriginal groups, as they expanded. This led to genetic admixture and the Zulu and Xhosa in South Africa have considerable genetic similarities with the Khoisan [Soodyall, 1993].

The R563Q mutation has been found in the Nguni and Sotho groups in South Africa at a high frequency, especially in hypertensives (see Chapter 5), but not in the Black African groups in the United Kingdom [Hartmann, 2007]. This chapter examines the frequency of the R563Q mutation in the Khoisan groups that are living in southern Africa and examines the following questions.

- Is the presence of this mutation in the Black African groups of South Africa a reflection of genetic admixture from the Khoisan?
- If the mutation is found in this group at what frequency and is it in Hardy Weinberg Equilibrium (HWE)?
- If present, does this mutation alter BP in this group, or the electrolytes and aldosterone levels in the Khoisan?

As discussed in Chapter 7, in order to determine if the R563Q mutation alters the parameters that determine the BP it is important to measure these parameters in individuals who are not taking any antihypertensive medication. Most of the subjects

with the R563Q mutation have severe resistant hypertension. This makes it impossible to get a true reflection of these parameters. The Khoisan are known to have normal to low BP [Nurse and Jenkins, 1977]. Thus, investigation of Khoisan subjects created the opportunity to determine the effects of the R563Q mutation in treatment naïve individuals.

METHODS

In collaboration with the University of the Witwatersrand, samples were collected from a group of Khoisan from the Northern Cape known as the Khomani San. An ongoing project to determine mitochondrial DNA and Y chromosome diversity has been approved by the University of the Witwatersrand provided it is accepted by the various Khoisan communities. The University of Cape Town Ethics Committee gave approval for association studies. The author was invited as the team medical officer in order to collect the blood samples and record the BP from the subjects in the Northern Cape.

DNA was available from a small cohort of Khoisan from Namibia [Rousseau et al., 2003] courtesy of Dr J. Rousseau. These subjects were judged to be unrelated on the basis of interviews but more distant inbreeding could not be excluded as the community was small. The DNA underwent PCR followed by restriction enzyme digestion to determine the presence of the R563Q mutation, as described in Chapter 3.

A random sample of DNA from the Khomani San was collected by driving into the communities and requesting people to be involved in the study. Subjects were self identified as Khomani San and gave the ethnicity and place of birth for each parent, to

the best of their knowledge. Close relatives were excluded in order to prevent over- or under-representation of the prevalence of the mutation.

BP was measured as previously described. Blood was taken for aldosterone, sodium, potassium and creatinine and DNA analysis. The blood was spun at 6000rpm for three minutes and the serum was stored in a different tube in an insulated container with ice-blocks (but was not directly placed on the block in order not to freeze them) until the samples could be placed in a refrigerator at 4^{o}C. The samples were then transported to the laboratory at GSH in an insulated container. This was to prevent haemolysis causing falsely raised potassium levels. Subjects receiving antihypertensive medication did not undergo biochemical analysis due to the influence of antihypertensives on electrolytes and aldosterone. DNA was extracted at the University of the Witwatersrand. The presence of the mutation was determined with PCR and sequencing as described in Chapter 3. All homozygous results were checked with different primers and sequenced, as previously described. HWE was calculated and, to determine if the deviation form equilibrium was significant, the Hardy Weinberg calculator was accessed from a website. The SPSS package was used for further statistical analyses. Student's t test was used for continuous variables and X^2 was used for categorical variables. Power was calculated from a freely available online calculator: Statistics calculators.

RESULTS

Namibian Khoisan

Eighty two samples from Namibia were analysed for the mutation. Fifteen (18%) subjects were heterozygous for the mutation, and one (1%) subject was homozygous.

The results were used to determine if the Namibian sample was in HWE (Figure 6.2). HWE is explained in the glossary (Appendix II). Briefly, the equation is used to determine if the actual number of heterozygous and homozygous individuals is similar to the expected frequencies. Significant deviation from the expected frequencies needs to be explained.

- **Total number of samples: 82**
- **Heterozygous: 15**
- **Normal (*RR*): 66**
- **Homozygous for the *Q* allele: 1**
- **$\therefore$ $p^2 = 66/82 = 0.804878$**
- **$\therefore$ $p = 0.8971499$**
- **And $q=1-p = 0.1028501$**
- **$\therefore$ $q^2 = 0.0105781 =\sim 1/100$**
- **And $q^2 = 1/82 = 0.0121951$ from the samples**
- **$2pq = 0.1845438$ using calculated figures**
- **$15/82 = 0.1829268$ from the samples**
- **Approximately equal at ~18% each**

Figure 6.2. Hardy Weinberg calculations for the Namibian Khoisan

This small random sample with a high prevalence of subjects heterozygous for the R563Q mutation and one homozygote did not have data on BP or any other phenotypic data. The Hardy Weinberg calculations suggested that the mutation may be in equilibrium in this population, but as it is a small sample additional subjects would be required before it is possible to determine if this population is truly in equilibrium.

Northern Cape Khomani San

85 Khomani San from the Northern Cape were analysed for the presence of the R563Q mutation. 51 (60%) were male, and 55 (65%) were normotensive (Table 6.1). 13 (15%) were heterozygous for the R563Q mutation and 3 (4%) were homozygous. The prevalence of hypertension in the normal group (negative) was similar to the combined homozygous and heterozygous (positive) group (Table 6.1).

Table 6.1. Frequency of hypertension in each group

	R563Q			
	Positive (%)	Negative	Total	X^2
Normotensive	12 (24)	37	49	
Hypertensive	4 (11)	32	36	
Total	16 (19)	69	85	0.129

Table 6.2 shows the results from the measurements that were taken form the Khomani San people from the Northern Cape. The positive group had a median age of seven years greater than the negative group. The serum potassium was not different between the groups, neither was the urinary excretion of sodium or potassium or the urinary sodium-creatinine ratio. The aldosterone levels were significantly lower in the positive group: the median was 52.3pmol/l vs. 96.2pmol/l (p=0.035 for the logarithm of the mean). However, the analysis is shown to be underpowered and more subjects will be required in order to be sure there is no difference. Figure 6.3 shows the calculations for the Hardy Weinberg equation for the frequency of the mutation in the Northern Cape Khomani San.

The observed frequencies of the genotypes in the Northern Cape Khomani San were different to the expected frequencies. In a sample of 85 with three homozygotes more than 13 heterozygotes were expected. The deviation from HWE was calculated and

the X^2 analysis is shown in Table 6.3. The small sample of Namibian Khoisan did not deviate significantly from the expected frequencies but the Northern Cape Khomani San deviated significantly (p=0.034).

Table 6.2. Variables in the positive and negative groups

	R563Q Positive			R563Q Negative			
	N	Mean / (Median**)	SD / (IQR)	N	Mean / (Median**)	SD / (IQR)	P value (Power)
Age	16	45	36;61	67	38	33;56	0.129*
SBP, mmHg	16	129	121;147	69	129	126;139	0.303* (0.325)
DBP, mmHg	16	76	10	69	81	11	0.075 (0.616)
Sodium, mmol l^{-1}	12	134	5	44	133	5	0.221 (0.256)
Potassium, mmol l^{-1}	10	3.9	0.4	43	3.9	0.4	0.431 (0.070)
Creatinine, µmol l^{-1}	12	65.9	13.2	44	69.9	16.3	0.221 (0.257)
Aldosterone, pmol l^{-1}	**12**	**96.2**	**54.5;191.7**	**44**	**52.3**	**31;118.1**	**0.035*** (0.079)
Urinary Sodium, mmol l^{-1}	8	54	29.5;146.0	27	52	40.5;125.8	0.372* (0.199)
Urinary Potassium, mmol l^{-1}	8	25	12.5;48.9	27	21.3	18.3;32.5	0.209* (0.256)
Urinary Creatinine, mmol l^{-1}	8	11	3;18	27	7	3.8;9.3	0.274* (0.517)
Sodium-Creatinine ratio	8	7.3	5.1;12.9	27	9.6	5.3;14.4	0.183* (0.233)

BP, blood pressure; N, number; SD, standard deviation; * P values for log transformed values, ** Medians and IQR are shown for data that required log transformation before analysis.

- **Total number of samples: 85**
- **Homozygotes (*QQ*): 3**
- **Heterozygotes: 13**
- **Normal (*RR*): 69**
- **$\therefore q^2 = 3/85 = 0.03529$**
- **$\therefore q = 0.18786$**
- **And p=1-q = 0.81213**
- **But in these data $p^2 = 69/85 = 0.81176$**
- **$\therefore$ p=0.90098**
- **$\therefore$ Expected frequency for heterozygotes:**
- **2pq= 2*0.18786*0.81213 = 0.30513**
- **Actual frequency in this sample: 13/85 = 0.15294**

Figure 6.3. Hardy Weinberg Calculations for the Northern Cape Khomani San

Table 6.3. Chi Squared Calculation for Deviation from HWE

	Genotype	Number	Expected	Frequency	Statistic
Namibian	RR	66	65.881	p=0.8963	
Sample	RQ	15	15.2378	q=0.1036	
	QQ	1	0.881		X^2 = 0.0199
	Total	82			P=0.888
Khomani	RR	69	67.0617	p=0.8882	
San	RQ	13	16.8764	q=0.1117	
	QQ	3	1.0617		X^2 = 4.4846
	Total	85			**P=0.034**
Total	RR	136	133.9285	p=0.8928	
	RQ	28	32.1428	q=0.1071	
	QQ	4	1.9285		X^2 = 2.7908
	Total	168			P=0.095

Comparison with Cape Town Normotensives

The Northern Cape Khomani San live a rural lifestyle, with the men going out to farm in the concessions that have been granted to the communities. The food that they do not produce on the farms they get from small shops that stock standard produce. Most of this produce was preserved food as the electricity supply to many of the areas was inadequate [Jones, personal observations]. Thus, the traditional diet that the Khoisan had has largely fallen away. The Khoisan that were sampled in the Northern Cape were compared with a group of normotensive subjects from the Western Cape in order to determine if there were any differences between the rural Khomani San normotensives and the urbanised Cape Town normotensives (Table 6.4). The R563Q status of the Cape Town normotensives was not known and the Khomani San was a group combining all the normotensives irrespective of the R563Q status. The subjects from Cape Town were included in a previous study requiring normotensive controls [Rayner et al., 2001]. These 87 normotensive subjects from Cape Town were a mixture of Black African (41) and Coloured (46) individuals and consisted of 26% males. The Khoisan were represented by a larger percentage of males (61%).

Table 6.4. Comparison of Northern Cape Khomani San and Cape Town normotensives

	Khomani San			Cape Town subjects			
	Number	Mean	SD	Number	Mean	SD	P value
			Physical Parameters				
Age, y	**48**	**41**	**16**	**87**	**55**	**10**	**<0.005**
SBP, mmHg	49	127	120;137	87	130	120;140	0.200*
DBP, mmHg	**49**	**75**	**72;83**	**87**	**80**	**75;90**	**0.010***
			Blood Measurements				
Potassium, mmol l^{-1}	**46**	**3.9**	**0.4**	**86**	**4.4**	**0.4**	**<0.005**
Creatinine, µmol l^{-1}	**49**	**65**	**56;78**	**86**	**76**	**69;83**	**0.001***
Aldosterone, pmol l^{-1}	**49**	**91.4**	**53.2;191.2**	**86**	**310**	**221.2;426.0**	**<0.001***
			Urine Measurements				
Sodium-Creatinine	**31**	**7.3**	**4.6;13.0**	**86**	**12.2**	**7.5;18.1**	**0.016***
Sodium, mmol l^{-1}	**31**	**53**	**26;130**	**86**	**116**	**70;153**	**0.001***
Creatinine, mmol l^{-1}	31	8	3;16	87	10	6;15	0.127*

* P values for log transformed data for which medians and IQR are displayed

The SBP was not different between these two groups, despite the thirteen year difference in age between them. The difference in DBP reached statistical significance but after adjusting for age this difference was attenuated (Table 6.5). An interesting observation was the lower potassium and aldosterone levels in the Northern Cape Khomani San suggesting innate overactivity of the sodium channels. The sodium-creatinine ratio was significantly higher in the Cape Town normotensives indicating that the sodium intake was higher in urbanised individuals. Despite the low sodium intake, the aldosterone level was lower in the Khomani San supporting innate ability to retain sodium. This has a long term impact on BP and aldosterone levels in the urbanised group. Adjusting for the difference in age did not alter the difference between potassium and sodium-creatinine ratio (Table 6.5). The aldosterone level was significantly different between the groups but this did not influence the differences in potassium level and sodium-creatinine ratio.

Table 6.5. Adjusting for age and aldosterone

	Age	Aldosterone
	P value	P value
DBP*, mmHg	0.09	
Aldosterone*, pmol l^{-1}	**<0.005**	
Potassium, mmol l^{-1}	**<0.005**	**<0.005**
Sodium-Creatinine*	**0.016**	**<0.005**

Footnote: This table shows the p values that were calculated after the variables were adjusted for age or aldosterone levels as these were significantly different between the groups. * Log transformed variables

Untreated Subjects

Many of the subjects (including the Khoisan, subjects from the family study and subjects from Johannesburg) that were assessed in this thesis were not on treatment for hypertension, either because they were normotensive or because they had not previously been assessed for hypertension. This created the opportunity to determine if there was a difference in biochemical characteristics and BP between those with and without the R563Q mutation. This analysis consisted of subjects from each of the chapters analysed in this thesis. The only excluding criterion for this analysis was the current use of antihypertensive medication. Despite the differences between the groups (such as the Khomani San and the Cape Town normotensives) all subjects were placed together and divided into two groups: a group with two copies of the normal β-ENaC (negative) and a group consisting of subjects either heterozygous or homozygous for the R563Q mutation.

There was a larger percentage of subjects with the mutation who were hypertensive (31% vs. 20%) but this was not significant until adjusted for the age difference (Table 6.6). The mean SBP, MAP and DBP were significantly higher in the subjects with the mutation; 134±19mmHg vs. 128±15mmHg (p=0.001); 99±13mmHg vs.95±9mmHg (p=0.013); and 81±11mmHg vs. 78±8mmHg (p=0.025) respectively. This difference

was present despite the eight year difference between the groups, the negative group was older (p<0.0005). The difference in BP was exacerbated by adjusting for age. Table 6.7 shows that there was no significant biochemical difference between the groups.

Table 6.6. BP for all untreated subjects

	Positive (55)		Negative (456)		P value	P value*
	Number	Percent	Number	Percent		
Hypertensive	**17**	**31**	**93**	**20**	**0.072**	**0.012**
	Mean	SD	Mean	SD		
Age	**43**	**15**	**51**	**13**	**<0.0005**	
SBP, mmHg	**134**	**19**	**128**	**15**	**0.001**	**<0.0005**
DBP, mmHg	**81**	**11**	**78**	**8**	**0.025**	**0.001**
MAP, mmHg	**99**	**13**	**95**	**9**	**0.013**	**<0.0005**

*P values after adjusting for age

Table 6.7. Biochemical parameters in the untreated subjects

	Positives			Negatives			
	N	Mean / Median*	SD / IQR*	N	Mean / Median*	SD / IQR*	P value
Potassium, mmol l^{-1}	36	4.1	0.4	75	4.1	0.4	0.436
Sodium, mmol l^{-1}	15	136	4.8	49	134	5.4	0.120
Creatinine, µmol l^{-1}	39	72	14	84	72	17	0.931
Aldosterone, pmol l^{-1}	39	142.0	106.9;233.0	84	156.3	81.4;247.3	0.490*
Urinary Sodium, mmol l^{-1}	8	52.0	40.5;125.8	28	46.5	27.5;131.0	0.432*
Urinary potassium, mmol l^{-1}	8	21.3	18.3;32.7	28	24.2	12.1;46.6	0.273*
Urinary sodium/creatinine	8	9.6	5.3;14.4	27	7.4	5.1;12.5	0.281*
FENa	8	4.3	2.4;6.1	27	4.0	2.9;6.6	0.458*

FENa, fractional excretion of sodium; *p values for log transformed variables and median and IQR are given.

DISCUSSION

A Common Mutation

The R563Q mutation was found at a high frequency in the two Khoisan groups that were sampled. Almost 20% of the subjects had the R563Q mutation in either the homozygous or heterozygous form. The fact that the R563Q mutation was found at such a high frequency in the Khoisan population supports the hypothesis that the

mutation in the Black and Coloured South Africans originates from the Khoisan. The Khoisan traditionally lived a sodium depleted lifestyle [Nurse and Jenkins, 1977], so if this mutation does increase sodium retention it would be expected to improve the chance of survival. This could explain the high frequency in this population. Another reason the frequency of this mutation could be high in this population is due to inbreeding. They are a small community and tend to be confined to small areas. This increases the risk of consanguineous mating which would increase the frequency of mutations.

Although the Hardy Weinberg calculation could be questionable with these small numbers it was considered worthwhile as they were random samples and represent small communities. The calculation could indicate if the samples were in the expected equilibrium. However, deviation may not be significant. It is known that the Khoisan live in small communities and thus the risk of non-random mating is high. There may have been very little migration in and out of the communities, but the fact that this mutation was not in equilibrium in a sample this size does not mean that there has been positive selection for this mutation. In a larger sample, deviation from HWE suggests that there is a selection pressure on a mutation. In this case it would be in favour of the mutation. Despite the small size of these samples the frequency of this mutation is important. These figures are in two random samples and are similar which support each other as true reflections of the frequency of the mutation in the Khoisan.

An excess of homozygotes (as was the case in the Northern Cape Khomani San) suggests that there could have been inbreeding, a selection pressure for the mutation or null alleles (i.e. deletions). The risk of another SNP that may have affected primer

annealing and extension was excluded by sequencing and using different primers to check the homozygous results. There is still debate as to whether or not the high frequency of the homozygotes is due to inbreeding or due to a selection pressure. Cannings and Edwards [1969] showed that, in small samples, the Hardy-Weinberg law did not correctly predict the actual genotypic frequencies. This suggests that the appearance of selection pressure in this sample could be a reflection of the sample size.

Functional Analysis in the San

The differences detected between the Northern Cape Khomani San with and without the R563Q mutation are shown in Table 6.2. The mutation did not associate with higher BP. The negative group was older than the positive group by a median of seven years but this was not significant. The DBP was higher in the negative group by a mean of 5mmHg which could be explained by the difference in age. The SBP did not display a similar trend. The lack of association with hypertension in this group could also be the result of other external factors, such as low sodium diet, and may not be relevant due to the small size of the sample. It is of interest that the aldosterone level was significantly lower in the group with the mutation, suggesting sodium channel over-activity. There was no difference in serum and urinary electrolytes between the groups.

When compared with a group of urbanised normotensives from Cape Town, the Northern Cape Khomani San normotensives had lower DBP but they were also younger (Table 6.5). The difference in DBP was attenuated by adjusting for age. The urbanised group were selected as age matched normotensive controls to compare with

a group of hypertensives, so would tend to be older to middle aged and have lower BP. The Khomani San were randomly selected for analysis and the hypertensives were excluded from this part of the analysis. This could explain the difference in age between the groups. The normotensive Khomani San had a significantly lower sodium intake than the urbanised group, as seen by the lower urinary sodium-creatinine ratio. They also had significantly lower potassium levels which may explain the low aldosterone levels, which are counterintuitive to the lower sodium intake. The low sodium intake with lower potassium levels and lower aldosterone levels could all be explained by an innately overactive sodium channel in the Khomani San.

The traditional Khoisan diet consisted of foraged and hunted foods which were high in potassium, lean protein, fibre and a balance of minerals [O'Keefe and Cordain, 2004]. This healthy lifestyle led to a low prevalence of hypertension, diabetes and hypercholesterolemia [Nurse and Jenkins, 1977]. The Northern Cape Khomani San live in communities which are made up of Khoisan people who have been relocated into farms. These communities do not live the traditional active hunter-gatherer lifestyle but eat a Westernised-type diet. In fact, it is not permitted to hunt without a permit and there are only permits for guns; none for bows and arrows. This means that those without guns may not hunt, and, as guns are expensive, many of these poverty-stricken people do not have access to food. This, within the arid African landscape, has created a community that struggles to survive.

Many families do not have access to electricity so fresh produce is not easily available. This means that they require food that is possible to store without a refrigerator. Most of these food products are in tin cans or are dried. These products

usually have added sodium chloride to preserve the foods and for flavour. This automatically increases the daily sodium intake which has been shown to increase the long term risk of hypertension and cardiovascular events [Tuomilehto et al., 2001].

This pseudo-westernised lifestyle has led to the presence of hypertension and, in a few cases, diabetes. Instead of the thin lithe bodies there is a fair amount of obesity [Jones, personal observations]. This has increased the incidence of the lifestyle disorders. The observed BP was higher than expected in this community, as was the incidence of hypertension. However, there was no difference in BP between the positive and negative groups. Those subjects that were on antihypertensives had their BP recorded and blood was taken for DNA but electrolytes, creatinine and aldosterone were not measured as these are influenced by antihypertensive medication which would invalidate the results.

In a group that was expected to have innately increased sodium reabsorption it would be expected that the aldosterone levels would be lower than the average ranges found in urbanised communities. This was the case, with 10 (17%) out of the 58 measured aldosterone levels being less than 31pmol/l. Thirty-one pmol/l is the level below which the laboratory does not report. The sodium and potassium levels were not different which reflects good compensation; as is expected in individuals that are clinically stable. However, there were many sodium levels that were lower than the normal clinical range (135-147mmol/l). There were 11 subjects who had a sodium level below 130mmol/l (the lowest was 117mmol/l) but only one of these subjects had the mutation. This subject had a sodium level of 128mmol/l and was heterozygous.

There were three homozygous subjects. Two of them were known hypertensives on treatment. They were both female. One was 67 years old and had a BP of 130/61mmHg and the other was 47 with a BP of 143/95mmHg. The third homozygous subject was 28 and was not known to have hypertension but her BP was 143/94mmHg. Her aldosterone was less than 31pmol/l, sodium was 132mmol/l and her potassium was 3.6mmol/l. Thus, all three homozygotes had raised BP in a community where this is not a common phenotype.

It was not possible to test renin levels as this test requires that the blood is stored frozen and is not defrosted until testing. This was not possible in the field. Field work in the Northern Cape involves being in the car for most of the daylight hours with no fridge or freezer so the samples were stored in an insulated container with ice-blocks. (The samples were not directly placed on the ice-blocks in order not to freeze them.) Furthermore, there was no dry ice for transport purposes. The samples were spun when there was access to electricity and stored until they could be placed in the refrigerator where they were stored at 4^{o}C until being transported back to Cape Town. A further complication of collecting samples in the field was the lack of ablution facilities at close range. Many of the subjects were found on the side of the road and refused to give urine samples, hence the small number of urine samples analysed.

The aldosterone and renin levels were previously found to be lower in Black African and Coloured normotensive subjects from Cape Town when compared with their White counterparts [Rayner et al., 2001]. The aldosterone was even lower in the Khomani San group, as was the potassium level. Sodium suppresses aldosterone and as the sodium intake is lower in the Northern Cape Khomani San the aldosterone level

was expected to be higher. A lower aldosterone in this group indicates an innately increased activity of the sodium channel. This is supported by the significantly lower potassium in the Northern Cape Khomani San. Pratt's team have repeatedly found that aldosterone levels are lower in Black African subjects when compared with Caucasians [Pratt et al., 1989; 1993; 1999]. The aldosterone appears to be even more suppressed in the Khomani San population in southern Africa.

Pratt et al. [2002] found that the lower aldosterone levels were not likely to be the result of higher ENaC function, as the subjects were not sensitive to amiloride. This group of young Black Americans had high levels of sodium retention resulting in aldosterone suppression. In The Northern Cape Khomani San the sodium intake was lower than the average urbanised individual and the serum aldosterone was lower, which would be explained if the ENaC activity was innately increased due to activating mutations. The R563Q mutation is a possible mutation and it is possible that this mutation hasn't altered the biochemical characteristics enough to reach statistical significance in a small sample. This supports the hypothesis that the R563Q mutation originates in the Khoisan. A mutation that completely alters the biochemical characteristics may not be sustainable but one that slightly alters the ability to survive is more likely to have a selection advantage.

It will be interesting to compare the mtDNA and Y chromosome clades and mutations. These data may show an association of the R563Q mutation with either the Khoikhoi or the San. The San, being a more hunter-gatherer group, may have had a higher prevalence of mutations in the sodium channel genes.

An interesting opportunity arose during this analysis. With so many subjects that were found to have the mutation and who were not taking antihypertensives it was possible to assess the difference in measurements between the groups with and without the R563Q mutation in an uninfluenced environment. The BP was significantly higher in the group with the mutation, with a mean difference of 6/3mmHg, despite the eight year age difference (positives: 43y and negatives: 51y). Adjusting for age increased the significance of the difference in BP. The biochemical parameters were not different between the groups but this could be due to the different results from the different groups, especially the Khomani San with their different sodium intake, i.e. population stratification. Thus, in all the untreated subjects analysed in this thesis the association of the R563Q mutation with hypertension remained significant.

CONCLUSION

The aboriginal group in southern Africa, the Khoisan, are most likely to be direct descendants of the original *Homo sapiens* that initially populated Africa. This group has been shown to have a high frequency of the R563Q mutation, which did not associate with hypertension in a small sample from this population. In one group analysed the mutation was in HWE but the equilibrium was not replicated in the second group analysed. However, the Hardy Weinberg Law may not apply to these small samples. Within the Khomani San there was no significant difference in the biochemical parameters that are affected by sodium channel activity, except the aldosterone level which was lower in those with the mutation. The lack of association with BP within this group could be related to the low sodium intake, when compared with the urbanised group. The high frequency of the R563Q mutation in the Khoisan groups, the history of intermingling between the Khoisan and the Black African

groups of Southern African and the lack of the mutation in other Black African groups previously studied suggest that the origin of the R563Q mutation in the South African Black population is the Khoisan. However, it needs to be acknowledged that alternative explanations as to the origins of the mutation are plausible and further data are required.

PART 3

THE R563Q MUTATION AND FUNCTION OF THE SODIUM CHANNEL

The Khoisan of Africa

A people requiring a sodium retentive genome

CHAPTER 7:

The R563Q Mutation Alters Phenotype in the Setting of Chronic Hypertension

INTRODUCTION

Patients that come to the GSH Hypertension Clinic are refractory to the standard levels of care in the primary and secondary health care systems. As a result of uncontrolled hypertension there is a high prevalence of target organ damage in these patients. Assessing the clinical characteristics of patients attending the Hypertension Clinic may determine whether the presence of the R563Q mutation of the β-ENaC is likely to predict a difference in levels of target organ damage and so may differentiate patients with the R563Q mutation from patients with EHT.

Intra- and inter-individual phenotypic variation increases the difficulty of assessing genetic polymorphisms. The index case with Liddle's syndrome was described by Liddle [1963], who also examined family members. Each subject presented slightly differently, despite having the same mutation. The index case had severe hypertension and hypokalaemia with alkalosis at 16 when she presented with symptomatic hypokalaemia. Her brother was asymptomatic but at the age of 16 was examined by Liddle as part of a follow up on the index cases and her family. He was found to have similar BP and potassium levels as his sister did at presentation. Liddle also listed the BP and potassium levels of close relatives, illustrating considerable variation between individuals, within a family, heterozygous for the same mutation (R566X). This phenotypic variation has been seen in other families [Melander et al., 1998].

The original index case with Liddle's disease developed chronic renal failure which responded well to renal transplantation [Botero-Velez, 1994]. After the transplantation her BP improved significantly, her aldosterone and renin levels returned to normal and she was normokalaemic. However, a biopsy of her kidneys did not reveal any major pathology and none of her family members developed renal failure. This case was a particularly severe presentation. Missense mutations outside the PY motif present with more subtle differences which, bearing in mind the differences in individuals with the R566X mutation, could be more difficult to illustrate.

This inherited hypertensive disorder illustrated the variation in blood pressure and target organ damage. Hypertension is a vascular disorder resulting from disrupted homeostasis of vascular resistance, volume and, occasionally, viscosity. The effects of an increase in BP are global as the vascular system is an integral part of every organ. However, there are a few organs that are particularly affected by hypertension. The heart muscle pumps against the pressure within the vasculature and so muscle mass and dilatation of the heart are influenced by BP. The kidneys are the major control organ for BP as they excrete sodium and water depending on the volumes and concentrations within the nephron. The nephron is highly sensitive to BP variation excreting renin when the BP decreases, resulting in an increase in BP (described previously).

In humans it is not possible to visualise the vasculature of the kidneys without an invasive procedure. It is however possible to see the vessels in the retina which give an indication of the effects of BP on the microvasculature. Thus, a clinical

examination detailing the retinal appearance of the microvasculature, the renal function (urea, creatinine and microalbumin-creatinine ratio) and the cardiac function (cardiothoracic ratio (CTR) and cardiac muscle hypertrophy) are considered good indicators of the effects of BP on microvasculature.

Each patient that is referred to the Hypertension Clinic undergoes an initial biochemical assessment which created the opportunity to determine if there was a difference in these parameters. Alderman et al. [1995] showed that renin levels are directly proportional to myocardial events in hypertensive patients. An overactive sodium channel would tend to decrease the renin and aldosterone levels and increase the potassium levels possibly resulting in fewer coronary vascular events. Thus, analysing the differences between these biochemical parameters and the macrovascular complications of hypertension (IHD, cerebrovascular disease and peripheral vascular disease) in the patients attending the Hypertension Clinic may illustrate the effects of the mutation and help to predict the chronic effects of the mutation.

Patients attending the Hypertension Clinic at GSH have access to amiloride, which is generally not available in South Africa as it is not registered in this country. Amiloride is brought into South Africa under section 21 of the Medicine Control Act. If a patient is confirmed to carry the R563Q mutation amiloride is added to their antihypertensive regimen. Amiloride is a potassium sparing diuretic which blocks the ENaC, decreasing sodium and water reabsorption [Rang et al., 1999].

Thus, as part of this study, the aims were to determine the levels of target organ damage in chronic hypertensives heterozygous for the R563Q mutation and compare with hypertensives without the mutation. Additionally, the opportunity arose to determine if there was a difference in biochemical parameters in the groups and to determine if there was a significant response to amiloride. However, this assessment was in the presence of multiple antihypertensives.

PATIENTS

All the patients known to GSH that were heterozygous for the R563Q mutation were selected to be part of this retrospective analysis. Age matched controls were selected from the GSH Hypertension Clinic where all Coloured and Black African patients are routinely screened for the R563Q mutation.

METHODS

A retrospective analysis of the clinical characteristics of the index cases known to GSH Hypertension Clinic, compared with age- and sexed-matched patients without the mutation, was performed. Folders of the index cases were accessed and the clinical characteristics at the time of presentation were recorded. Age and sex matched R563Q normal controls were selected from the Hypertension Clinic and their clinical presentation was compared with those with the mutation. The concentrations of potassium, urea, creatinine, aldosterone and renin were determined from serum taken on referral. The aldosterone-renin ratio (ARR) was calculated from the measured values. Urine was analysed for albumin and creatinine concentrations and the microalbumin-creatinine ratio was calculated.

Sokolow-Lyon (SL) criteria were calculated from the electrocardiogram (ECG) as the amplitude of the S wave in millimetres in lead V1 plus the amplitude of the R wave in millimetres in either lead V5 or V6 (depending on which is the larger). The CTR was determined from the chest X ray as a ratio of the largest transverse diameter of the heart on the erect posteroanterior film compared with the inner diameter of the thoracic cage on the same film. BPs were recorded from the folders and MAP was calculated as one third of the pulse pressure plus the DBP.

Results were converted to categorical variables. Left ventricular hypertrophy was determined by a Sokolow-Lyon value of greater than 35 and left ventricular enlargement is present if the CTR is greater than 0.5. Microalbuminuria was defined as a microalbumin-creatinine ratio between 3mg/mmol and 30mg/mmol and a ratio greater than 30mg/mmol was considered macroalbuminuria. A raised urea level was 7mmol/l or more and a raised creatinine level was greater than 100µmol/l for females and greater than 120µmol/l for males. An aldosterone concentration of less than 255pmol/l was defined as suppressed [Rayner et al., 2003].

IHD was present if the patient reported having had an ischaemic event or if there were signs of IHD on the ECG (ST segment changes or q waves). The patient was defined as having had a cerebrovascular event if the patient reported an event or if clinical examination revealed neurological deficit suggestive of such an event. A history of peripheral vascular disease was present if the patient reported claudication, if they had had peripheral vascular surgery or the peripheral vasculature was compromised on examination. Signs of congestive heart disease were present if the patient had class 2 or more dyspnoea according to the New York Heart Association classification, if

there were signs on physical examination or if there was evidence of pulmonary oedema on the chest X ray. Hypertensive retinopathy was present if there were signs of hypertensive vascular changes on fundoscopy. The GFR was calculated using the formula described in the Chapter 3. A GFR of less than 60ml/min/1.73m^2 was classified as CKD.

The response to amiloride was determined by recording the BP and potassium level from the first visit to the Hypertension Clinic and the latest recordings while taking amiloride. The mean change for all patients on amiloride was calculated.

Continuous results that were not normally distributed were log transformed before analysis in order to allow analysis with the student's t test. The groups were divided into those with and without pathology or abnormal criteria. This highlighted the difference between the groups.

RESULTS

Initially, 136 Caucasian hypertensive subjects were tested for the presence of the R563Q mutation but none were found to have the mutation [Rayner et al., 2003] so the presence of the mutation is not routinely determined in this population group. In total, 855 non-Caucasian patients have been tested for the mutation; 561 Coloureds, 278 Black Africans and 16 of other ancestry. 44 patients attending the Hypertension Clinic were known to be heterozygous for the R563Q mutation; 18 (6.5%) Black African and 26 (4.6%) Coloureds. All these patients were selected for this analysis. Out of those known not to carry the mutation, 52 age matched control patients (563Q normal) were selected to compare with those carrying the mutation. Figure 7.1 shows

the percentages of patients who were receiving the various antihypertensives on referral to the Hypertension Clinic. The antihypertensives represent the treatment available for hypertension in the primary and secondary hospitals and could either be as a single agent or a fixed combination medication. Table 7.1 lists the quantitative variables that were assessed with the median and IQR as they deviated significantly from the normal distribution. The P values are means of the log transformed variables.

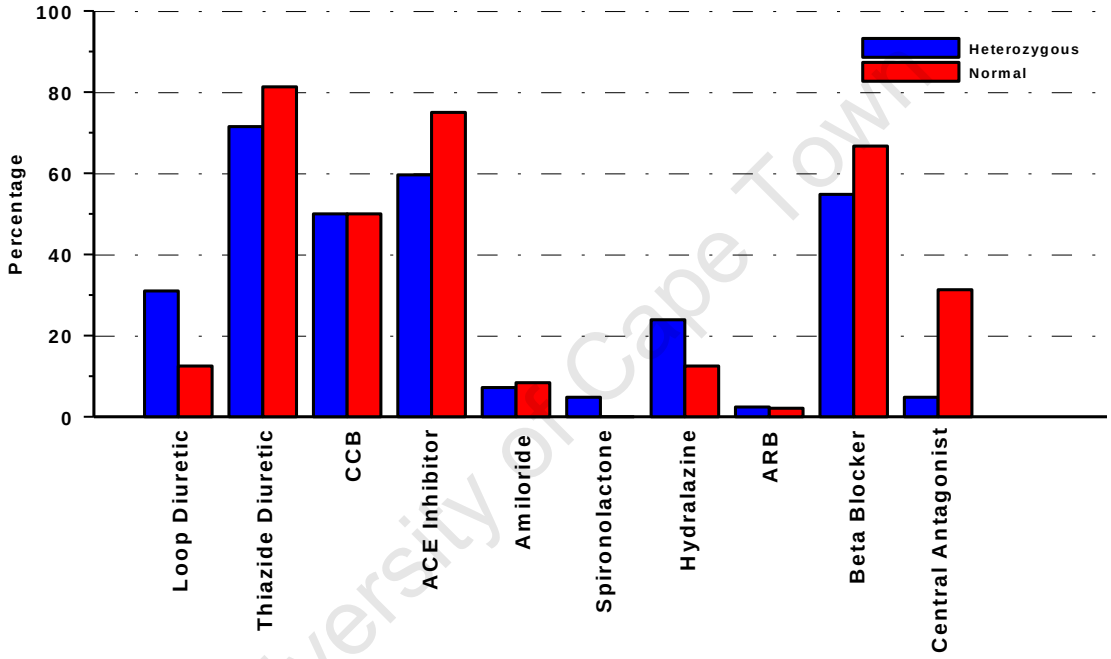

Figure 7.1. Percentages of patients taking the different antihypertensives on referral to the Hypertension Clinic
(P=0.032 for loop diuretics and p=0.007 for central acting antihypertensives.)

There was no significant difference between those with and without the mutation in the GSH Hypertension Clinic in age, renin, aldosterone and electrolytes; although, the potassium was slightly lower in the group with the R563Q mutation, and approached statistical significance (p=0.054). The urea and creatinine were significantly higher in the group with the mutation (p=0.016 and p=0.024 respectively) (Table 7.1). These two groups displayed a similar clinical picture. There was no significant difference in

BP between the two groups which is supported by the minimal difference in ECG Sokolow-Lyon criteria (showing cardiac hypertrophy) and CTR (showing enlargement).

Table 7.1. Quantitative variables assessed

	R563Q Heterozygous (44)			R563Q Normal (52)			P value*
	N	Median / Mean	IQR / SD	N	Median / Mean	IQR / SD	
Age**, y	44	53	14	52	52	14	0.385**
Years on treatment	35	10	3;23	44	5	2;15	0.087
Age at onset of HPT, y	36	39	31;45	44	38	30;44	0.617
Aldosterone, pmol l^{-1}	41	219	140;466	52	256	201;365	0.160
Renin, mmol l^{-1}	41	19	7;39	49	16	6;36	0.327
Potassium, mmol l^{-1}**	**44**	**4.1**	**0.6**	**52**	**4.3**	**0.5**	**0.054****
ARR	41	17.9	6.2;32.0	49	15.2	5.0;42.6	0.442
ECG SL criteria, mm	36	28	25;40	50	34	23;41	0.405
CTR	38	0.51	0.49;0.58	48	0.53	0.50;0.57	0.202
albuminuria, mg $mmol^{-1}$	35	3.0	0.9;26.5	47	4.4	0.8;12.5	0.167
urea, mmol l^{-1}	**44**	**6.2**	**4.4;8.4**	**52**	**5.3**	**3.8;6.3**	**0.016**
creatinine, µmol l^{-1}	**44**	**101**	**74;118**	**52**	**82**	**74;98**	**0.024**
SBP, mmHg	44	160	150;192	52	170	150;191	0.324
DBP**, mmHg	44	102	23	52	101	19	0.423**
MAP**, mmHg	44	125	24	52	125	20	0.436**

Footnote: N, number of individuals assessed for each variable; ARR, aldosterone renin ratio; SL, Sokolow Lyon criteria; ECG, electrocardiogram; CTR, cardiothoracic ratio; BP, blood pressure; MAP, mean arterial pressure; HPT, hypertension. *P values were calculated after log transformation. **Normally distributed variables with means ±SD and p values calculated from original data.

Table 7.2 shows the presence of pathology in the heterozygous and normal groups. The markers of renal function (urea, creatinine and GFR) all indicate that the renal function in those patients with the mutation was worse than those without the mutation. There was a difference in small vessel damage, as seen by the larger numbers of those with hypertensive retinopathy, in the normal group, but this did not reach statistical significance (p=0.063) for presence of hypertensive retinopathy of any grade. The trend across grades of hypertensive retinopathy was different (p=0.007) (Figure 7.2).

Table 7.2. The frequency of pathology in relation to the R563Q mutation

	Total	Heterozygous (%)	Normal (%)	P value
ECG LVH	34	13 (36)	21 (42)	0.582
Left Ventricular Enlargement	49	19 (50)	30 (63)	0.245
Albuminuria (>3.5mg/mmol)	44	17 (53)	27 (57)	0.425
Microalbuminuria	33	11 (32)	22 (48)	0.241
Macroalbuminuria	11	6 (18)	5 (11)	0.347
Urea (>7mmol/l)	**27**	**18 (41)**	**9 (17)**	**0.010**
Elevated Creatinine	**23**	**14 (32)**	**9 (17)**	**0.045**
Ischaemic Heart Disease	23	7 (17)	16 (31)	0.089
Cerebrovascular Event	9	4 (10)	5 (10)	0.635
History of Peripheral Vascular Disease	3	0	3 (6)	0.165
Hypoaldosteronism (<255pmol/l)	53	27 (66)	26 (50)	0.125
Hypertensive Retinopathy	61	26 (70)	35 (88)	0.063
Signs of Congestive Cardiac Failure	18	10 (24)	8 (15)	0.204
GFR<60ml/min/1.73m^2	**24**	**15 (34)**	**9 (17)**	**0.029**

Footnote: GFR, glomerular filtration rate

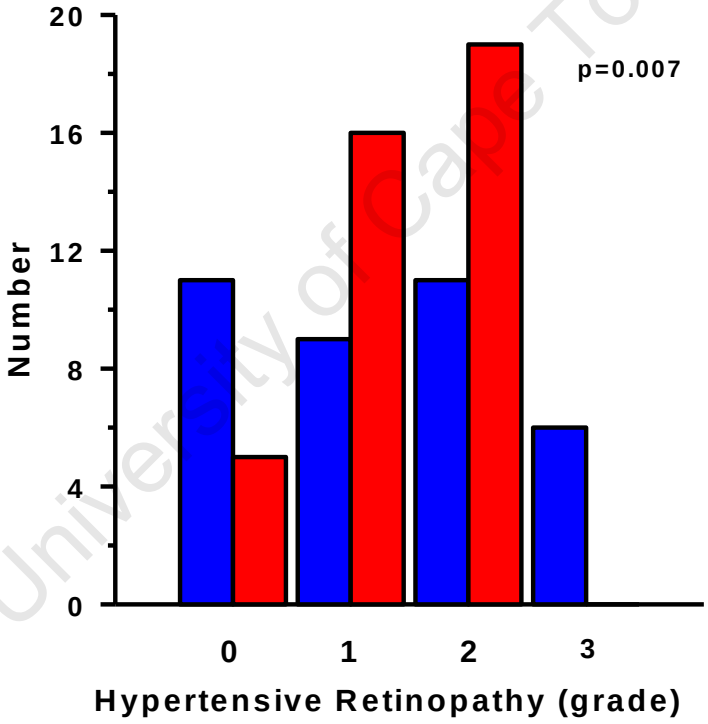

Figure 7.2. Grade of hypertensive retinopathy between heterozygous (blue) and normal (red) patients. The p value is for the difference in trend between the groups.

Microvascular disease was present in a large portion of the entire group (70% of the heterozygotes and 86% of the normal) suggesting long standing hypertension. There were two patients heterozygous for the R563Q mutation who underwent a renal

transplant as a result of CKD. These two patients were excluded and the analysis repeated to determine if this altered the results (Table 7.3). Removing these two patients decreased the differences in GFR but the heterozygous group had persistently elevated urea levels and less overall retinopathy.

Table 7.3. Variables without the patients who received a renal transplant

	Heterozygous	Normal	P value
	Median (IQR)	Median (IQR)	
Potassium, mmol l^{-1}	**4.2 (3.7;4.3)**	**4.3 (3.9;4.6)**	**0.025***
Urea, mmol l^{-1}	**6.0 (4.3;8.1)**	**5.3 (3.8;6.2)**	**0.045***
	Number (%)	Number (%)	
Elevated Urea	**16 (38)**	**9 (17)**	**0.023**
Decreased GFR	13 (31)	9 (17)	0.120
Retinopathy	**24 (69)**	**35 (88)**	**0.046**

*P values for log transformed data

Twenty two heterozygous patients were taking amiloride as part of their antihypertensive regimen. The average decrease in BP (Table 7.4) for all the patients on amiloride was 36/17mmHg, ($p<0.0001$ for SBP and DBP). Nine of these patients reached the target blood pressure on this treatment regimen. Furthermore, the patients with measured potassium levels increased while on amiloride (0.6mmol/l, $p=0.002$) as was expected of an agent that inhibits sodium channel activity.

Table 7.4. Summary of BP and potassium levels at presentation and on amiloride treatment

	Initial		Latest		Change	
Patient	BP, mmHg	Potassium, mmol l^{-1}	BP, mmHg	Potassium, mmol l^{-1}	BP, mmHg	Potassium mmol l-1
1	172/86	4.9	126/60	5.3	46/26	0.4
2	160/100	3.7	148/80		12/20	
3	169/91	3.0	135/82	3.8	34/9	0.8
4	180/130	4.2	138/110		42/20	
5	160/90	3.7	118/85	4.1	42/9	0.4
6	154/96	4.7	114/80		40/16	
7	176/100	3.9	139/82	4.6	37/18	0.6
8	222/100	4.2	153/85	4.4	69/15	0.2
9	210/92	2.9	152/75	3.5	58/17	0.6
10	142/90	4.2	140/95	5.0	2/+5	0.8
11	180/110	3.6	148/85	3.9	32/25	0.3
12	190/90	3.9	110/69	4.1	80/21	0.2
13	210/90	4.2	162/65	5.2	48/25	1.0
14	137/94	4.4	132/90	4.5	5/4	0.1
15	162/100	4.1	128/78	6.6	34/22	2.5
16	208/130	3.7	149/101	4.4	59/29	0.7
17	153/95		133/84		20/11	0.0
18	142/99	3.8	146/80	4.1	+4/19	0.3
19	144/94	4.3	104/70	5.3	40/24	1.0
20	180/115	3.7	142/94	4.8	38/21	1.1
21	169/90		140/68		29/22	0.0
22	190/104	4.1	140/85	4.3	50/19	0.2
Mean	**172/99**	**4.0**	**136/82**	**4.6**	**37/17**	**0.6**

DISCUSSION

This thesis has analysed the differences in clinical and laboratory characteristics between the heterozygous and normal patients presenting to the Groote Schuur Hypertension Clinic. Patients that attend the clinic are likely to have a similar history of prolonged hypertension resistant to the standard secondary level care for high blood pressure. This was found to be true. The patients heterozygous for the R563Q mutation had been hypertensive for a median of ten years and the normal patients for five years, but this difference was not significant. All the medications were being taken by similar percentages in the normal and heterozygous patients except for the central acting antihypertensives (more for normal patients) and loop diuretics (more for heterozygous patients). Resistant hypertension and the presence of multiple

antihypertensive medications provide a similar environment in which to compare subjects with and without the R563Q mutation. The two groups were well matched for age and gender.

These data suggest that individuals heterozygous for the R563Q mutation with hypertension are more susceptible to CKD. The mean BP for the patients in this analysis did not reach target levels and inadequately treated hypertension has been shown to be associated with elevated creatinine levels and a decrease in renal function [Coresh et al., [2001]. The heterozygous patients did not have mean BP significantly different from those patients without the R563Q mutation but more heterozygous patients had an estimated GFR suggestive of CKD than those without (34% vs. 17%, p=0.029). The urea supported the difference in GFR but there was no difference in the presence of albuminuria (Table 7.3). Thus, despite similar degrees of hypertension, the patients attending the hypertension clinic that were heterozygous for the R563Q mutation had an increased prevalence of CKD.

Heterozygous patients had higher urea and creatinine levels than those without the mutation, but this could be due to the selection criteria. All heterozygous patients that are known to GSH were assessed. The patients that were selected for comparison were hypertensive patients attending the Hypertension Clinic who had been tested for the mutation. None of the comparative patients had been through other units whereas two patients with the mutation had had kidney transplants and others had been referred to the clinic for investigation for Liddle's syndrome. Selecting all the patients with the mutation may have lead to bias. When the two transplant patients were excluded from the analysis the difference in renal function decreased between the two

groups. The GFR indicating CKD was no longer different between the groups but the presence of a raised urea was still significantly higher in the heterozygous group. Interestingly, the difference in potassium levels became significant when the two transplant patients were excluded: the heterozygous group had a lower potassium level than those without the mutation. CKD requiring a transplant is likely to increase the potassium level. The potassium in the transplant patients was in the upper range of the normal levels, which could account for the difference when they were excluded.

Saydah et al. [2007] reported the prevalence of CKD, from the NHANES data, to be 16.8% of the adults in the United States, between 1999 and 2004. CKD was higher in the Black African population (19.9%) and cardiovascular disease and diabetes were the major contributing factors. Afshar et al. [2007] reported that hypertension was the cause of CKD in 13.5% of cases in Iran and that hypertension, for more than five years, was associated with smaller kidneys. Data for South Africa [Naicker, 2003] are lacking but the higher prevalence in the Black Americans suggests that chronic renal disease could be linked to different population groups. The prevalence of the R563Q mutation, and its association with hypertension, may explain some of the CKD in the South African population. The mutation appears to be associated with renal disease in this cohort. Further support for this is that two patients with the mutation required renal transplantation for end stage renal disease.

Rayner and Becker [2006] found that microalbuminuria was present in 25.4% of hypertensives in private practices in Cape Town which is lower than the 41% present in the group of hypertensive patients analysed here. Patients referred to GSH all present with severe resistant forms of hypertension which could explain this

difference. The presence of CKD would tend to increase the potassium level in these patients. Despite this the potassium level was lower in the heterozygous group, possibly supporting the hypothesis that the mutation influences sodium channel activity.

Renin and aldosterone levels and the presence of hypoaldosteronism were not significantly different between the groups. However, these serum levels were determined while patients were taking their antihypertensive treatment which influences the levels. Traditionally, increased plasma renin activity has been associated with deteriorating renal function [Baldoncini et al., 1999] and inhibition of the RAAS has been shown to improve renal function [Furumatsu et al., 2008]. In some subjects treated with RAAS inhibitors a phenomenon was observed where aldosterone levels increased after the initial suppression. This 'aldosterone escape' was associated with a decline in GFR [Schjoedt et al., 2004]. In this data set there is no evidence to suggest that heterozygous subjects are more likely to exhibit this phenomenon; both groups had similar levels of aldosterone and renin after similar periods on treatment. Despite the similarities the heterozygous group presented with more renal impairment and lower potassium levels which would support an increase in ENaC activity. However, the increased use of loop diuretics in the heterozygous group distorts the effects the mutation could have on the potassium level.

It is interesting that the heterozygous subjects were prescribed more loop diuretics as a group. This could be a reflection of the poorer renal function in this group. As the renal function decreases thiazide diuretics are less effective and patients are changed

to loop diuretics. It could also be the result of fluid retention but there was no difference between the groups in prevalence of signs for congestive cardiac failure.

The heterozygous group appears to have lower levels of microvascular disease, as evidenced by less hypertensive retinopathy, 70% vs. 86%, but this was not statistically significant (p=0.063). It did become significant when the two patients requiring a renal transplant were removed from the analysis because they both had grade III hypertensive retinopathy (p=0.046). The trend for hypertensive retinopathy was also different between the groups (Figure 7.2) and retinopathy is an independent predictor of damage due to BP [Duncan et al., 2002]. In a group of Nigerian hypertensives [Nwankwo et al., 2007] hypertensive retinopathy was found to be a risk factor for the development of impaired renal function so it was predicted that the heterozygous group would have greater levels of hypertensive retinopathy when they were found to have more CKD. Unexpectedly, this was not the case. A possible explanation for this is a protective effect during the initiation of the disease. Treatment would distort the biochemical picture at the time of analysis, but before treatment there may have been considerable differences in the renin and aldosterone profiles, as suggested by the analysis in Chapter 8.

Despite the microvascular differences the macrovascular events did not differ greatly between the two groups, but the normal group did have more events. 31% of the normal group had a history of IHD compared with 17% of the heterozygotes. There was no difference in those who had had strokes (for which hypertension is the commonest risk factor in South African general practice [Connor et al., 2005]) and very few overall reported a history of peripheral vascular disease. Overall, 40% of this

group had ECG criteria for left ventricular hypertrophy. Again, this is much higher than the group studied by Rayner and Becker [2006] (18.9%), probably for similar reasons. These results suggest that there is not a significant difference between the heterozygous and normal patients in the presence of vasculopathy.

Studies assessing the differences in biochemical and physical parameters between a mutation and the normal variant are scarce due to the low numbers of sodium channel mutations found at high frequencies. Staessen at al. [1999] conducted a meta-analysis of the literature on the M235T variant of the AGT gene. They found that the T allele was associated with hypertension but not with atherosclererotic or microvascular disease, including renal disease. This meta-analysis illustrates the difficulties of associating point mutations with disease. The T594M and G442V mutations were also found at high frequencies in the African population in London and Dong et al. [2001] tried to determine if the BP and biochemical parameters between those with and without these mutations were different. They showed that the T594M mutation was associated with hypertension but there was no difference in electrolytes, creatinine and renin and aldosterone and the variants did not appear to interact. The association with hypertension of the T594M mutation was not replicated in South Africa [Nkeh et al., 2003]. This could indicate that the T594M mutation is in LD with another mutation in the sodium channel in the African population in London but not with the South African Black African population.

This analysis was in treated hypertensive patients, which distorts the picture when trying to determine a difference in the biochemical parameters affected by sodium channel activity. However, determining the difference in biochemical parameters in

this environment was expected to be complicated by the presence of antihypertensive medications and from this additional analysis no definite conclusions were drawn.

Initially, on observing the BP of the R563Q heterozygous patients in response to amiloride, it appeared that amiloride had a significant effect on hypertensives with the R563Q mutation. However, the patients at the Hypertension Clinic are referred for specialist opinion as the secondary hospitals have not managed to control the BP with the standard antihypertensives available. The secondary hospitals are inundated with patients and often do not have the time to give a full assessment or give the required lifestyle change advice. When patients arrive at GSH they are given a full examination and biochemical assessment. If there are any indications patients are investigated for secondary causes of hypertension. Lifestyle modification advice is given at the Hypertension Clinic and most patients are seen by a dietician. The patients are given optimal antihypertensive medication at the correct doses and schedules.

Amiloride has been suggested as the specific drug treatment for subjects with mutations in the sodium channel [Baker et al., 2002]. Those authors found that subjects with the T594M mutation of the β-ENaC had similar BP on amiloride when compared with two drugs. When the amiloride was stopped the BP increased significantly (17/8±4/2mmHg) and returned to within normal ranges (140/88mmHg) after the amiloride was restarted. This decrease in BP is less than the observed decrease in BP of the subjects presented here.

Carter et al. [2001] studied the effects of amiloride on nine subjects with refractory hypertension. These subjects were given add-on therapy for two weeks of 5mg amiloride daily and then two weeks of 5mg amiloride twice a day. The BP was determined at the beginning of the trial and after the amiloride treatment. An independent electrophysiological analysis divided these subjects into a group of three subjects with a hyperactive ENaC and six subjects with normal ENaC activity. Those subjects with the hyperactive ENaC had a significant decrease in BP (24/15±7/6mmHg). Those with normal ENaC activity did not have a significant decrease in BP. This confirms that amiloride is specifically useful in subjects with increased sodium channel activity. This study, similar to the cases presented here, was add-on therapy but the mean decrease in BP was even greater in the cases presented above.

Another study [Saha et al., 2005] found that amiloride, as add-on therapy (to a calcium channel blocker and a diuretic) for nine weeks, significantly decreased the BP by 9.8/3.4±1.6/1.0mmHg. This was part of a randomised double blind trial and 21 subjects were included in this arm. The patients with the R563Q mutation had a decrease in BP of 36/17mmHg, but this was not a controlled analysis and other treatments were instituted. It is interesting that the decrease in BP in all three studies presented here for subjects on amiloride was less than the decrease observed in the subjects with the R563Q mutation.

CONCLUSION

Patients heterozygous for the R563Q mutation with severe hypertension appear to be more susceptible to CKD compared with age and sex matched patients with severe

hypertension. The R563Q mutation does not allow differentiation between EHT and LRHT at initial biochemical assessment while chronically treated patients are on medication. The presence of the mutation does not predict increased vascular or cardiac disease, but there may be a protective effect resulting in a lower prevalence of hypertensive retinopathy than expected. Furthermore, there is a clinically significant decline in BP for R563Q heterozygous patients on amiloride that warrants a controlled clinical trial to assess the true effect of amiloride on subjects with the mutation.

CHAPTER 8

Electrolyte Excretion and Blood Pressure Response to an Acute Saline Challenge

INTRODUCTION

The high prevalence of the R563Q mutation in hypertensives suggests that it is an activating mutation. The susceptibility for hypertension is thought to be due to an increased propensity to reabsorb sodium. Thus, the aim was determine if there was a difference in response to sodium challenge between R563Q heterozygous and normal subjects suggesting that the sodium channel is overactive in the presence of the R563Q mutation.

Most authors [Overlack et al., 1993; Poch et al., 2001; Gill et al., 1991] have defined salt sensitivity in terms of BP response to acute or chronic sodium restriction or loading. In other words, in salt sensitive individuals BP drops to a greater extent during times of restriction and rises to a greater extent during salt loading. In terms of feasibility of assessing salt sensitivity, chronic salt loading or restriction over two to four weeks will be fraught with problems in controlling sodium intake outside the premises of metabolic units, unless there is very strict supervision of the diet.

Weinberger et al. [2001] proposed that salt sensitivity should be determined by the BP response to acute intravenous sodium loading followed by acute deprivation and diuresis. He suggested that a decrease in MAP of 10mmHg or more between the BP at the end of salt loading and the end of salt deprivation as salt sensitive and a difference

of less than 5mmHg as salt resistant. Between these values was defined as indeterminate. The use of BP in defining salt sensitivity (while important) is fraught with problems related to measurement and variability of BP requiring large numbers of subjects. Furthermore it is not a direct measurement of sodium channel activity. Rydstedt et al. [1986] used an acute saline challenge to show that individuals with EHT had blunted natriuresis. The saline suppression test is used to diagnose primary aldosteronism [Mulatero et al., 2006], suggesting that this test is a valid means of illustrating salt sensitivity.

It is important to assess a mutation *in vivo* as this is where the potential effects of a mutation exist. *In vitro* analyses are important to determine how a mutation potentially alters protein activity but any functional effect of the mutation is within the cells in which the protein is expressed. Hence the importance of the saline challenge. The effect of a mutation can also be a long term cumulative effect which will not be evident in a once off functional measurement of, for example, a single cell's sodium current. (This is a method used to illustrate alterations in sodium channel activity.) The sodium excretion, BP response, renin and aldosterone were assessed during an acute saline challenge in order to determine if the mutation created an environment sensitive to sodium. Analysing sodium excretion creates the opportunity to focus on the underlying pathophysiological mechanisms. The subjects with the R563Q mutation were expected to excrete less sodium, more potassium and have a decreased urinary sodium-potassium ratio due to intrinsically increased sodium channel activity. In addition, baseline aldosterone levels would be significantly lower and suppress more after the saline challenge.

METHODS

Subjects over 21 years of age, whose R563Q status was known from previous studies, [Rayner et al., 2003; Family analysis, Chapter 4], were contacted. Patients were excluded if they had a known history of cardiac or renal disease; were taking antihypertensives to avoid the effects of these drugs on sodium excretion and the RAAS; or had severe hypertension with a SBP greater than 180mmHg or a DBP greater than 100mmHg at commencement of the challenge. All subjects gave signed informed consent for the procedure (Appendix III).

All subjects fasted from 22.00 hours on the evening before the challenge until the end of the procedure, to standardise sodium intake. *Ad libitum* water was allowed until an hour before the procedure to maintain hydration. During the challenge the subjects remained strictly nil by mouth until completion.

On arrival a brief history was taken, and subjects had their height and weight measured, bladders were emptied and urine was analysed with hand-held rapid tests (Dipstix®) to determine the presence of protein and blood. Subjects were then allocated a bed and an individual number. After 10 minutes at rest patients had their BP measured and the mean of three readings was recorded. Blood was taken for electrolytes, creatinine, renin and aldosterone. A litre of 0.9% sodium was infused over 60 minutes.

BP was measured every hour for four hours after the start of the procedure. The mean of three stable readings was recorded. After one hour bladders were emptied again and the volume measured. Urine was taken for analysis. A second litre of 0.9%

sodium was infused over 60 minutes. Two hours after the start of the procedure the drip was removed, bladders emptied and volumes recorded. Urine was sent for analysis to determine sodium, potassium and creatinine levels, and blood creatinine and electrolyte concentrations were measured.

After three hours bladders were emptied, volume measured and urine sent for analysis. At four hours blood was taken for renin and aldosterone levels. Again bladders were emptied, volume measured and urine sent for analysis. Subjects were discharged. A summary of the saline challenge measurements is given in Figure 8.1.

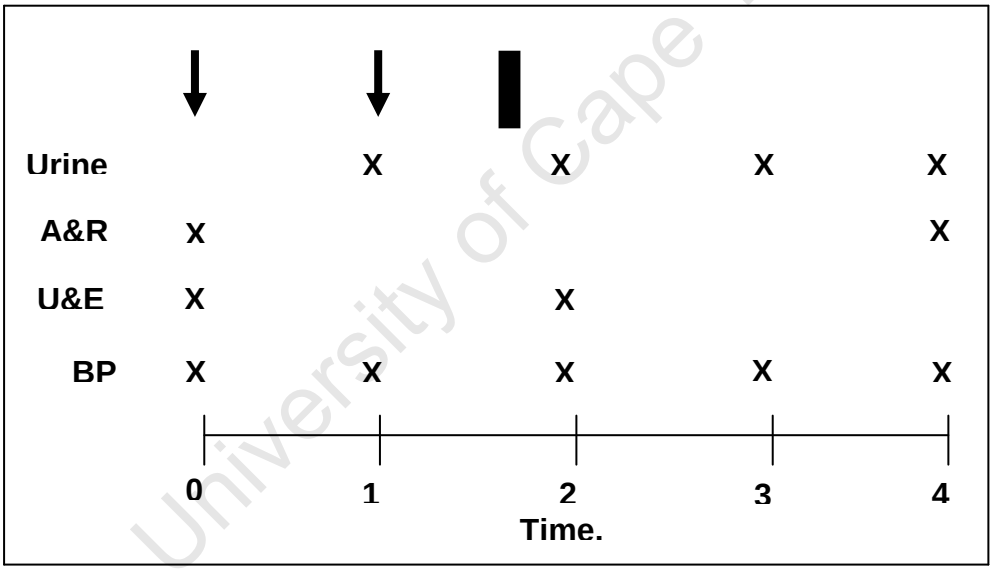

Figure 8.1. Summary of measurements at each time interval. Arrows indicate initiation of each litre of normal saline and the black bar indicates when the infusion stopped. (U&E, urea, electrolytes and creatinine; A&R, aldosterone and renin)

Continuous variables that were not normally distributed were log transformed before analysis with the student's t test and assessing in the multivariate analysis. In the tables the medians and IQR are shown for data that were not normally distributed and in the graphs means and standard deviations are displayed in order to ensure

consistency. The differences between the variables in the heterozygous and normal groups were compared. Paired samples T test was used to compare the differences between baseline and measurements during the challenge of serum electrolytes and renin and aldosterone levels. General Linear Models were fitted to compare the response to saline in electrolytes and renin and aldosterone between the heterozygous and normal groups. Pillai's Trace Test from the Repeated Measures Analysis was used to assess the significance of differences between groups and to indicate power.

RESULTS

The baseline characteristics of the groups with and without the R563Q mutation are shown in Table 8.1. Twenty four subjects completed the study and were analysed; 13 (54%) individuals were heterozygous for the R563Q mutation and 11 (46%) were normal; 15 (63%) subjects were male and 23 (96%) were Coloured. Two subjects from each group were found to be hypertensive at initial assessment and were advised accordingly.

Table 8.1. Baseline characteristics of the heterozygous and normal groups

R563Q	Heterozygous (13)		Normal (11)		P value
	Mean / Median*	SD / IQR	Mean / Median*	SD / IQR	
Age, y	35	30;40	33	24;37	0.970*
Height, cm	172.5	9.44	164.5	9.903	0.060
Weight, kg	75	58;90	73	59;80	0.670*
BMI, kg m^{-2}	24.6	5.4	26.18	5.65	0.500
SBP, mmHg	135	139;142	134	126;144	0.836*
DBP, mmHg	82	77;92	81	74;88	0.580*
MAP, mmHg	99	97;109	98	92;109	0.680*
Pulse, b min^{-1}	62	61;70	70	64;80	0.076*
Potassium, mmol l^{-1}	4.1	0.4	4.3	0.3	0.101
Sodium, mmol l^{-1}	**141.5**	**1.8**	**139.6**	**1.8**	**0.017**
Aldosterone, pmol l^{-1}	**217.2**	**120.8**	**332.8**	**153.8**	**0.051**
Renin, mmol l^{-1}	9.8	6.5;27.5	22.6	16.3;38.3	0.081*
Creatinine, µmol l^{-1}	72	14	69	14	0.606

BMI, body mass index; *P values were calculated from log transformed data and medians and IQR are displayed.

Sodium Handling

The serum sodium levels at the start and end of the infusion were significantly higher in those with the mutation (Table 8.2). This was despite the similar urinary sodium-creatinine ratio between the groups (the means were 23.8±13mmol/l for the heterozygotes and 29.4±18.3mmol/l for the normal group). The change in serum sodium during the challenge was significantly different between those with and without the mutation (p=0.001). Throughout the challenge the serum sodium was higher in the heterozygotes (Figure 8.2 A). The difference in serum sodium was exacerbated by the infusion of sodium indicating that those with the mutation were less able to handle the sodium load. The urinary excretion of sodium at each time was not significantly different between the groups (Table 8.3).

Table 8.2. Serum sodium at the start and completion of the saline infusion

	R563Q Heterozygous (13)	R563Q Normal (11)	P Value
Hour	Mean ± SD, mmol l^{-1}	Mean ± SD, mmol l^{-1}	
0	**142±1.81**	**140±1.8**	**0.020**
2	**143±2.06**	**141±1.76**	**0.008**

Table 8.3. Urinary Excretion of Sodium during the saline infusion

	R563Q Heterozygous (13)	R563Q Normal (11)	
Hour	Median (IQR), mmol	Median (IQR), mmol	P Value*
1	19.8(6.1;32.9)	17.4(11.5;34.2)	0.305
2	46.5(14.8;70.5)	24.0(19.6;50.1)	0.052
3	44.0(17.1;53.2)	23.4(12.5;43.9)	0.093
4	38.7(21.0;45.9)	23.1(17.6;26.5)	0.458
Total**	156.4 (77.9;194.4)	83.1(70.4;151.2)	0.099

*P values calculated from log transformed data. ** Total sodium excreted during the challenge.

The total urinary excretion of sodium (p=0.004 for trend) was higher in the group with the mutation (Figure 8.2 B). Throughout the four hours the heterozygous group excreted more sodium and the sodium excretion plateaued when the infusion was stopped. In contrast the normal group had a blunted natriuretic response to the saline

challenge, compared with the heterozygotes and sodium excretion declined in the two hours after the infusion was discontinued. The sodium-potassium ratio was also significantly higher in the heterozygotes ($p<0.0005$ for trend).

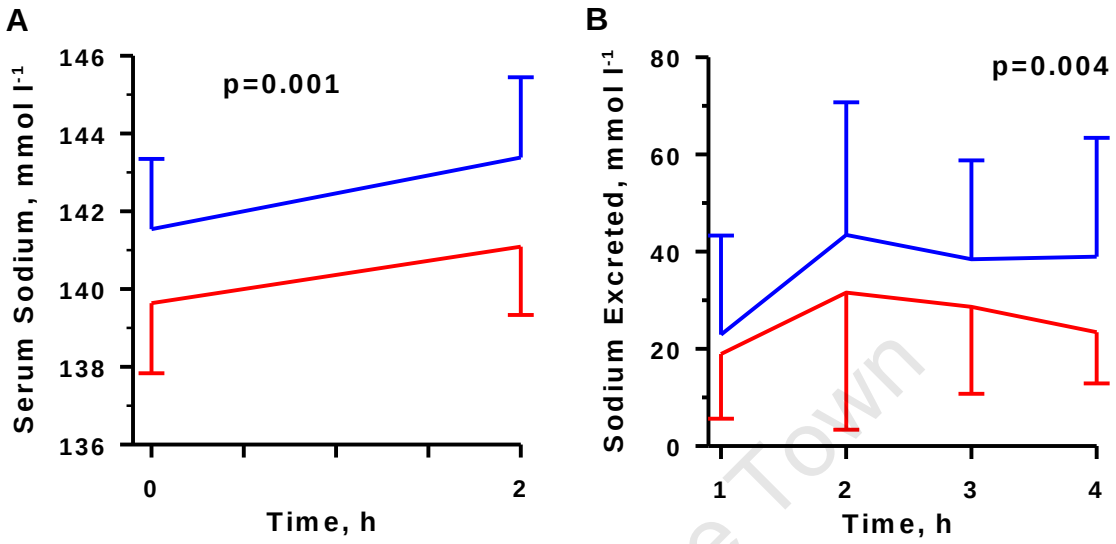

Figure 8.2. Serum (A) and urinary (B) sodium levels during the saline challenge with standard deviations (SD) (Subjects heterozygous for the mutation are shown in blue and the normal subjects are shown in red, p values are for trend, as assessed by the general linear model. This will apply to all the figures in this chapter.)

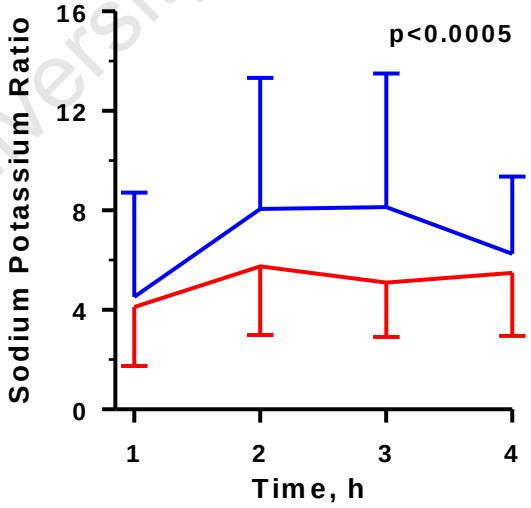

Figure 8.3. Relationship of sodium with interquartile ranges (IQR) to potassium excretion during the saline challenge

Potassium Handling

Serum potassium levels at the start and end of the infusion were not different between the groups. Similarly potassium excreted at each hour during the infusion was not different between the two groups except for an isolated difference at four hours when the excreted potassium was higher in the heterozygous group (p=0.047) (Table 8.3). However there was no difference in the total amount of potassium excreted between the groups (Figure 8.4). Thus the highly significant difference in sodium-potassium ratio (p<0.0005) (Figure 8.3) was likely to be the result of the difference in excreted sodium.

Table 8.4. Total potassium excreted during the saline infusion

	R563Q Heterozygous (13)	R563Q Normal (11)	
Hour	Median (IQR), mmol	Median (IQR), mmol	P Value*
1	4.1 (2.3;8.0)	4.4 (3.0;6.4)	0.756
2	6.9 (3.7;7.8)	5.7 (3.3;6.8)	0.843
3	5.4 (3.6;6.6)	6.8 (2.5;8.2)	0.972
4	**6.4 (4.7;7.7)**	**4.3 (3.1;5.5)**	**0.047**
Total**	26.1(16.4;29.8)	19.3(9.7;30.5)	0.176

*P values calculated from log transformed data. ** Total potassium excreted during the challenge.

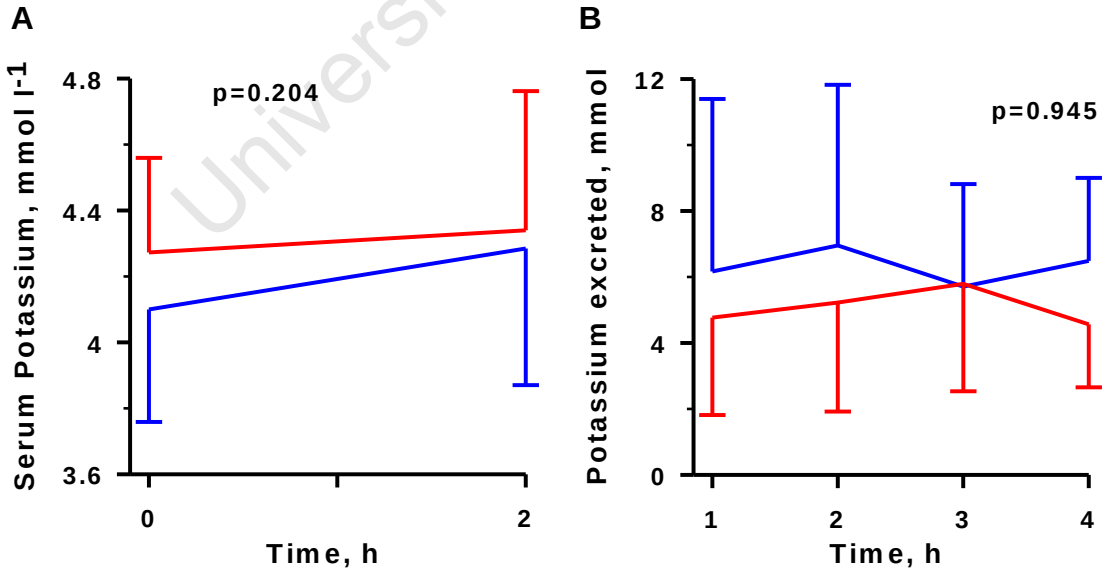

Figure 8.4. Comparison of potassium handling during the saline challenge. (A) Measured serum potassium levels, (B) Calculated excreted potassium, with IQR.

Aldosterone and Renin

The aldosterone concentration was lower in the heterozygous group at the start of the saline infusion and the difference approached statistical significance (Table 8.4). The difference in aldosterone was less marked at the end of the study but the overall trend during the study between the groups was highly significant (p<0.0005) (Figure 8.5), A similar trend was seen with plasma renin except the control group had a greater suppression at 4 hours (Table 8.4 and Figure 8.5).

Table 8.5. Aldosterone and renin levels and ratio at the start and finish of the challenge

	Hour	R563Q Heterozygous (13) Mean ±SD / Median (IQR)*	R563Q Normal (11) Mean ±SD / Median (IQR)*	P value
Renin, mmol l^{-1}	0	9.8 (6.5;27.5)	22.6 (16.3;38.3)	0.081*
Aldosterone, pmol l^{-1}	**0**	**217.2±120.7**	**332.8±153.8**	**0.051**
Renin, mmol l^{-1}	4	8.9 (6.5;9.7)	6.1 (5.1;8.7)	0.321*
Aldosterone, pmol l^{-1}	4	59 (55;64)	70 (47;86)	0.167*

*P value calculated from log transformed data with medians and IQR's.

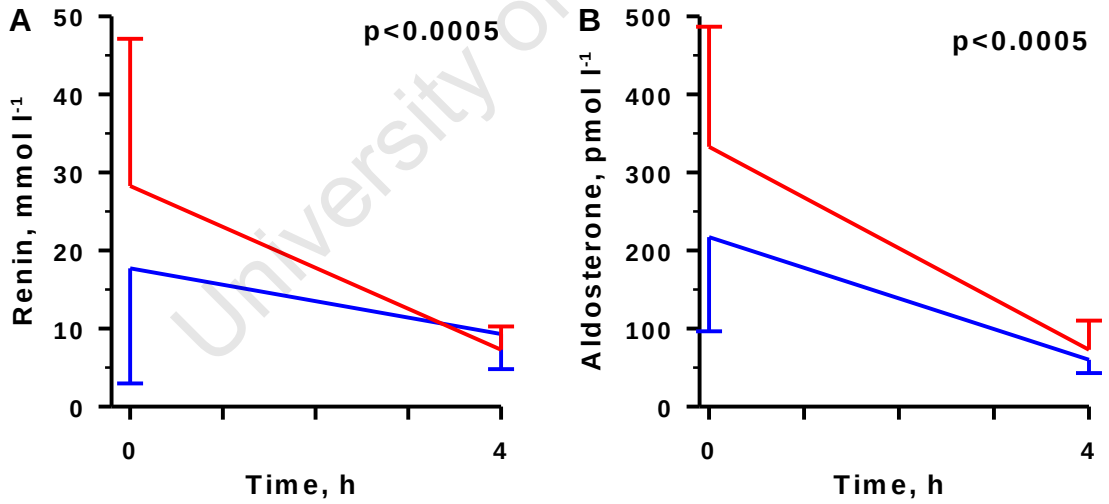

Figure 8.5. Serum renin (A) and aldosterone (B) levels with IQR during the challenge

Volumes

Some subjects were not able to pass urine at each time point. The urine volume and electrolyte concentrations were recorded as zero for that time point and were excluded from the calculation of the mean. The volumes excreted at each time point were

similar except at 4 hours, when the heterozygous subjects excreted a significantly higher volume (Table 8.5). The total volume excreted as a fraction of the volume infused was not different between the groups. The total output for both groups was similar. There was a significant difference in the way the volumes were excreted (p=0.001 for trend) during the four hours (Figure 8.6).

Table 8.6. Urine volumes excreted at each hour and the fraction of infused fluid excreted

Hour	R563Q Heterozygous (13) Median (IQR), ml	R563Q Normal (11) Median (IQR), ml	P Value*
1	170 (90;310) n=12	135 (70;205) n=10	0.388
2	330 (130;475)	338 (153;488) n=10	0.841
3	290 (85;340)	205 (90;305)	0.499
4	**180 (110;260)**	**100 (88;110)**	**0.036**
Total	1000 (440;1405)	750 (380;1055)	0.461
Fraction	0.5 (0.2;0.7)	0.4 (0.2;0.5)	0.398

Footnote: Where the number of samples differs from the total number of participants the different 'n' is shown. * P values for log transformed data.

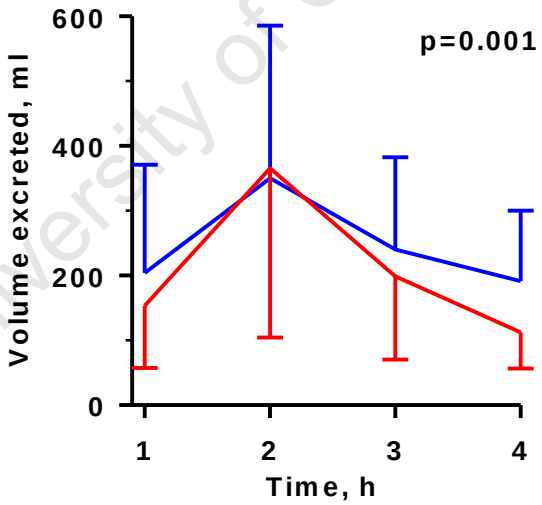

Figure 8.6. Volumes excreted with IQR, over the four hours after the start of the saline infusion.

The heterozygotes had a steady increase in urine output during the infusion, whereas the normal group were initially slower but the excretion was equal by the time the

infusion ceased. The normal group had a steeper response to the fluid infusion with greater a decline after cessation of the fluid infusion.

Blood Pressure and Pulse

Baseline BP was not different between the groups and there was no rise in BP in response to saline infusion in either group. There was a possible trend for higher SBP and DBP in the heterozygotes (figure 8.7) during the experiment. Interestingly there was a lower pulse rate in the R563Q heterozygous subjects at baseline and this trend became highly significant throughout the study. (p=0.002 for trend) (Figure 8.8).

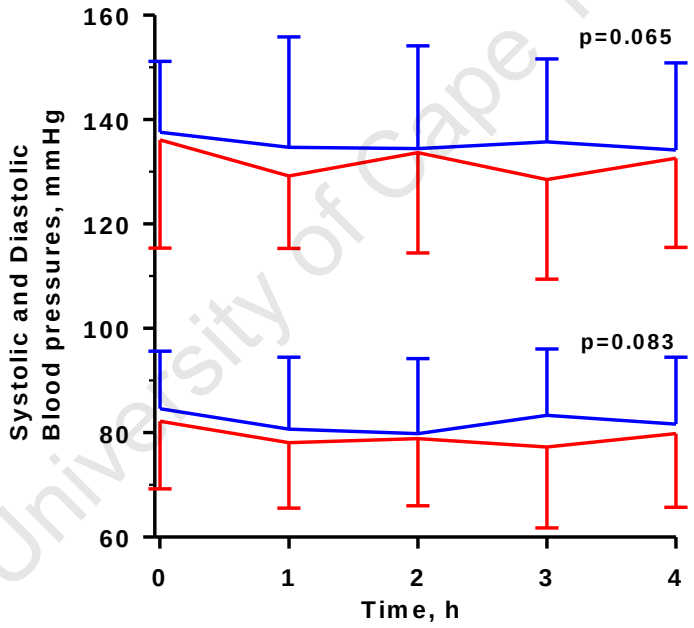

Figure 8.7. BP changes with IQR at each hour during the saline challenge

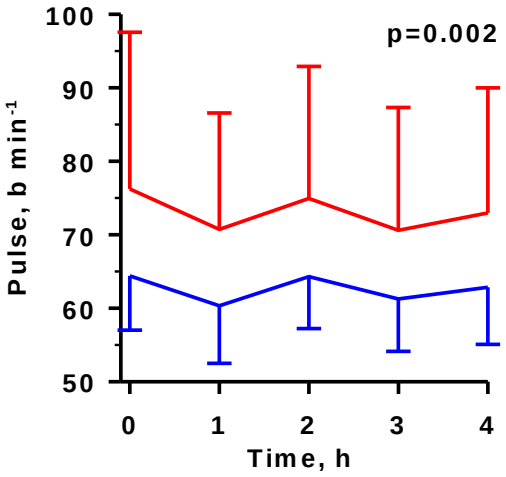

Figure 8.8. Pulse with IQR at each hour during the saline challenge

Summary

The difference between serum concentrations in electrolytes and aldosterone and renin levels from the start and the second measurement between the groups are shown in Table 8.6. Table 8.7 summarises the results from the saline challenge and presents the power for each analysis.

Table 8.7. Paired samples T test P values

	R563Q Heterozygous	R563Q Normal
Aldosterone 0,4, pmol l^{-1}	**<0.0005**	**<0.0005**
Renin 0,4, mmol l^{-1}	**0.031**	**<0.0005**
Sodium* 0,2, mmol l^{-1}	**0.005**	0.075
Potassium* 0,2, mmol l^{-1}	0.195	0.578

Table 8.8. Summary of saline challenge results

Variable	Time point	R563Q heterozygous	GLM p value	Observed Power
Serum sodium, mmol l^{-1}	0, 2	↑, ↑	**0.001**	**0.946**
Excreted sodium, mmol			**0.004**	**0.668**
Urinary sodium-potassium ratio			**<0.005**	**0.997**
Serum potassium, mmol l^{-1}			0.207	0.240
Excreted potassium, mmol	4	↑	0.945	0.087
Serum renin, mmol l^{-1}			**<0.005**	**0.986**
Serum aldosterone, pmol l^{-1}			**<0.005**	**1.000**
Urine volumes	4	↑	**0.001**	**0.954**
SBP, mmHg			0.065	0.558
DBP, mmHg			0.083	0.526
Pulse, b min^{-1}	1	↓	**0.002**	**0.968**

Footnote: The table displays the time point at which the variable was significantly different between the groups and whether the subjects with the mutation had a mean that was significantly higher (↑) or lower (↓) at that time point. The p value is for the difference assessed by the general linear model (GLM) between the groups.

DISCUSSION

The patients from the Hypertension Clinic are poorly controlled hypertensives with target organ damage. These patients were unable to participate in the saline challenge due to renal failure, congestive cardiac failure or the inability to stop their antihypertensive medication. It was, however, possible to find members of their families with the R563Q mutation and other people not being treated for hypertension in whom it was possible to infuse two litres of a 0.9% sodium solution. The saline challenge described the biochemical differences in response to an acute saline challenge between the heterozygotes and normal subjects. The saline challenge tested for salt sensitivity in an out-patient setting as it only required the subjects to be in the hospital for four hours. It also created the opportunity to measure the physiological determinants of salt sensitive BP. There was a significantly different response to saline between those with and without the mutation, the results of which are summarised in Table 8.7.

The salient findings in the saline challenge were:

1. <u>At baseline</u>

- The BPs were similar between the normal and heterozygous subjects (p=0.836 for SBP and p=0.580 for DBP).
- The pulse rate was lower in the heterozygotes (borderline significant, p=0.076).

- Serum sodium was significantly higher in the heterozygotes (p=0.017).
- Serum aldosterone was lower in the heterozygotes (borderline significant, p=0.051).
- There was no difference in serum potassium (p=0.101).

2. <u>During the challenge</u>

- There was no rise in BP after saline challenge in either group and there was no significant differences in BP during the experiment (p=0.065 for SBP and p=0.083 for DBP).
- The pulse was significantly different due to the infusion (p=0.002).
- The aldosterone level was suppressed to a greater degree in the normal subjects during the infusion (p<0.0005).
- The sodium excretion (p=0.004) and sodium-potassium ratio (p<0.0005) were higher in the heterozygotes, contrary to expectations.
- Potassium excretion was higher four hours into the saline challenge in the heterozygotes (p=0.047), but overall potassium excretion was the same.

The saline challenge was performed on subjects who were normotensive or never treated mild hypertensives as many antihypertensives interfere with the RAAS, altering the response to saline and renin and aldosterone concentrations. This would also tend to reduce the differences in BP between the groups. The mean baseline BP was within the normal range (138±14/85±11mmHg for the heterozygous group and 136±21/82±13 mmHg for the normal group) and only two subjects in each group were found to be hypertensive. In addition vascular changes resulting from long standing severe hypertension were not expected to influence the response to the saline infusion or any baseline differences between the groups.

The baseline results in this experiment are very suggestive of innate overactivity of the ENaC and chronic sodium overload in the R563Q heterozygous group. Despite no difference in baseline sodium levels intake as determined by urinary sodium-creatinine ratio the baseline serum sodium was significantly higher and the plasma aldosterone concentration suppressed. The significantly lower pulse rate could be explained by reduced sympathetic and increased vagal tone due to stimulation of baroreceptors in response to sodium overload. Furthermore during the challenge the aldosterone levels were suppressed and the serum sodium level increased to a greater degree in the heterozygous group. The change in serum potassium was not significant during the saline infusion between the two groups. Neither was there a difference within each group between the serum potassium levels at the beginning and the end of the infusion. The total excretion of potassium was not significantly different between the groups over the four hour period. The sodium-potassium ratio was significantly higher in the subjects with the mutation, which is a reflection of the higher sodium excretion.

Contrary to our hypothesis the total sodium excretion was higher in the heterozygotes after saline challenge. On reflection, although the ENaC is the final determinant of sodium regulation by the kidney, it is important to note that only between 2-5% of sodium reabsorption is regulated by the ENaC. As discussed previously it appears that the R563Q subjects are chronically sodium overloaded and the increased sodium excretion in response to saline challenge could have occurred at a variety of other sodium regulatory sites in the kidney. For instance Gill et al. [1991] found that salt sensitive subjects had an increased urinary dopa (a metabolite of dopamine) excretion. Dopamine is known to cause natriuresis at the level of the proximal convoluted tubule

[Baum and Quigley, 1998]. Atrial natriuretic peptide (ANP) is also a natriuretic hormone than is increased in volume overloaded states [Guyton and Hall, 1996] with vasorelaxant effects [Bolli et al., 1987]. ANP inhibits sodium reabsorption, increases GFR, inhibits aldosterone and renin release, increases nitric oxide activity (another vasodilator) and inhibits the vasoconstrictor activity of angiotensin II [Feehally et al., 2007]. This suggests that ANP is an alternative mechanism controlling sodium homeostasis in patients with chronic sodium overload [Ferri et al., 1994]. However, neither of these parameters was measured and further studies are required to determine if the R563Q mutation influences dopamine and ANP response to a saline infusion. The use of lithium may have also help determine the site of sodium excretion in this experiment.

Volume or water excretion was different between the two groups. The heterozygotes were slower to respond to the saline infusion. The initial response in the heterozygous group was a steady increase in excreted volumes but by two hours the normal group had a similar output. When the saline infusion ceased the normal group decreased the volume excreted but the heterozygotes were slower to diminish the volumes excreted. By four hours the volumes excreted were significantly different; the heterozygotes had a higher volume excreted. However, the total volume excreted over the four hours and the fraction of the infused volume that was excreted was no different between the groups. These differences are of interest but remain unexplained in this experimental model, but may be due to an expanded intravascular volume as previously discussed or already increased number of sodium channels at the cell surface.

Heterozygous individuals had a non-significant higher mean SBP than the normal individuals. There was large intra- and inter-individual variation of BP which made it difficult to determine a significant difference in such a small sample. Furthermore, differences in BP in the acute setting have been difficult to determine, and has involved pre-loading with dietary sodium [Luft et al., 1991] or volume depleting the subjects with intravenous furosemide [Weinberger et al., 2001]. Higher BPs would be expected in an overactive sodium channel, but the BP did not increase or differ between the groups, with the saline infusion, but it is important to note this may not be the case if patients with hypertension were included in the sample. Alternatively to detect BP changes more extreme stressors such as sodium overload followed by volume depletion by furosemide may be required. In support of this it is noted that the analysis for blood pressure was underpowered and the differences that are observed may become statistically significant should there be higher numbers. It is also observed that all non-significant results were underpowered in this analysis.

CONCLUSION

There are several lines of evidence to support that subjects with the R563Q mutation have functional overactivity of the ENaC, in this experiment. Firstly, aldosterone, an important marker of volume status, was suppressed and, taken in conjunction with the significantly higher serum sodium levels and slightly lower serum potassium levels, supports the hypothesis that the ENaC with the R563Q mutation is inherently overactive but does not provide proof of this. Contrary to our expectations, urinary sodium excretion was higher in R563Q heterozygous subjects. This probably reflects that, in the face of chronic sodium overload, these subjects excreted sodium by other counter-regulatory mechanisms in the kidney.

CHAPTER 9

The R563Q Mutation Does Not Alter the Sodium Current in the *Xenopus* Expression System

INTRODUCTION

The association of the R563Q mutation with hypertension and the differences exhibited in the saline challenge are not sufficient to determine the isolated physiological effects of a mutation. Phenotypic variation, such as that seen in the saline challenge, could be as the result of a mutation in LD with the discussed mutation. The effect seen in the saline challenge could thus be the result of a mutation other than the R563Q mutation that is inherited with it. Thus, despite a mutation being only one of the differences in each individual's genome, it is important to determine whether the isolated mutation is associated with a difference in function which could then have an effect on phenotype.

The most commonly used method to determine a difference in activity of the sodium channel is by expressing the protein in *Xenopus* oocytes. This method is currently the most sensitive method available and has been used by many teams to show a difference in sodium channel activity when a mutation causes Liddle's syndrome [Schild et al., 1995; Hansson et al., 1995b; Tamura et al., 1996]. This method has not shown a difference in sodium channel activity for mutations that do not interfere with the PY motif [Ambrosius et al., 1999]. Unfortunately, this method does not illustrate the biochemical effects of changes to the protein and is purely a means of determining the change in sodium fluctuation through the channel.

METHODS

The contents of this chapter represent work that was organised and completed in Dr Stephen J. Tucker's Laboratory of the Oxford Centre for Gene Function, Department of Physiology, Anatomy and Genetics, Oxford, England. Dr Lijun Shang assisted with the measurements. The author was involved in the planning of the project and visited the Oxford Centre for Gene Function in order to observe and learn the technique, particularly the defolliculation and the electrophysiological measurements.

The Oxford Centre for Gene Function has ethical approval to use *Xenopus laevis* for this technique.

Oocyte Preparation

An adult female *Xenopus laevis* underwent a laparotomy under anaesthesia to remove the oocytes. After surgical removal of the oocytes she was killed in a humane way. Oocytes were treated with collagenase type II and manually defolliculated.

Mutant DNA Preparation

The mutated DNA sequence was introduced into the rat genome via mutagenesis of the β-ENaC subunit cloned into the pBF oocyte expression vector. Complementary RNA was synthesised *in vitro* and 5-8ng was injected into mature oocytes which were then incubated overnight in Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.40 mM $NaHCO_3$ and 20 mM HEPES (N-(2 hydroxyethyl) piperazine-N-2-ethanesulfonic acid) at pH 7.2).

Electrophysiology

The two electrode voltage clamp technique was used to measure the sodium current in a recording solution consisting of 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$ and 10 mM HEPES(NaOH) at pH 7.2. Sodium channel activity was assessed by measurement of the amiloride sensitive current. The amiloride sensitive current was the difference in sodium current at a membrane potential of -100mV in the recording solution with and without 5µM amiloride. Between five and eight oocytes from the same animal were used for each experiment and repeated with oocytes from a different animal. The mean current with standard error of the mean is recorded.

RESULTS

There was no significantly recordable difference in amiloride-sensitive sodium channel activity between the *Xenopus* oocytes with the wild-type and the R563Q variant of the β-subunit gene. The results are shown in Table 9.1 and illustrated in Figure 9.1.

Table 9.1. Sodium Currents in the Wild-Type and R563Q variant when expressed in *Xenopus* oocytes

	Wild type	R563Q Variant	Control (uninjected)	P value
Number	18	18		
Mean, µA	12.35	11.96	<0.2	0.104
Standard Error	0.89	0.93		
Blocked with 30µmol amiloride	>95%	>95%		

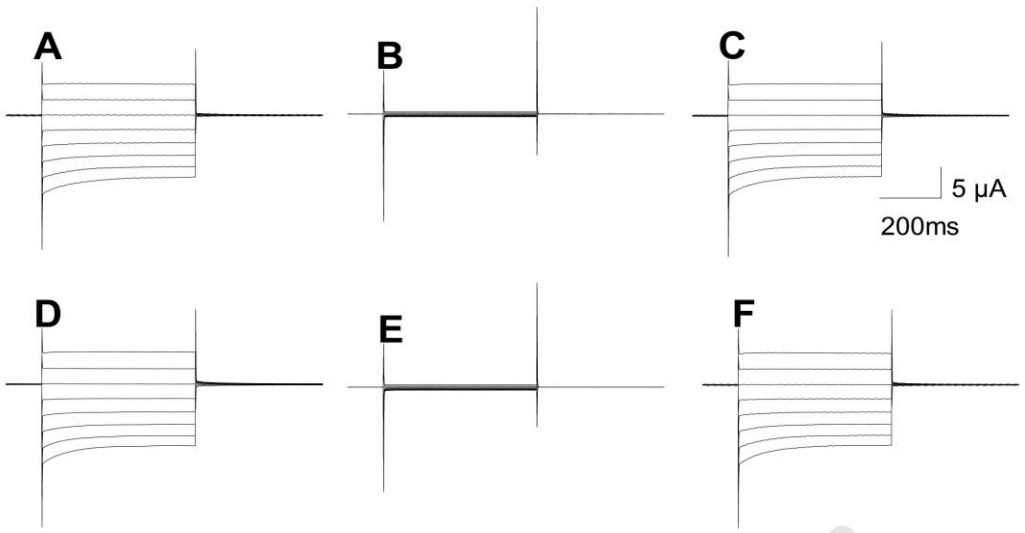

Figure 9.1. Currents detected. *A)* currents for wild-type ENaC. *B* and *C,* respectively, show blockade with amiloride and recovery. Similarly, *D, E* and *F* show currents for β-ENaC-R563Q.

DISCUSSION

The most commonly used technique to determine a difference in sodium current is expression in *Xenopus* oocytes [Valentijn et al., 1998] although this method has not been found to be helpful in determining differences in mutations that do not alter the PY motif [Schild et al., 1996]. In this study, the method did not show a difference in sodium channel activity, between wild-type oocytes and oocytes with the R563Q mutation. An activating mutation of the sodium channel could alter the sodium current in this method but this would probably only be seen if there was a large change in current, as is seen with the R566X mutation [Schild et al., 1995]. Thus it appears that the potentially more subtle effects of missense mutations, when compared with those that cause Liddle's Disease, are not as easily elicited using the available physiological techniques.

Expression of the ENaC in *Xenopus* oocytes is an accepted and well defined method to illustrate differences in ion channel activity. This method utilises the patch clamp technique to determine the direct effects of a mutation on the sodium channel. Patch clamping involves attaching a micropipette and sealing it to the cell membrane. (The micropipette usually contains a solution that is matched to the ionic composition of the solution in which the cells are incubated.) It is then possible to determine the current through the single ion channels in that patch of membrane. *Xenopus* oocytes do not express sodium channels, (the control sodium current was negligible at <0.2μA) so innate channel activity is not likely to influence the results. They do express Nedd4 [Staub et al., 1996], allowing internalisation of the channels that are inserted into the cells. In this experiment, both wild-type and mutant currents (which did not differ) were completely blocked by amiloride indicating that the recorded current was the result of the amiloride-sensitive ENaC.

Another benefit of the *Xenopus* expression system is that it permits the determination of interactions between proteins (such as ENaC and Nedd4-2 [Bhalla et al., 2005]). This creates the opportunity to determine if there is a difference in channel activity due to different protein interactions, such as SGK1 or S1A [Alvarez de la Rosa et al., 1999]. The *Xenopus* expression method may also permit the use of more than one mutation within a subunit. This would enable the use of a mutation with known effects, such as locking the channel in the open state, after which any changes would indicate a change in channel number. Use of more than one mutation with a subunit could also determine interactions between mutations.

There are some limitations to the expression system. Since mRNA needs to be injected into the oocytes to express the protein it is not practical to use this method for large numbers of oocytes, such as may be required to determine a subtle difference resulting from missense mutations outside the PY motif of the ENaC genes. A further limitation observed by Costa et al. [1994] is that it is not possible to change solutions quickly due to the vitelline membrane on the oocyte increasing diffusion time to the cell membrane, which decreases the sensitivity of the measurements when assessing the peak macroscopic and timing of currents. Furthermore, the large surface area of the cell decreases the reliability of the initial one to two milliseconds after a voltage change. Small changes in sodium channel activity are thus difficult to determine in this system (or in fact any system currently available).

The R563Q mutation lies just within the cytoplasm, following the second transmembranous domain. Current differences could be the result of a change in N, Po or the conductance through the channel pore (which is constant for sodium channels). The position of the mutation suggests that it is more likely to influence N rather than Po. Surface N is influenced by the insertion into the membrane, removal from the membrane and length of time that it remains within the membrane (half life). The *Xenopus* expression system is better able to determine differences due to changes in channel Po rather than N so this system is perhaps not an optimal method to determine differences due to the R563Q mutation.

Other techniques that have been used to determine differences in channel activity are summarised in Table 9.1. Ma et al. [2004] showed that B lymphocytes could also be used for patch clamp techniques and Snyder et al. [1995] labelled the channel with a

FLAG epitope in the extracellular domain and used an anti-FLAG antibody to show that β-R566X was found at higher concentrations at the cell surface. Bubien et al. [2001] showed that antibodies directed against the ENaC itself also show the increase in N in lymphocytes. They also showed that patch clamping of lymphocytes revealed an increase in sodium current in lymphocytes and an increase in Po of the channels. Intracellular abundance of channels has been shown by antibodies directed against each subunit and visualised using immunofluorescence [Loffing et al., 2000]. Baker et al. [1998b] described using nasal potential difference to illustrate the difference in sodium channel activity in a family with Liddle's disease. Nasal potential difference measurements are not sensitive enough to show the small differences that may be present in mutations that do not cause the full Liddle's phenotype. In fact, none of these methods may be able to show a difference in a cell with or without the R563Q mutation but it may be possible to increase the sensitivity of these experiments for the R563Q mutation by using interactions of more than two proteins, as described by Bhalla et al. [2005].

Table 9.2. Physiological Methods for Determining a Difference in Function

Method	Reference
Xenopus Oocyte Patch Clamping	Schild et al. [1995]
B Lymphocyte Patch Clamping	Ma et al. [2004]
FLAG labelling with Anti-FLAG antibody	Snyder et al. [1995]
Radiolabelled antibodies to the surface ENaC	Bubien et al. [2000]
Radiolabelled antibodies to the cytoplasmic ENaC	Loffing et al. [2000]
Nasal potential Difference	Baker et al. [1998]

All of the above methods describe the physiological effects *in vitro*. This is very useful to delineate interactions and individual mutations, but practically, a mutation is just one of many genetic differences within each individual and the effects of a mutation need to be assessed within the individual. The saline challenge enabled the illustration of the effects of the mutation *in vivo* (Chapter 8) and assessing the clinical

characteristics of the hypertensives helped to delineate the long term effects. As the expression technique above did not delineate a difference in sodium channel conductance between the wild-type channel and the channel with the R563Q mutation other evidence suggesting a difference in the protein were sought. Figure 9.2 illustrates that the R563Q mutation is in a highly conserved region of the β-ENaC. This arginine is preserved in all mammals, which indicates that this amino acid could be crucial to function or structure of the channel.

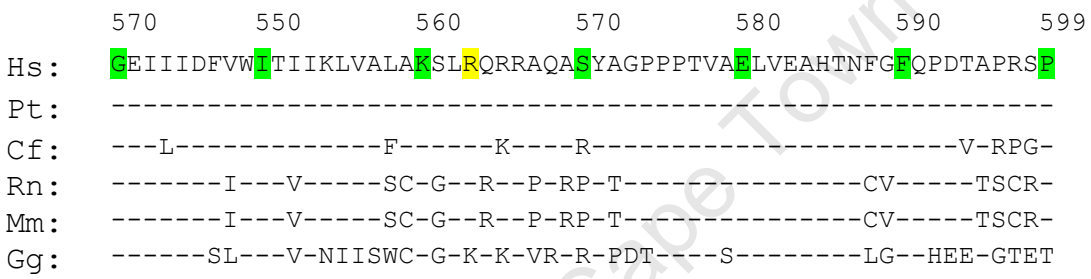

Figure 9.2. Sequence homology (synteny) in exon 13 of the β-ENaC between species. Amino acids that differ from the human sequence are shown. In *Homo sapiens sapiens* (Hs) the sequence includes amino acids from 570 to 599. The numbered amino acids are shaded in green and amino acid 563 in yellow. *Pt, Pan troglodytes* (chimpanzee); *Canis familiaris* (dog); Rn, *Rattus norvegicus* (rat); Mm, *Mus musculus* (mouse); Gg, *Gallus gallus* (chicken). (*Information from NCBI Sequence Viewer v2.0)*

Arginine is a positively charged amino acid and is often important in active regions of a protein. Arginine and lysine (both positively charged) usually maintain the charge balance of proteins. Of note, the deviation from arginine in the chicken is to lysine. This suggests that the presence of a positively charged amino acid in this position is essential for protein structure and function.

PIP_3 (phosphoinositol 3,4,5, trisphosphate) was shown by Pochynyuk et al. [2005] to interact with the γ-ENaC at the conserved positive amino acids just distal to the second transmembranous domain. This then regulates the signalling cascades of the γ-ENaC. The interaction of PIP_3 and γ-ENaC at these positive amino acids appear to influence channel gating rather than internalisation and mutations in this region influenced the sodium current. Interestingly, the amino acid that is particularly susceptible to this alteration is an arginine (in a similar place to the β-ENaC R563Q mutation). Other possible proteins that interact with the ENaC in this region are PKC and SGK1. These intracellular proteins are potentially capable of phosphorylating certain amino acids causing down regulation of the channels [Alvarez de la Rosa et al., 2004; Cui et al., 1997]. However, this remains to be shown.

Missense mutations may have an effect *in vivo* that is not easily reflected in *in vitro* experiments. Multiple factors influence BP within each individual, such as diet and genetics, as discussed earlier (Chapters 1 and 2). These factors may interplay with this mutation to make the heterozygous subjects more susceptible to hypertension. Other genetic mutations that could influence the effects of this mutation are yet to be determined as are the means of interaction.

CONCLUSION

It was however evident from these studies that this simple use of the *Xenopus* expression system did not show a difference in sodium conductance between wild-type and the R563Q variant of the β-ENaC.

CHAPTER 10

Interesting Cases

1) An R563Q Homozygous Patient

HISTORY

Patient X is a 54 year-old Coloured female, whose family originates from the Western Cape. Her father died at age 29 due to a haemorrhagic stroke. Her mother died at age 85 due to complications from hypertension and diabetes mellitus. One of her sisters died at age 57 due to complications from hypertension and diabetes mellitus, with chronic renal failure. One of her brothers has hypertension (and is known to the Hypertension Clinic) and the other has hypertension and diabetes mellitus. Most of her aunts and uncles died from the complications of hypertension and diabetes mellitus.

She had fairly well controlled hypertension for the last 15 years and was hypercholesterolaemic. She was taking 2.5mg indapamide, 4mg perindopril, 20mg simvastatin and 150mg asprin once a day to control her BP and decrease her cholesterol level. She has not had any surgical problems but has cervical spondylosis for which she took Arthrotec® on one occasion. She did not take over-the-counter medications and seldom has headaches. She has tried to improve her diet and usually eats chicken or fish, seldom red meat. She said she has a weakness for salt and often eats tinned foods.

EXAMINATION

General examination was normal. All pulses were present, regular and had good volume. There was no radio-femoral delay and no carotid bruit. The BP averaged

155/80 with a wide range (Table 10.1) on the automated monitor when she arrived at the clinic for assessment. There was no postural difference in BP or a difference between the right and left arms. The chest, cardiac, neurological and abdominal examinations were normal; the kidneys were not palpable, and there were no renal artery bruits. Fundoscopy revealed early silver wiring. The ECG revealed that she was in normal sinus rhythm with no signs of left ventricular hypertrophy. Standard laboratory investigations were normal and are shown in Table 10.1.

Table 10.1. Clinical and laboratory features for Patient X

Clinical Examination		**Laboratory Investigations**	
BMI, kg m-2	30	Potassium, mmol l^{-1}	4.2
Waist Circumference, cm	97	Creatinine, µmol l^{-1}	55
Neck Circumference, cm	33.5	Random Glucose, mmol l^{-1}	4.8
Mean SBP, mmHg	142	Random Cholesterol, mmol l^{-1}	4.8
Highest SBP, mmHg	175	Aldosterone, pmol l^{-1}	152.5
Lowest SBP, mmHg	155	ARR	10.1
Mean DBP, mmHg	72	Renin, mmol l^{-1}	15.1
Highest DBP, mmHg	86	Urine Creatinine, mmol l^{-1}	10
Lowest DBP, mmHg	80	Urine microalbumin, mg $mmol^{-1}$	15.4
Sokolow Lyon (ECG), mm	19	Microalbumin-creatinine ratio	1.5
Cornell (ECG)	1584		
ECG Axis, degrees	60		
Aortic Width, cm	3.6		
CTR	0.46		
Pulse, b min^{-1}	66		

The presence of the R563Q mutation was determined using standard PCR and RFLP methods as described in Chapter 3. The result was checked with sequencing and the PCR was repeated with different primers. Interestingly, all methods confirmed that this patient appeared to be homozygous (i.e. has two copies) for the variant allele. Figure 10.1 shows the sequence for a homozygous individual. The amplified region of exon 13 of the β-ENaC sequence (around nucleotide 110 in Figure 10.1) is CTACAG not CTACGG (normal). (Note there is a peak only for adenosine, no glutamine, i.e. homozygous.)

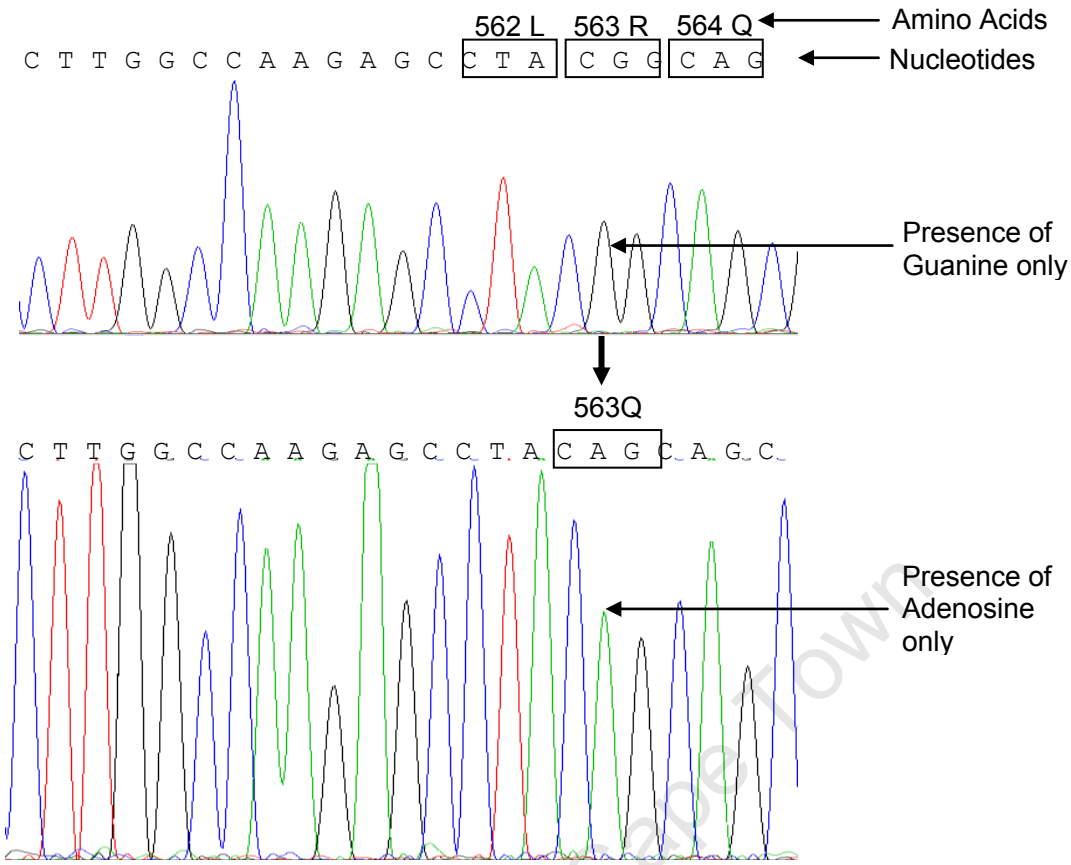

Figure 10.1. Sequence of the β-ENaC gene showing normal (above) and the homozygous transition (bold arrow) causing the R563Q mutation

After Patient X came to the Hypertension Clinic amiloride was added to her antihypertensive regimen, as her SBP was not sufficiently controlled (it reached 175mmHg). She was also given dietary and lifestyle advice. Six months later she reported that she has been receiving amiloride from the GSH pharmacy and her BP had settled at 128/80.

DISCUSSION

This case study shows the presenting characteristics of the first identified homozygous person with the R563Q mutation examined at the Hypertension Clinic. Interestingly she did not differ phenotypically from patients who are heterozygous for the mutation. Her aldosterone and renin levels were not markedly suppressed, but she

was on a diuretic and an angiotensin-converting enzyme inhibitor. Both these agents would tend to interfere with the renin and aldosterone levels. This makes the aldosterone level unreliable in assessing the effects of the mutation on the RAAS. Also, both agents alter potassium handling so the potassium level is not a true reflection of sodium channel activity.

This case study presents the features of an individual homozygous for the R563Q mutation which has been associated with hypertension in the heterozygous form. It appears that Patient X has inherited two copies of the variant allele as no normal copy was seen. While this is the likely scenario, in the absence of data from her biological parents it is possible to surmise that alternatively she may have inherited an allele with a large deletion spanning the entire amplified region which would result in only one variant of the channel (the one with the R563Q mutation). Importantly, it was predicted that the homozygous form would present as a more extreme case of hypertension with hypokalaemia. She appeared not to. The only characteristic that this subject presents with is a suppressed aldosterone level. As she did not stop her antihypertensive treatment before assessment the aldosterone and potassium results are poor reflections of sodium channel activity but stopping her medication would not be ethical unless in a tightly controlled environment. Ambrosius et al. [1999] found nine subjects homozygous for the β-G442V mutation. They were all hypertensive which resulted in the association of this mutation with hypertension in this study group. Unfortunately that is all the information that is given on these homozygous subjects, and there are few discussions of the effects of heterozygosity versus homozygosity on phenotype for the ENaC in the literature.

After four months on 5mg amiloride Patient X's BP was within the normal range. She reported no symptoms of a postural drop in BP or any side effects possibly related to the amiloride.

This case study further illustrates the difficulties of associating a mutation *in vivo* with biophysical properties and shows that the R563Q mutation is compatible with life in the homozygous form.

2) The R563Q mutation causes Liddle's syndrome in pregnancy

INTRODUCTION

Liddle's syndrome presents with hypertension, hypokalaemia, metabolic alkalosis and low renin and aldosterone levels. The full phenotype is not generally recorded in subjects with the R563Q mutation. However two cases of Liddle's syndrome during pregnancy are described. The presence of the full phenotype is suggestive that the R563Q mutation results in an overactive sodium channel.

1) Liddle's Case 1

Liddle's Case 1 was referred to the GSH Hypertension Clinic from the Cardiac Clinic. She was 36 years old, unemployed and not married. She did not smoke or consume alcohol. Her parents live in the Eastern Cape. Her mother was alive and well, her father had diabetes mellitus and hypertension and her sister also had hypertension and diabetes.

She was diagnosed with gestational hypertension when she presented in August 2004 at seven months pregnant to the ante-natal unit at GSH. She was found to have a

potassium level of 2.1mmol/l and an aldosterone level of 73pmol/l. She also had atrial tachycardia and evidence of peripartum cardiomyopathy, and was referred to the Cardiac Clinic. Her potassium was aggressively supplemented and she was treated for cardiac failure. Amiloride 5mg was added because of suspected Liddle's syndrome, and later she was confirmed to be R563Q heterozygous. She was later assessed at the GSH Hypertension Clinic when she was found to be well and taking all her medication (bromocriptine 2.5mg, enalapril 10mg and atenolol 50mg twice a day and amiloride 5mg and nifedipine 30mg daily), and her baby was thriving. The peripartum laboratory results are shown in Table 10.2 2 confirm the presence of Liddle's syndrome with severe hypokalaemia in the face of highly suppressed renin and aldosterone concentrations in the absence of liquorice use.

Table 10.2. Liddle's Case 1: Blood Results from the time of presentation

Date (2004)	**08/08**	**08/09**	**08/12**	**08/13**	**08/14**	**08/23**	**11/15**
Sodium (mmol l^{-1})	136	135	137	143	138	134	143
Potassium (mmol l^{-1})	**2.6**	**2.9**	**3.1**	**4.5**	**3.3**	**5.9**	**3.6**
Urea (mmol l^{-1})	2.2	2.2	2.3	3.9	5.1	11.2	
Creatinine (µmol l^{-1})	58	49	43	57	60	112	
Glucose (mmol l^{-1})			4.2			5.1	
Cholesterol (mmol l^{-1})						7.9	
Uric Acid (mmol l^{-1})	0.39	0.5				0.74	
Calcium (mmol l^{-1})	2	2.04					
Magnesium (mmol l^{-1})	0.81	0.72					
Phosphorus (inorganic) (mmol l^{-1})	1.37	1.49					
Total Bilirubin (µmol l^{-1})	11	9					
Alanine Transaminase (U l^{-1})	9	9					
Aspartate Transaminase (U l^{-1})	25	26					
Lactate Dehydrogenase (U l^{-1})	1092	979					
U-Potassium (mmol l^{-1})	19.1						
U-Creatinine (mmol l^{-1})	4						
Iron (µmol l^{-1})			22.8				
Transferrin (g l^{-1})			2.4				
Transferrin saturation (%)			38				
Ferritin (µg l^{-1})			326				
CRP (mg l^{-1})			23.8				
Haptoglobin (g l^{-1})			0.92				
Aldosterone (pmol l^{-1})			**73**			524	
Renin (mmol l^{-1})			13			1665	
Aldosterone-Renin Ratio			5.6			0.3	

On Examination she was found to have a BMI of 26.6kg/m2. All pulses were present and equal at a regular 60b/min. Her BP was low, at 90/60mmHg and she did not have pedal oedema. She did not have any signs of congestive cardiac failure but her apex beat was pressure overloaded and there was a fourth heart sound. Chest, abdominal and neurological examinations were normal but she did have grade II hypertensive retinopathy. Her ECG showed that left ventricular hypertrophy and left atrial enlargement were present, but there were no repolarisation changes. Se also had an echocardiogram which showed a thickened left ventricle with poor contractility and ejection fraction. Mild mitral and tricuspid regurgitation were present.

Due to her low BP the nifedipine was stopped and the atenolol was decreased to 50 mg daily. On 15/11/2004 she retuned for follow up at the Hypertension Clinic. She was found to be heterozygous for the R563Q mutation and her BP was 120/100mmHg. Her pulse was 120b/min and regular and she was generally doing well and did not have any problems. She wished to return to the Eastern Cape to her parents and so was discharged to Cecelia Makiwane Hospital.

2) Liddle's Case 2

Liddle's Case 2 was a 25 year old Xhosa female who had previously been well. She had had two pregnancies in which she was found to have pregnancy induced hypertension and had had one miscarriage. She was pregnant (42 weeks) at the time of referral to GSH in 2001 and had recently developed gestational hypertension without proteinuria. The BP and biochemistry surrounding her delivery are recorded in Table 10.3. The results are highly suggestive of Liddle's syndrome – hypertension,

hypokalaemia, and suppressed renin and aldosterone, and she was not taking any liquorice-containing agents.

Table 10.3. Liddle's Case 2: BP and biochemical parameters surrounding the delivery

Date	4/03	5/03	6/03	7/03	8/03	9/03	10/03	11/03
BP (mmHg)	130/90	120/80	150/100	160/110	160/82	160/90	170/100	150/90
Clinical Event			**C/sxn**	**endometritis**				
Sodium (mmol l^{-1})			139	145	142	142	147	143
Potassium (mmol l^{-1})			2.1	2.5	2.7	2.8	2.7	2.8
Urea (mmol l^{-1})			1.2	1	1.2	1.4	1.1	1.2
Creatinine (µmol l^{-1})			55	61	50	47	47	
Aldosterone (pmol l^{-1})				101				
Renin (mmol l^{-1})				<0.1				
ARR				>1000				

Footnote: C/sxn, caesarean section

Induction of pregnancy was not successful and she underwent a caesarean section on 6 March after which she developed endometritis which was treated with antibiotics. During this time she developed profound hypokalaemia which did not respond to potassium supplements. She died suddenly at home shortly after discharge, possibly due to cardiac arrhythmias related to hypokalaemia. Due to the interest in this case she was investigated further and found to be heterozygous for the R563Q mutation.

DISCUSSION

These two cases that presented with Liddle's syndrome were both heterozygous for the R563Q mutation. The explanation for the full expression of the phenotype is speculative, but it is known that progesterone has a similar structure to aldosterone and has some mineralocorticoid activity. The concentrations are markedly increased in pregnancy with levels reaching 320-700nmol/l in the third trimester. (The normal ranges depend on the stage of the menstrual cycle but range from 0.6nmol/l to

110nmol/l.) The pathway for progesterone and aldosterone production is shown in Figure 10.2.

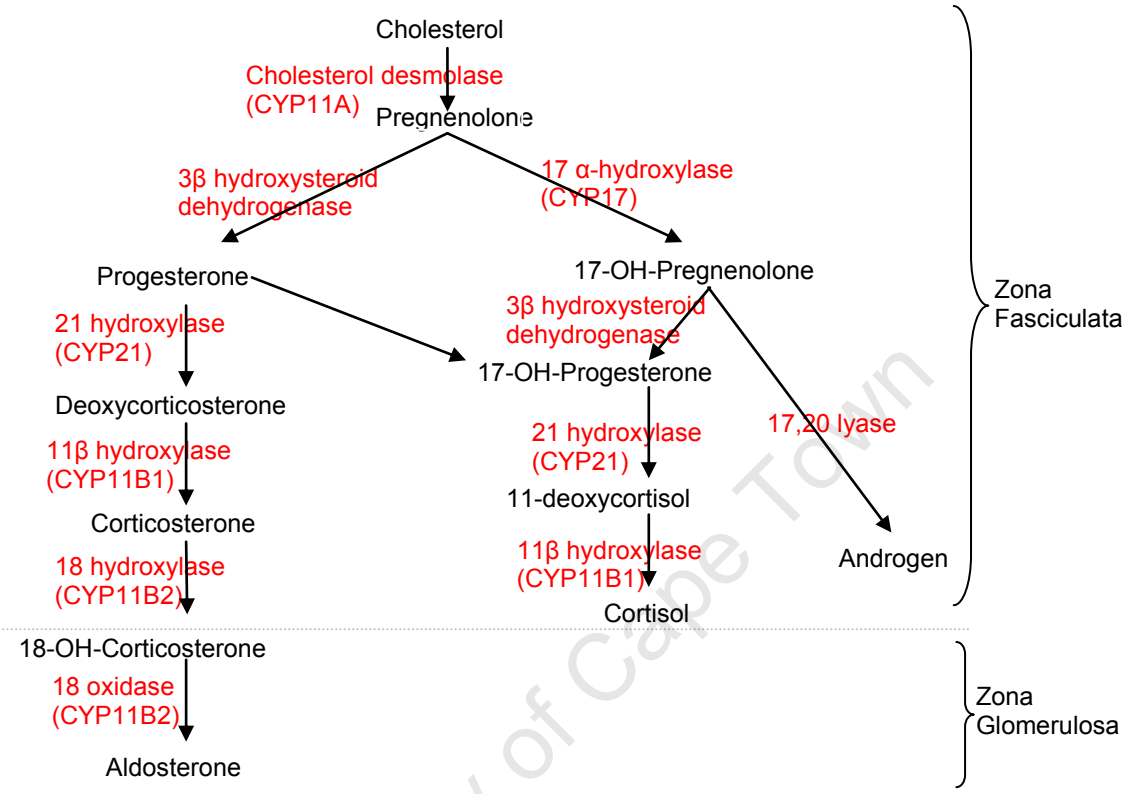

Figure 10.2. Steroid production from cholesterol in the adrenal gland (Enzymes required are highlighted in red.)

Progesterone has a high affinity for the mineralocorticoid (MR) receptor, higher than aldosterone, but the agonist effect of progesterone on the MR receptor is only 27% of the effect of aldosterone on the MR receptor [Quinckler et al., 2002]. Thus progesterone, at high concentrations, effectively blocks the MR receptor from aldosterone, having a comparative antagonistic effect on the MR receptor. This antagonistic effect is probably countered (during pregnancy) by the potent inhibition of progesterone on 11-β-hydroxysteroid dehydrogenase type 2 (11-β-HSD-2)

[Quinckler et al., 1999] which will result in a build up of cortisol, which has agonist effects on the MR receptor (see Figure 1.1) [Feehally et al., 2007].

The S810L mutation of the MR receptor alters the conformation of the receptor making it sensitive to the effects of progesterone [Geller et al., 2000], exacerbating hypertension during pregnancy. Rafestin-Oblin et al. [2003] showed that cortisone and 11-dehydrocorticosterone bound to and activated the MR receptor with the S810L mutation resulting in hypertension. This suggests that progesterone could be involved in the hypertension associated with this mutation because of the inhibition of 11-β-HSD-2. The difference in MR receptor and progesterone interaction during pregnancy could explain why these two female patients with the R563Q mutation presented with Liddle's syndrome. Aldosterone is innately suppressed in subjects with the R563Q mutation (see Chapter 4) which would usually tend to up-regulate the receptor, making it sensitive to stimulation by mineralocorticoids, glucocorticoids and progesterone. High concentrations of progesterone would also result in high concentrations of progesterone metabolites, like 11β-hydroxyprogesterone which has been found to act as a mineralocorticoid [Rafestin-Oblin et al., 2002]. Together, these factors would result in an overactive MR causing increased transcription of the ENaC subunits which may be poorly down-regulated due to the R563Q mutation. This hypothesis is yet to be shown physiologically but these two cases do provide further evidence that the R563Q mutation is an activating mutation. However the occurrence of the R563Q mutation and Liddle's syndrome is not universal as in the study by Dhanjal et al there were no cases of Liddle's syndrome occurring in pregnant patients with the mutation. The reasons for the differences in phenotypic expression in pregnancy are not readily explained.

3) R565Q: A Novel Mutation of the ENaC causing Liddles's Syndrome

Patient Y

Patient Y was a 65 year old Caucasian male who was referred to the Hypertension Clinic with uncontrolled hypertension and hypokalaemia. He was diagnosed with hypertension in 1950 when he was 20 years old but the first time he was noticed to have profound hypokalaemia was in 1998 when he was 58 years old. He used to smoke (for 25years) but never consumed alcohol and ate a normal healthy diet. There was no family history of cardiovascular disease or hypokalaemia. He had been treated with 100mg spironolactone daily and 100mg losartan for his BP and had been taking up to 10 tablets of Kloref®, each one containing 6.7mmol/l of potassium when dissolved in 500ml of water, to try maintaining normal serum potassium concentrations.

Recently, Patient Y had been breathless, with low energy levels and proximal muscle weakness. He had had a recent episode of atrial flutter, at the same time his BP was noted to be 236/138, and he was treated with adenosine. His potassium level was noted to be 4.1mmol/l at this time. When he was seen at the Hypertension Clinic his pulse was 75bpm and his BP was 190/90mmHg. There was no pathology related to hypertension but his neurological examination revealed generalised muscle weakness that was subsequently diagnosed as chronic demyelinating polyradiculopathy. At this time his potassium and aldosterone levels were within the normal range (Table 10.4). He also had an abdominal CT scan which was normal. However, due to poor BP control and previous profound hypokalaemia he was referred for investigation for sodium channel mutations. The R563Q mutation was not found in this patient but he was heterozygous for a novel mutation of the 565th amino acid. The mutation found

was a guanine to adenine transition resulting in conversion of arginine to glutamine (R565Q). The nucleotide change is shown in Figure 10.3, and is two amino acids away from the R563Q mutation.

Table 10.4. Laboratory findings for Patient Y

	Patient Y	Normal Ranges
Potassium, mmol l^{-1}	4.5	3.3-5.3
Sodium, mmol l^{-1}	138	135-147
Cholesterol, mmol l^{-1}	4.7	3.3-5.3
Aldosterone, pmol l^{-1}	297	255-860
Renin, mmol l^{-1}	38	5-47
Aldosterone-Renin ratio	7.8	<70
Calcium, mmol l^{-1}	2.28	2.05-2.56
Phosphate, mmol l^{-1}	0.96	0.8-1.4
Magnesium, mmol l^{-1}	0.76	0.65-1.1
Alkaline phosphatase, mmol l^{-1}	31	40-120
Total protein, g dl^{-1}	63	60-85
Albumin, g dl^{-1}	38	35-52
Urine albumin-creatinine ratio	0.4	<0.02

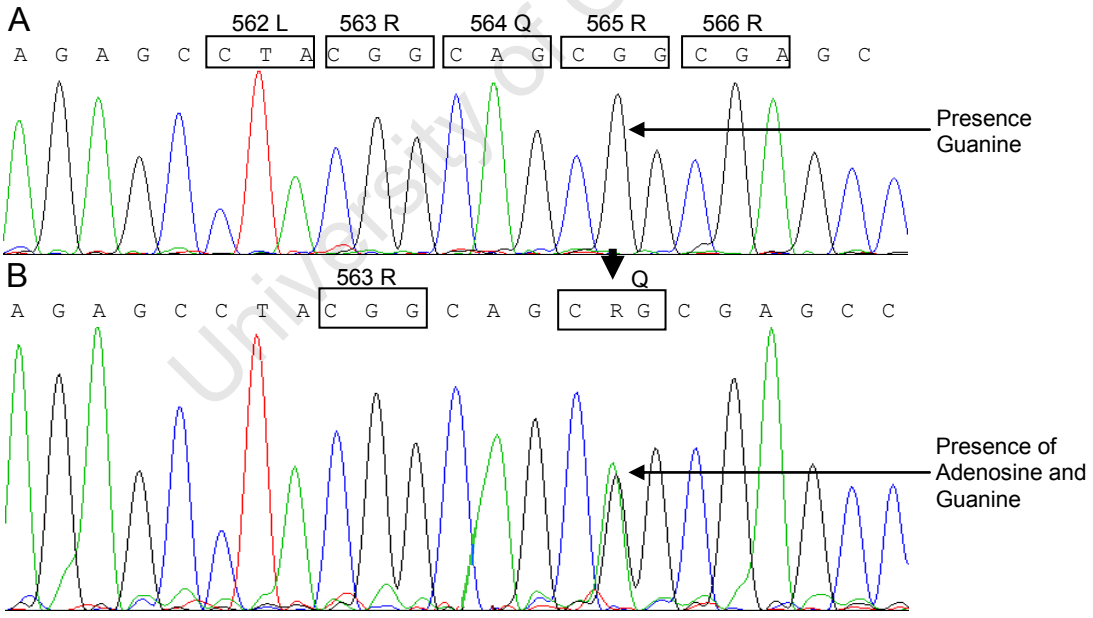

Figure 10.3. Sequence for Patient Y showing the G>A transition of base 1694 (B) from the normal sequence (A).

The finding of the mutation in the sodium channel prompted the initiation of amiloride in this patient in 2008 which normalised his potassium and BP without the need for spironolactone or potassium supplements.

DISCUSSION

This patient presented with un-investigated severe hypokalaemia and uncontrolled hypertension at the age of 65 years. On amlodipine, spironolactone and losartan his aldosterone level was normal (297pmol/l), which essentially excludes primary aldosteronism and supports the diagnosis of Liddle's syndrome. The patient was started on amiloride when he was found to have a mutation in the β-subunit of the sodium channel. The R563Q mutation was not found in this patient as was expected, because it has not previously been reported in subjects with only Caucasian ancestry.

The mutation that this patient has is two amino acids downstream of R563Q mutation. This region of the β-ENaC is highly conserved in mammals (see Figure 9.2, Chapter 9) and mutations in this region may result in phenotypic differences. Finding the R565Q mutation in a patient with full blown Liddle's syndrome lends support to the hypothesis that the R563Q mutation may cause functional changes in the ENaC. It is interesting that the amino acid substitution is the same as that of the R563Q mutation. The R565Q mutation also results in the change from the basic amino acid to a neutral amino acid which has the potential to alter the three dimensional configuration of the channel changing the degradation of the channel by certain processes, as previously discussed.

PART 4

CONCLUDING REMARKS

The Serengeti Plains

A scarce African landscape across which people and animals have had to migrate

CHAPTER 11

General Discussion and Synopsis

SYNOPSIS

In this thesis a total of 2440 subjects were assessed for the presence of the R563Q mutation, either by personal examination or by accessing their records. 1057 of these subjects were genotyped for the R563Q mutation by restriction enzyme digestion or sequencing of the β-ENaC for the purposes of this thesis. The remainder (1383) had previously been assessed. Forty five of these individuals were included in the family study (Chapter 4) in which 22 were families visited. Forty four were included in the clinical assessment (Chapter 7). Twenty four of the 1057 subjects selected for this thesis gave consent for a saline infusion (Chapter 8) to assess the response of the sodium channel. The search for the origin of the mutation took the author across South Africa. Subjects were recruited from Johannesburg, Cape Town and the Northern Cape and represent multiple population groups: Caucasians (178), Coloured (829), Khoisan (167), Sotho (261), Venda (17), Xhosa (424) and Zulu (519). Five subjects were homozygous for the R563Q mutation, 167 were heterozygous and one new mutation (R565Q) was discovered. The author further collaborated with a team in England to assess the function of the mutated protein.

1. HYPERTENSION

a) The R563Q mutation was significantly more common in the hypertensive population of Coloured or Black African ancestry compared with the normotensive controls (Chapters 4 and 5).

b) Within families the mutation strongly associated with both hypertension and blood pressure levels (Chapter 4).

c) The BP was significantly higher in the positive group when pooling all R563Q positive and normal subjects, either normotensive or untreated hypertensive (Chapter 6).

d) In the small Khoisan sample the R563Q mutation was extremely common but did not associate with hypertension, presumably because of their low sodium diet. The R563Q mutation may have offered a survival advantage in the arid areas of South Africa where sodium was a scarce resource (Chapter 6).

e) Within hypertensives the R563Q mutation was associated with an increased risk of CKD compared with comparable age and sex matched hypertensive controls (Chapter 7).

f) R563Q hypertensives treated with amiloride had a significant and clinically relevant improvement in BP levels (Chapter 7).

g) The R563Q mutation was not associated with the Liddle's phenotype except in two cases during pregnancy (Chapter 10). The mutation was previously associated with hypertension during pregnancy [Dhanjal et al., 2006].

Thus the association with hypertension has been considerably strengthened by this thesis and the effect appears to be clinically relevant with a significant BP response to amiloride (a specific inhibitor of the ENaC). Furthermore, the previous observations that the mutation does not cause full blown Liddle's disease is confirmed apart from isolated cases during pregnancy. The possible association with CKD deserves further study as it may be an explanation for the higher prevalence of CKD in indigenous populations.

2. ORIGINS

Evolution allows for selection of variants that will improve survival. A mutation in a gene that alters function will not be sustainable if it decreases an individual's ability to survive or reproduce. If, however, a mutation has no affect on the individual's survival or reproductive ability then the mutation will not be favoured and will not increase in frequency. Only if the mutation improves survival will it be selected for and will increase in frequency in further generations. The ability for a mutation to alter survival will depend on the environment in which the individual lives. If the mutation improves survival in one environment, it may decrease survival in another environment with differing circumstances. The R563Q mutation illustrates this point by being associated with hypertension in the urbanised Black African and Coloured groups but not in the Khoisan population.

The R563Q mutation has been found in Black African and Coloured groups in South Africa, who both have Khoisan genetic admixture. The mutation has not been found in the Black African population that has been screened for in London [Hartmann, 2007] who are usually migrants from central and northern Africa. This suggests that the mutation did not originate from the Black African population from central Africa and strongly suggests that this mutation arose in the Khoisan. This is a plausible hypothesis as the Khoisan often lived in extremes of salt scarcity where an intrinsically active ENaC would provide a survival advantage.

In the Khoisan the R563Q mutation is found at a high frequency (see Chapter 6). The lower sodium excretion illustrates that they had a lower dietary intake of sodium. This could account for the lack of difference in the BP and sodium and potassium levels, as

could the small sample size. The frequency of the mutation also warns that hypertension could be a serious problem in these populations as the sodium intake increases with the westernisation of Africa.

3. FUNCTIONAL STATUS

a) This thesis has demonstrated strong circumstantial evidence for intrinsic over activity of the β-ENaC in subjects with the R563Q mutation. This missense mutation is found in what is generally considered the unconserved region of the carboxy terminal. While this region is not part of the highly conserved PY motif it does contain certain amino acids that are conserved across the species, including the arginine at position 563 (Chapter 9), suggesting that it is important for structure and function.

b) The saline challenge (Chapter 8) supports the hypothesis that the R563Q mutation increases the activity of the sodium channel. Those with the mutation had intrinsically higher serum sodium levels and suppressed aldosterone levels which suppressed to a lower level during the challenge, suggesting an innately overactive channel. An overactive sodium channel would result in an increase the activity of control mechanisms, (e.g. Na+/K+ ATPase being stimulated by dopamine which is increased with higher concentrations of sodium [Pestana et al., 2001; Vieira-Coelho et al. 2000]) in order to ensure normal homeostasis. This is illustrated by the normal potassium levels and increased sodium excretion.

The BP were not different between the groups, which is not surprising as the small changes in BP in an acute setting usually vary widely between individuals making it

difficult to detect a change. This has previously been observed by Weinberger et al. [2001]. The saline challenge did not prove that the mutation increases the activity of the sodium channel but did illustrate the effects of the mutation in its normal environment. This is important to improve our understanding of the effects of the R563Q mutation.

c) This thesis was unable to prove that the mutation resulted in intrinsic over activity of the channel as the oocyte expression system used in this study did not show that cells expressing the sodium channel with the R563Q mutation had a different sodium conductance when compared with the wild-type channels. This method is a basic method with limitations as discussed in Chapter 9. A more extensive analysis would be required to assess the physiological effects of this mutation completely.

d) The Khoisan data are interesting in that there was an isolated decrease in aldosterone levels in the subjects with the mutation. This is interesting as an increase in activity of the channel could only disturb the aldosterone level as the sodium and potassium balance is essential for cellular function and disturbances would not be homeostatically acceptable, so a multitude of mechanisms are in place to ensure these electrolytes are within the normal range.

e) The presence of Liddle's disease presenting in two pregnant patients with the R563Q mutation is further evidence that the mutation alters the activity of the sodium channel. During pregnancy there is an excess of progesterone which activates the mineralocorticoid receptor, albeit at a lower efficacy than aldosterone, and thus has the potential to create an environment where there is excessive activation of the

ENaC. In susceptible patients, with impaired down-regulation of the channel or increased up-regulation of the ENaC, this could impair sodium homeostasis.

IMPORTANCE AND RECOMMENDATIONS

This thesis challenges the current understanding of the genetics of hypertension and tends to support the hypothesis proposed by Platt [1947]. There is evidence in this thesis that the R563Q mutation is a single genetic defect that is the major contributor to hypertension in these subjects (Chapter 4). Mendelian forms of hypertension have been described but do not account for the majority of EHT, and are influenced by environmental factors. The R563Q mutation is found frequently in resistant hypertensives (Chapter 5) and so could account for a major proportion of EHT in South Africa. This is contrary to the current teaching of hypertension as a multifactorial disorder influenced by multiple genes and environmental factors.

The major importance of this thesis is the impact it could have on the policies in Hypertension Clinics in South Africa. A major result of the findings in this thesis is the opportunity it could create to improve treatment plans for hypertensive individuals heterozygous for the R563Q mutation. The observation that amiloride improves BP in patients from the Hypertension Clinic at GSH suggests that amiloride is the treatment of choice in these patients (Chapter 5). However, further studies are recommended before such a policy is put into practice. It is recommended that, once the presence of the mutation has been determined in hypertensive patients, careful monitoring for CKD is implemented. It would also be advised that young family members of patients with the mutation, who develop hypertension, are screened for the mutation. If the

mutation is found in a pregnant woman there should be careful monitoring for the development of Liddle's syndrome and hypertension in pregnancy.

A further important finding, resulting in a change in clinical practice, is that the R563Q mutation is found in multiple ethnic groups in South Africa and is associated with hypertension within these groups (Chapter 5). These findings suggest that it would be recommended to screen patients referred to Hypertension Clinics for resistant hypertension of unknown origins in all major centres in South Africa.

It is further recommended that lifestyle modifying behaviour is taught to the Khoisan who have been found to succumb to lifestyle disorders on exposure to environmental stressors. Previously the Khoisan groups have been found to have a low prevalence of the disorders of lifestyle [Nurse and Jenkins, 1977] but this appears to be changing and they may be a highly susceptible group due to a difference in adaptive requirements.

Lifestyle modifying behaviour should be taught in all the population groups in South Africa because polymorphisms such as the R563Q mutation are found more frequently in the Black African groups. This creates a genotype more susceptible to interaction with environmental factors, such as sodium intake, that can alter phenotype.

FURTHER RESEARCH

This project contributes considerably to strengthening the association and linkage of the R563Q mutation to hypertension. It does not however provide robust

physiological evidence of the increased activity of the mutation. There are a few further areas that could be explored to try and show physiologically and biochemically that this mutation does actually affect the activity of the channel.

Function

1) Physiologically, the activity of the ENaC can be illustrated by expressing the channel in *Xenopus* oocytes [Schild et al., 1995]. This method has been employed as an estimate of the function of the channel with and without the R563Q mutation in Oxford, using rat DNA. There was no direct obvious effect of the mutation using this crude method (Chapter 9). However this method could be extended and an interaction with hormones could be determined. The channel could be expressed with the R563Q mutation in human DNA and with an alanine instead of the arginine or glutamine. (Arginine is a positively charged amino acid and glutamine is a negatively charged amino acid whereas alanine is neutral.) The charge of the amino acids could affect the three dimensional configuration of the protein and thus the activity and interaction with other proteins.

Expression of the channel with and without the mutation and some of the proteins that are known to influence channel activity (such as SGK1 and S1A) may show a difference in interaction with these proteins. There may be more subtle differences that are not visible with the current methodology.

2) The functional expression techniques may not be able to show a difference but it could be possible to see a difference when using imaging techniques [Blazer-Yost et al., 2004]. Immunofluorescence could be used to show a difference in channel number

at the cell surface or show a difference in channels that are contained within the cell. Direct imaging has the advantage of visualising the difference within the cell expressing the protein, but can still be difficult to show subtle differences.

3) Another method of showing a difference in interaction with different proteins is the yeast two hybrid system [Jahn et al., 1997]. This system uses a yeast cell with a library of the complementary DNA of the hormones that are available in the cortical collecting duct cells. The ENaC DNA then would be inserted into the yeast cell. After incubating the yeast cell any proteins that interact would bind together.

This method can determine if there is a difference in the interaction of two proteins (e.g. the ENaC and S1A) or it can find out if there is an interaction with various unknown proteins. This method is not able to determine a change in function due to a mutation but it could determine if there was a difference in binding ability between proteins and the ENaC with or without the R563Q mutation.

4) Crystallisation techniques have been used to show a difference in protein configuration leading to an alteration in function. The sodium channel has not been crystallised but if it were it could be possible to show a difference in protein configuration between the normal and the protein with glutamine at position 563. Many efforts have been made to crystallise the protein and the closest result is crystallisation of the acid-sensing-ion channel [Jasti et al., 2007]. (This channel is related to the ENaC.) Unfortunately, when this channel was crystallised the intracellular portion was not part of the crystallisation process. The R563Q mutation is found in the intracellular carboxy-terminal domain which means that the

intracellular portion would need to be crystallised. This method is, thus far, not a feasible option, as many teams have been trying to crystallise the ENaC.

5) The response to a specific inhibitor of the sodium channel could be elicited. There are two drugs available to humans that are known to inhibit the sodium channel: amiloride and triamterene. Triamterene was used by Liddle [1963] to treat his original patients. They responded well to triamterene when included with a low sodium diet. Amiloride was shown to control BP in patients in London with the T594M mutation of the β-ENaC [Baker et al., 2002]. The T594M mutation was not shown to be associated with hypertension in South Africa [Nkeh et al., 2003] and so is not routinely determined in the hypertensive patients at GSH. The R563Q mutation has repeatedly been associated with hypertension but amiloride is not registered in South Africa and so is not available, except for a few patients with the R563Q to treat their hypertension. A formal controlled clinical trial is warranted to assess the response to amiloride in patients with the R563Q mutation. This could strengthen the association and could enable registration of amiloride in South Africa, so improving the treatment of hypertension.

6) The association of the mutation with CKD seen in Chapter 7 is not completely explained in these data. Further research into the association with CKD using prospective analyses is warranted. The possible mechanism for this association could be determined by ongoing clinical assessments of the patients and by kidney biopsy. The clinical assessments need to be initiated before the onset of hypertension and CKD.

Origins

The frequency with which this mutation is found in the Khoisan groups assessed suggests that this mutation originated from this population. This could well have been an adaptation to the harsh environment in which they lived. However the frequency only suggests the origins and more evidence would be required to prove that this mutation did in fact originate from this group.

Two methods are a possibility to strengthen the hypothesis:

1) This mutation could be compared with the frequency of the clades within the mitochondrial DNA and Y chromosome, similar to the comparison by Lane et al. [2002]. The Y chromosome and mitochondrial DNA illustrate some of each individual's genetic background. The clades that are known to be from the Khoisan are the L1d and L1k clades from the mitochondrial DNA [Salas et al., 2002]. If there is a strong association with these clades and the R563Q mutation it could suggest that this mutation did originate from the Khoisan. This method may also show an association of the R563Q mutation with either the Khoikhoi or the San.

2) Another method is to look at the microsatellite markers in the β-ENaC gene itself, such as the *Alu* insertion polymorphisms discussed by Stoneking et al. [1997]. Similarities between the haplotypes with the mutation between the Xhosa, Zulu, Coloured and Khoisan could also suggest that this mutation originates from the Khoisan.

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APPENDICES

Appendix I: Quantitative Transmission Disequilibrium Testing

The transmission disequilibrium test was developed by Spielman et al. [1993] as a direct test of linkage between a marker and a disease in a population which already shows association. The test uses the genetic data from an affected offspring and heterozygous parents. It tests the number of times the heterozygous parents transmit a marker or the alternate allele to an affected child. One of the strengths of the test is that it does not require data from unaffected siblings or other affected family members.

It is a form of X^2 analysis testing the significance of linkage between a disease and a genetic marker. The pure X^2 test is inadequate in a situation where there is a lack of independence between individuals, i.e. family members. This analysis will not show a significant result in a situation where there is association without linkage.

In diseases that are of later onset the TDT is inadequate as parental data are usually not available. Spielman and Ewens [1998] modified the TDT to the sib TDT (S-TDT) to allow comparison between affected and unaffected siblings. S-TDT requires that there is at least one affected and one unaffected sibling and that there is a difference in marker status between the siblings. This enables the test to determine if there is a difference in marker frequency between affected and unaffected siblings, but requires larger numbers than the TDT.

The TDT was designed for dichotomous traits. However, many disorders are assessed as continuous traits (e.g. BP for hypertension) and, in general, quantitative phenotypes are more informative than dichotomous traits, providing stronger statistical evidence of association in linkage studies.

The QTDT analysis [Abecasis et al., 2001] allows the use of continuous variables in family based association studies. It does not require the parental genotypes. It can compare within family and between family effects. The QTDT assesses linkage, LD and population admixture from all offspring. It does this to model the phenotypic similarities that are common in the family data. The test is not affected by confounding variables like population stratification, and can include nuclear families of any size and composition. It also takes into account the degrees of the relationships. The QTDT analysis is available via the *Abecasis* homepage.

Appendix II: Glossary

Hardy Weinberg Equilibrium shows whether or not gene frequencies have changed within a population. If the criteria for equilibrium are met the allele frequencies will equal 1. If frequencies have changed in a population it indicates that evolutionary pressure has occurred. This will alter the frequency of one of the alleles.

If there are two alleles in a population: *A* and *a*, and the frequency of *A* is *p* and the frequency of *a* is *q* then $p + q = 1$. As people have two copies at each locus the possible combinations are $p^2 + 2pq + q^2 = 1$.

There will be no change in frequency in the different generations if certain criteria are met.

1) A large population – decreases the risk for random error
2) Random mating – i.e. no selection of mate
3) No Mutation at this allele
4) No migration – no influx or efflux of this allele
5) No natural selection – for or against the allele

If these criteria are met then the frequencies of alleles *A* and *a* will remain constant throughout the generations.

Heterozygosity refers to the inheritance of two different copies of an allele at the same locus on a pair of homologous chromosomes.

Homozygosity refers to the inheritance of two copies of the same variant at a single locus on a pair of homologous chromosomes.

Intermediary phenotype is a genetic trait that influences a certain multifactorial phenotype discriminating the phenotype between individuals.

Linkage disequilibrium (LD) is high between two markers if there has been no recombination between them, which means that they must be physically very close to one another on a chromosome. One can do a statistical test of LD if the genotypes of the individuals at the two markers are known.

Mendelian Forms of Hypertension refer to inherited forms of hypertension with a clear autosomal dominant or recessive or X-linked pattern of inheritance. This form of hypertension develops when a single genetic variant causes the disorder.

Mutation refers to any inheritable change in the DNA sequence.

Nasal Potential Difference assesses the voltage across nasal epithelium, which correlates with the transport of sodium and chloride across cell membranes.

Nedd 4-2 is a protein that functions as an ubiquitin ligase that has WW domains and is able to interact with the ENaC PY motif. Nedd stands for Neuronal Precursor Cell Expressed Developmentally Downregulated.

Open probability (Po) refers to the chance that a channel is able to conduct a substance through the pore from one side of a membrane to the other.

Polymorphism is the difference in DNA sequence between individuals. The different sequence is considered a polymorphism when it is too common to be considered a new mutation. A **single nucleotide polymorphism** (SNP) occurs when one base differs from the usual base at that particular position.

Population Stratification refers to differences in allele frequencies between cases and controls due to systematic differences in ancestry rather than association of genes with disease. It has been proposed that false positive associations due to stratification can be controlled by genotyping a few dozen unlinked genetic markers.

PY motif is the proline rich motif that contains a tyrosine residue which is known to bind to proteins bearing tryptophan rich (WW) domain.

Appendix III: Consent and Ethics Approval

III.1 Molecular Studies in the Khoisan

REQUEST FOR MOLECULAR STUDIES (DNA)
UNIVERSITY OF CAPE TOWN

Dr ESW Jones Groote Schuur Hospital New Main Building Nephrology Unit E12 Workshop 021 404 2292	Research Ethics Committee Groote Schuur Hospital Observatory 7925 Old Main Building E52-24 021 404 6338 021 406 6492	*DNA number:* *Volume:* *Result:*

Surname:	First Name:
Sex:	Date of Birth:
Number of children:	
Ethnic Origin: (both your mother and father)	
Contact Address:	Tel:
Hospital Address:	Tel:
Additional family history:	
Clinical Details:	
Other:	

Consent for Blood Pressure Measurement

I consent for my blood pressure to be taken using a standard non-invasive blood pressure measuring device. □
I have been informed that there are no complications of measuring blood pressure.
I consent for my DNA results to be compared with my blood pressure measurements. □
I consent to have my blood tested for aldosterone and renin levels, (normal hormones in the blood) and area and electrolytes. □
I consent for a sample of my urine to be tested for creatinine and sodium levels. □

Subject signature _____

Witnessed consent _____

Date _____

BP 1: BP 2:

III.2 Molecular Studies with the Hypertension Clinic

REQUEST FOR MOLECULAR STUDIES (DNA)

Inherited Metabolic Disease Laboratory R6.35

Division of Chemistry
Room 6.35 Level 6
Fulmouth Building
CT Medical School
Observatory 7925

Tel: (021) 406 6219 Fax: (021) 448 8150

Blood should be drawn in 1 plastic EDTA Tube (Purple top) +/- 5ml each.
Each tube should be inverted to mix and should be clearly labelled with the patient's name and DOB

Keep blood in fridge at 4°C until able to send to laboratory

Please <u>DO NOT</u> send specimens on ice or frozen.

<u>Please fill in all the information requested:</u>

Surname:_____
First Name(s):_____
Family name: _____

Sex: M ☐ F ☐ Date of Birth: (DD/MM/YY)_____

Number of children: _____

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

Contact Address: _____
Town: _____ Tel: _____

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

Hospital or Address: _____
Town: _____ Tel: _____

Reason for Referral (Clinical diagnosis):
_____
Additional disorders (apparent or previously treated):
_____
Additional family history
_____
Other: _____

CONSENT FOR DNA ANALYSIS AND STORAGE

1. I, _____, request that an attempt be made using genetic material to assess the probability that I might have inherited a disease-causing mutation in the genes for **HYPERTENSION.**

2. I understand that the genetic material for analysis is to be obtained from my blood cells.

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

   Or

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

   (a) possible re-analysis

   (b) analysis for the benefit of my immediate family members

   (c) research purposes, subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remains confidential.

4. The results of the analysis carried out on this biological sample will be made known to me, via my doctor, if and when available, if there is compelling evidence that this information will improve my or my family's medical care. Only then will the information be recorded in my medical records and I will undergo genetic counselling.
   In addition, I authorise that they may be made known to: (DELETE WHERE NOT APPLICABLE)
   other doctors involved in my care.
   The following family members:_____
   other:_____

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

6. I understand that I may withdraw my consent for any aspect of the above at

any time without this affecting my future medical care.

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

Date:_____

Patient signature_____

Witnessed consent_____

Dr ESW Jones
Groote Schuur Hospital
New Main Building
Nephrology Unit
E12 Workshop
021 404 2292

Research Ethics Committee
Groote Schuur Hospital
Observatory 7925
Old Main Building
E52-24
021 404 6338
021 406 6492

III.3 PATIENT INFORMATION AND INFORMED CONSENT
R563Q Sodium Channel Activity Study

INTRODUCTION

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

WHAT IS THE PURPOSE OF THE STUDY

The purpose of this study is to assess the function of a mutation of the epithelial sodium channel gene, R563Q, which we think predisposes people to salt sensitive hypertension. We would like to confirm this.

We will look to see if this mutation causes a change in activity that can be controlled by amiloride.

HOW MAY THE STUDY BENEFIT YOU?

There may be no direct benefit for you but the results may improve your treatment and the treatment of other patients in the future.

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

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

HAS THE STUDY RECEIVED ETHICAL APPROVAL?

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

WHAT ARE MY RIGHTS AS A PARTICIPANT IN THE TRIAL?

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

MAY THE TRIAL PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

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

WHAT ARE THE RISKS INVOLVED?

In rare circumstances the saline test may cause fluid overload and you may become breathless but this can be treated with an injection of a diuretic. In experienced hands this is very unlikely to occur and is easily treated. If you are known to have heart disease or kidney disease you should not have the saline test. This study will be performed by an experienced medical practitioner, Dr Jones, who will administer the saline, and any diuretic should it be required. She will be assisted by a registered nurse during the procedure. Emergency resuscitative equipment will be available at all times.

Saline is an isotonic (same as the blood concentration) salt solution that is frequently used in hospital for intravenous therapy.

INSURANCE AND FINANCIAL ARRANGEMENTS

All the study doctors are covered by insurance for medical liability. You will be paid R200.00 to participate in the study for transport costs and time spent at the study centre.

SOURCE OF ADDITIONAL INFORMATION

For the duration of the study you will be under the care of Dr Jones, and you are entitled to speak to her before entering the study.

CONFIDENTIALITY

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

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

INFORMED CONSENT

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

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

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

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

Patient's Name**:** **…….**(print)

Patient's Signature:……Date: ……

Study Doctor's Name: …… (print)

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

Witness Name: …… (print)

Witness Signature: ……

Dr ESW Jones
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021 404 2292

Research Ethics Committee
Groote Schuur Hospital
Observatory 7925
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III.4 REC Khoisan Approval

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty

Research Ethics Committee

Room E52-24 Groote Schuur Hospital Old Main Building

Observatory 7925

Telephone [021] 406 6338 • Facsimile [021] 406 6411

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09 November 2007

REC REF: 269/2006

Dr ESW Jones
Nephrology, E12
Groote Schuur Hospital

Dear Dr Jones

PROJECT TITLE: THE ACTIVITY OF THE R563Q MUTATION OF THE B ENaC

Thank you for your letter to the Research Ethics Committee dated 18th October 2007.

It is a pleasure to inform you that the Ethics Committee has **noted and approved** an aliquot of the DNA sample that is going to be collected to test for the R563Q mutation in the βENaC gene. It is also noted that blood sample for aldosterone, rennin and electrolytes and a urine sample for urea-creatinine ratio to determine if they are affected by the presence of mutation will be taken.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROF M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

III.5 REC Initial Approval

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
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Telephone [021] 406 6338 • **Facsimile** [021] 406 6411
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24 August 2006

REC REF: 269/2006

Dr ESW Jones
Nephrology E12
NGSH

Dear Dr Jones

PROJECT TITLE: THE ACTIVITY OF THE R563Q MUTATION OF THE BENaC

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Your comments to the queries raised are noted with thanks.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Convention on Harmonisation Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Please quote the REC. REF in all your correspondence.

Yours sincerely

DR. M. BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS