https://theses.gla.ac.uk/

Theses Digitisation:

https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses
https://theses.gla.ac.uk/
research-enlighten@glasgow.ac.uk

MOLECULAR AND CLINICAL STUDIES OF CORTICOSTEROID BIOSYNTHESIS AND REGULATION IN HYPERTENSION

By

ELLEN MARIE FREEL BSc (Hons) MB ChB (Hons) MRCP (UK)

©Ellen Marie Freel, 2006

Thesis submitted for the degree of Doctor of Philosophy to the University of Glasgow

Glasgow Cardiovascular Research Centre

University of Glasgow

126 University Place

Glasgow

July 2006

ProQuest Number: 10391043

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

ProQuest 10391043

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.
This work is protected against unauthorized copying under Title 17, United States Code
Microform Edition © ProQuest LLC.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

GLASGOW
UNIVERSITY
LIBRARY:

Declaration:

I declare that this thesis has been composed and written entirely by me. It has not been submitted previously for a higher degree.

I carried out the work described in this thesis under the supervision of Professor John MC Connell and Dr Eleanor Davies in the MRC Blood Pressure laboratories at the Western Infirmary, Glasgow.

Ellen Marie Freel

July 2006

Acknowledgements:

I would like to thank my supervisors, Dr Eleanor Davies and especially Professor John Connell for their advice, practical assistance and support during my time in clinical research and in the writing of this thesis. I also acknowledge the invaluable help of Professor Robert Fraser for his words of wisdom, scientific and literary advice.

My thanks also go to all the members of the MRC blood pressure group for their support, practical advice and good humour in the laboratory over the past few years. In particular, I thank Mary Ingram, Bob Fraser and Dr Ian Morton for performing biochemical assays and Elaine Friel, Morven Campbell and Dr Gordon Inglis for help with genotyping. Thank you also to Professor Anne White and Dr Rob Oliver for their help with ACTH assays. Most of all, I thank Marianne Barr and Donna Wilkinson for their immense contribution to sequencing of the CYP11B locus and their patience whilst I (almost) mastered the art of the PCR!

I also gratefully acknowledge the nursing staff of wards E3/4 of the Western Infirmary for their assistance with my patient studies and indeed to all patients and volunteers who participated.

I would like to sincerely thank the Wellcome Trust for awarding me a Junior Clinical Fellowship and so providing the funding for this work in its entirety.

Finally, my thanks go to friends, family and medical colleagues who have been and continue to be an invaluable source of love, support, patience and good humour and without whom this thesis would not have been possible.

Table of Contents:

Chapter 2 Materials and Methods

Index of tables:

Index of figures

Publications:

Original Articles:

Freel EM, Shakerdi L, Friel EC, Wallace AM, Davies E, Fraser R and Connell JMC. The Origin of Circulating 18-oxo and 18-hydroxycortisol in normal human subjects 2004 *J Clin Endocrinol Metab* 89 (9): 4628-4633

Freel EM, Ingram M, Bernhardt R, Wallace AM, Fraser R and Connell JMC. Endogenous Corticosteroid Production in Subjects after Bilateral Adrenalectomy 2006 *J Clin Endocrinol Metab* (submitted)

Freel EM, Ingram M, Friel EC, Davies E, Fraser R, Brown M, Samani N, Caulfield M, Farrall M, Munroe P, Domiczak A and Connell JMC. Phenotypic Consequences of Variation across the Aldosterone Synthase and 11β-hydroxylase Locus in a Hypertensive Cohort 2006 *Hypertension* (submitted)

Review Articles:

Freel EM and Connell JMC. Mechanisms of Hypertension: The Expanding Role of Aldosterone 2004 *J Am Soc Nephrol* 15:1993-2001

Freel EM and Connell JMC. The Resurgence of Aldosterone in Hypertension and Cardiovascular disease 2006 *Curr Hyperten Rev* 2:21-32

Freel EM and Connell JMC. Primary Aldosteronism: Common but Controversial 2005 *Nature Clin Prac:Endocrinol & Metab* 2:111-115

Presentations to Learned Societies:

Endogenous Corticosteroid Production in Subjects after Bilateral Adrenalectomy (2006)
American Endocrine Society
Boston, USA.
Winner of a Travel Award for overseas presenters 2006

Phenotypic consequences of Variation across the CYP11B Locus in Hypertensive Subjects (2005)
British Hypertension Society
Cambridge, UK
Shortlisted for Clinical Science Young Investigator Prize

Variation at the Aldosterone Synthase Locus (CYP11B2) and Adrenal 11beta Hydroxylation in a Hypertensive Cohort (2005)
American Endocrine Society
San Diego, USA

Aldosterone Through the Ages: The Expanding Role in Hypertension and Cardiovascular Disease (2004)
Caledonian Society for Endocrinology Prize Lecture
Peebles Hydro

The Expanding Role of Aldosterone in Hypertension (2004)
Scottish Hypertension Research Group
St Andrews

Effect of Variation at the Aldosterone Synthase locus (CYP11B2) on Adrenal 11-beta hydroxylation: Pilot Data from the MRC BRIGHT Study (2004)
Society for Endocrinology Annual Meeting
Royal College of Physicians, London
Winner of Young Endocrinologist award for best oral presentation

The Origin of Circulating 18-Oxocortisol and 18-Hydroxycortisol in Normal Human Subjects (2004)
American Endocrine Society
New Orleans, USA

Abbreviations:

3βHSD	3β-hydroxysteroid dehydrogenase
11β-HSD	11β-Hydroxysteroid dehydrogenase
17-OH-P	17-Hydroxyprogesterone
17-OH-PREG	17-Hydroxypregnenolone
18-OH-B	18-Hydroxycorticosterone
AC	Adenylyl (adenylate) cyclase
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropin
ACTH-R	ACTH receptor
A'dione	Androstenedione
AIP	Aldosterone induced protein
Aldo	Aldosterone
AME	Apparent mineralocorticoid excess
Ang I	Angiotensin I
Ang II	Angiotensin II
AP-1	Activator protein 1
ARR	Aldosterone-renin ratio
AT1 or 2	Angiotensin II receptor 1 or 2
ATF-1	Activating transcription factor 1
ATP	Adenosine triphosphate
B	Corticosterone
BAH	Bilateral adrenal hyperplasia
BHS	British Hypertension Society
BMI	Body mass index
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
$[Ca^{2+}]_i$	Intracellular free calcium concentration
CaMK	Ca^{2+}-calmodulin-dependent protein kinase
lipoid CAH	Congenital adrenal lipoid hyperplasia
cAMP	3', 5'-Cyclic adenosine monophosphate
CBP	CREB binding protein

cDNA	Complementary deoxyribonucleic acid
CHIF	Corticosteroid hormone induced factor
Cl^-	Chloride ions
CMOI/II	Corticosterone methyl oxidase type I or II
CNS	Central nervous system
CRE	cAMP-response element
CREB	cAMP-responsive element binding protein
CRH	Corticotrophin-releasing hormone
CYP11A1	Gene encoding side-chain cleavage enzyme
CYP11B1	Gene encoding 11β-hydroxylase
CYP11B2	Gene encoding aldosterone synthase
CYP17	Gene encoding 17-hydroxylase
CYP21	Gene encoding 21-Hydroxylase
CYP450	Cytochrome P450 enzymes
DAG	1,2-Diacylglycerol
DBD	DNA binding domain
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DOC	11-Deoxycorticosterone
DOCA	Deoxycorticosterone acetate
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminotetraacetic acid
EMSA	Electrophoretic mobility shift assay
E	Cortisone
ENaC	Epithelial sodium channel
EtOH	Ethanol
F	Cortisol
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GCMS	Gas chromatography mass spectrometry
G protein	Guanine nucleotide-binding regulatory protein

GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GSH	Glucocorticoid suppressible hyperaldosteronism
GTP	Guanosine triphosphate
HDL	High-density lipoproteins
HPA	Hypothalamic-pituitary-adrenal axis
HPLC	High performance liquid chromatography
HRE	Hormone response element
IC	Intron conversion polymorphism
IP_3	1, 4, 5, Inositol triphosphate
K^+	Potassium ions
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LV	Left ventricle
LVEDP	Left ventricular end-diastolic pressure
LVH	Left ventricular hypertrophy
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
Na^+	Sodium ions
NAC	N-acetylcysteine
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
Na^+/K^+-ATPase	Sodium, potassium-adenosine triphosphatase
nfH_2O	Nuclease free water
NGFIB	Nerve growth factor-induced clone B
NMDA	*N*-Methyl-D-asparate
NO	Nitric oxide
NOS	Nitric oxide synthase
NURR1	NUR-related factor 1
P	Progesterone
$P450_{11ß}$	11ß-Hydroxylase
P450c17	17α-Hydroxylase
P450c21	21 α -Hydroxylase

$P450_{aldo}$	Aldosterone synthase
P450scc	Side-chain cleavage enzyme
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PREG	Pregnenolone
QTL	Quantitative trait loci
RAS	Renin-angiotensin system
RAAS	Renin-angiotensin-aldosterone system
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
S	11-Deoxycortisol
SAP	Steroidogenesis activator peptide
SCP-2	Sterol carrier protein-2
SF-1	Steroidogenic factor 1
Sgk1	Serum and glucocorticoid-regulated kinase 1
SIP	Steroidogenesis-inducing protein
SNP	Single nucleotide polymorphism
StAR	Steroidogenic acute regulatory protein
TAE	Tris-acetic EDTA buffer
TBS	Tris-buffered saline
TE	Tris-HCl EDTA buffer
THAldo	Tetrahydroaldosterone
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol
THS	Tetrahydrodeoxycortisol
UTR	Untranslated region
VSMC	Vascular smooth muscle cell
WHR	Waist-hip ratio
ZF	Zona fasciculata
ZG	Zona glomerulosa

Summary:

Hypertension is a common disorder affecting a large heterogeneous patient population. Despite improved understanding of its pathophysiology and availability of effective treatment strategies, hypertension remains a major, potentially modifiable, risk factor for cardiovascular disease.

Aldosterone, the principal human mineralocorticoid, is increasingly recognised as playing a significant role in cardiovascular morbidity and its role in hypertension has recently been re-evaluated with studies that suggest that increased aldosterone concentration (as defined by an elevated aldosterone to renin ratio – ARR) is a key phenotype in up to 15 per cent of hypertensive subjects. We have previously reported that polymorphisms of the gene (C to T conversion at position –344 and intron conversion (IC) in intron 2) encoding aldosterone synthase (CYP11B2) are associated with hypertension, particularly in individuals with a high ARR. In normotensives, the T and IC alleles associate with raised basal and ACTH-stimulated levels of the 11-deoxysteroids, deoxycorticosterone and 11-deoxycortisol which are substrates for the enzyme 11ß-hydroxylase, encoded by the adjacent and homologous gene CYP11B1.

This has led to speculation that the T and IC alleles of CYP11B2 are in linkage disequilibrium (LD) with functional variants in CYP11B1 resulting in reduced efficiency of this enzyme. In order to maintain cortisol production, positive feedback to the pituitary leads to a subtle but more pronounced adrenocorticotrophic hormone (ACTH) drive to the adrenal cortex and enhanced synthetic capacity of the zona glomerulosa resulting in increased

production of aldosterone and suppression of renin. Thus in such individuals, there should be recognisable, genotype dependent, changes in the pattern of adrenal steroid production as well as alteration in the cortisol/ACTH relationship.

In chapter 3, by sequencing the entire CYP11B locus in a cohort of normotensive subjects, I demonstrated that there was indeed tight LD across this locus and identified a number of novel polymorphisms in both genes linked to the T and IC alleles. Whilst most of these were in non-coding regions, making analysis of their functional effects difficult, two were in the promoter region of CYP11B1 and reduce transcriptional activity of this enzyme in vitro (reported elsewhere).

In chapter 4, the effect of CYP11B2 genotype on intermediate corticosteroid phenotype was explored further in a large, severely hypertensive cohort. In this population, cortisol production was unaffected by CYP11B2 genotype but there was a significant elevation of the ratio of deoxycortisol to cortisol in subjects homozygous for the –344T allele suggesting reduced efficiency of 11ß-hydroxylase. In addition, excretion of aldosterone was found to correlate strongly with that of ACTH-dependent steroids (total cortisol and adrenal androgens) in TT subjects. This suggests that in such subjects, there is an important common regulatory influence on aldosterone, cortisol and androgen production that is likely to be ACTH.

The association of CYP11B2 polymorphisms as well as the novel SNPs in 5' untranslated region (UTR) of CYP11B1 with alterations in cortisol/ACTH relationship as well as on aldosterone was assessed in a cohort of hypertensives homozygous for the -344T allele. In chapter 5, I report that TT

homozygotes demonstrated diminished suppressibility of ACTH with dexamethasone leading to a decreased cortisol/ACTH ratio. These findings are in keeping with a mild increase in ACTH secretion in order to maintain cortisol production. Additionally, aldosterone exhibited diurnal variation and correlated closely with cortisol levels only in TT homozygotes. This supports further the proposal that, in TT individuals, ACTH contributes to the regulation of aldosterone production. In addition, the novel alleles of CYP11B1 (-1888T and –1858G) identified in chapter 3 resulted in similar changes in cortisol/ACTH relationship and in aldosterone regulation as the –344T± IC alleles of CYP11B2. These alleles have been reported to result in reduced transcription of CYP11B1 and so provide a plausible explanation for the altered efficiency of 11ß-hydroxylation described in several populations.

The hybrid corticosteroids, 18-oxocortisol and 18-hydroxycortisol are found in the urine of individuals with primary aldosteronism (PA) due to glucocorticoid remediable Aldosteronism (GRA) and Conn's adenoma where they play a diagnostic role. However, they have also been identified in normal individuals. In chapter 6, I explored the origins of these steroids in order to provide a better understanding of corticosteroid physiology and production. I showed that 18-oxocortisol is produced in normal subjects by the action of zona glomerulosa aldosterone synthase on recirculating cortisol whilst 18-hydroxycortisol is produced by the action of zona fasciculata 11ß-hydroxylase on locally produced cortisol. Subsequently, 18-oxocortisol was used as a marker of aldosterone synthase activity and was detectable in subjects who had previously undergone bilateral adrenalectomy (chapter 7). 18-hydroxycortisol was also found in easily detectable amounts and its

production was independent of ACTH suppression by exogenous glucocorticoid supporting the concept of extra-adrenal aldosterone synthase activity.

In summary, I have shown that there is strong LD across the CYP11B locus. In particular, variants in the 5' UTR of CYP11B2 (–344T) are in tight LD with a number of polymorphisms across this and the adjacent CYP11B1 locus. These polymorphisms (of which -1888T and –1858G are likely candidates) result in altered expression of the enzyme product of CYP11B1, 11ß-hydroxylase. In turn, this leads to changes in hypothalamic pituitary adrenal (HPA) axis activity consistent with a subtle increase in ACTH-mediated adrenal stimulation to maintain cortisol production. Finally, these data are also consistent with a contribution of ACTH to the production of aldosterone (in -344T & CYP11B1 -1888T/ –1858G) leading to the eventual phenotype of hypertension with aldosterone excess.

Chapter 1: Introduction

1.1 Blood Pressure – a historical perspective

The study of hypertension stemmed from one of the single most influential discoveries in medical science- the circulation of the blood (William Harvey, 1578-1657)(1). It was not until a century later, however, that the Reverend Stephen Hales made the first quantitative measure of blood pressure. Hales measured arterial pressure by inserting a brass tube into the crural artery of a horse and connecting this to a vertical glass tube(2). Consequently, the first arterial blood pressure measurements were recorded in feet of water- in this case, 8 feet 3 inches. Whilst this was a key discovery, it was clearly not a practical method for blood pressure measurement in man. However, it was not until 172 years later that the Russian military surgeon Nicolai Korotkoff reported a reliable, simple and reproducible method of measuring systolic and diastolic pressure in man using an inflatable cuff to compress an upper limb artery and listening to the sounds created by the arterial flow on release of the compression(3). One hundred years later, Korotkoff's auscultatory method remains the principal technique of blood pressure measurement in man.

Despite an initially slow start, progress in hypertension research has accelerated in an exponential fashion over the past 100 years. In particular, there have been major advances in understanding the underlying biochemistry and physiology as well as, more recently, genetic mechanisms. Observational studies in large populations have identified hypertension as a major health problem in the developed world and highlight the importance of a better understanding of its pathophysiology and eventual treatment to minimise its consequences.

1.2 Clinical relevance of hypertension

Hypertension is the commonest indication in the Western world for chronic, lifelong pharmacological treatment(4). Despite improved understanding of its pathophysiology and availability of effective treatment strategies, hypertension remains a major, potentially modifiable, risk factor for cardiovascular disease (Table 1.2a). Over the past 15 years, age-adjusted rates of stroke incidence have risen slightly and the rate of decline in coronary disease has slowed (5). The incidences of end-stage renal disease and cardiac failure have also increased (5). Inadequate control of blood pressure within a hypertensive population is thought to be a major contributor to these worrying trends. It is clear that any insight into underlying mechanisms, which can subsequently make a positive impact upon treatment regimes, would be desirable.

1.2.1 Definition of Hypertension

This has always been a controversial area. Historically, it was argued by Platt that hypertension was an entity distinct from normotension(6) whilst Pickering claimed that blood pressure was on a continuous spectrum and hypertension was a quantitative deviation from the norm(7). Clearly, Pickering has been proven correct and it is now accepted that blood pressure is indeed a quantitative trait that is highly variable. In population studies, blood pressure has a normal distribution that is slightly skewed to the right(5). This obviously makes the definition of hypertension problematic. Moreover, as there is no specific level of blood pressure where cardiovascular and renal complications start to occur then an exact definition is somewhat arbitrary but necessary for

		Stroke		Myocardial Infarction		Peripheral arterial disease	
Age (years)	Hypertensive status	Men	Women	Men	Women	Men	Women
35-64	Normal	0	0	4	1	2	1
	Mild	1	1	8	2	4	1
	Definite	4	2	10	3	5	3
65-94	Normal	3	1	7	4	6	3
	Mild	5	4	17	7	7	3
	Definite	12	9	21	9	9	5

Table 1.2a. Incidence of atherosclerotic cardiovascular events (as age-adjusted annual rate per 1000) according to hypertensive status.
Data taken from 30-year follow up of Framingham study

patient assessment and treatment. It should also be noted that there remains a strongly positive correlation between blood pressure and the risk of cardiovascular disease, renal disease and mortality even within the normotensive range(8).

The recently published British Hypertension Society (BHS) Guidelines(9) have based classification of blood pressure levels upon those previously published by the World Health Organisation- International Society of Hypertension(10). Hypertension, according to the BHS, is defined as a systolic blood pressure of 140 mm/Hg or greater and/or a diastolic blood pressure of 90mm/Hg and above in subjects who are not taking anti-hypertensive medication (Table 1.2b). Within the normal blood pressure category 3 subgroups exist- optimal, normal and high/normal as well as in the hypertension category – grade1, grade2 and grade 3.

For a brief period, these classifications were universally accepted resulting in reduced confusion and providing consistent advice to clinicians around the world. However, in 2003, the Joint National Committee VII (JNC VII)(11) in the United States published another classification system stating that the risk of cardiovascular disease began at 115/75mm/Hg and calling systolic pressures of 120-139 mm/Hg and diastolic pressures of 80-89 mm/Hg 'pre-hypertension.'

1.2.2 Prevalence of Hypertension

Obviously, given the changing definition of true hypertension and the lack of clear distinction between 'normotension' and hypertension, exact prevalence is difficult to estimate. The most recent data, using a cut off of 140/90 mm/Hg

Category	Systolic blood pressure (mm/Hg)	Diastolic blood pressure (mm/Hg)
Blood pressure		
Optimal	<120	<80
Normal	<130	<85
High/normal	130-139	85-89
Hypertension		
Grade I (mild)	140-159	90-99
Grade II (moderate)	160-179	100-109
Grade III (severe)	>180	>110
Isolated systolic hypertension		
Grade 1	140-159	<90
Grade 2	>160	<90

Table 1.2b. Classification of blood pressure according to the British Hypertension Society (9)

estimates the age-adjusted prevalence of hypertension in the UK to be around 42% in individuals aged 35-64 with only 24% of these individuals on anti-hypertensive treatment(12) (Figure 1.2a, Table 1.2c). Unsurprisingly, these data represent an increase in prevalence from earlier data which were based on more generous definitions of hypertension (>160/95 mm/Hg). Nonetheless, in both cases, there is a similar association between age and the prevalence of hypertension with a 20-fold increase in prevalence in the oldest age group compared with their counterparts in the lowest age group.

In the United States, data from the fourth survey conducted by the National Health and Nutrition Examination Survey (NHANES) seem to indicate that, contrary to earlier reports, hypertension prevalence is increasing with an overall age-adjusted prevalence of 28.7% compared with 25% in 1988(13).

1.2.3 Incidence of Hypertension

There is a considerable lack of studies examining the incidence of hypertension in the general population. Probably the best data still derives from the Framingham Heart Study(14). Briefly, this large-scale, prospective study examining factors related to development of vascular disease has followed a cohort of over 5000 men and women for over forty years with biennial examinations at the study clinic in Framingham, USA. This study helped identify hypertension as a major cardiovascular risk factor as well as quantify its atherogenic cardiovascular disease potential(15). The incidence of hypertension increases with age in men from 3.3 per cent per year at ages 30-39 to 6.2 per cent at ages 70-79 and in women from 1.5 per cent at ages 30-39 to 8.6 per cent at ages 70-79(15;16) (Figure 1.2b). This trend is

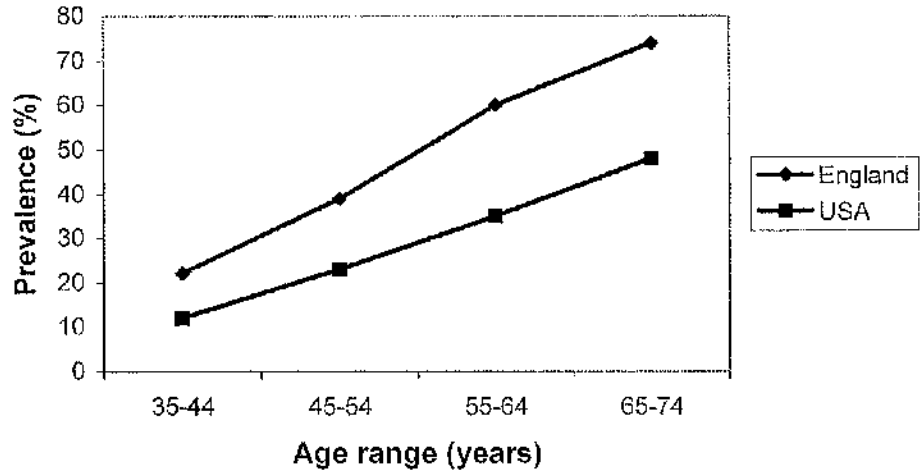


Figure 1.2a. Hypertension prevalence in England and North America, men and women combined, by age group.
Adapted from ref (12).

Prevalence (%)				
Country	**All**	**Men**	**Women**	**On treatment (%)**
North America	27.6	30.4	24.8	44.4
USA	27.8	29.8	25.8	52.5
Canada	27.4	31.0	23.8	36.3
Europe	44.2	49.7	38.6	26.8
Italy	37.7	44.8	30.6	32.0
Sweden	38.4	44.8	32.0	26.2
England	41.7	46.9	36.5	24.8
Spain	46.8	49.0	44.6	26.8
Finland	48.7	55.7	41.6	25.0
Germany	55.3	60.2	50.3	26.0

Table 1.2c. Hypertension prevalence and treatment among subjects age 35-64 in 6 European countries and North America.
Adapted from ref (12).

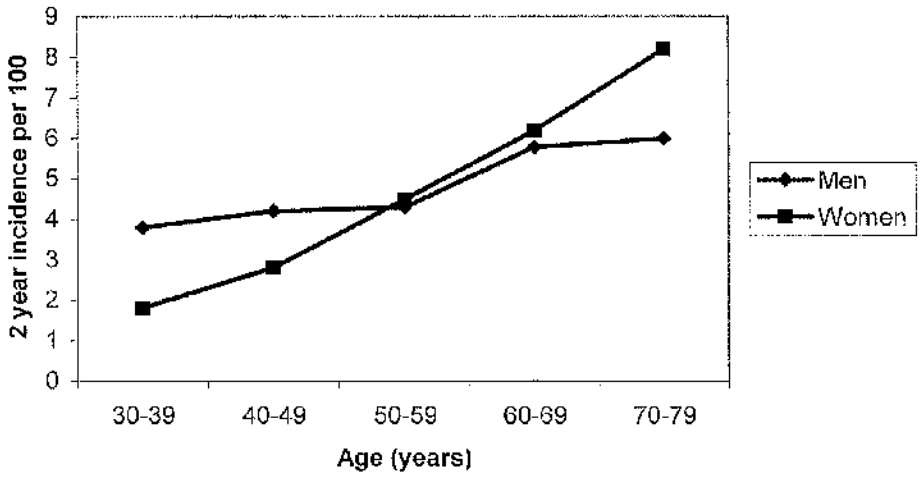


Figure 1.2b. Two year incidence of hypertension per 100 by sex and age based on a pooling of 15 bienniums
Adapted from ref 16

consistently observed across other populations(17). It has become increasingly understood that the incidence of hypertension is affected by other factors such as body weight (18), alcohol intake(19), smoking(20) and physical activity(21).

1.2.4 Morbidity and Mortality from Hypertension

Hypertension affects around one billion people worldwide and is the most common treatable risk factor for cardiovascular disease in patients aged over 50(22). Both systolic and diastolic blood pressure levels have been shown to be positively and continuously related to the risk of stroke across a wide range of levels worldwide(23). Amongst middle-aged individuals, lowering diastolic blood pressure by 5mm/Hg for a prolonged period associates with a 35-40 per cent lower risk of stroke with no lower level below which the risks of stroke do not continue to decline(23) (Figure 1.2c). Levels of blood pressure are positively associated with both intra-cerebral haemorrhage and cerebral infarction, but the relationship is stronger with haemorrhagic stroke.

It is also well established that blood pressure levels are positively and continuously related to the risks of major coronary heart disease (death or non-fatal myocardial infarction) (24) (Figure 1.2d). This association is slightly less strong than that of blood pressure and stroke but remains constant across a broad range of blood pressure levels. One recent prospective cohort study demonstrated that hypertensive men had 50 per cent more myocardial infarctions than normotensive men and their mortality for coronary heart disease was doubled(25).

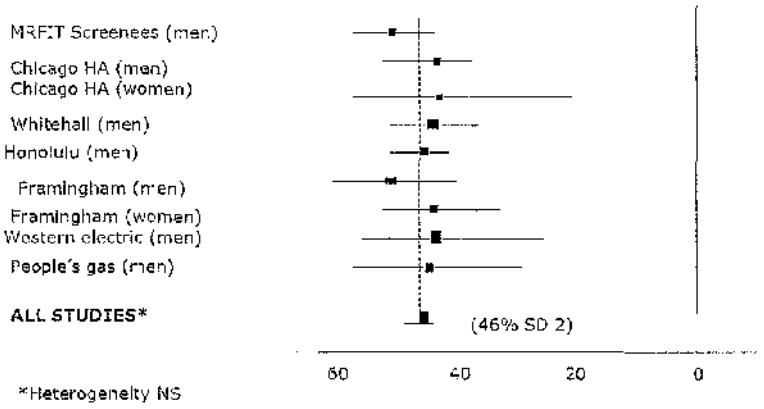


Figure 1.2c. Estimates from 7 studies of eventual difference in stroke risk associated with a 7.5mm/Hg reduction in DBP
Solid squares represent estimated difference in risk (adjusted for age, cholesterol and smoking) in each study; sizes of squares are proportional to number of events in each study and 95% CIs are denoted by horizontal lines. The broken vertical line represents a 46% difference in risk, which was the weighted mean difference in risk for all studies combined.

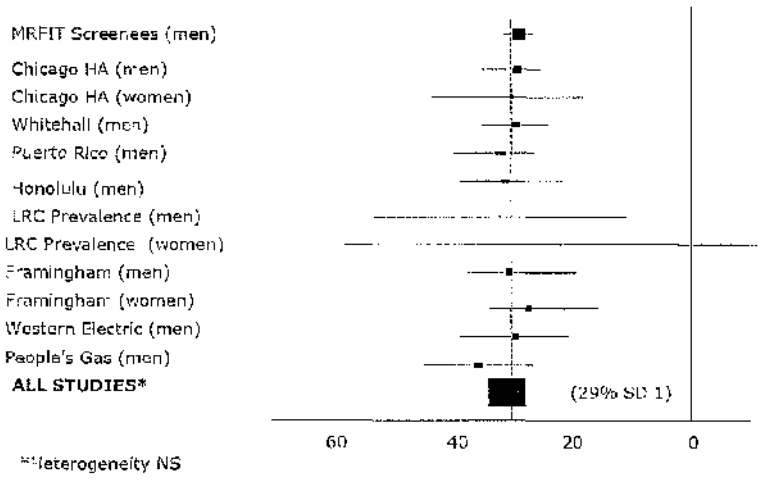


Figure 1.2d. Estimates from 9 studies of eventual difference in coronary heart disease risk associated with a 7.5mm/Hg reduction in DBP.
Broken vertical line represents total difference in risk of 29%.

Hypertension also contributes to the development of heart failure and renal disease but the strength of these relationships is less well established than those for stroke and coronary heart disease(23). Nonetheless, there is evidence to suggest that patients with a history of hypertension have a six-fold greater risk of heart failure than normotensive subjects(26). In renal failure, each 5 mm/Hg lower level of DBP is associated with at least a one-quarter lower risk of development of end-stage renal disease (27). As well as its significant vascular effects, elevated blood pressure leads to a variety of so-called 'end-organ' effects, i.e. the presence of hypertension has deleterious effects on a number of important body tissues. Organs susceptible to the adverse effects of hypertension include the heart, kidney, retinal vessels, the cerebral circulation as well as the general systemic arterial circulation.

1.2.5 Importance of Drug Treatment in Hypertension

In light of the information outlined previously, it is not surprising that large benefits, in terms of avoided cardiovascular disease, are expected from the treatment of hypertension. This is perhaps best appreciated by considering the number of deaths from stroke or coronary heart disease that could be prevented by a 9/5 mm/Hg reduction in population blood pressure (Table 1.2d).

Many large-scale morbidity and mortality trials have compared different classes of anti-hypertensive drugs. The Blood Pressure Lowering Trialists' Collaboration has conducted two major meta-analyses of blood pressure lowering agents (28;29). In the first, 'newer' therapies such as treatments

	Annual mortality		Deaths avoided by 9/5 mm/Hg reduction in population BP*	
Country	Stroke	Coronary Heart Disease	Stroke	Coronary Heart Disease
UK	76308	169481	26000	36000
USA	149972	509412	51000	107000

Table 1.2d. Annual mortality from stroke and coronary heart disease, and number of deaths likely to be avoided by a reduction in the population blood pressure of 9/5 mm/Hg
*(adjusted for age, smoking and blood cholesterol)
Adapted from ref (24)

targeting the renin-angiotensin system or calcium channel blockers were compared with 'conventional' therapies (diuretics/beta-blockers). The conclusion made here has been that newer therapies are as effective but no more so than conventional therapies at reducing stroke, coronary heart disease or all cause mortality. The second meta-analysis reviewed 29 major trials published as of 2003, with over 700,000 years of patient follow-up. As with the previous conclusion, this demonstrated that the main benefit from anti-hypertensive therapy is blood pressure lowering *per se,* with little evidence to suggest additional benefits specific to a class of drug regarding major cardiovascular outcomes.

However, recent data from the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) challenges this(30). The ASCOT trial involved >19000 hypertensive patients with at least three other cardiovascular risk factors, randomised to receive old conventional treatment (beta-blockers/diuretics) or newer treatments (calcium channel blocker/angiotensin converting enzyme (ACE) inhibitor). The trial was halted early because of a significant benefit in terms of cardiovascular mortality in the amlodipine/perindopril treated arm despite similarities in blood pressure reduction between the two groups.

There is a lack of definitive evidence on optimal targets for blood pressure lowering. The hypertension optimal treatment (HOT) study provides the best evidence to date on desired targets(31). It reports that the optimal blood pressure for reduction of major cardiovascular events is 139/83 mm/Hg but that reduction below this value causes no harm. Overall however, benefits from treatment of hypertension are not seen because the control of hypertension remains poor in European countries. This is particularly true of

the UK where it is controlled in only 10 per cent of the hypertensive population(32).

1.3 Mechanisms of Hypertension

Despite many years of research, the underlying aetiology of the vast majority of cases of hypertension is unknown. Such individuals are said to have 'essential' or 'primary' hypertension and account for between 95-99 per cent of all cases of hypertension. The remaining cases are classified as 'secondary' hypertension- i.e. where hypertension arises due to a biochemical or mechanical pathology which is potentially reversible and the hypertension curable.

1.3.1 Secondary Hypertension

Secondary causes of hypertension are widely accepted to be relatively uncommon. Although there is a lack of recent data, several published series from the past three decades have reported a prevalence of 1.1-11 per cent(33-37). This variation in prevalence is due to a combination of differences in the populations screened and differences in the type of screening tests used.

The causes of secondary hypertension are numerous and outlined in table 1.3a. The majority of causes are due to hormone excess, renal disease or vascular disease such as renal artery stenosis.

Organ	Example
Kidney	Autosomal Dominant Polycystic Kidney Disease
	Renal parenchymal disease: chronic glomerulonephritis, chronic interstitial nephritis, reflux nephropathy, amyloidosis
Arterial disease	Renal artery stenosis (atheromatous or 2° to fibromuscular dysplasia)
Adrenal gland	Medulla: phaeochromocytoma
	Cortex: cortisol overproduction
	Aldosterone overproduction
	Other mineralocorticoid excess syndromes
Drugs	Oral contraceptive pill
	Glucocorticoids
	Cyclosporin
	Non-steroidal anti-inflammatory agents
	Carbenoxolone
Others:	Acromegaly
	Hyperthyroidism
	Acute Intermittent Porphyria

Table 1.3a. Secondary causes of hypertension

1.3.2 Essential Hypertension

Essential hypertension is a multi-factorial disorder thought to be caused by the interaction of an individual's genetically determined risk of developing hypertension with environmental factors including diet, levels of physical activity and other modifiable factors (37) (Figure 1.3a)

1.3.3 Lifestyle factors and essential hypertension

A variety of lifestyle factors have been shown to directly influence blood pressure levels at both an individual and population level (38)(Table 1.3b).

Of these, the most important are excess body fat, alcohol consumption, physical activity and a variety of dietary constituents including salt, potassium, and a complex of fruits, vegetables and saturated fat as well as n3 fatty acids. The role of stress in sustained blood pressure elevation remains controversial other than through possible influences of coping mechanisms that determine dietary, drinking, exercise and smoking habits(39).

Moderate changes in combinations of some of these factors have additive effects on blood pressure reduction in all grades of hypertension. Indeed, the effects are often as large as those seen with antihypertensive drug therapy, but with a greater potential to simultaneously reduce the risk of cardiovascular disease by mechanisms other than blood pressure reduction. Cook et al., using data from a number of observational studies and randomised controlled trials, estimated that reducing the average population diastolic blood pressure by as little as 2 mm/Hg by lifestyle measures would decrease the prevalence of hypertension by 17 per cent, with a six per cent reduction in risk of coronary heart disease and a 15 per cent reduction in the risk of stroke(40).

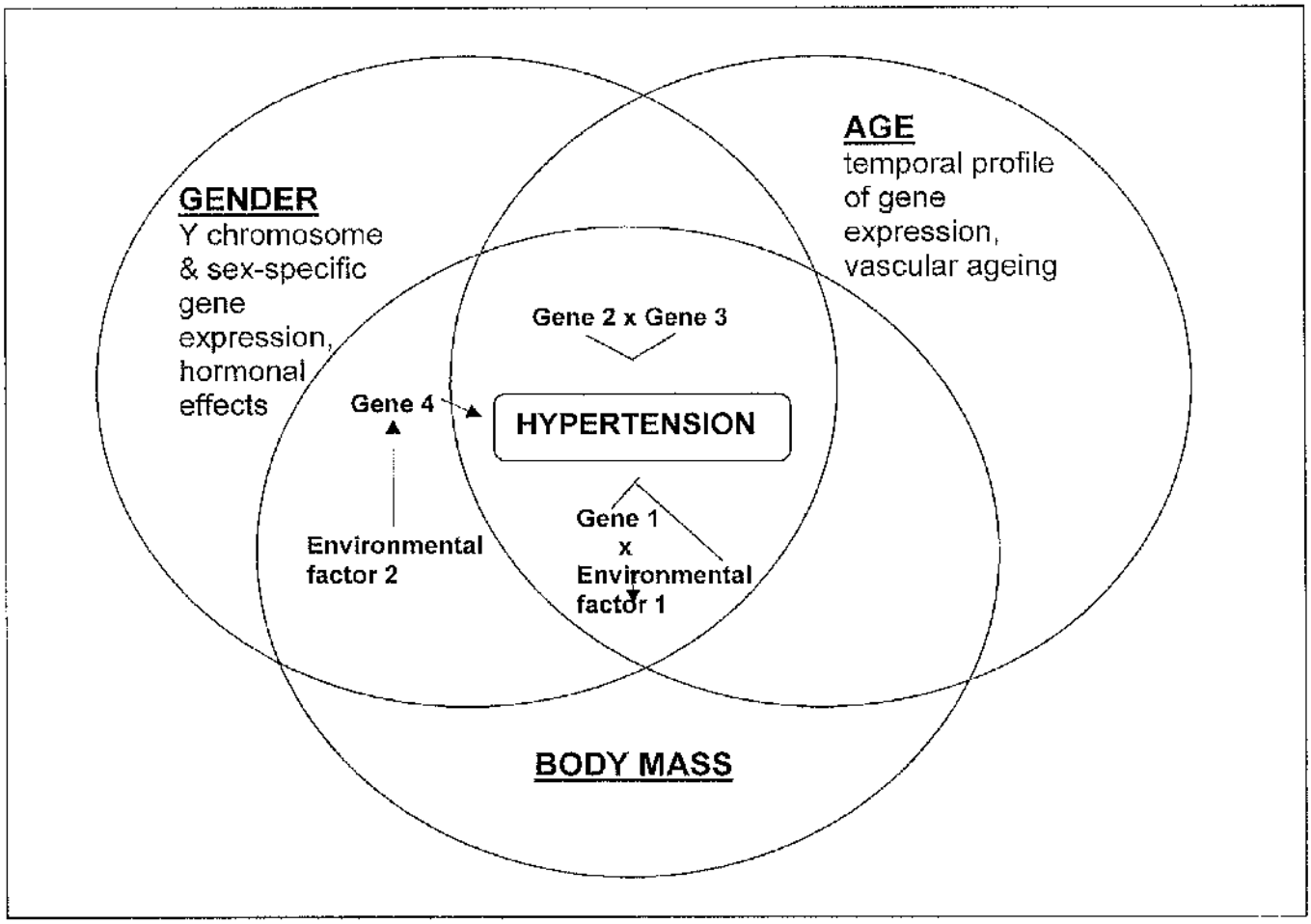


Figure 1.3a The multifactorial model of essential hypertension.
Gene-gene and gene-environment interactions may be dependent on a background context of factors such as age and sex.

	Men		Women	
Variable	**SBP**	**DBP**	**SBP**	**DBP**
Lifestyle factors				
BMI	0.39‖	0.33‖	0.41‖	0.26‖
Exercise	0.03	0.11‡	0.17†	0.14‡
Cigarettes (g/wk)*	0.06	-0.04	-0.05	0.05
Smoking status	-0.09	-0.09	0.05	0.07
Alcohol intake¶	0.14‡	0.14‡	0.10	0.10
Drinking status	0.07	-0.04	0.01	0.00
Unhealthy eating	0.01	0.07	0.00	0.07
Healthy eating	0.07	0.03	0.03	0.04
Stress Measures				
Job stress	0.03	0.06	0.01	0.08
Home/work stress	0.08	0.09	0.01	0.1

Table 1.3b. Pearson correlation co-efficients among lifestyle, stress and blood pressure

*non-smokers included

¶ non-drinkers excluded from average weekly alcohol intake

‡ $p<0.05$ † $p<0.01$ ‖ $p<0.001$

Adapted from (39)

1.3.4 Renin-angiotensin system in essential hypertension

The renin-angiotensin system (Figure 1.3b) is central to blood pressure regulation in man. Briefly, the precursor peptide, angiotensinogen, is cleaved by renin (produced by renal juxtaglomerular cells) to produce angiotensin I. In turn, this is converted (predominantly within pulmonary vasculature) by the Angiotensin Converting Enzyme (ACE) into angiotensin II (Ang II). This system is described in more detail in section 1.6.2. Ang II increases blood pressure by various mechanisms including constriction of resistance vessels, stimulating aldosterone synthesis by the adrenal gland (discussed in detail 1.6.2), renal tubular sodium absorption (directly and indirectly via aldosterone), stimulating thirst and release of anti-diuretic hormone and enhancing sympathetic outflow from the brain(41). Locally produced Ang II plays both an autocrine and paracrine role in maintaining cardiovascular homeostasis, acting via specific receptors.

In particular, Ang II induces cardiac and vascular cell hypertrophy and hyperplasia directly by activating the angiotensin II type 1 receptor (AT1) and indirectly by stimulating the release of several growth factors and cytokines(42). The role of angiotensin II type 2 receptors (AT2) has been less clear but recent evidence suggests that activation of this receptor leads to vasodilation, growth inhibition and cell differentiation(43), hence opposing the biological effects of AT1 receptor activation.

1.3.5 Genetic factors in essential hypertension

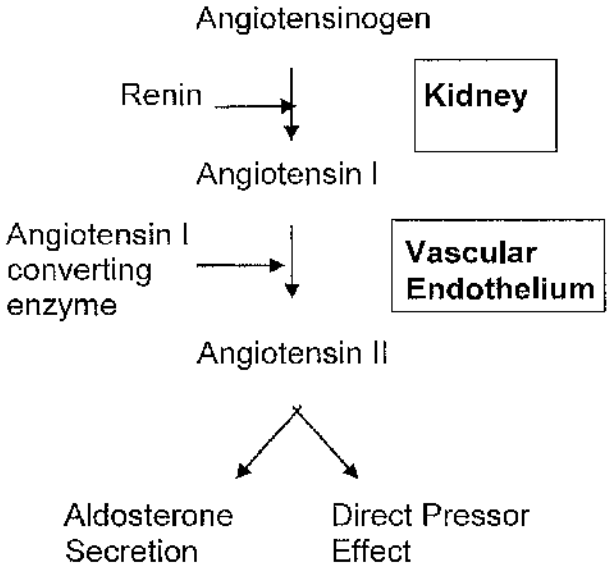


Figure 1.3b. The renin-angiotensin system

It is clear from many sources of evidence that there is a considerable genetic influence on the development of hypertension. Twin studies have demonstrated greater concordance of blood pressure between monozygotic than dizygotic twins (44) and population studies show greater similarity in blood pressure within families than between families (45). This latter observation cannot only be attributed to a common environment as adoption studies demonstrate greater concordance of blood pressure among biological siblings than adoptive siblings living in the same household (46). A comparison between essential hypertension and 'Mendelian' hypertension (due to a single gene disorder) is outlined in table 1.3c.

Studies of rare single-gene disorders resulting in hypertension (Mendelian hypertension) can give some insight into underlying pathophysiological mechanisms (Figure 1.3c). This is particularly true of adrenal steroid biosynthesis and two particular syndromes of aberrant production of adrenal steroids resulting in hypertension (Glucocorticoid Remediable Aldosteronism and 11β-hydroxylase deficiency) will be considered later.

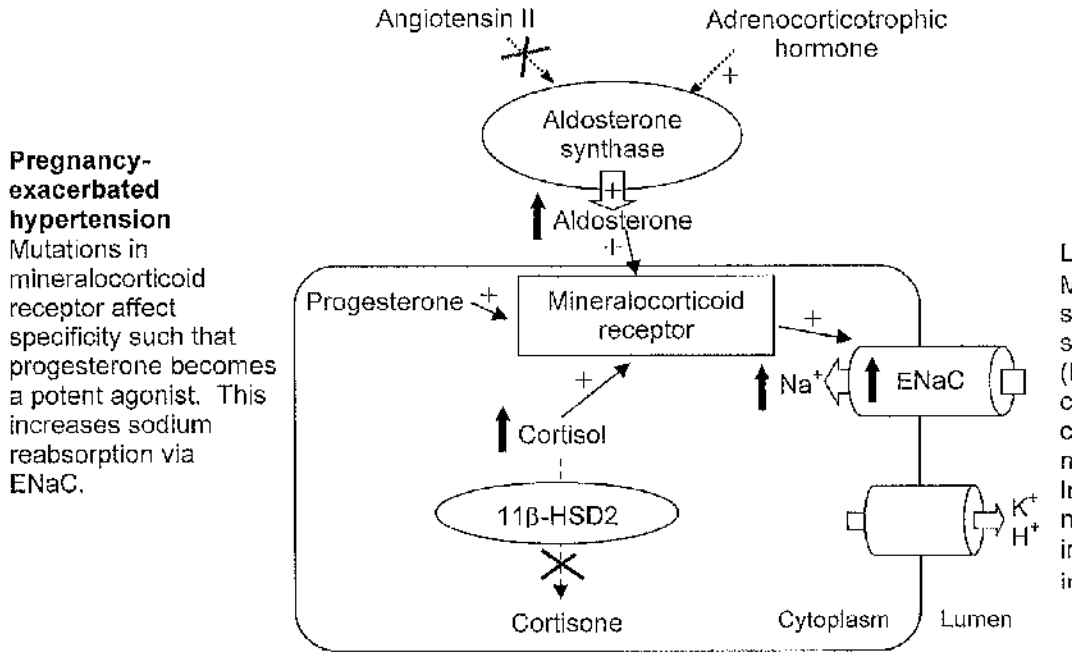
However, it is clear that, in the overwhelming majority of cases, essential hypertension results from a complex interaction of genetic, environmental and demographic factors. It is estimated that between 30-40 per cent of BP variation in a population has an underlying genetic basis(47) and so it is clear that a better understanding of the genetic basis of essential hypertension could result in significant benefits such as tailoring treatment regimes and developing preventive strategies. The use of genome-wide linkage analysis has found statistically significant linkage of blood pressure with several chromosomal regions(41). These findings suggest there are multiple genetic

MENDELIAN	ESSENTIAL
Rare Minimal contribution to global disease burden.	**Common** Affects about 20% of subjects in industrialised societies.
Monogenic Mutations in one gene are necessary and sufficient.	**Oligogenic or polygenic** Subtle polymorphisms in multiple - possibly many - genes increase susceptibility.
Simple genetic syndrome Effect of causative gene is large. Interactions with other genes are of secondary importance.	**Complex genetic trait** Gene-gene interactions are important and result in clinically relevant effects on blood pressure.
Unifactorial Gene defect is highly penetrant.	**Multifactorial** Effects of genes are important in the context of environmental and dietary factors.
Relative risk is high Risk of syndrome in 1st degree relative of affected person is much higher than the general population.	**Relative risk is lower** Risk of hypertension in 1st degree relative of affected person is about 2-5 fold higher than the general population.

Table 1.3c. Comparison between Mendelian and essential hypertension

Cortical collecting duct

Glucocorticoid remediable aldosteronism (GRA)
Increased aldosterone production from abnormally regulated chimaeric aldosterone synthase gene increases sodium reabsorption via ENaC.



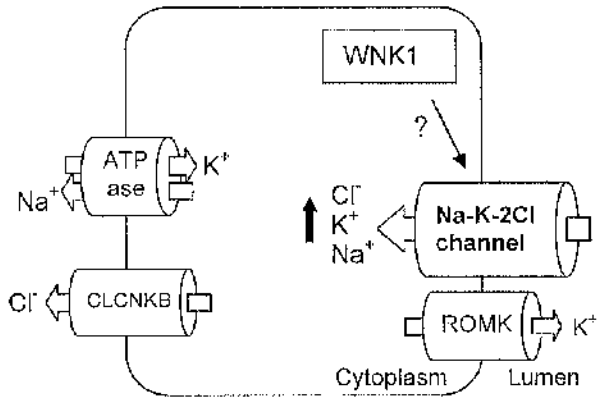
Pregnancy-exacerbated hypertension
Mutations in mineralocorticoid receptor affect specificity such that progesterone becomes a potent agonist. This increases sodium reabsorption via ENaC.

Liddle's Syndrome
Mutations in β and γ subunits of epithelial sodium channel (ENaC) impair clearance of channels from cell membrane. Increased channel number causes increased sodium influx.

Apparent mineralocorticoid excess (AME)
Mutations in 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) impair conversion of cortisol to cortisone. Increased cortisol levels activate mineralocorticoid receptor and increase sodium reabsorption through ENaC.

Thick ascending loop

Gordon's syndrome;
Pseudohypoaldosteronism type IIC (PHA IIC)
A mutant lysine-deficient kinase type I (WNK1) is the cause. Mechanism is currently unknown but may be dysregulation of Na-K-2Cl channel.



Distal convoluted tubule

Gordon's syndrome;
Pseudohypoaldosteronism type IIB (PHA IIB)
Mutant lysine-deficient kinase type 4 (WNK4) causes increased Na^+ and Cl^- influx via sodium-chloride cotransporter (NCCT), and decreased K^+ efflux via inwardly-rectifying potassium channel (ROMK).

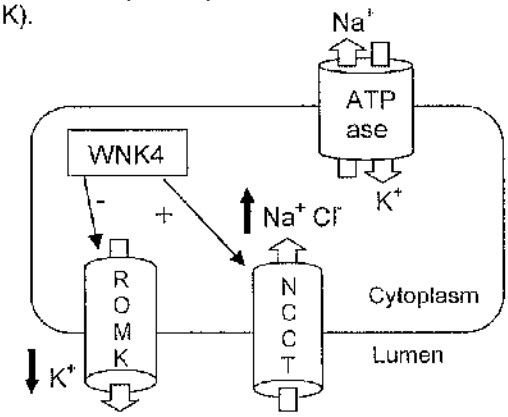


Figure 1.3c. Molecular mechanisms of Mendelian hypertension

loci, each with small effects on blood pressure in the general population. Linkage analysis in hypertension has a number of shortcomings most especially due to locus heterogeneity (more than one causal gene) and clinical heterogeneity. Indeed, genome-wide scans have yielded loci linked to hypertension in almost every chromosome(48)! Nonetheless, as long as these are interpreted with caution, genome wide scans are useful tools in identifying candidate genes for further analysis.

The candidate gene approach typically compares the prevalence of hypertension or the level of blood pressure among individuals of contrasting genotypes at candidate loci in pathways known to be involved in blood pressure regulation. Numerous genes have been linked to essential hypertension but, to date, no single gene is consistently linked to or associated with this complex disorder. The major genes still hypothesised to contribute to essential hypertension are listed in Table 1.3d (49). A few will be discussed further.

(a) Renin-angiotensin system genes

The idea of a candidate gene has been most readily studied in the renin-angiotensin system. Animal model studies have clearly demonstrated that variations in the renin gene affect blood pressure and that the renin gene is directly involved in the development of hypertension (50). However, as yet there is no convincing evidence linking the renin locus to human essential hypertension.

The most promising results have been obtained with the angiotensinogen gene located on chromosome 1q 42-43. The link between angiotensinogen and the development of hypertension has been demonstrated in human

	Statistical approach	
Gene or protein	**Linkage +/-**	**Association +/-**
Renin-angiotensin-aldosterone system or Na^+ volume		
Angiotensin-converting enzyme (ACE)	0/1	12/14
Angiotensinogen (AGT)	3/4	13/9
Aldosterone synthase (CYP11B2)	1/1	6/7
AT 1 receptor		6/6
Adducin (ADD1)	0/1	5/3
Adducin (ADD2)		0/1
Atrial natriuretic peptide		2/5
Human natriuretic peptide receptor (A)		1/1
Human natriuretic peptide receptor (B)		1/2
Renin (REN)	3/2	2/2
Protein kinase lysine deficient 4 (WNK 4)	1/1	
11ß Hydroxysteroid dehydrogenase type 2(11ßHSD2)		2/2
Adrenergic		
$ß_2$-Adrenoceptor		3/4
$ß_3$-Adrenoceptor		3/1
Dopamine D2 receptor gene		2/0
α-Adrenoceptor		1/1
Vascular		
Endothelin-1 gene		3/1
Nitric oxide synthase, endothelial (NOS3)	0/2	5/10
Nitric oxide synthase, inducible (NOS2A)		1/1
Metabolic		
Glycogen synthase		1/1
Insulin receptor	0/1	3/0
Lipoprotein lipase		1/2
Apolipoprotein C-III		1/1

Table 1.3d Genes hypothesised to be involved in essential hypertension. Number of pertinent positive (+) and negative (-) published studies in humans since 1997. Two statistical approaches have been reported: linkage and association (see text)

studies where plasma angiotensinogen levels co-segregate with blood pressure in families (51) as well as transgenic mouse models which develop hypertension when over-expressing angiotensinogen (52). Specific mutations in this gene have been associated with essential hypertension. The most commonly occurring is the M235T polymorphism which encodes a change in amino acid from methionine to threonine (53). This polymorphism is associated with raised circulating levels of angiotensinogen, hypertension (54) and pre-eclampsia (55). The results from some studies suggest that the association with essential hypertension is limited to men(56) or to non-overweight hypertensives (57). Moreover, recent haplotype analysis suggests that the mutation acts as a marker linked to the actual aetiological mutation(58).

The gene for angiotensin-1-converting enzyme (ACE) has been cloned, mapped to chromosome 17 and a polymorphism in intron 16 of the gene characterised(59). This polymorphism consists of the presence or absence of an Alu repeat which can be detected by PCR amplification of intron 16 (insertion (I)/deletion (D) polymorphism). The I/D polymorphism accounts for approximately 50 per cent of the variance in plasma ACE levels in normal populations, with DD homozygotes having the highest plasma ACE levels, II homozygotes the lowest and heterozygotes intermediate levels (60). Again, evidence that the ACE I/D polymorphism is associated with hypertension is inconclusive. A number of studies have shown no association between this polymorphism and hypertension (61;62), whilst more recent investigation has suggested a link with blood pressure variation in men only(63;64). In an attempt to clarify this controversial area, a meta-analysis of studies involving

ACE polymorphism with cardiovascular parameters has been published (65). Significant odds ratios for disease susceptibility with the DD genotype of 1.3 were reported for coronary heart disease, 1.4 for myocardial infarction and 1.5 for both diabetic and non-diabetic renal disease. Significantly, no association was found for left ventricular hypertrophy or hypertension in non-diabetic subjects.

(b) Adducin genes

Adducin is a cytoskeletal protein that interacts with Na-K ATPase.Adducin exists within the cell as a heterodimer comprising various combinations of its α,β and γ subunits (termed ADD1, 2 and 3 respectively). Adducin variants associated with both rat and human hypertension show greater affinity for proximal renal tubular Na-K ATPase resulting in an increase in its membrane expression and activity(66). Initial positive association studies in humans(67) have been followed by significant linkage in hypertensive siblings(68) and have identified a Gly460Trp variant (with an allele frequency of 0.13-0.16 in controls)in ADD1 in association with hypertension. One study identified an approximate 50-70 per cent increase in hypertension risk in older, heavier Caucasians carrying the 460Trp variant (odds ratio 4.2)(69). Phenotypic studies identify a significant increase in salt retention associated with the 460Trp variant(70) providing a plausible pathophysiological mechanism. Additionally, 460Trp homozygotes more frequently have low-renin hypertension and have a greater capacity for sodium reabsorption.

Polymorphisms have also been described in the ADD2 and ADD3 genes but these, by themselves, have no effect on blood pressure or renal sodium handling. However, there is a suggestion that a polymorphism in ADD3

accentuates the effect of ADD1 460Trp suggesting epistatic effects between these loci(71).

(c) Role of WNK kinase polymorphisms

The WNK kinases are a small group of serine/threonine kinases with unique catalytic domains that lack the lysine residue used in other kinases to co-ordinate ATP (hence, With No K [WNK]). Their closest homologues are found within the mitogen-activated protein kinase (MAPK) pathway suggesting a role in signalling. To date, four WNK kinases have been identified in different chromosomal locations and share 84-92 per cent homology across their catalytic domains(72). Two WNK isoforms, WNK1 and WNK4, have been identified as the disease genes for a rare monogenic hypertension syndrome (Gordon's syndrome or pseudohypoaldosteronism type 2 [PHA2]) implicating them in salt homeostasis by the kidney(73).

WNK4 is located on chromosome 17 and several lines of evidence have implicated a region of human chromosome 17 that harbours a gene influencing blood pressure(74;75). However, as yet, no plausible candidate SNP within the WNK4 gene has been identified and studies are ongoing within this field(76;77).

In summary, these variants seem to only modestly affect blood pressure (if at all) and other candidate genes have not shown consistent associations with blood pressure or hypertension in larger populations(78); thus, demonstration of common genetic causes of hypertension in the general population remain elusive.

1.3.6 Sub-groups in essential hypertension

Over the years, there has been a substantial effort to categorize large heterogeneous groups of essential hypertensives into smaller homogeneous sub-groups based on hormonal responses to biological stimuli. In the mid 1980s Williams and Hollenberg(79) described a subgroup of hypertensives in whom changes in sodium intake failed to produce the anticipated reciprocal changes in adrenal (aldosterone) and renal vascular responses to angiotensin II infusions. Such individuals, who have normal/high renin levels, have been termed 'non-modulators'. Non-modulators tend to be older than modulators and there is evidence to suggest that non-modulation has a genetic basis. In one study of hypertensive individuals, 81 per cent with a positive family history of hypertension were non-modulators (80). A previous study of hypertensive sibling pairs showed that non-modulation tended to aggregate within families and is independent of sodium intake (81). In common with low-renin essential hypertension, non-modulators also demonstrate salt sensitivity. In contrast to low-renin hypertension, however, non-modulators clinically respond best to ACE inhibitors as opposed to diuretics (Table 1.3e) (82).

One of the earliest classifications was that of low-renin hypertension(83). In this form of hypertension, subjects exhibit low plasma renin activity (PRA) which does not respond normally to sodium restriction, and maintain basal aldosterone levels which, whilst not elevated, are inappropriate for the principal trophin, angiotensin II. Such a hormonal profile may be due to increased responsiveness of aldosterone to angiotensin II(84;85), although not all patients share this abnormality (42). Classically, low-renin subjects have sodium sensitive hypertension, which tends to respond better to diuretics than agents that block the RAA system. Low-renin hypertension is

	LOW RENIN	NON-MODULATORS
Plasma renin level	Low	Normal-high
Salt sensitive	Yes	Yes
Anti-hypertensive	Diuretics	ACE inhibitors
Phenotype	Elderly	Males>Females
Response to Ang II	Normal	Reduced

Table 1.3e. Sub-groups in essential hypertension

found more frequently among black and elderly populations (86).Thus it is clear that production of aldosterone and regulation of sodium states are important features amongst large sub-groups of subjects with hypertension.

1.4 The Adrenal Gland

The first anatomical description of the adrenal gland was made in 1563 By Eustachius(87). The first differentiation of the adrenal gland into cortex and medulla was made by Huchke and in the 19th century Arnold first described the concentric zones we now use to describe the adrenal cortex (88). However, ideas about the function of the adrenal gland were slower to develop but were pioneered by observations by Thomas Addison(89). It was six years later that Addison published his authoritative account of the effects of adrenal disease in man(90). Experimental confirmation of these clinical findings came the following year when Brown-Séquard demonstrated the detrimental effect of adrenalectomy in various animal species(91). It was not until 1930 however, that the efficiency of adrenal cortex extracts in maintaining the health and growth of adrenalectomised cats was noted (92) and these extracts used to successfully treat patients with Addison's disease (93).

1.4.1 The Anatomy of the Adrenal Gland

Each adrenal gland represents a functionally distinct endocrine gland within a single fibrous capsule. The adrenal glands are situated on the superior poles of the kidneys and normally weigh four grams in healthy adults (Figure 1.4a). The outer capsule consists of loose fibrous tissue and surrounds the outer

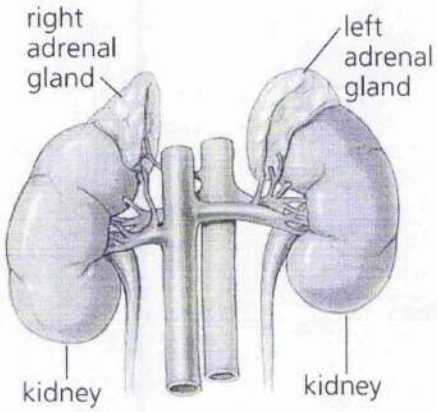


Figure 1.4a. The gross anatomy of the human adrenal glands

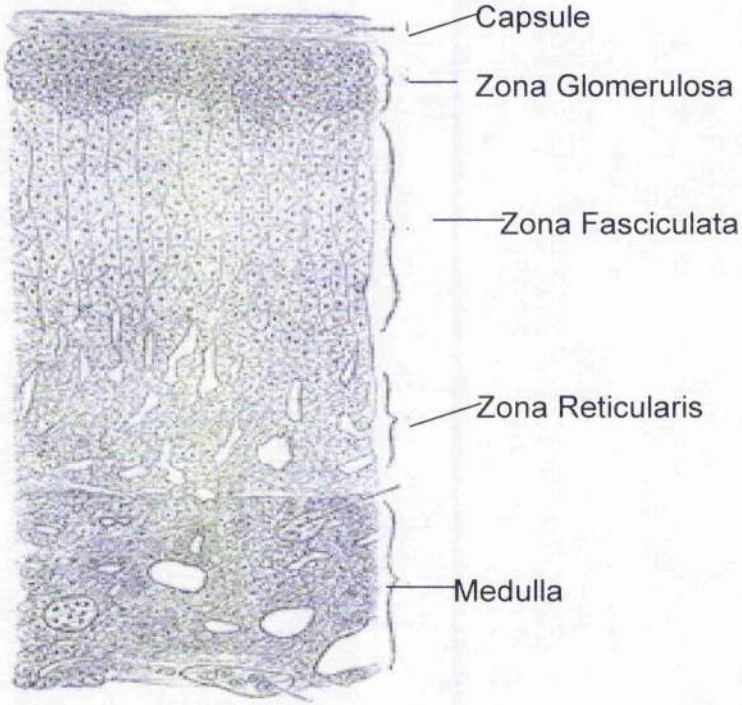


Figure 1.4b. Histology of human adrenal gland

cortex of the adrenal. The cortex accounts for 90 per-cent of the weight of the adult adrenal and is derived from the mesenchymal cells adjacent to the urogenital ridge. The inner adrenal medulla is derived from cells of the neural crest which migrate into the adrenal during the third month of fetal life. Its cells secrete the catecholamines, adrenaline, noradrenaline and dopamine; its function will not be discussed further.

The adrenal cortex is divided into three roughly concentric zones based on the light microscopic appearance of the cells (88) (Figure 1.4b). The outer zone, the zona glomerulosa, constitutes 15 per cent of the cortex and consists of poorly demarcated islands of cells lying subjacent to the capsule of the gland. In the majority of instances these islands do not form a continuous layer. The glomerulosa cells are small and have a low cytoplasm: nuclear ratio and an intermediate number of lipid inclusions. The adjacent zone is the zona fasciculata which constitutes 75 per cent of the cortex. It is not well demarcated from the glomerulosa and cells from the fasciculata may penetrate the glomerulosa to contact the capsule. Cells in this zone have a high cytoplasm: nuclear ratio, are large and have a foamy, vacuolated cytoplasm because of the numerous lipid deposits. The innermost zone of the cortex is the zona reticularis, and is sharply demarcated from the fasciculata and the medulla. Cells in this zone have a compact, lipid-poor cytoplasm and lie in anastomosing cords separated by sinusoidal spaces (94).

1.4.2 Corticosteroid Synthesis in the Adrenal Cortex

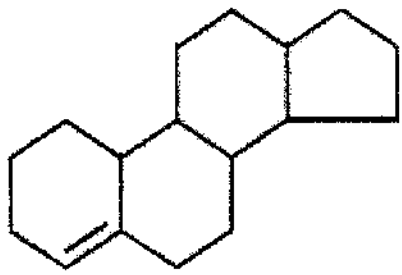


Figure 1.4c Cyclopentanophenanthrene ring structure

The adrenal cortex is the site of synthesis of a number of important steroid hormones in man. Steroid hormones are derived from the cyclopentanopentane ring (Figure 1.4c).

The starting point for the synthesis of all adrenal corticosteroid hormones in man is cholesterol. Cholesterol can be synthesized *de novo* from acetate, mobilized from intracellular pools or imported from plasma lipoproteins by steroidogenic tissues. About 80 per-cent of cholesterol is provided by circulating lipoproteins, principally low-density lipoprotein (LDL) which acts via a cell surface receptor (95). The LDL particle undergoes receptor-mediated endocytosis to be internalised by the cell and cholesteryl esters are released for use as steroidogenic substrates (96). Under normal conditions, a further 20 per-cent of cholesterol is synthesised *de novo* from acetyl coenzyme A (95).

Three major groups of steroid hormones are synthesised in the adrenal cortex in man. The outer zona glomerulosa is the exclusive site of synthesis of aldosterone whilst the zonae fasciculata and reticularis synthesise cortisol and the adrenal androgens dehydroepiandrosterone (DHEA) and androstenedione. The most abundant product of the adrenal cortex, dehydroepiandrosterone sulphate (DHEAS), is formed exclusively in the zona reticularis(94).

Synthesis of the various adrenal corticosteroids is compartmentalised within the cells of the adrenal cortex as a result of the subcellular location of the enzymes involved (Figure 1.4d). The biosynthetic pathways for the conversion of cholesterol to aldosterone, cortisol and adrenal androgens are outlined in

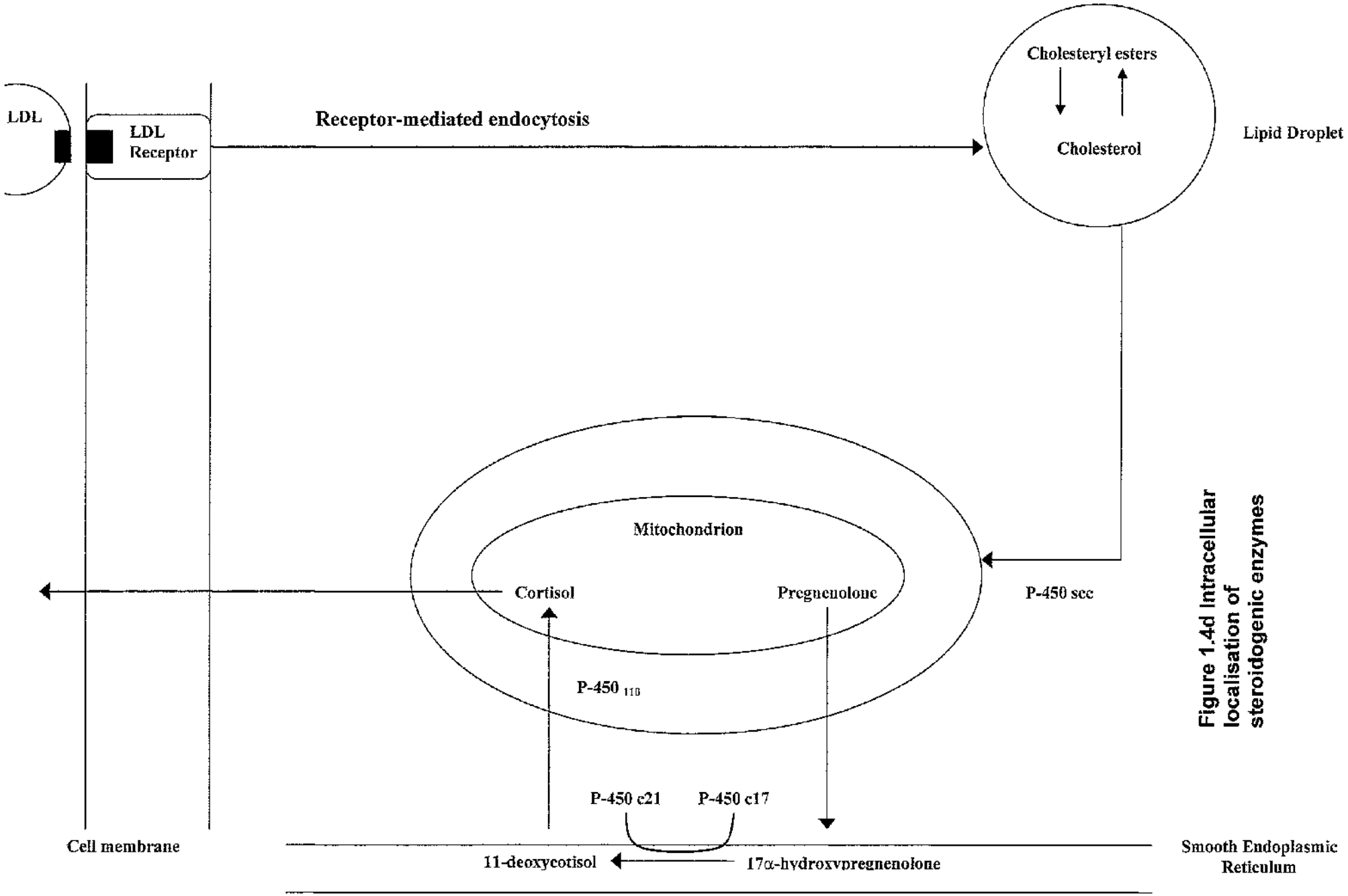


Figure 1.4d Intracellular localisation of steroidogenic enzymes

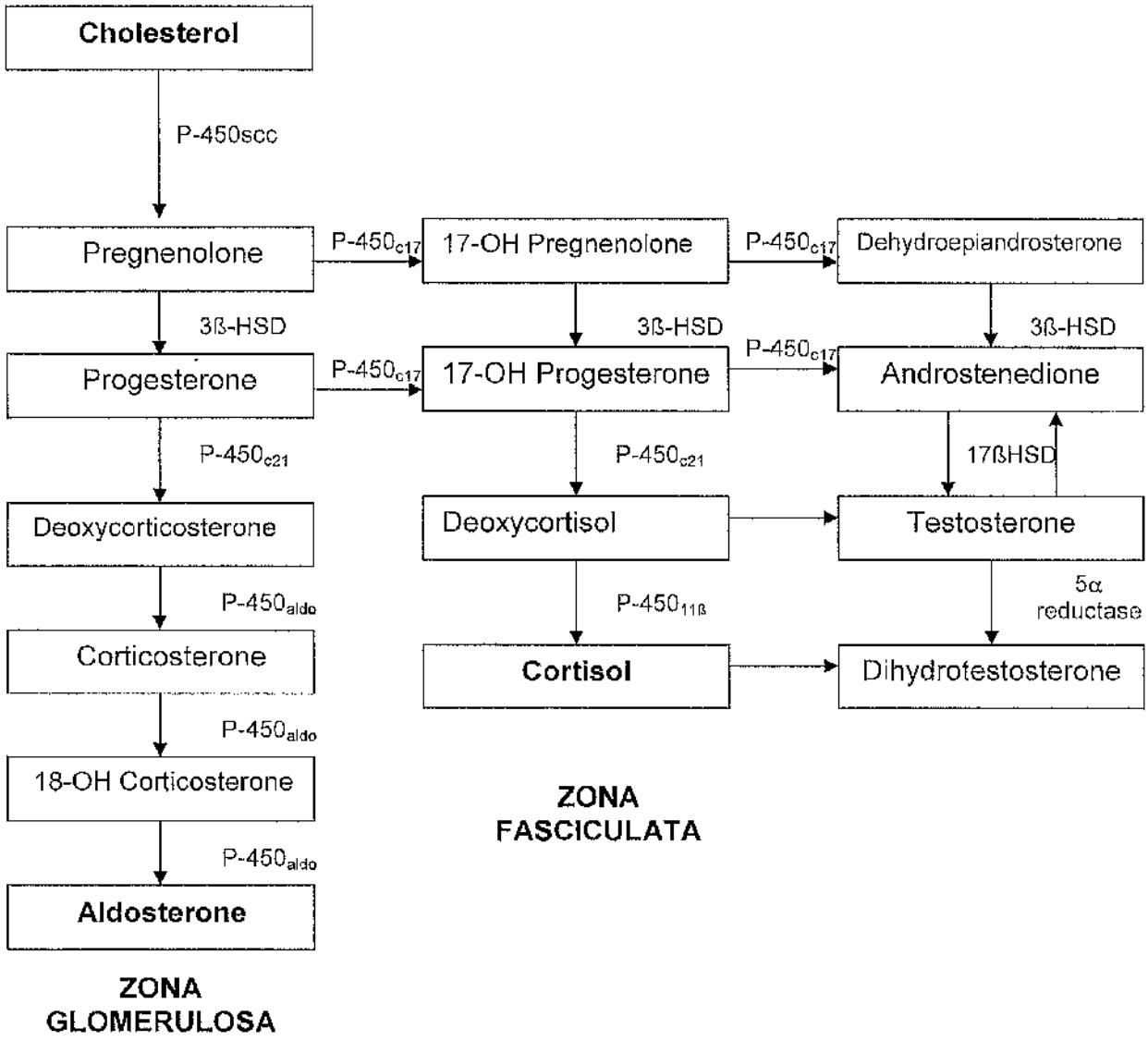


Figure 1.4e. Corticosteroid biosynthesis in the adrenal cortex
Many of the biosynthetic reactions are catalysed by cytochrome P450 enzymes:

scc- side chain cleavage
c17- 17α-hydroxylase
c21- 21α-hydroxylase
aldo- aldosterone synthase
11ß- 11ß-hydroxylase

3ß/17ß-HSD: 3ß / 17ß hydroxysteroid dehydrogenase

figure 1.4e. The aldosterone synthetic pathway is discussed in detail in section 1.6.

The synthetic action of the steroidogenic enzymes is made possible by the existence of a coupled enzyme system that transfers electrons to the P-450 enzymes as reducing equivalents and allow the final hydroxylation step to occur. In the human adrenal, two such systems exist, both coupled to the action of the steroidogenic P-450 enzymes. The first is based on adrenodoxin, a non-haem iron binding protein that exists in soluble form in the mitochondrial matrix and in man is linked to the activity of $P\text{-}450_{scc}$, $P\text{-}450_{11ß}$ (11ß-hydroxylase) and $P\text{-}450_{aldo}$ (aldosterone synthase)(97). Linked to adrenodoxin is the enzyme adrenodoxin reductase which accepts electrons from NADPH in the first step of the reduction cascade. This so called 'electron shuttle' system is illustrated in Figure 1.4f. NADPH donates electrons to an adrenodoxin reductase and converts it to its reduced state. This then acts to convert adrenodoxin to a reduced state that in turn transfers reducing equivalents to the P-450 enzyme.

A second system exists coupled to the activity of $P\text{-}450_{c17}$ and $P\text{-}450_{c21}$ (98). This system utilises a flavoproten distinct from adrenodoxin reductase. This enzyme, P-450 reductase, transfers two electrons from NADPH to $P\text{-}450_{c17}$ and $P\text{-}450_{c21}$. In addition, cytochrome b5 may also donate electrons to $P\text{-}450_{c17}$ and $P\text{-}450_{c21}$ (99) (Figure 1.4g).

Cholesterol is converted to pregnenolone by the enzyme $P\text{-}450_{scc}$ located on the inner mitochondrial membrane. This step is the rate-limiting step in steroidogenesis and involves two oxidations at the C20 and C22 positions followed by the removal of isocaproic acid from the C20 position (100). This

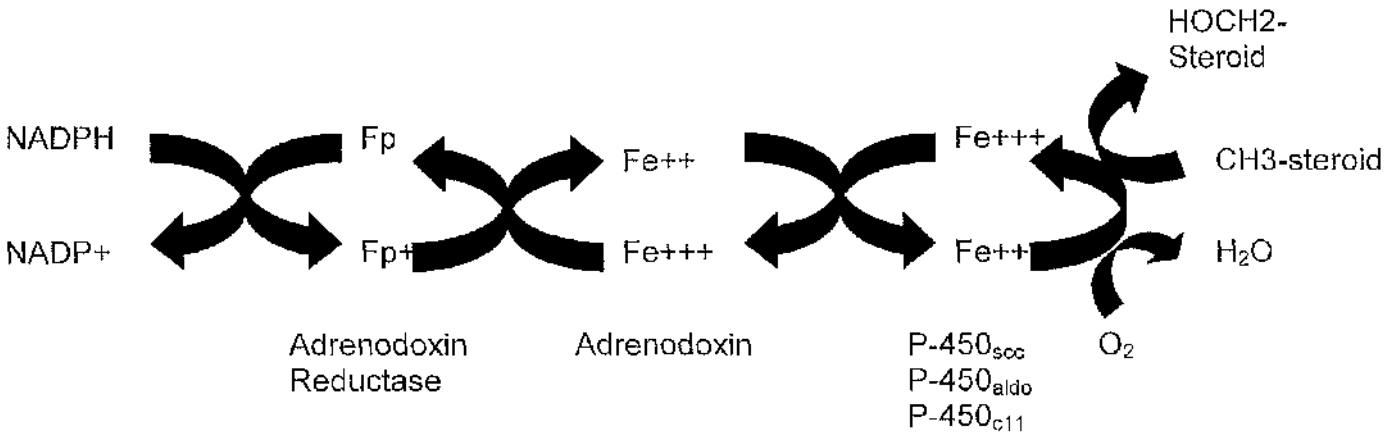


Figure 1.4f. Electron shuttle system for P-450$_{scc}$, P-450$_{aldo}$ and P-450$_{c11}$.

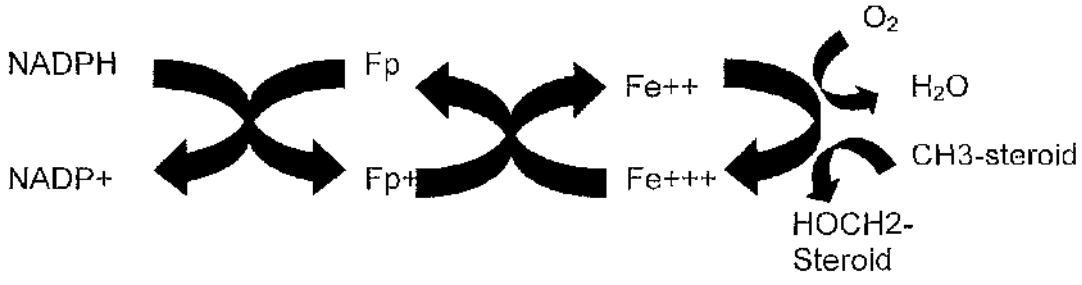


Figure 1.4g. Electron shuttle system for P-450$_{c17}$ and P-450$_{c21}$.

enzyme, the so-called 'Side-chain Cleavage' enzyme is encoded by a single gene found on chromosome 15 and is found in all steroidogenic tissues (101). This cholesterol transport process is hormonally regulated via cAMP and synthesis of the Steroidogenic Acute Regulatory (StAR) protein in response to trophic stimuli(102). The StAR protein is thought to be located within the mitochondria of adrenocortical cells and is key in the binding and transport of cholesterol to the inner mitochondrial membrane (the sub-cellular location of P-450_{scc})(103;104).

The newly synthesised pregnenolone is returned to the cytosolic compartment where a series of microsomal enzymes convert it to 11-deoxycortisol (S). Pregnenolone is converted to progesterone by the enzyme 3ß-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase, a non P-450 enzyme encoded by a gene on chromosome 1 (105). A number of isoforms of this enzyme have been described with tissue specific expression (106).

The enzyme P-450_{c17} is encoded by the gene CYP17A on chromosome 10 (107) which transcribes a single mRNA message and protein product. The degree of P-450_{c17} activity helps determine which pathway the reaction products take. In the zona glomerulosa P-450_{c17} activity is absent and therefore pregnenolone is converted to progesterone as a precursor in the formation of aldosterone. In the zona fasciculata pregnenolone undergoes hydroxylation of the C-17 position and the product, 17α-hydroxyprogesterone undergoes subsequent 21-hydroxylation to 11-deoxycortisol and then 11-hydroxylation to produce cortisol. However, some pregnenolone may be converted to progesterone before being converted to 17α-hydroxyprogesterone and subsequently cortisol. If the action of the enzyme is

to cleave the two-carbon chain at C-21 in addition to the hydroxylation of pregnenolone (i.e. 17α-hydroxylase 17,20 lyase) the product is DHEA – a major adrenal androgen and the precursor of other adrenal androgens (94). Whether 17-hydroxycorticosteroids are formed by this enzyme system is determined by the relative electron supply from the P-450 reductase system. This system donates electrons to $P\text{-}450_{c17}$ in competition with $P\text{-}450_{c21}$ and the relative excess or defecit of donated electrons can determine the degree of $P\text{-}450_{c17}$ activity (108). In the zona glomerulosa there is no $P\text{-}450_{c17}$ activity.

The next step in the sequence is the conversion of 17α-hydroxyprogesterone to 11-deoxycortisol (S) by the enzyme P-450c21. This enzyme resides in the smooth endoplasmic reticulum of the cell and is encoded by the gene CYP21A which lies on chromosome 6 in the midst of the Major Histocompatibility Complex region. It lies in tandem with a highly homologous pseudogene, CYP21P, which has no known function and can participate in gene conversion events to cause the clinical syndromes of steroid 21-hydroxylase deficiency (109).

Once formed, 11-deoxycortisol is transported to the inner mitochondrial membrane where the final hydroxylation step to form cortisol, the principal human glucocorticoid, is formed. This step is catalysed by the enzyme $P\text{-}450_{11ß}$ (11β-hydroxylase) whose gene, CYP11B1, is found on the long arm of chromosome 8 (8q22)(110). In addition to the formation of cortisol, $P\text{-}450_{11ß}$ is also thought to catalyse the formation of corticosterone, 18-hydroxy-11-deoxycorticosterone and 19-hydroxy-11-deoxycorticosterone from 11-deoxycorticosterone in the zona fasciculata, emphasising the close

relationship between this enzyme and $P\text{-}450_{aldo}$ (aldosterone synthase) in their catalytic properties (111).

The gene for 11β-hydroxylase (CYP11B1) and its closely related homologue, CYP11B2 (encoding aldosterone synthase) are of central importance in the biosynthesis of aldosterone and in the pathogenesis of hypertensive disorders of adrenal metabolism and are discussed further in section 1.6.5.

1.4.3 Regulation of Adrenal Steroid Secretion

Control of the rate of synthesis of cortisol and the adrenal androgens is determined by factors outwith the adrenal gland. Principally, these include interactions between the hypothalamus, anterior pituitary and adrenal glands as well as neural stimuli and other factors such as stress. The normal regulation of human cortisol secretion is summarised in figure 1.4h.

1.4.4 Adrenocorticotrophin (ACTH)

The principal secretory stimulus to the cortisol-producing cells of the adrenal cortex is ACTH. ACTH is synthesised as part of a large precursor molecule (241 amino acids), pro-opiomelanocorticotrophin (POMC), which also contains other peptides, including melanocyte-stimulating hormone. The gene for POMC is located on chromosome 2 (112;113). The gene product of the POMC gene undergoes significant post-translational processing to produce multiple peptides. In the anterior pituitary, the products of this enzymatic cleavage are ACTH, β-lipotrophin and the joining (J) peptide. ACTH is a 39 amino acid peptide, of which the first 24 are conserved in all species studied.

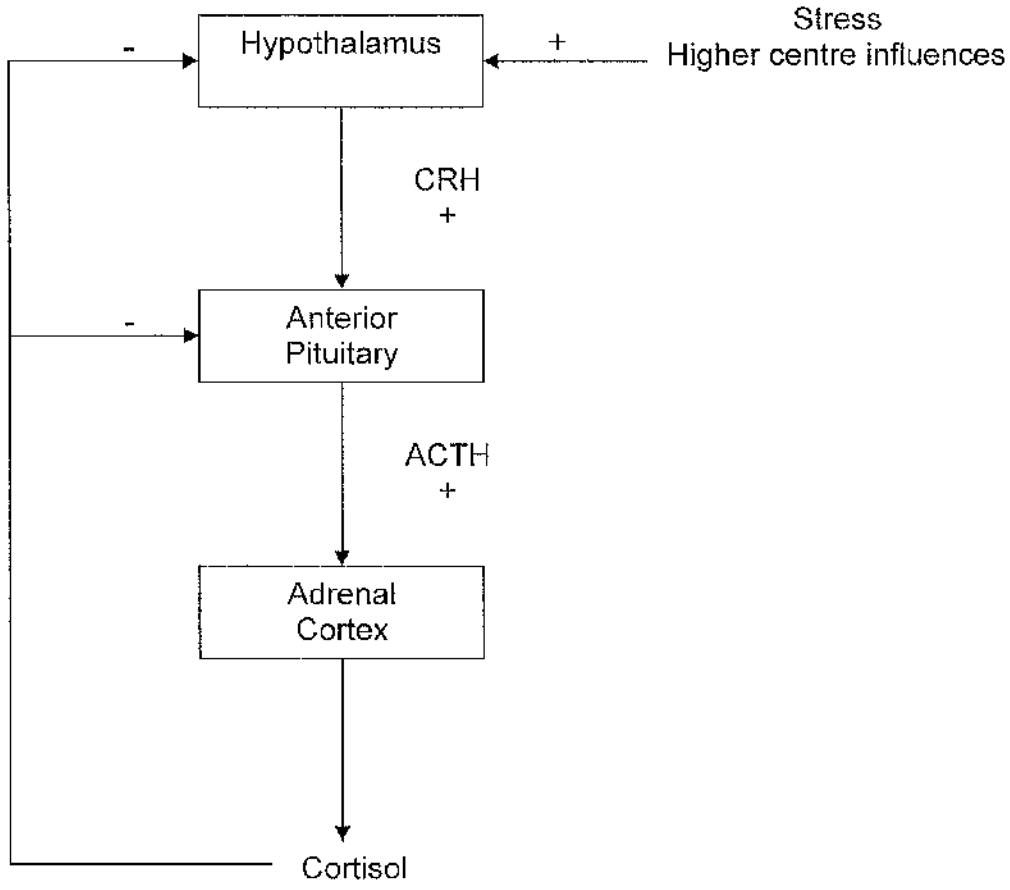


Figure 1.4h. Control of cortisol secretion in man

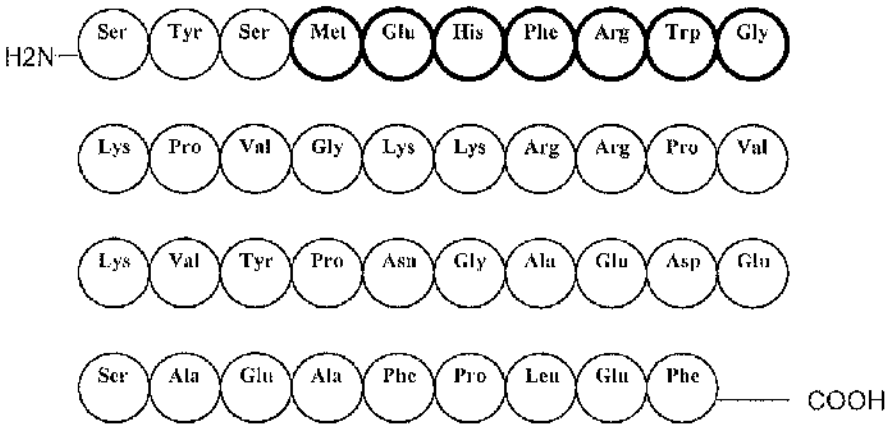


Figure 1.4i. Amino acid sequence of human ACTH.
Bold residues indicate those common to ACTH, alpha and beta MSH.

The biological activity of ACTH is conferred by the first 18 NH_2-terminal amino acids (114) (Figure 1.4i).

The ACTH peptide also contains the precursor molecule for one of the melanocyte-stimulating hormones (α-MSH) the effect of which can be seen when ACTH is present in gross excess in Nelson's syndrome (skin pigmentation as a result of chronically elevated plasma ACTH levels from an ACTH-secreting pituitary adenoma).

ACTH release from the pituitary gland is pulsatile with brief episodic bursts of ACTH release throughout a 24 hour period. Release of ACTH is greater in the early hours of the morning due to pulses of greater amplitude (115). The increase in amplitude of the pulses occurs after 3-4 hours of sleep and reaches a maximum prior to waking and for 1 hour after waking before declining over the course of the day (115). Plasma cortisol levels mirror these changes in ACTH secretion but the longer clearance time for cortisol results in a slower decline in plasma cortisol relative to ACTH (116). This so-called 'nyctothemeral' or 'diurnal' rhythm is maintained in the physiological state but lost in disease states where ACTH or cortisol secretion becomes autonomous, e.g. Cushing's disease (ACTH-secreting pituitary adenoma) or a cortisol-secreting adrenal adenoma/carcinoma.

Under normal circumstances, ACTH and cortisol regulate their own secretion by means of a negative feedback loop whereby high levels of cortisol inhibit the secretion of ACTH and corticotrophin releasing hormone (CRH; which regulates ACTH secretion). However, ACTH secretion and consequently plasma cortisol levels can be influenced by inputs from 'higher' centres. For example, hypoglycaemia, fever and mental stress all raise ACTH and cortisol

levels and are important sources of variation in day-to-day measurement of plasma levels.

The primary action of ACTH on the cells of the zona fasciculata is to increase cortisol secretion by increasing its synthesis; intra-adrenal storage of cortisol is minimal (117;118). ACTH depletes adrenal cholesterol content to an extent which corresponds with increased synthesis (119). ACTH acts by binding to a high-affinity cell-surface receptor, and each adrenocortical cell is said to possess approximately 3600 ACTH-binding sites (94). In turn, ACTH binding promotes the generation of cyclic AMP (cAMP) via adenylate cyclase, which subsequently activates protein kinase A and leads to the phosphorylation of a number of proteins and increase in nuclear mRNA production.

The effects of ACTH can be divided into acute (within minutes) and chronic (after several hours). The acute effect of ACTH is to increase conversion of cholesterol to pregnenolone, the rate-limiting step of cortisol synthesis, by activation of P-450_{scc} (120). Using polyclonal antibodies directed against the various steroidogenic enzymes it has been demonstrated that ACTH treatment of bovine adrenal cortex cells results in a 4-5-fold increase in the rate of synthesis of P-450_{scc}, P-$450_{11\beta}$, P-450_{c21}, P-$450_{17\alpha}$, StAR and adrenodoxin 24-36 hours after ACTH treatment. ACTH and cAMP treatment lead to similar increases in the levels of translatable mRNA of all of these enzymes (120) and this is thought to be due to an increase in transcription of the corresponding genes. These increases in mRNA synthesis can be detected as soon as 4 hours after ACTH treatment. Similar results have been found using human fetal adrenal cells (121).

The chronic effects of ACTH involve increased synthesis of most of the enzymes in the steroidogenic pathway as well as actions on cell growth (118;120). When there is a prolonged deficiency of ACTH, such as following hypophysectomy, the level of steroidogenic enzymes and RNA synthesis falls in association with atrophy of the adrenal cortex. These changes are reversed by ACTH administration but may require several days to return to normal (94). Using nuclear run-on assays, it has been demonstrated that ACTH administration results in increased transcription of the various steroidogenic enzyme genes, and that the increased mRNA synthesis leads to chronically enhanced enzyme production (121). Moreover, the addition of cycloheximide, an inhibitor of RNA translation, to ACTH-stimulated bovine adrenocortical cells inhibits production of steroidogenic enzyme species, but the total enzyme levels remain normal. Therefore, it would appear that ACTH, acting via cAMP generation, generates short-lived protein factor(s), which may interact directly with steroidogenic genes to influence their expression.

1.5 Adrenal Glucocorticoids

Glucocorticoids exert effects on every system in the body, although their name derives from their effects on carbohydrate metabolism. In common with all steroid hormones, glucocorticoids produce their effects via binding to soluble intracellular receptor proteins. The steroid is thought to enter the cell by passive diffusion and once inside binds to the specific glucocorticoid receptor (GR), to form a complex capable of activating transcription of target genes.

1.5.1 Glucocorticoid Receptors

Glucocorticoid receptors belong to the steroid/thyroid/retinoid/orphan receptor superfamily. Binding of cortisol to the GR leads to activation of *cis*-acting elements located in or near hormone responsive genes (122). On binding steroids the receptor and its associated molecules ('chaperonins'(123)) dissociate and the steroid-receptor complex can travel to the cell nucleus. Dissociated from the satellite proteins, the steroid-receptor complex can then bind as a dimer to a specific region of DNA termed the hormone response element (HRE). HRE are present in many genes and fall into five groups-including the glucocorticoid/progesterone responsive element (GRE/PRE), which also acts as the mineralocorticoid-responsive element, the estrogen-responsive element (ERE) and the thyroid-responsive element (TRE)(Table 1.5a).

Binding to these sites is through the highly conserved 'zinc-finger' region of the receptor which, if altered, leads to depression of DNA binding (124). Thus bound, the steroid-receptor complex leads to transcriptional activation of the bound gene by switching on enhancer regions of the gene(125). It is through this series of actions that steroid hormones exert their effects in man.

1.5.2 Physiological Actions of Glucocorticoids

The pleiotropic effects of corticosteroids are outlined in Figure 1.5a. Arguably, the effects of cortisol on carbohydrate/lipid metabolism as well as the immune system are the most biologically significant.

Hormone-responsive element	Consensus sequence
GRE-PRE-MRE-ARE	5'-AGGACANNNTGTACC-3'
ERE	5'-NGGTCANNNTGACCN-3'
VDRE	5'-TTGGTGACTCACCGGGTGAAC-3'
TRE	5'-GGGTCATGACAG-3'
RARE	5'-AGGACATGACCT-3'

Table 1.5a. Consensus sequences of gene hormone-response elements
GRE-glucocorticoid responsive element, MRE-mineralocorticoid responsive element, ARE-androgen responsive element, ERE-estrogen responsive element, VDRE-vitamin D responsive element, TRE-thyroid responsive element, RARE-retinoic acid responsive element.
N- any nucleotide

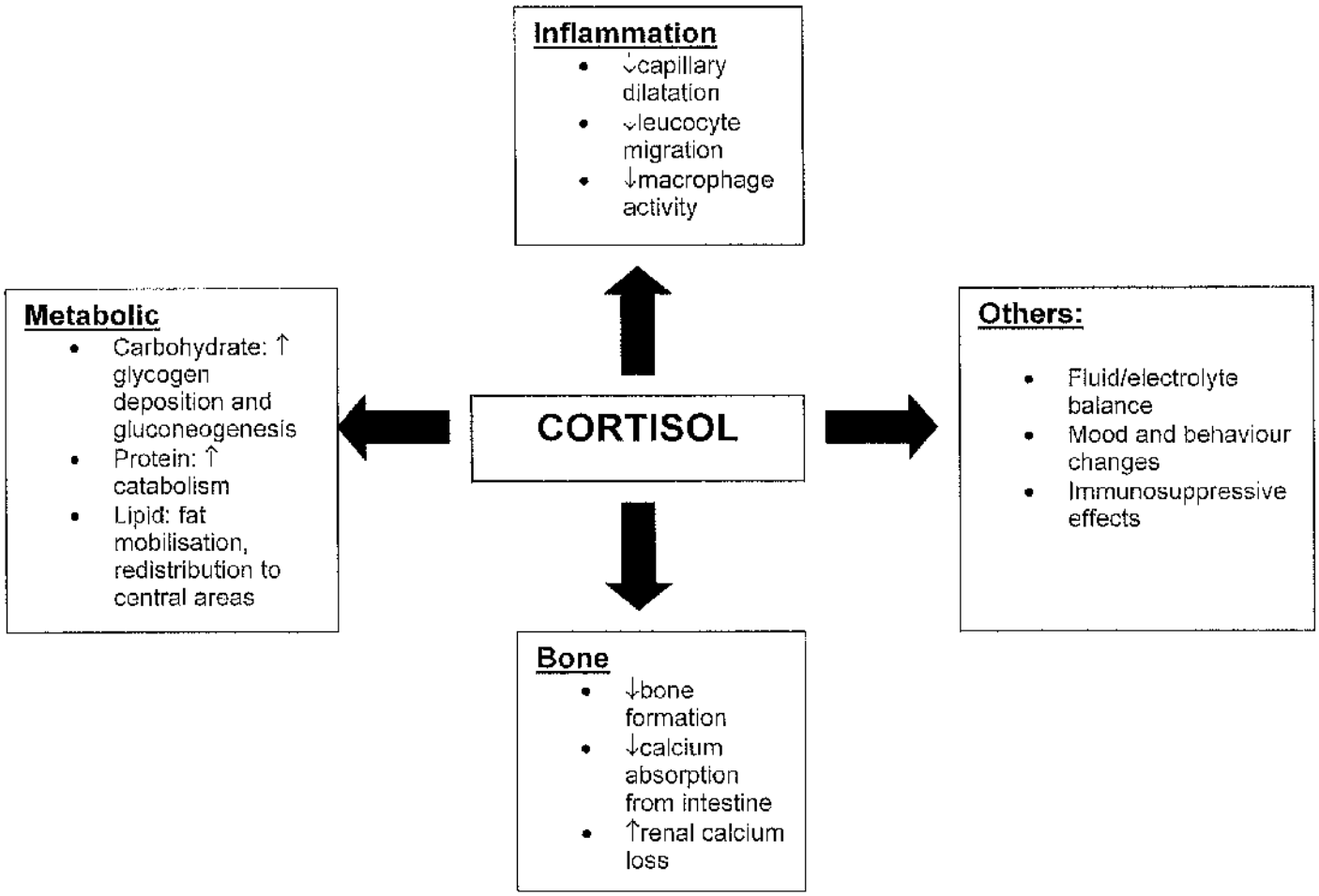


Figure 1.5a. The pleiotropic effects of corticosteroids

Glucocorticoids have weak mineralocorticoid activity under normal physiological conditions. Cortisol has similar affinity for the mineralocorticoid receptor (MR) as aldosterone but in ordinary circumstances is prevented from binding the receptor by the 11ß-hydroxysteroid dehydrogenase system(126). This system is discussed in more detail in section 1.6.6.

1.5.3 Glucocorticoids and Hypertension

Evidence of glucocorticoid modulation of blood pressure comes largely from patients with glucocorticoid excess or deficiency. Around 75 per-cent of patients with Cushing's syndrome (glucocorticoid excess) have hypertension (127)and subjects with glucocorticoid deficiency are almost always hypotensive.

However, the mechanisms underlying glucocorticoid-mediated hypertension are unclear since cortisol has only very weak mineralocorticoid activity under normal physiological conditions. Moreover, whilst the sodium and water-retaining properties of cortisol are ameliorated by use of the mineralocorticoid antagonist, spironolactone, this has no effect on the rise in blood pressure(128). In addition, Clore et al (129) found that the glucocorticoid antagonist RU-486 did not modify cortisol-induced elevations in blood pressure despite blockade of cortisol-induced hyperinsulinemia. Thus, the hypertension produced by cortisol is not simply explained through classic steroid actions.

The mechanism(s) by which cortisol raises blood pressure in humans is unclear; cardiac output is increased, but this is not essential for the rise in blood pressure, (130) and sympathetic activity is decreased(131-133).

Cortisol has a variety of effects on kidneys, heart, brain, blood vessels, and body fluid volumes, but it is not clear which of these are causal rather than coincidental or simply modulators of the rise in blood pressure.

Functional abnormalities of the adrenal cortex were suggested as a cause for essential hypertension many years ago (134); indeed, early studies reported that adrenal cortex hyperplasia was a feature of many hypertensive individuals at post mortem examination (135;136). Additionally, a number of abnormalities of urinary excretion, plasma levels and clearance of several adrenal steroids in hypertensive patients have been identified over the years(137-140). No single defect in adrenal corticosteroid biosynthesis has been identified but it is relevant to consider briefly two rare monogenic syndromes involving 11β-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) that cause hypertension and help identify candidate mechanisms (Table 1.10c).

(a) Glucocorticoid remediable aldosteronism (GRA) is a rare autosomal dominant condition characterised by hypertension and aldosterone excess that is regulated by ACTH rather than Ang II. The molecular basis of this condition was first described in 1992(141). In GRA, a chimeric gene is created containing the 5' promoter sequence of *CYP11B1* and functional elements of *CYP11B2* resulting in aldosterone production under control of ACTH (Figure 1.5b).

(b) 11β-hydroxylase deficiency is a rare cause of congenital adrenal hyperplasia accounting for 5 to 8% of cases(142). In this autosomal recessive disorder, mutations in CYP11B1 result in impaired activity of 11β-hydroxylase leading to accumulation of the steroid precursors 11-deoxycortisol and

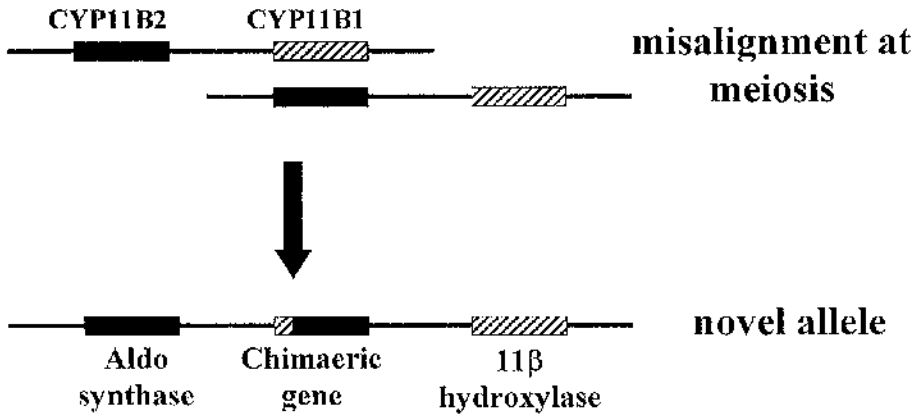


Figure 1.5b. Molecular basis of Glucocorticoid Remediable Aldosteronism (GRA)

deoxycorticosterone (Figure 1.5c). This leads to mineralocorticoid hypertension in approximately two thirds of cases.

In light of these rare syndromes, it is of interest that impaired activity of the enzyme 11β-hydroxylase has also long been reported to be a feature of subjects with essential hypertension. In 1985, de Simone et al demonstrated that ACTH-stimulated plasma levels of deoxycorticosterone (DOC) were increased in hypertensives compared to controls(143); a finding similar to that of Honda and colleagues 10 years earlier(144). More recently, we have observed a similar phenomenon in hypertensive patients from Italy, in whom the ratio of 11-deoxycortisol to cortisol (a marker of 11β-hydroxylase activity) was elevated (145). Finally, animal models of hypertension confirm an aetiological role for the CYP11B locus. The Dahl hypertensive rat is characterised, phenotypically, by salt sensitive hypertension in which there is altered production of β-adrenal 18-hydroxylated steroids; this is accounted for by mutations at the CYP11B1 and CYP11B2 locus(146).

Thus, these studies provide evidence of alteration of adrenal cortex 11ß-hydroxylase activity in essential hypertension. The exact nature of this abnormality is unclear, but a hypothesis linking this finding to individuals with hypertension with aldosterone excess and the underlying genetic explanation for this forms much of the work of this thesis.

1.6 Aldosterone

Aldosterone, the principal human mineralocorticoid, is produced in the zona glomerulosa of the adrenal gland. After its isolation from bovine adrenal glands by Simpson and Tait in 1953 (147), progress into aldosterone research

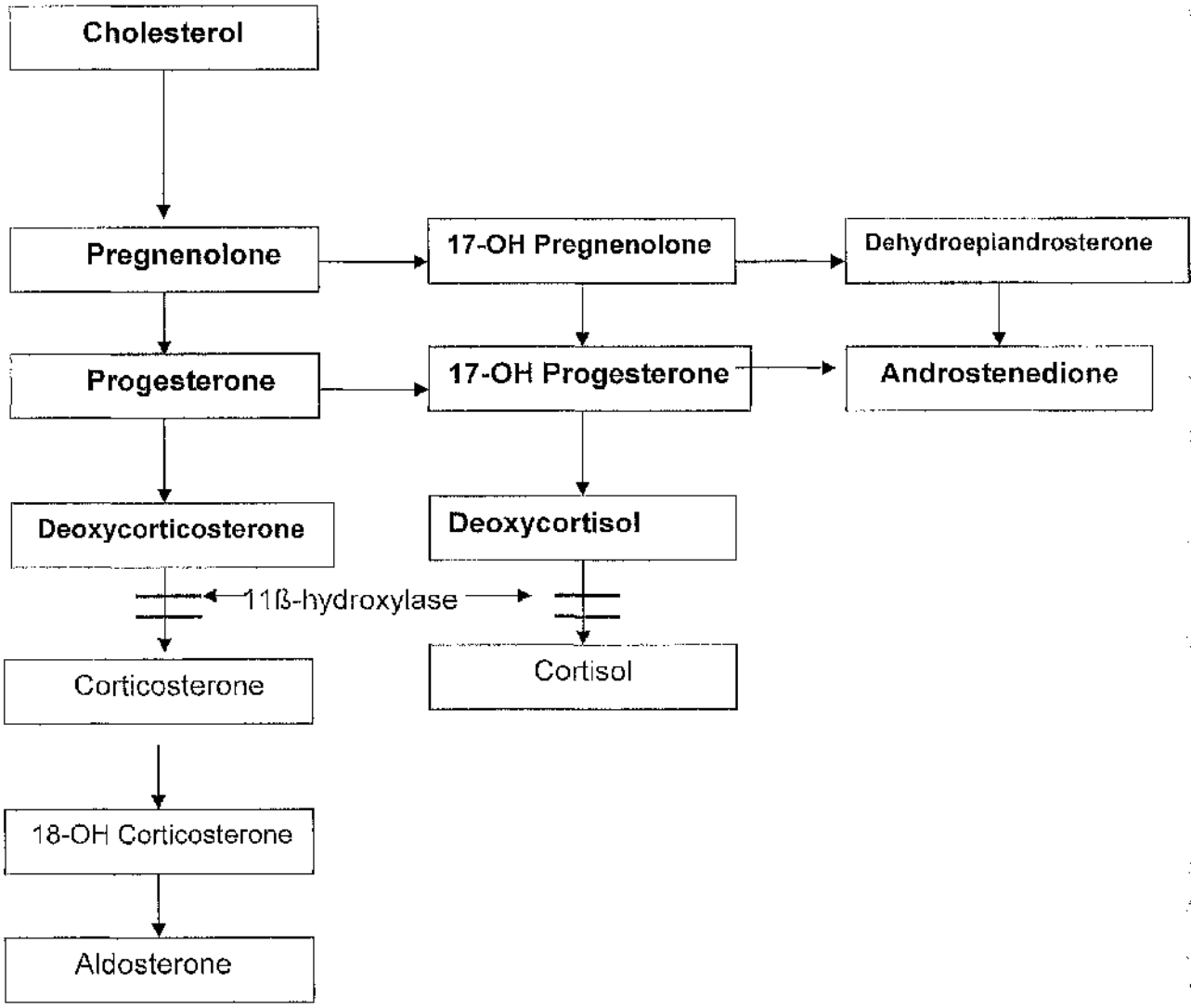


Figure 1.5c. Deficiency of steroid 11ß-hydroxylase in man.

Impaired cortisol synthesis results in an increase in ACTH drive to the adrenal gland resulting in cortical hyperplasia as well as an increase in steroid precursors and adrenal androgens (shown in bold).
Aldosterone synthesis is also defective but excess DOC production proximal to the synthetic defect leads to sodium retention with suppression of the renin-angiotensin axis.

was initially fairly rapid. Within months, its structure was reported(148) and more sensitive and specific bioassays developed (149). Only 2 years later, Jerome Conn described hyperaldosteronism arising from a benign adrenal tumour, leading to the identification of a syndrome characterised by hypertension and hypokalaemia that bears his name (150).

It was subsequently suggested by Conn in 1964 that Primary Aldosteronism (PA) could account for up to 20% of cases of hypertension and hypokalaemia was a late manifestation (151).This was never widely accepted and, until recently, PA was regarded as a relatively rare cause of secondary hypertension accounting for around 1% of cases (152). However, more recent evidence discussed in section 1.10 may at least partly vindicate Conn's views.

1.6.1 Biosynthesis of Aldosterone

Aldosterone is synthesised exclusively in the cells of the zona glomerulosa. As has already been described in section 1.4.2, aldosterone is the end product of a series of reactions starting with the conversion of cholesterol to pregnenolone by P-450_{scc} in the inner mitochondrial membrane with the assistance of the StAR protein. Pregnenolone is then converted to progesterone by the enzyme 3ß-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase and progesterone converted to 11-deoxycorticosterone (DOC) by P-450_{c21} in the smooth endoplasmic reticulum of the cell. In the zona glomerulosa, there is no significant expression of P-450_{c17} (153) and the result is the production of 17-deoxycorticosteroids (11-deoxycorticosterone). In the zona fasciculata, not all progesterone undergoes 17α-hydroxylation by P-450_{c17} (154) and as a result both DOC and 11-deoxycortisol are formed. In the

human adrenal, both the zona fasciculata and zona glomerulosa can convert DOC to corticosterone. Indeed, in man, the major source of corticosterone is the zona fasciculata. However, it is the formation of aldosterone from DOC through corticosterone which is unique to the cells of the zona glomerulosa.

1.6.2 11β-hydroxylation in the Adrenal Cortex

The steps involved in the conversion of DOC to aldosterone and 11-deoxycortisol to cortisol are often referred to as the 'late reactions' or 'late pathways' in adrenal steroid synthesis. The conversions of 11-deoxycortisol to cortisol and DOC to corticosterone requires the addition of an 11β-OH group at the C11 position of both DOC and 11-deoxycortisol (Figure 1.6a). The 11β-hydroxylase step is carried out by the enzyme 11β-hydroxylase ($P\text{-}450_{11\beta}$). This enzyme is able to 11-hydroxylate both substrates in man and is encoded by a single gene on chromosome 8, CYP11B1 (110). Analysis of a panel of somatic cell hybrids coupled with *in situ* hybridisation to metaphase chromosomes has localised this gene to chromosome 8q22 (155). *In vitro* expression of human 11β-hydroxylase cDNA in COS-7 cells has shown that the conversion of DOC to aldosterone by 11β-hydroxylase is negligible although it can form corticosterone and a little 18-hydroxycorticosterone (Table 1.6a) (156). Thus, this provides good evidence that human 11β-hydroxylase is responsible for the formation of cortisol and corticosterone in the zona fasciculata but is unable to perform the necessary reactions for the conversion of DOC to aldosterone.

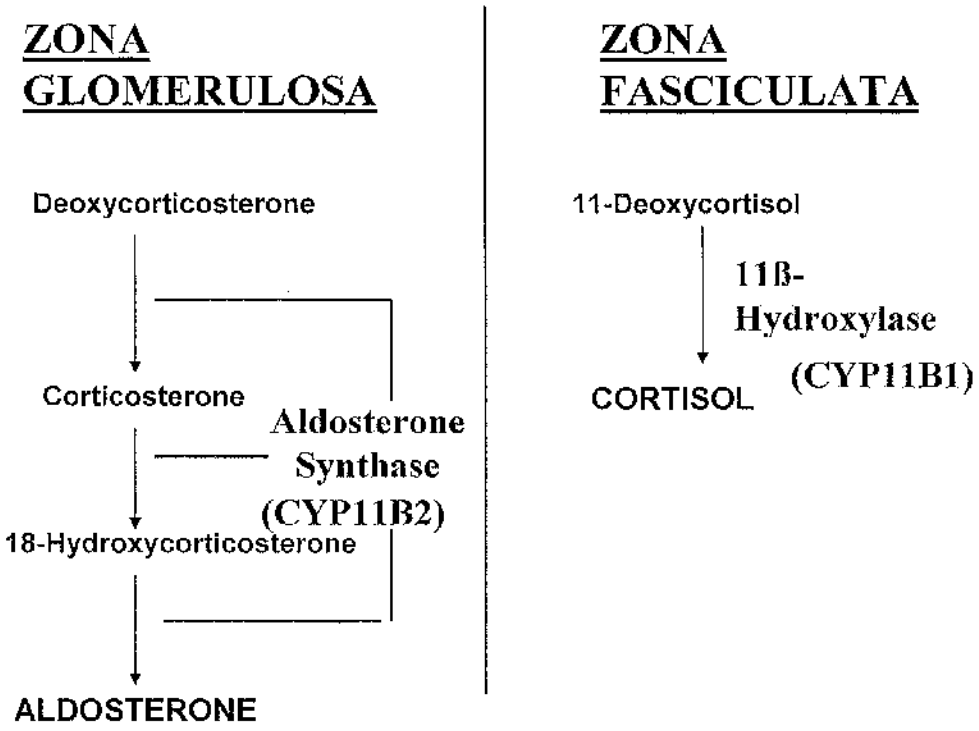


Figure 1.6a. The terminal steps in the biosynthesis of aldosterone and cortisol within the adrenal cortex.

Substrate	Product	Hydroxylase activity	
		$P\text{-}450_{11\beta}$	$P\text{-}450_{aldo}$
DOC	Corticosterone	482	438
	18-OH-corticosterone	2.7	14
	Aldosterone	<0.02	2
11-deoxycortisol	Cortisol	411	393
	18-OH-cortisol	1.1	7
	18-oxocortisol	<0.02	1.8
Corticosterone	18-OH-corticosterone	1.5	10.6
	Aldosterone	<0.02	0.9
Cortisol	18-OH-cortisol	0.7	4
	18-oxocortisol	<0.02	0.4

Table 1.6a. Hydroxylase activity of P-450s expressed in COS-7 cells.
Results are expressed in pmol/mg of protein.
Adapted from ref 156.

1.6.3 Regulation of aldosterone production

Three major stimulants of aldosterone secretion have been identified in man: angiotensin II (AngII), plasma potassium (K+) and ACTH. The classical renin angiotensin system (RAS) is outlined in Figure 1.3b. This consists of a precursor protein, angiotensinogen, produced by the liver, and two enzymes, renin (first described in the juxtaglomerular cells of the kidney) and angiotensin I converting enzyme (ACE) which is widely distributed but found predominantly in pulmonary vascular endothelium.

Renin is an aspartyl protease which has only one substrate *in vivo,* angiotensinogen, and cleaves this protein to release angiotensin I (AngI). Angiotensin I is converted into the octapeptide Ang II in the pulmonary circulation by ACE. Once formed, Ang II acts directly on the adrenal cortex to stimulate aldosterone secretion as well as producing a direct pressor response through increased resistance vessel tone(37). Secretion of renin (and hence activation of the RAS) is increased under conditions of reduced sodium intake, sodium loss or reduced extracellular fluid volume. Hence, the RAS is important in acute cardiovascular homeostasis, i.e. the response to head-up tilt or blood loss and in the regulation of aldosterone secretion in response to salt depletion.

Renin is thought to have no direct biological (non-enzymatic) function, although a prorenin or renin receptor has recently been identified (157). Ang I has no other known function other than as a substrate for ACE. Ang II produces most of its effects on aldosterone secretion via the type I angiotensin II receptor (AT_1) (Figure 1.6b). Binding to this G –protein coupled receptor results in activation of phospholipase C. In turn this leads to

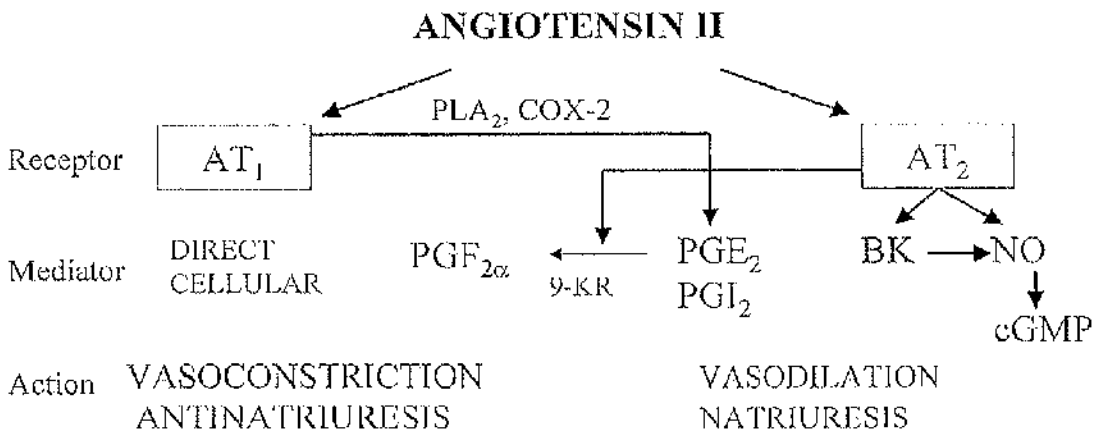


Figure 1.6b. Schematic depiction of the regulation of blood pressure and renal function by the AT_1 and AT_2 receptor.
PLA_2-phospholipase A2; COX-2-cyclooxygenase-2; BK-bradykinin; NO-nitric oxide; cGMP-guanosine 3', 5' monophosphate; PGE_2-prostaglandin E_2; $PG1_2$-prostacyclin and $PGF_{2\alpha}$ -prostaglandin $F_{2\alpha}$.

hydrolysis of phosphatidyl inositol biphosphate producing inositol triphosphate (IP3) and diacylglycerol (DAG) causing a rapid rise in intracellular calcium and activation of aldosterone synthesis by activating transcription factors such as activating transcription factor (ATF)-1, ATF-2 and cAMP-response element (CRE)-binding protein (CREB)(158;159). Recent studies have identified the transcription factor NURR-1 as a key regulator of CYP11B2 transcription that responds to Ang II; its expression is upregulated in aldosterone-secreting tumours (160). Concurrently, there is a decrease in membrane potassium permeability and resultant rapid depolarisation of the cell(161). Voltage-dependent calcium channels open and there is entry of extra-cellular calcium which results in opening of calcium-dependent potassium channel and repolarisation of the membrane. This rise in intracellular calcium correlates well with aldosterone production(162). In addition, DAG activates protein kinase C which then acts on cellular proteins to alter gene expression, most notably at the side-chain cleavage step(163) and the aldosterone synthase step(153).

In man, studies have defined the sensitivity threshold to intravenous Ang II infusion (164). The threshold varies from 0.3 to 1.0ng AngII/kg body weight min^{-1} in individuals consuming 100-200 mEq of sodium. A low sodium diet increases this sensitivity and the magnitude of response to Ang II rises by up to threefold. The threshold sensitivity is often reduced to 0.3 AngII/kg body weight min^{-1}, an infusion rate that produces no measurable change in circulating Ang II levels (165). Potassium loading also increases the maximum aldosterone response to Ang II infusion, but has only one third of the effect of changes in sodium intake (166). With prolonged Ang II infusion, aldosterone

secretion decreases, possibly secondary to whole body potassium depletion(167).

Recently, the functions of another major Ang II receptor, AT_2, have been partially characterised(168;169) (Figure 1.6b). Ang II stimulation of the AT_2 receptor generally opposes Ang II actions mediated via the AT1 receptor, most notably by promoting vasodilation and growth inhibition. Vasodilation mediated by the AT2 receptor is though to occur via the production of bradykinin and nitric oxide mainly in peripheral microvessels (e.g. coronary and mesenteric vessels)(170).

Besides the conversion of Ang I to Ang II by means of ACE, accumulating evidence indicates the existence of an alternative (ACE-independent) pathway for generation of Ang II. It has been suggested that chymotrypsin-like protease (chymase), which is a potent and specific Ang II-forming serine protease that is not affected by ACE inhibitors, plays a functional role in Ang II formation in the human vascular tissue (171) and in the carotid artery of the dog (172). Furthermore, chymase may be involved in Ang II synthesis in the human heart (173;174) and kidney (171). The possible contribution of non-ACE pathways to *in vivo* Ang II formation remains to be established, because most approaches used so far have not made it possible to extrapolate from the *in vitro* to the *in vivo* situation. Clarification of the mechanisms of the tissue Ang II formation in humans, especially in pathological conditions, may open new possibilities for therapeutic interventions.

Potassium is a powerful direct stimulus to aldosterone secretion; very small increments, which do not alter plasma levels perceptibly, raise aldosterone secretion rate(166). The effects of extracellular potassium and AngII are

synergistic, so that the prevailing potassium determines the concentration/effect relationship for AngII-mediated aldosterone production(175). Brief changes in dietary potassium intake can substantially modify the adrenal response to acute potassium administration: high dietary potassium intake enhances responsiveness, while low potassium reduces it (176).

Potassium acts at the plasma membrane of the zona glomerulosa by modifying the membrane potential. Increasing potassium concentration leads to depolarisation of the cell membrane and activation of voltage dependent calcium channels (177). The resultant calcium influx leads to a sustained rise in cytosolic calcium present for the duration of the stimulus which correlates with aldosterone production(162). Ang II and potassium therefore regulate CYP11B2 transcription through common calcium-dependent signalling pathways and also through common transcription factors(178).

ACTH may also increase aldosterone secretion in a dose-dependent manner (179). However, this response is abolished as a result of pre-treatment with ACE inhibitors implying that Ang II may be necessary for the actions of ACTH on aldosterone secretion(180). Moreover, like cortisol, the response of aldosterone to ACTH is impaired with age with the result of increased circulating ACTH levels still within the physiological range (181). However, it has been generally accepted that this ACTH-mediated increase in aldosterone levels is temporary and after 24 hours of continuous ACTH infusion, aldosterone levels fall back to normal or even sub-normal levels in healthy individuals(182). This effect is thought to be due, in part, to sodium retention, and thus suppression of Ang II formation, caused by the ACTH-

mediated elevation in plasma concentrations of intermediates such as DOC, corticosterone and cortisol itself(182). However, this is not the entire reason as sodium restricted individuals given ACTH exhibit the same rise and fall in plasma aldosterone despite minimal sodium retention(183). The mechanisms whereby ACTH increases aldosterone secretion are speculative but are thought to be similar to those in the zona fasciculata (163). Of interest, individuals with Cushing's disease (pituitary driven ACTH excess) demonstrate no such decline in aldosterone levels despite continuous ACTH stimulation (184). Furthermore, recent evidence shows that POMC-knockout mice have abnormal adrenal morphology with reduced (although detectable) levels of aldosterone, further suggesting that ACTH is required for normal aldosterone secretion(185).

In normal subjects, a variety of other amines and peptides exert a minor influence on aldosterone secretion (Table 1.6b). Atrial Natriuretic Peptide (ANP) is probably the most important of these and levels of ANP negatively correlate with plasma renin, indicating that ANP acts as an antagonist of aldosterone secretion(186). In patients with primary aldosteronism volume expansion results in increased secretion of ANP from the heart. The catecholamines (adrenaline, noradrenaline) as well as acetylcholine, dopamine and vasoactive intestinal peptide (VIP) act as stimulatory factors.

1.6.4 Aldosterone Synthase Activity in the Adrenal Cortex

In order to produce aldosterone from the zona glomerulosa, the precursor steroid DOC must undergo sequential 11-hydroxylation, 18-hydroxylation and

Control factors	Effect	Receptor/mechanism
Atrial natriuretic peptide	Inhibitory ↓ aldosterone	ANP receptor ↓ pregnenolone
Adrenaline, noradrenaline	Stimulatory ↑ aldosterone	ß-adrenergic receptors
Acetylcholine	Stimulatory ↑ aldosterone	Muscarinic receptors
Vasoactive intestinal peptide	Stimulatory ↑ aldosterone	Synergises with ACTH
Dopamine	Inhibitory ↓ aldosterone	Tonic inhibition via dopamine receptor

Table 1.6b. Factors other than angiotensin II, potassium and ACTH involved in the regulation of aldosterone secretion.

18-methyl oxidation. A single enzyme, aldosterone synthase, catalyses all three reactions.

In addition to describing the presence of a gene for 11β-hydroxylase in man, Mornet has also described the presence of a second gene, CYP11B2, with a high degree of homology to CYP11B1 in human genomic clones (110). The nucleotide sequence of CYP11B1 and CYP11B2 are 95 per cent identical in coding regions and 90 per cent identical in the intronic regions. The predicted amino acid sequence of the gene products of CYP11B1 and CYP11B2 are 93 per cent identical. Initially, analysis of mRNA from human adrenal glands showed that there was strong hybridisation of a CYP11B1-specific probe to the adrenal mRNA but no detectable hybridisation of a CYP11B2-specific probe, suggesting that CYP11B2 was a pseudogene with no function or was expressed at very low levels in man. Later, a cDNA clone was isolated from tissue from a patient with idiopathic hyperaldosteronism which was shown to be the transcript of CYP11B2 and to possess the ability to convert DOC to aldosterone (187). This was the first evidence that CYP11B2 may encode a functionally important gene product with aldosterone synthase activity, i.e. P-450_{aldo}. A similar report of P-450_{aldo} expression in aldosterone-producing adenoma tissue showed that P-450_{aldo} did indeed catalyse aldosterone synthesis from DOC and the protein product had a molecular mass of 48.5 kDa compared with 50kDa for P-$450_{11\beta}$ (188).

Curnow et al (153) provided the final confirmation that P-450_{aldo} was entirely responsible for aldosterone synthesis in the human zona glomerulosa. This was through the use of reverse transcriptase-PCR (RT-PCR), which revealed CYP11B2 transcripts in normal adrenals. However, the level of transcription

was shown to be low when compared to CYP11B1 transcripts in normal adrenals and the levels of transcription of CYP11B2 seen in aldosterone-secreting tumours.

Subsequently, the coding sequences for CYP11B1 and CYP11B2 were amplified from an aldosterone-secreting tumour tissue and expressed in COS-1 cells along with pCD-Adx (a plasmid containing the cDNA for adrenodoxin which is poorly expressed in COS-1 cells)(189). These cells were then incubated with their respective radiolabelled substrates ([^{14}C] DOC or [^{3}H] 11-deoxycortisol). Cells transfected with CYP11B1 converted 11-deoxycortisol almost entirely to cortisol although a small amount of cortisone was also formed due to intrinsic 11ß-hydroxysteroid dehydrogenase activity. DOC was converted to corticosterone and 11-dehydrocorticosterone but no aldosterone was detected. In contrast, cells transfected with CYP11B2 produced substantial amounts of corticosterone and 18-hydroxycorticosterone from DOC and aldosterone was easily detectable. With 11-deoxycortisol as the substrate CYP11B2 produced cortisol, cortisone and a third steroid, 18-hydroxycortisol. We have subsequently shown similar results, using a hamster cell line (V79) stably transfected with CYP11B1 and CYP11B2 transcripts (see chapter 6).

1.6.5 Molecular genetics of CYP11B1 and CYP11B2

The genes encoding CYP11B1 and CYP11B2 are very similar and lie on chromosome 8q21-22 (110;155;190). The organisation of introns and exons within each gene is also very similar with each consisting of nine exons and a coding region which spans approximately 7 kilobases of DNA (Figure 1.6c).

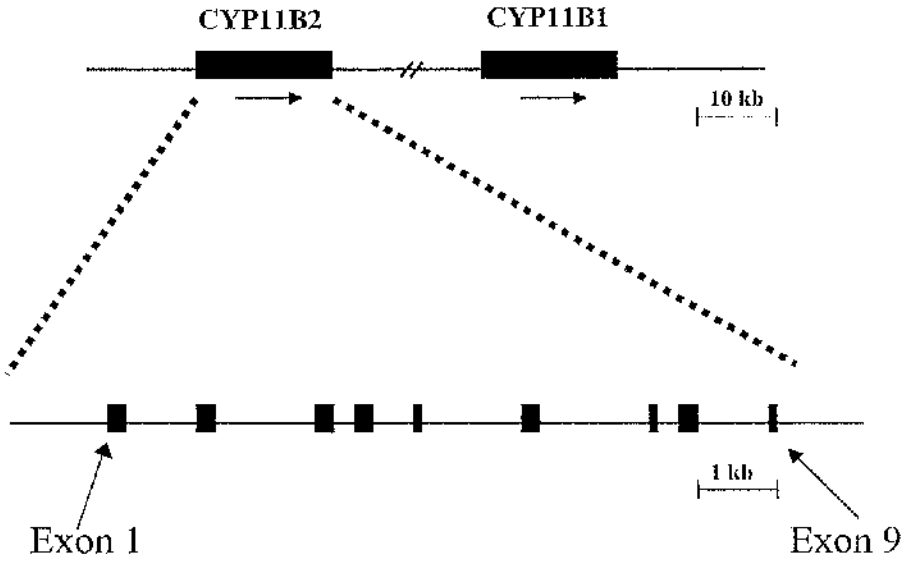


Figure 1.6c. Organisation of human CYP11B genes and the structure of CYP11B2 on chromosome 8.

The nucleotide sequence of the exons is 95 per cent identical and that of the introns approximately 90%. The putative proteins encoded by CYP11B1 and CYP11B2 each contain 503 amino acids including a 24-residue signal peptide. These sequences are 93 per-cent similar to each other and 75 per-cent similar to the predicted sequence of bovine P-$450_{11\beta}$ and 36 per cent identical to that of CYP11A. The positions of the introns of CYP11B1 and B2 are identical to those of CYP11A (gene for P-450scc)(191). As a result, these genes are grouped into a single family within the cytochrome P-450 gene superfamily (192).

Both CYP11B1 and B2 possess a TATA box variant at position –35 to –29 (GATAAAA) relative to the transcription initiation site. Both possess a palindromic sequence identical to sequences seen in cAMP- responsive genes (TGCGTA), i.e. a cAMP response element (CRE) which binds to a cAMP-responsive element binding protein (CREB), downstream from the TATA box. Factors that bind to TATA boxes (193) and cAMP response elements (194) have been identified. Clinical and biochemical observations of *in vitro* responses to ACTH suggest that cAMP is responsible for the regulation of steroidogenic enzyme synthesis (120) through increased transcription of steroidogenic enzyme cDNA (121) and expression of cDNA constructs *in vitro* has shown that cAMP regulates the transcription of CYP11B1 (195). It is thought that the generation of cAMP by adenylate cyclase leads to activation of cAMP-dependent kinase and subsequent phosphorylation of CREB which binds to the CRE with resultant increased gene expression.

Cell-specific expression of steroidogenic enzymes may arise due to sharing of a specific promoter element in these genes. This theory has been supported by the discovery of a AGGTCA motif in the promoter regions of murine $P450_{scc}$, $P450_{c21}$, $P450_{aldo}$ and bovine $p450_{11ß}$ which binds a protein termed steroidogenic factor 1 (SF-1) or, alternatively, adrenal-4-binding protein(196). SF-1 is a member of the orphan nuclear receptor superfamily. It was first isolated from an adrenal cDNA library, and subsequently shown to be expressed predominantly within major steroidogenic organs such as the adrenals and gonads(197). SF-1 is the mammalian homologue of the Fushi taruzu (ftz-f1) gene in *Drosophila melanogaster* which, in humans, is located on chromosome 9q33(198). This gene encodes a nuclear protein of approximately 54kDa which binds to target gene promoters and recognises variations of the DNA sequence PyCAAGGTCA as a monomer (199;200). The creation of ftz-f1 knockout mice has revealed the critical role of SF-1 in adrenal and gonadal development. These animals die of glucocorticoid insufficiency within 7 days post partum due to inadequate adrenal development(201;202). As well as regulating the major enzymes in adrenal steroidogenic pathways, SF-1 additionally regulates the transcription of both the ACTH receptor promoter(203) and the steroidogenic acute regulatory protein (StAR)(204;205), both essential early determinants of steroid production within the adrenal.

Aldosterone synthase activity is confined to the zona glomerulosa cells and therefore expression of CYP11B2 must be regulated in a manner different from that of CYP11B1. In primary cultures of human zona glomerulosa cells, Ang II markedly increases the level of both CYP11B1 and B2 transcripts

(153). ACTH increases CYP11B1 mRNA levels(153) and causes an acute rise in CYP11B2 activity which is apparently reversed by chronic administration(206). Recent evidence suggests that CYP11B1 and B2 do not respond similarly to SF-1(207). When human H295R cells are co-transfected with human CYP11B2 or CYP11B1 reporter constructs and an expression plasmid encoding SF-1, CYP11B1 reporter constructs are activated by SF-1 whereas CYP11B2 reporter constructs are actually inhibited. Thus, human CYP11B2 expression is regulated quite differently from genes for other proteins involved in steroid biosynthesis including StAR and CYP11B1 which are all positively regulated by SF-1.

Differences in gene transcription may result from important divergences in structure of the promoter regions of CYP11B1 and B2. In the 5' untranslated region (UTR) of both genes, outwith the immediate vicinity of the transcription initiation site, the nucleotide sequence of the two genes diverges quite considerably. These regions are only 48% identical, the difference arising principally due to the presence of a long palindromic sequence in CYP11B2 from –1734 to-1001(156). Deletion analysis of 5' flanking DNA of CYP11B1 suggests that regions between –1093 and –505 significantly decrease reporter activity. In contrast, reporter constructs containing from –2105 and –65 of the human CYP11B2 5' flanking region are thought to inhibit promoter activity, although a previous analysis of human CYP11B2 reporter constructs in murine Y-1 cells disagrees with these findings(208). These differences in the promoter regions between genes clearly result in important alterations in promoter-dependent regulation of gene expression, particularly the differential

response to cAMP effects, and may also have a role in determining the site-specific expression of the genes.

In the mouse, CYP11B1 and CYP11B2 lie in tandem with CYP11B2 on the left if the genes are being transcribed right to left (Figure 1.6c)(209). Evidence from studies of patients with congenital hypoaldosteronism due to aldosterone synthase deficiency (corticosterone methyloxidase II deficiency; CMO)(210)and GRA suggest that the same arrangement exists in man. Studies of large restriction fragments generated by rare cutting restriction enzymes and separated by pulsed field electrophoresis suggest that the two genes are located in close proximity, approximately 40 kilobases apart(211;212).

In summary, in man two highly homologous genes encoding steroidogenic enzymes lie together on chromosome 8q21-22 approximately 40 kilobases apart. One gene, CYP11B1, encodes the enzyme 11ßhydroxylase ($P\text{-}450_{11ß}$) which is expressed throughout the adrenal cortex but principally catalyses the formation of cortisol and corticosterone in the zona fasciculata. The transcription of CYP11B1 is regulated by ACTH via cAMP and it is unable to catalyse the formation of aldosterone. The other, CYP11B2, is expressed only in the zona glomerulosa in normal human adrenal tissue and catalyses the synthesis of aldosterone from DOC. CYP11B2 is regulated *in vitro* by Ang II and its promoter region differs considerably from that of CYP11B1, a difference which may reduce responsiveness to cAMP. These genes are clearly of central importance in the synthesis of cortisol and aldosterone and mutations altering the activity of their gene products and the intermediate

hypertensive phenotype that may be the result of this forms much of the focus of this thesis and is discussed in detail in section 1.10.

1.7 Aldosterone and its effects in man

Traditionally, the principal target organ for aldosterone was said to be the kidney; mineralcorticoid receptors (MRs) are found in high concentration in the renal distal nephron as well as other epithelial sites such as the colon and ducts of sweat and salivary glands(94). However, MRs have also been identified in non epithelial sites such as heart, brain, vascular smooth muscle, liver and peripheral blood leucocytes (94).

1.7.1 Mechanism of action of aldosterone-the genomic effects

Aldosterone binds to the MR, an intracellular receptor similar to the GR receptor and also belonging to the steroid/thyroid/retinoid/orphan receptor superfamily(213). The DNA binding regions of the two receptors are 94 per cent identical with 57 per cent homology at the C-terminal ligand binding domain and <15 per cent homology at the N-terminal region (Figure 1.7a)(213). Once bound, the ligand/receptor complex translocates to the nucleus and acts as a transcription factor by direct interaction with DNA regulatory elements (the classical genomic effect of aldosterone)(125). The mechanism by which the ligand/receptor complex exerts its effects is discussed in detail in 1.5.1.

The gene product(s) resulting from the interaction of aldosterone/MR complexes binding to DNA regulatory elements are termed Aldosterone-induced Protein(s) (AIP). AIP may have effects on the apical membrane,

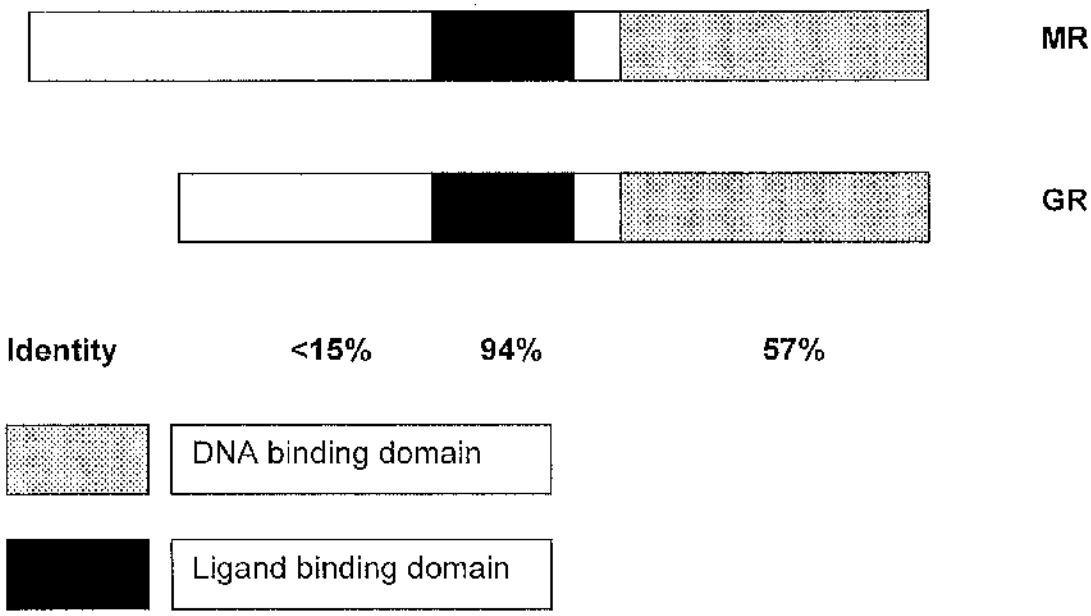


Figure 1.7a. Schematic representation of human mineralocorticoid (MR) and glucocorticoid (GR) receptors

cellular energy production and/or the basolateral Na/K-ATPase pump resulting in increased sodium reabsorption and potassium and hydrogen ion excretion (214). (Figure 1.7b)

Recently, a serine/threonine kinase, serum glucocorticoid-regulated kinase (sgk) has been identified as an AIP resulting in an increase in activity of the epithelial sodium channel (ENaC) located apically in the collecting tubules and distal convoluted tubule of the nephron (215). Aldosterone causes phosphorylation and activation of sgk which in turn increases ENaC activity by an increase in the number of channels at the cell surface (216). The principal ENaC inhibitory accessory protein is Nedd 4 (Neuronal precursor cells Expressed Developmentally Downregulated). This ubiquitin protein ligase binds to the C tails of β and γ sub-units of ENaC leading to channel internalisation and degradation. Recent work suggests that the stimulatory action of sgk on ENaC is mediated through phosphorylation of serine residues on Nedd4. Such phosphorylation reduces the interaction between Nedd4 and ENaC, leading to elevated ENaC cell surface expression (217).

Another mediator of aldosterone action is Kirsten Ras GTP-binding protein 2A (Ki-RasA). Expression of this protein is induced during the early phase of aldosterone action and appears to be necessary for the effects of aldosterone on sodium transport in renal epithelial cells. The exact mechanism by which it mediates this is unclear but Ki-RasA appears to have dual contrasting effects on the ENaC channel decreasing the number of channels but keeping channels open (218).

Other mediators of aldosterone action include the lipid kinase, PI3K and corticosteroid hormone-induced factor (CHIF). Whilst PI3K is not an actual

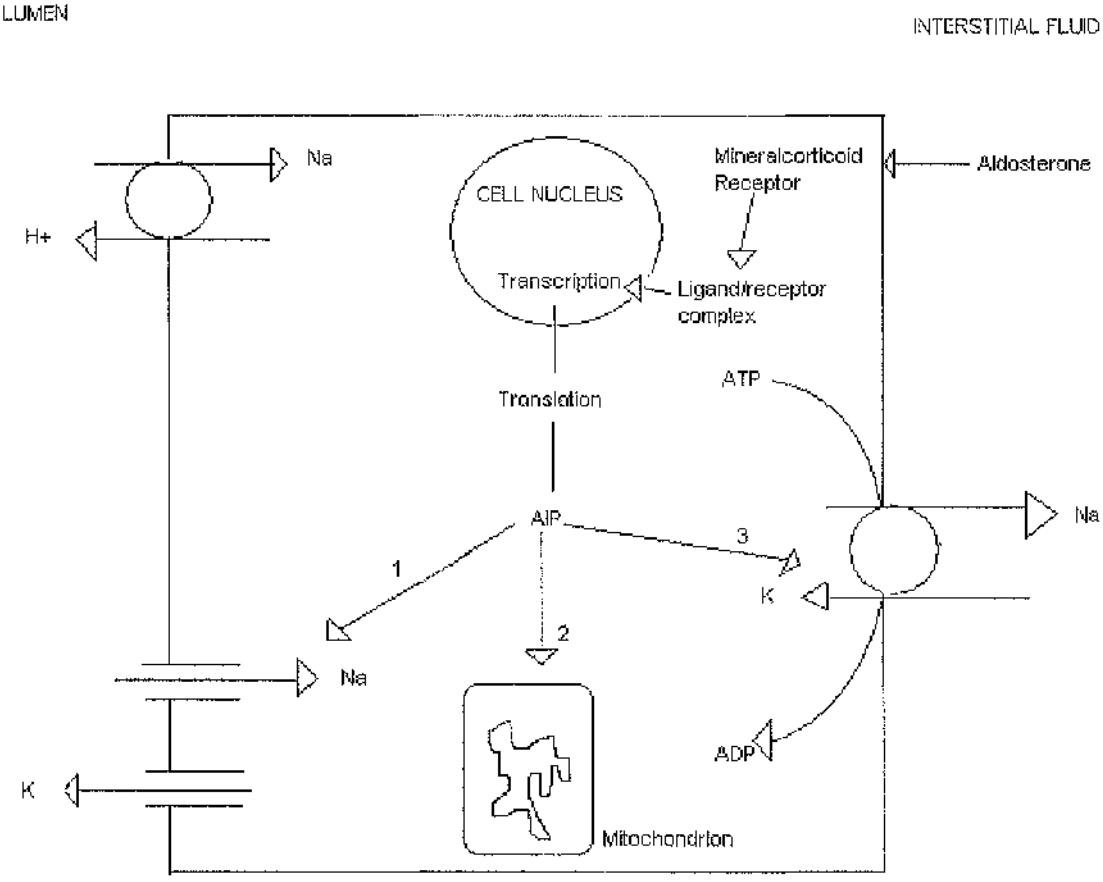


Figure 1.7b. Intracellular effects of aldosterone on distal collecting tubule of nephron

AIP – Aldosterone-induced protein(s)

1 – Effect of AIP on apical membrane sodium channel
2 – Effect of AIP on mitochondrial energy production
3 – Effect of AIP on energy-dependent Na/K-ATPase

AIP its activity is increased by aldosterone and insulin in the kidney and inhibition of PI3K reduces both the early and late actions of aldosterone(219). CHIF is expressed in the basolateral membranes of epithelial cells of the distal colon and nephron and belongs to a family of proteins known to regulate ion channels and transport proteins (219). Their mechanism of signal transduction is unknown.

1.7.2 Non-genomic effects of aldosterone

It is now accepted that, as well as classical genomic effects through ligand/receptor binding of DNA regulatory elements, aldosterone also exerts rapid, non-genomic, effects. Non-genomic effects are associated with rapid activation (occurring within minutes) in the absence of a need for transcription or protein synthesis(220). Because of the brief response time, it is presumed that non-genomic actions are initiated at the membrane level, and membrane signaling transduction pathways have been intensively studied. These studies suggest the existence of novel steroid hormone receptors or possibly classical receptors embedded in the membrane that initiate the non-genomic signal cascade although to date none have been found. However, two recent studies have demonstrated a role for intra-cellular calcium as well as protein kinase C activity as a potential mechanism of action despite the elusive nature of the membrane receptor(221;222).

This has led to investigation of aldosterone action in tissues other than the kidney. Reports of rapid, non-genomic, effects of aldosterone have been described in smooth muscle, cardiac muscle, skeletal muscle, colonic epithelial cells and myocardial cells(82). These effects have been linked to the

development of increased systemic vascular resistance and so could, theoretically, contribute to hypertension and cardiovascular disease

1.7.3 11ß-hydroxysteroid dehydrogenase system

Studies of the *in vitro* receptor affinity of GR and MR have revealed that the affinity of GR for steroids mirrors their *in vivo* therapeutic potency whilst the MR has equal affinity for aldosterone, cortisol and corticosterone in both adrenal tissue and recombinant MR studies (213;223). In normal subjects, circulating cortisol concentrations are 100-1000 times that of aldosterone and would thus be expected to exert important mineralocorticoid effects well in excess of aldosterone at normal plasma concentrations. This is clearly not the case *in vivo* as the 11-β-hydroxysteroid dehydrogenase system (11β-HSD) acts as a gatekeeper to prevent activation of the MR by much higher available levels of cortisol(126) (Figure 1.7c). The type 2 isoform of this enzyme is found in the renal distal nephron as well as other aldosterone sensitive target tissues (colon, salivary glands and placenta) and converts cortisol to its inactive metabolite, cortisone, which has no affinity for the MR. The type 1 isoform of this enzyme was cloned in 1989(224) but only later was recognised to act *in vivo* predominantly as a NADPH dependent reductase performing the opposite role to 11β-HSD2(225) (i.e. generating cortisol from inactive cortisone). 11β-HSD1 is found in many tissues including liver, adipose, gonads, brain and vasculature(226). These tissues are abundant in glucocorticoid rather than mineralocorticoid receptors.

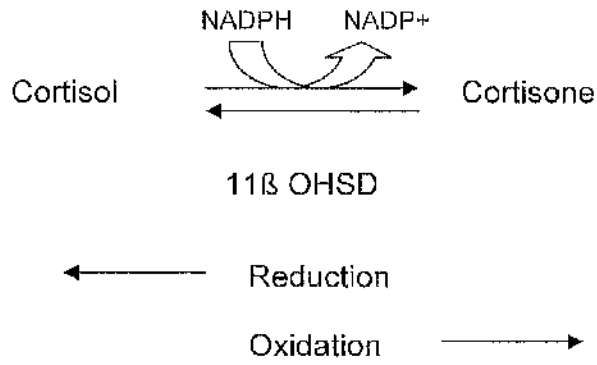


Figure 1.7c. 11ß-hydroxysteroid dehydrogenase (11ßHSD) activity.

1.7.4 Aldosterone and the renal tubule

The best-characterised physiological effect of aldosterone is to increase the reabsorption of sodium in the kidney and at other secretory epithelial sites at the expense of potassium and hydrogen ions (227). The major sites of aldosterone-induced sodium and potassium transport are luminal cells of the cortical collecting tubules and the distal convoluted tubule. The apically located epithelial sodium channel (ENaC) is the major determinant of renal sodium reabsorption (228). Its availability in open conformation at the apical membrane of the cell is increased by aldosterone and also by vasopressin, glucocorticoids and insulin; elevated intracellular levels of calcium and sodium down-regulate it (229).

Aldosterone also regulates hydrogen ion excretion by the kidney in the distal nephron. Hydrogen ion secretion is through a sodium-insensitive route since it occurs principally in the intercalated cells of the collecting tubule. This segment of the nephron exhibits little or no aldosterone-induced sodium transport, and so aldosterone-induced natriuresis and hydrogen ion secretion appear to be independent events. This effect is mediated via an effect of aldosterone on the activity of the ATP-dependent apical hydrogen ion pump and parallel regulation of the basolateral membrane Cl^-/HCO_3^- exchanger(230).

The net effect of aldosterone on the renal tubule is therefore to promote sodium retention at the expense of potassium and also to promote hydrogen ion excretion by the kidney. This explains the clinical features observed in cases of primary aldosterone excess, i.e. plasma hypokalaemia, alkalosis, a raised exchangeable sodium content and low total body potassium (231).

1.7.5 Non-epithelial actions of aldosterone

MRs have been localised in a number of non-epithelial tissues, particularly in the cardiovascular system and central nervous system (CNS). While the properties of the MRs in these tissues are largely similar, the effects they mediate are extremely diverse. In contrast to its established effects on electrolyte balance in epithelial tissue, aldosterone in the cardiovascular system promotes cardiac hypertrophy, fibrosis and abnormal vascular endothelial function. These effects are described more fully in section1.8.

In the CNS, aldosterone appears to regulate blood pressure, salt appetite and sympathetic tone. There are relatively few MRs in the CNS and these are concentrated predominantly in the hippocampus, the septum and the cerebellum (232). In contrast to epithelial cells, MRs in the CNS do not appear to co-localise with 11ß-OHSD2(233). However, high levels of 11ß-OHSD2 are found in the subcommisural organ (SCO) and amygdala(234-236). The SCO is involved with the central regulation of aldosterone secretion while the amygdala MR has been implicated in the control of salt appetite. The lack of 11ß-OHSD2 in MR-rich areas suggests that the majority of brain MRs are likely to be occupied by glucocorticoid.

1.8 Aldosterone as a cardiovascular hormone

As result of a series of *in-vitro*, animal and large-scale clinical studies, the role of aldosterone in the pathophysiology of cardiovascular disease is now recognised more fully. Aldosterone is now recognised as an important cardiovascular hormone contributing to a number of pathophysiological

mechanisms in the heart and other organs (Figure 1.8a). As a consequence of this, aldosterone antagonists are increasingly used in cardiovascular disease.

1.8.1 Traditional effects of aldosterone on the heart

Congestive cardiac failure (CCF) causes activation of the renin-angiotensin-aldosterone system (RAAS). The consequent aldosterone excess leads to sodium retention and expansion of extra-cellular volume, further impairment of haemodynamic response and a fall in cardiac output. Thus, a vicious cycle is created whereby reduced renal perfusion causes further activation of the RAAS(237). This 'secondary aldosteronism' is an important cause of potassium depletion in this circumstance.

Moreover, loop diuretics, one of the primary treatments to relieve congestive symptoms, are potent stimulators of the RAA system(238). Indeed, some researchers have proposed that activation of the RAAS in patients with CCF is a result more of diuretic treatment than the disease process itself (239).

In addition to these well-recognized adverse effects of aldosterone excess on electrolyte and fluid balance, studies over the past decade have demonstrated more direct effects of aldosterone on myocardial and vascular function.

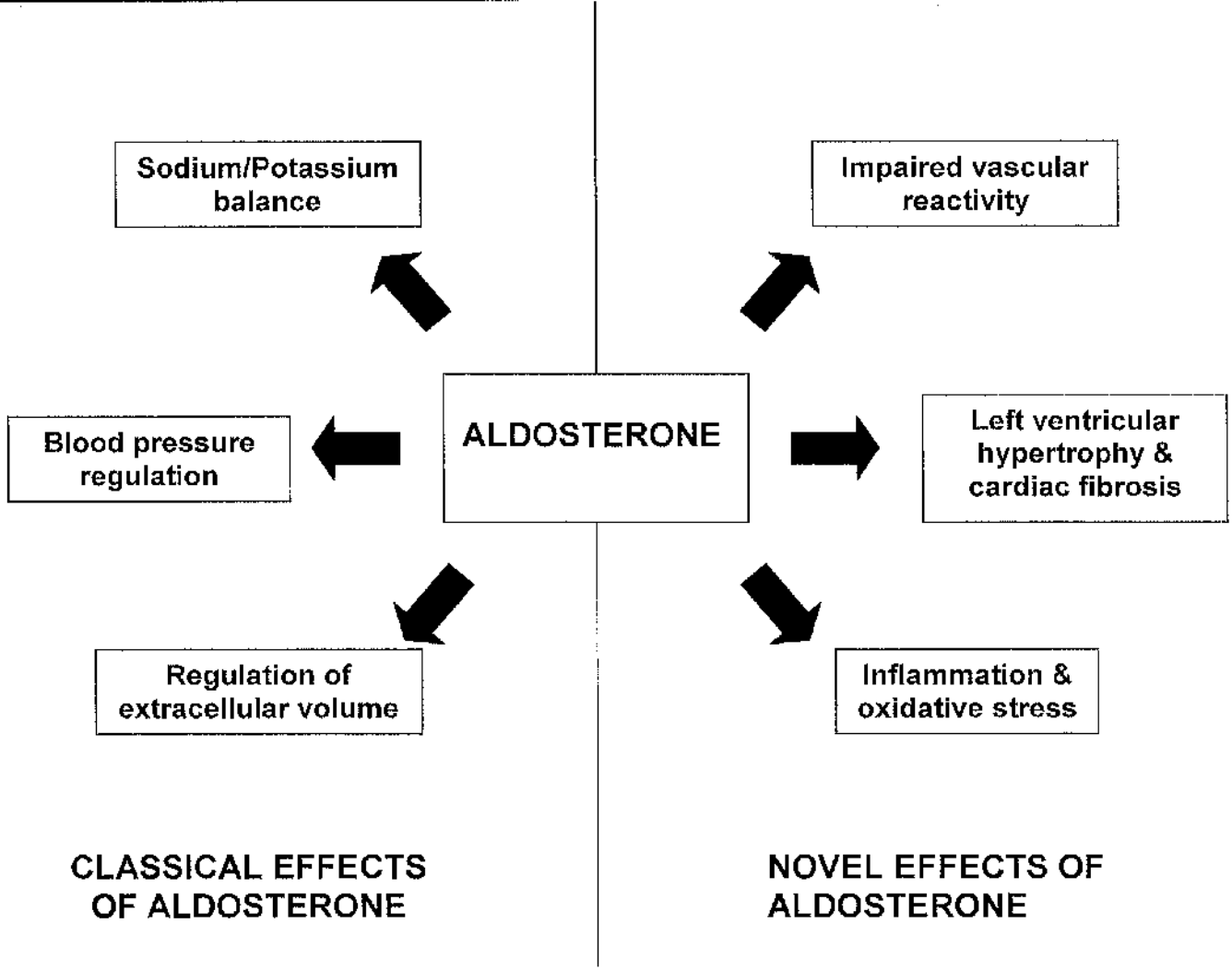


Figure 1.8a. Widespread effects of aldosterone within the cardiovascular system.
Traditional effects are on the left whilst more recently described adverse effects are outlined on the right.

1.8.2 Pathological effects of aldosterone on the heart

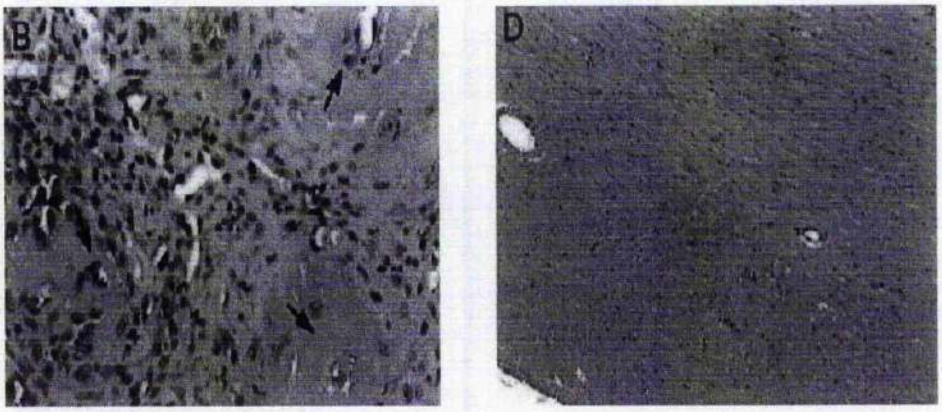
In 1988 Karl Weber and colleagues pioneered experimental work examining the effects of aldosterone on the myocardium(240). In a rat model of renovascular hypertension they observed fibrosis not only of the hypertrophied left ventricle but also of the non-hypertrophied right ventricle. This implied that fibrosis might be a result of humoral rather than haemodynamic factors.

Cardiac fibrosis can also be demonstrated in rat models of PA (uninephrectomized salt-fed rats infused with aldosterone) (241) (Figure 1.8b). Moreover, despite severe persistent hypertension, cardiac hypertrophy and fibrosis are blocked by doses of spironolactone insufficient to ameliorate the hypertension (154).

Several other investigators have confirmed and developed the findings of Weber's group (242). Thus, if considered together, these observations suggest that aldosterone can cause cardiac fibrosis independent of its effect on blood pressure or on the development of ventricular hypertrophy.

Crucially, these pro-fibrotic actions of aldosterone only develop in animals fed a high salt diet(243). The effect is not observed if dietary salt is restricted underscoring the importance of salt with mineralocorticoids in this pathophysiological effect. The mechanism that subserves this interaction between sodium status and mineralocorticoid related damage is unknown.

A novel hypothesis to partly explain this phenomenon has been proposed by Funder and colleagues(244). They propose that cardiovascular damage described above can be accounted for not by aldosterone per se but by inappropriate activation of the MR by normal levels of glucocorticoids. It is



Aldosterone+ salt

Aldosterone+ salt+ eplerenone

Figure 1.8b. Effects of aldosterone + salt on rat myocardium

Photomicrograph of myocardium in rats treated with aldosterone and salt with and without eplerenone. Note the presence of vasculopathy including perivascular inflammatory cell infiltrate in rats treated with aldosterone and the absence of these findings in rats treated with aldosterone and eplerenone

argued that 11-ßOHSD2, whilst markedly reducing amounts of locally available cortisol, cannot reduce levels to those well below locally available aldosterone. Thus cortisol levels remain 10-fold higher than aldosterone occupying the majority of the MR. Moreover, a by-product of 11-ßOHSD2 activity is the generation of NADH. Crucially, they maintain that high levels of NADH (which reflect high 11-ßOHSD2 activity) inhibit the activity of glucocorticoid-bound MR(245). When intracellular NADH falls, in response to enzyme blockade or generation of reactive oxygen species (as a result of tissue damage), the MR is activated by glucocorticoids, mimicking the cardiovascular effects of inappropriate aldosterone for salt status. Hence, a second function of the enzyme 11-ßOHSD2 is to modulate MR activation by altering local redox state.

1.8.3 Mechanism of aldosterone-induced myocardial damage

The exact mechanism by which aldosterone causes tissue damage remains controversial; several mechanisms have been proposed and all may contribute to a variable extent. Firstly, evidence from animal and human subjects suggest that aldosterone is a pro-inflammatory hormone which causes cardiac peri-vascular inflammation(82). After several weeks of treating rats with aldosterone and salt, macrophages, lymphocytes and proliferating endothelial and vascular smooth muscle cells and fibroblasts are found in the perivascular space of intra-mural coronary arteries as well as areas of cardiomyocyte necrosis in both ventricles(246). This is accompanied by fibrosis, as demonstrated by a significant increase in ventricular collagen volume fraction. Moreover, there are concurrent increases in mRNA for pro-

inflammatory mediators and cytokines such as Intercellular Adhesion Molecule1 (ICAM-1), Monocyte Chemoattractant Protein (MCP-1) and Tumour Necrosis Factor alpha (TNFα) (247). Crucially, development of all these changes can be prevented by co-administration of selective aldosterone receptor antagonists such as eplerenone(248).

Aldosterone also exerts a direct pro-fibrotic action by stimulating collagen production by cardiac fibroblasts. Application of aldosterone to rat cardiac fibroblasts *in vitro* has been shown to increase collagen synthesis significantly(249). Whether this direct pro-fibrotic effect is solely responsible, or occurs via reparative fibrosis secondary to cell necrosis and death accounting for the original observations by Weber et al (aldosterone induced, blood pressure independent cardiac fibrosis) is yet to be determined.

Chronic aldosterone and salt treatment increases the expression of NADPH oxidase which, in turn, catalyses the formation of the superoxide anion(246). These changes are mainly seen in inflammatory and endothelial cells in the perivascular space of the myocardium. Spironolactone and the two antioxidants, pyrrolidine dithiocarbamate and N-acetylcysteine attenuate all of these responses, suggesting that aldosterone and salt induce a pro-inflammatory phenotype, at least in part by increasing oxidative stress (247).

However, for aldosterone to act directly on the heart to induce fibrosis, 11β-HSD2 would also have to be present in order to allow aldosterone access to the mineralocorticoid receptor. 11β-HSD2 has never been detected in rat cardiac fibroblasts (250). Therefore, it could be argued that aldosterone contributes to the development of myocardial fibrosis indirectly, through its actions on systemic sodium retention, expansion of extravascular space and

hypervolaemia. On the other hand, 11β-HSD2 is present in the vasculature suggesting that these pro-fibrotic effects are in fact peri-vascular (251).

1.8.4 Effects of aldosterone on the vasculature

Several human studies have demonstrated that aldosterone is pro-atherogenic and promotes endothelial dysfunction (252). One possible mechanism by which this occurs is by decreasing nitric oxide (NO) availability through superoxide generation. Superoxide anions inactivate NO and produce the highly toxic peroxynitrite radical ($ONOO^-$).

Aldosterone has been shown to reduce NO bioactivity in an in-vitro study of rat smooth muscle(253). In a study of healthy volunteers, intravenous aldosterone significantly attenuated endothelium-dependent vasodilatation (as measured by venous occlusion plethysmography) in response to acetylcholine (an endothelium-dependent vasodilator). Moreover, in a placebo-controlled clinical study of heart failure patients, administration of spironolactone significantly increased forearm blood flow response to acetylcholine. This improvement was shown to be associated with improved NO bioavailability (254).

Aldosterone-associated effects on the vasculature lead to a reduction in arterial compliance and, therefore, impaired baroreflex activity(255). As a consequence, patients with hypertension and cardiac failure demonstrate impaired autonomic control of cardiac and vascular responses. In one study of treated heart failure patients, there was a negative correlation between plasma aldosterone concentrations and arterial compliance of the aorta and its major branches(256).

1.8.5 Extra-adrenal production of aldosterone

As has already been discussed, the main source of circulating aldosterone is the adrenal zona glomerulosa. However, recent studies have demonstrated that tissues other than the adrenal cortex express enzymes required for corticosteroid biosynthesis, such as aldosterone synthase (CYP11B2) and 11β-hydroxylase (CYP11B1) and so it is possible that aldosterone can be synthesised in a range of other tissues (257). In particular, whilst there is strong evidence to support aldosterone production in the brain (258), research into the heart has proved to be less conclusive. A number of published studies fail to agree as to whether cardiac production of corticosteroids actually occurs(259;260). Mineralocorticoid receptors are known to be expressed in cardiac tissue as well as brain and blood vessels(94). Thus, locally produced aldosterone could potentially act in a paracrine fashion to produce some of the effects described above. Given the implications of this for treatment of cardiovascular disease and the suggestion by some that cardiac aldosterone synthase expression may be increased in heart failure (261), this is an important issue that requires further consideration.

Most recently, Gomez-Sanchez et al. addressed this issue by measuring cardiac and plasma levels of aldosterone and its precursor, corticosterone in rats under varying conditions (260). They have concluded that aldosterone concentrations in the heart reflect those in the circulation and are far lower than others have previously reported.

Investigators from my group have used our previously validated, highly sensitive quantitative RT-PCR method to measure CYP11B1 and CYP11B2

mRNA levels in cardiac tissue of several rat models of cardiovascular pathology (congestive heart failure, genetic hypertension and cardiac hypertrophy). Cardiac CYP11B1 and CYP11B2 mRNA transcript levels from all groups were never greater than 10^2 copies per microgram of total RNA (Figure 1.8c) and therefore too low to be detected reproducibly(262). These data suggests that cardiac corticosteroid production is of little or no pathological or physiolological significance.

Thus, in concordance with the conclusions of Gomez-Sanchez et al., it is more likely that cardiac aldosterone derives from the adrenal cortex via the circulation. It has been suggested that the high concentrations of aldosterone that have been reported in heart tissue could be due to extraction of aldosterone from the circulation rather than local synthesis. This has been confirmed in rats where endogenous aldosterone production was ameliorated by a combination of adrenalectomy and dexamethasone treatment. Circulating and cardiac levels of aldosterone were barely detectable in these rats in comparison with control animals(263).

1.8.6 Aldosterone escape

This describes the failure to suppress the synthesis of aldosterone during treatment with angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs). This is a common phenomenon with reports of aldosterone levels returning to baseline as soon as 3 days after initiation of ACE inhibitor therapy in patients with acute myocardial infarction (264). In the RESOLVD Pilot study (265), heart failure patients given both enalapril and candesartan demonstrated a significant fall in aldosterone levels at 17 weeks

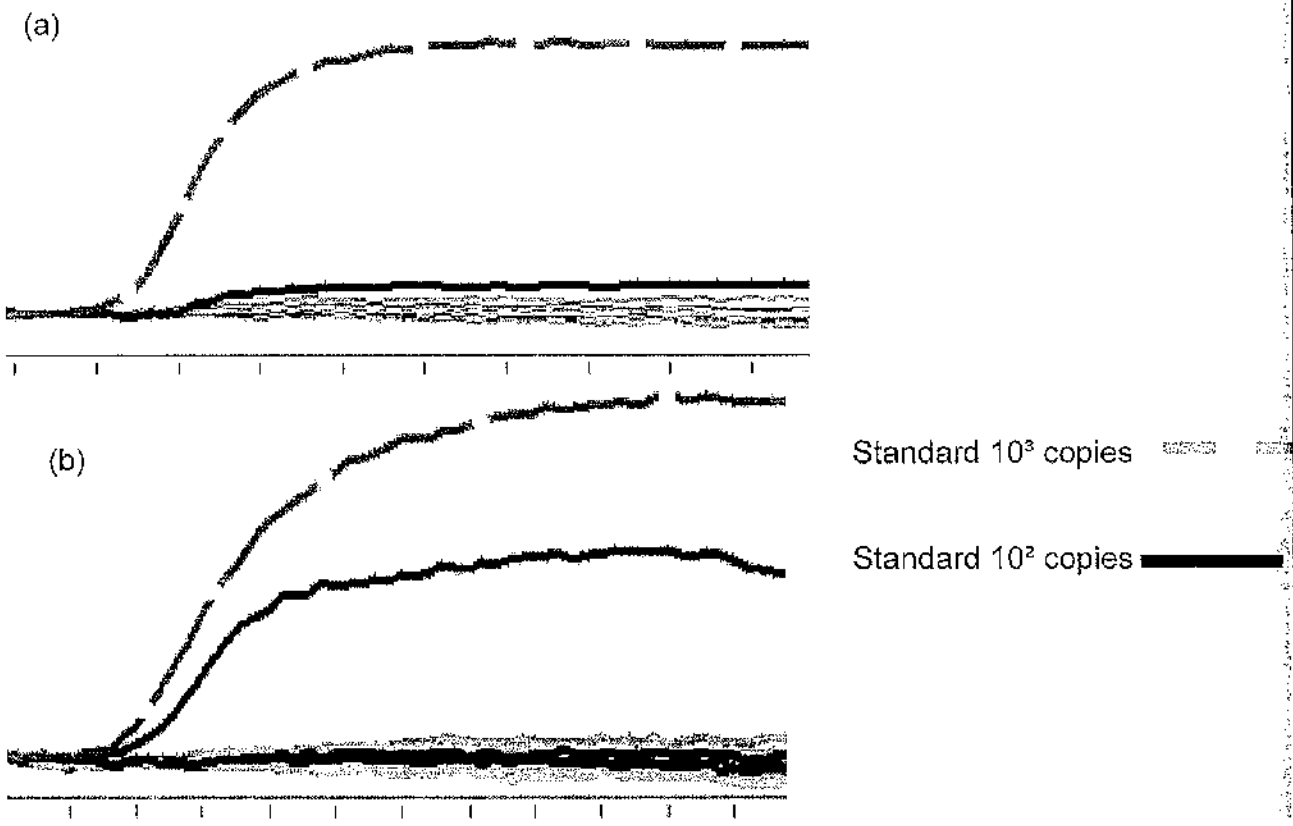


Figure 1.8c . Real-time polymerase chain reaction amplification of β-actin mRNA in rat cardiomyocytes.

(a) CYP11B2 transcripts (b) CYP11B1 transcripts.

In both cases, gene expression undetectable at <100 copies/mg total RNA.

compared to those given either agent alone, but mean aldosterone levels had returned to baseline by 43 weeks, even with maximum doses of both agents (Figure 1.8d). This is not surprising given the importance of plasma potassium as well as Ang II in the regulation of aldosterone production. This is further illustrated by the finding of continued aldosterone production in angiotensinogen knockout mice (266).

The relationship between aldosterone levels and clinical outcomes has been assessed in large-scale clinical studies. In a sub-population of 534 post-myocardial infarction (MI) patients participating in the SAVE trial who were randomised to captopril or placebo, mean aldosterone levels were lower among patients who remained free of cardiovascular events over 2 years compared to those who died, developed heart failure or had a further MI(267). In the CONSENSUS trial, amongst patients who received enalapril those who had a large decrease in aldosterone levels at 6 weeks had approximately half the 6-month mortality rate of those with smaller reductions in aldosterone (268). It has also been reported that aldosterone escape is associated with reduced exercise capacity in patients with congestive heart failure(269) and decreased vascular compliance (270)despite ACE inhibitor therapy.

The role of aldosterone escape in patients with type 2 diabetes mellitus and diabetic nephropathy has also been evaluated. One study demonstrated the presence of aldosterone escape in 40 per cent (18/45) of patients with nephropathy being treated with an ACE inhibitor. Addition of spironolactone o this cohort significantly reduced urinary albumin excretion without alteration in blood pressure(271). Taken together, these data suggest that insufficient suppression of plasma aldosterone with ACE inhibitor treatment (i.e.

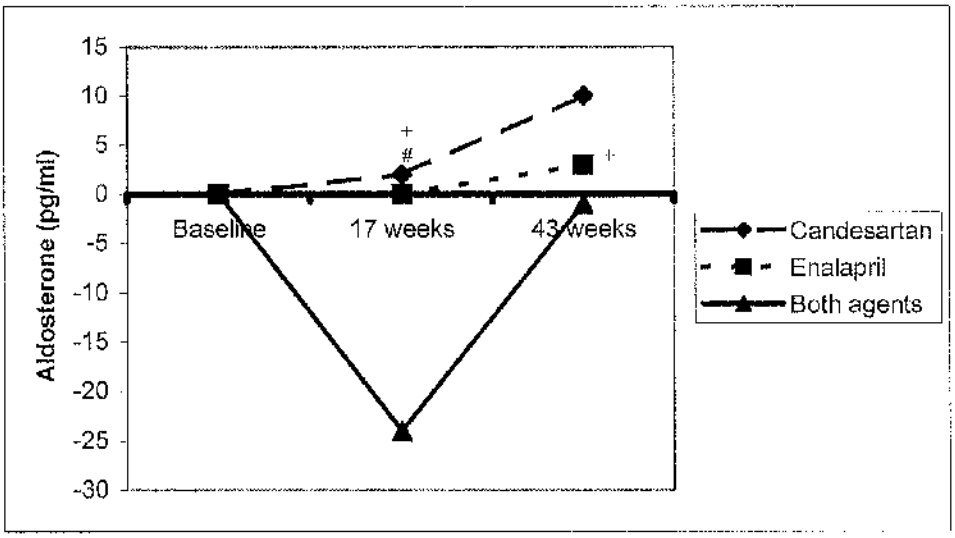


Figure 1.8d. Changes in plasma aldosterone concentrations at 17 and 43 weeks for candesartan, enalapril and candesartan + enalapril.
+p<0.05 compared with baseline, # p<0.01 compared with enalapril
Adapted from ref 264

aldosterone escape) correlates with poor outcome and that more specific blockade of aldosterone effects could be beneficial.

1.8.7 Aldosterone blockade in cardiovascular disease

The first evidence of the additional benefit of aldosterone blockade in heart failure came from the Randomized Aldactone Evaluation Study (RALES) published in 1999(272). In this population of patients with chronic moderate-to-severe heart failure treatment with the aldosterone antagonist spironolactone, given in addition to ACE inhibitor therapy significantly lowered mortality (RR 0.70, 95% CI 0.60-0.82, $p<0.001$) through a reduced risk of death from progressive heart failure and sudden cardiac death. A sub study of sodium retention demonstrated that the dose of spironolactone used in the study (25-50mg) had no apparent diuretic effect. The authors concluded therefore that the cardioprotective effect of aldosterone blockade contributed to the reduction in mortality rate.

To provide a mechanistic explanation for these benefits a further sub-study of the RALES population looking at serological markers of collagen turnover (procollagen type 1 carboxy-terminal peptide (PICP), procollagen type 1 amino-terminal peptide (PINP), procollagen type II amino peptide (PIIINP)) was performed(273). The procollagen markers decreased in the spironolactone group over 6 months but remained unchanged in the placebo cohort, indicating that benefits of aldosterone blockade paralleled the reduction in cardiac fibrosis.

More recently, the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival (EPHESUS) study has evaluated treatment with the highly selective aldosterone antagonist eplerenone (274). This was initiated in 6632 patients with acute MI complicated by heart failure. A major difference in the patient cohort of this study compared to RALES was pharmacotherapy- 75% of patients received beta-blockers vs. 10% in RALES. At a mean follow-up of 16 months, treatment with eplerenone was associated with a 15% reduction in overall mortality and a 17% reduction in cardiovascular mortality. Hence, the myocardial protective effect of aldosterone blockade is maintained even in the presence of optimal therapy and in patients close to the acute phase of myocardial infarction.

In summary, the above evidence provides a compelling argument for an important role for aldosterone in the clinical deterioration of patients with heart failure and myocardial infarction complicated by heart failure. Although the exact mechanism by which aldosterone exerts its adverse cardiovascular effects remains unresolved, it is now generally accepted that aldosterone receptor antagonists have a significant role in the treatment of severe heart failure and post acute MI complicated by heart failure.

1.9 Disorders of mineralocorticoid production

In the majority of clinical syndromes charcterised by disordered mineralocorticoid production, mineralocorticoid hormones are produced in excess leading to hypertension. Mineralocorticoid hypertension is probably the commonest cause of secondary hypertension in man, accounting for around 10 per cent of cases (section 1.10). There are a number of clinical

Cause	Subtype	Mineralocorticoid in excess
Primary Aldosteronism	Adrenal adenoma Bilateral adrenal hyperplasia GRA Adrenal carcinoma	Aldosterone
Congenital Adrenal Hyperplasia	17α hydroxylase deficiency 11ß hydroxylase deficiency	Deoxycorticosterone Corticosterone
Liddle's syndrome		
11ß Hydroxysteroid Dehydrogenase deficiency	Apparent mineralocorticoid excess Liquorice /Carbenoxolone	Cortisol
Miscellaneous (rare)	Ectopic ACTH syndrome Glucocorticoid resistance DOC secretion	Deoxycorticosterone Corticosterone Aldosterone

Table 1.9a. Classification of Mineralocorticoid excess syndromes

syndromes resulting in mineralocorticoid hypertension and these are summarised in Table 1.9a.

1.9.1 Primary aldosterone excess

Excessive production of aldosterone can occur due to an aldosterone producing adenoma (APA), idiopathic hyperaldosteronism due to bilateral adrenal hyperplasia (BAH) and glucocorticoid remediable aldosteronism (GRA; discussed in 1.5.3). This will be discussed in more detail in section 1.10.

1.9.2 Congenital adrenal hyperplasia (CAH)

(a) Deficiency of 11ß-hydroxylase

Clinical deficiency of 11ß-hydroxylase was first noted in 1951(275) and it is characterised by hypertension in the face of impaired cortisol synthesis. Although there is impaired 11ß-hydroxylase activity, aldosterone synthesis is not impaired although plasma aldosterone may be low due to suppression of renin synthesis (276). In these patients hypertension is relieved by administration of glucocorticoids. Other features of clinical 11ß-hydroxylase deficiency are due to adrenal androgen excess, i.e. masculinisation of the female genitalia, premature growth spurt and early closure of the bony epiphyses with subsequent short stature. Figure 1.5c summarises the underlying mechanism which is due to a combination of increased cortisol and aldosterone precursors such as DOC and 11-deoxycortisol (mineralocorticoid hypertension) and ACTH drive to the adrenal to maintain cortisol production resulting in excess androgens.

Classical 11ß-hydroxylase activity is rare with an incidence of 1/100000 births in the general Caucasian population (277). It is inherited as an autonomic

recessive condition and so is more common in populations which are relatively inbred, e.g. Jews of Moroccan descent living in Israel (incidence of 1/5000-1/7000 births)(278).

Multiple polymorphisms within CYP11B1 accounting for clinical 11ß-hydroxylase deficiency have been reported and continue to be detected. The clinical syndrome is mainly caused by mutations of the coding region, clustering at exons 2,6,7 and 8(279).

In Moroccan Jews, almost all affected subjects have the same disease allele, R448H (280). R448 is adjacent to C450 which is presumed to be ligand of the haem iron atom in the P-450 complex and is highly conserved in all eukaryotic p-450 enzymes. This would suggest that C450 is of great importance in the function of 11ß-hydroxylase and a mutation of this amino acid abolishes enzyme activity (281).

(b) Deficiency of 17α-hydroxylase

First described in 1966, 17α-hydroxylase deficiency is an uncommon cause of mineralocorticoid dependent hypertension(282). 17α-hydroxylase ($P450_{C17}$) is active in the zona fasciculata where it is a key enzyme involved in the synthesis of cortisol and the adrenal androgens. In the gonads, $P450_{C17}$ catalyses the formation of testosterone and oestrogens because of its combined 17α-hydroxylase/17,20 lyase activity. In man, $P450_{C17}$ is not expressed in the zona glomerulosa. Absence of 17α-hydroxylase leads to impaired synthesis of 11-deoxycortisol and cortisol in the adrenal, with resultant increased ACTH secretion from the pituitary (Figure 1.9a). Synthesis of oestradiol and testosterone are similarly impaired with a resulting rise in LH and FSH secretion. The combined effects of excess ACTH and gonadotrophin

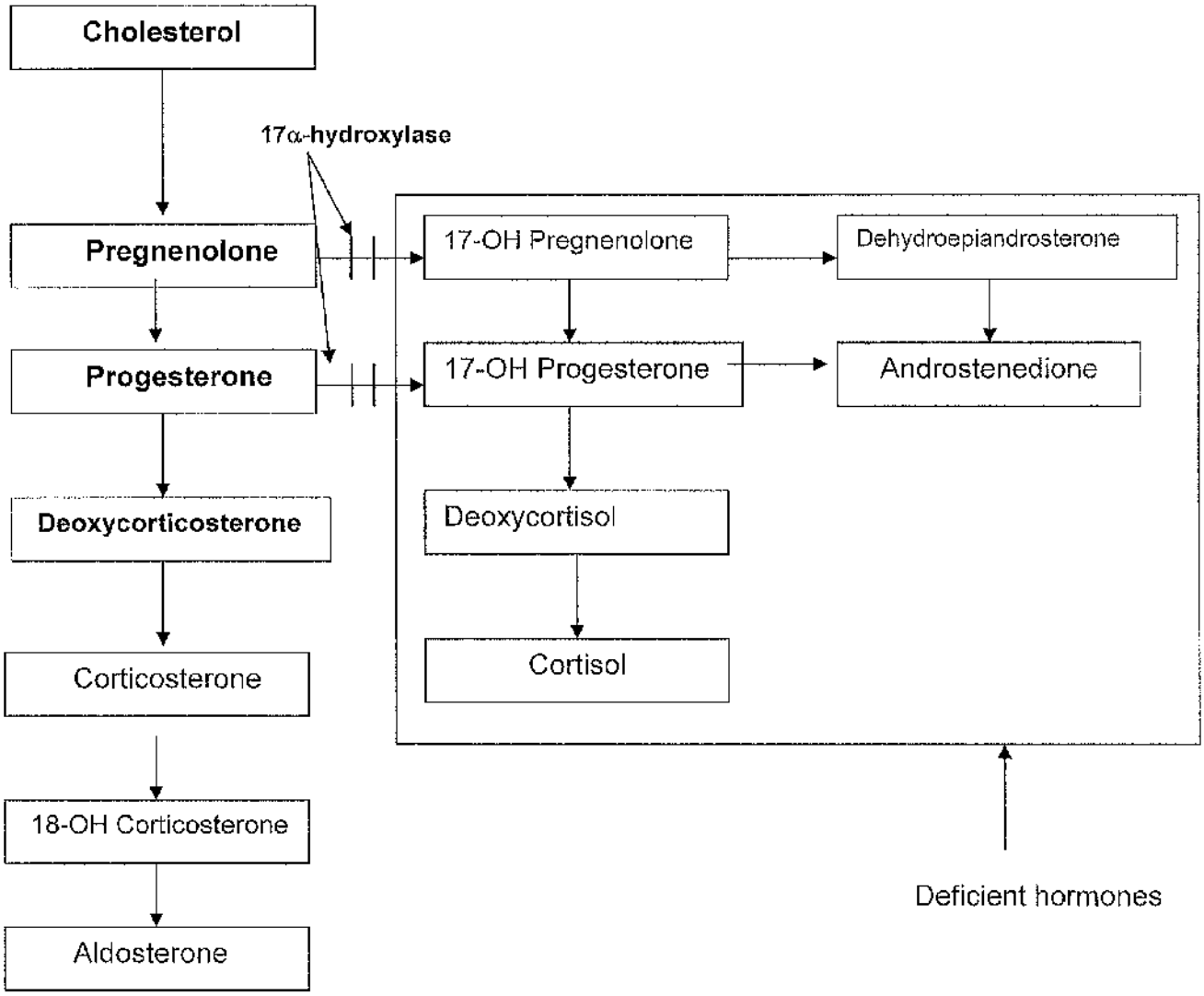


Figure 1.9a. Deficiency of steroid 17α-hydroxylase in man.
Termination of steroid biosynthesis at this site leads to impaired synthesis of cortisol and adrenal androgens which results in an increase in ACTH-driven synthesis of DOC in the zona fasciculata (steroids in bold).
Gonadal steroidogensis is also impaired and secondary sex characteristics fail to develop.
Aldosterone synthesis is normal because 17α-hydroxylase is not expressed in the zona glomerulosa. Excess DOC in the fasciculata leads to sodium retention with suppression of the renin-angiotensin axis and hypertension.

secretion are two-fold. Firstly, there is a build up of immediate precursors of $P450_{C17}$ such as progesterone and pregnenolone. Secondly, increased ACTH secretion results in activation of conversion of DOC to corticosterone and 18-hydroxyDOC in the zona glomerulosa. DOC, and to a lesser extent, 18-hydroxyDOC have sodium retaining properties which suppress renin secretion and as a result plasma aldosterone levels are suppressed. Signs of glucocorticoid deficiency are usually absent and the very high levels of corticosterone probably compensate for the lack of cortisol (283).

The clinical features of 17α-hydroxylase deficiency include hypertension and, in females, lack of secondary sexual characteristics. In XY genetically male fetuses there is a variable degree of pseudohermaphroditism and secondary sex characteristics do not develop at puberty. Indeed, affected males are often raised as females (283).

Most mutations causing this disorder are found in the structural gene of CYP17 as shown in table (284) and some are located in the introns or regulatory elements of the CYP17 gene(285).Many of these CYP17 mutations are defective in both 17α-hydroxylation and 17,20-lyase activities, suggesting that the same residues are required for both enzymatic activities(286).

1.9.3 Liddle's Syndrome

This was first described by Liddle in 1963 (287)when he reported a family in which the siblings appeared to have features of aldosterone excess (early onset hypertension and hypokalaemia) but with suppressed plasma renin and aldosterone levels. It is now known that this syndrome is inherited as an autosomal dominant trait and occurs due to mutations of the gene encoding

the β or γ subunit of the EnaC of the distal nephron(288) (Figure 1.3c). This results in constitutive activation of this receptor (sodium retention and potassium excretion) irrespective of circulating mineralocorticoid levels (usually suppressed). The clinical and laboratory signs include chronic hypokalemia, increase of urinary potassium excretion with sodium retention and reduction of plasma renin activity and of circulating levels of Ang II and aldosterone. In addition, ten mutations causing Liddle's syndrome have been identified and described on the SCNN1B gene at locus 16p12(289) and one mutation on the SCNN1G gene at locus 16p12(196). All the mutations identified to date either alter or delete (290) a highly conserved PY motif at the C-terminal end of the channel which is involved in its normal regulation. Interestingly, in the proband of one of Liddle's original cases, renal transplantation resulted in normalisation of blood pressure and electrolyte abnormalities(291). In practice, however, using antagonists at the sodium channel such as amiloride or triamterene usually effectively treats this condition(291).

1.9.4 Deficiency of 11ß-hydroxysteroid dehydrogenase (11ß-HSD)

Apparent mineralocorticoid excess (AME) is a rare syndrome of hypertension and hypokalaemia associated with suppression of plasma renin activity and low plasma concentrations of aldosterone and other known mineralocorticoids (292) (Figure 1.3c). 11ß-HSD activity is reduced or absent such that cortisol overwhelms the MR causing cortisol-mediated mineralocorticoid hypertension. The gene encoding 11ß-HSD is 6.2kb long, comprises 5 exons and is located on chromosome 16q22 (293). Less than 100 cases of AME have been

reported with >30 different mutations identified clustered in exons 2-5 (294). Biochemical diagnosis of AME can be made by measuring the ratio of cortisol to cortisone as indicated by the ratios of their urinary metabolites (THE: THF ratio abnormally low)(295). Administration of dexamethasone causes reversal of the clinical features (by suppression of ACTH-dependent cortisol secretion), which are reproduced by administration of physiological doses of cortisol (295).

Deficiency of 11ß-HSD can also occur as a result of ingestion of liquorice or carbenoxolone (used for the treatment of peptic ulcer disease). The active component of liquorice is glycyrrhetinic acid which has been shown to inhibit the activity of 11ß-HSD 2 in the renal tubule(296). Carbenoxolone is a semi-synthetic hemisuccinate deriviative of glycyrrhetinic acid and has its effect through a mechanism analogous to that of liquorice(297).

1.9.5 Hypoaldosteronism

Primary hypoaldosteronism is a rare autosomal recessive condition due to mutations in the aldosterone synthase gene (CYP11B2). Prior to characterisation of this gene, this disease was termed corticosterone methyl oxidase (CMO) deficiency. In both cases, the disease is sub-categorised into type 1 and type 2 depending on the associated biochemical phenotype (298). In type 1 deficiency, patients have low to normal levels of 18-hydroxycorticosterone and undetectable aldosterone whereas in the type 2 variant, 18-hydroxycorticosterone levels are high with only mildly reduced or even normal levels of aldosterone. The reason for the variation in phenotype

is unknown, especially since the same mutation in CYP11B2 has been identified in both variants (299).

The clinical phenotype associated with aldosterone synthase deficiency is also variable but its severity tends to be inversely associated with age at diagnosis (300). Whilst it may be asymptomatic in older children and adults, in infancy it may present with failure to thrive, vomiting and dehydration with hyponatraemia, hyperkalaemia and metabolic acidosis. Such children can be treated successfully with mineralocorticoid replacement therapy such as fludrocortisone.

1.10 Primary Aldosteronism (PA)

Primary aldosteronism can be defined as over-production of aldosterone independent of its major regulator, Ang II. The first case of PA was reported by Dr Jerome Conn in a patient with intractable hypertension and hypokalaemia with biochemical evidence of aldosterone excess and an adrenal tumour on abdominal imaging(150). Subsequent adrenalectomy proved curative and led to the identification of a syndrome initially thought to account for<1% of cases of hypertension.

1.10.1 Prevalence of PA

A number of studies around the world over the past decade have reported a marked but highly variable increase in the detection of PA with prevalence

AUTHOR	COUNTRY	NUMBER SCREENED	SETTING	PREVALENCE (%)
Hiramatsu et al. (298)	Japan	348	Hypertension Unit	2.5
Gordon et al (299)	Australia	199	Hypertension Unit	8.5
Lim et al (297)	UK	115	Primary care	14
Fardella et al (300)	Chile	305	Hypertension Unit	9.5
Rayner et al (302)	South Africa	216	Hypertension Unit	32
Schwartz et al (303)	USA	505	Primary care	40
Olivieri et al (304)	Italy	287	Primary care	32

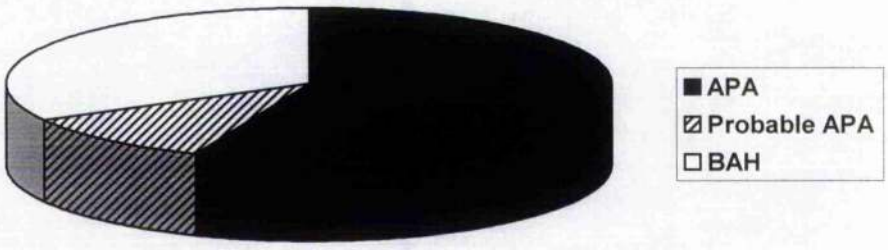
Table 1.10a. Increasing prevalence of Primary Aldosteronism worldwide.

rates ranging from 3 to 40% (Table 1.10a)(301-307). Gordon and colleagues in Brisbane, Australia provided initial evidence that use of the aldosterone to renin ratio (ARR) identifies a higher percentage of hypertensive individuals with aldosterone excess than previously thought. After the introduction of the ARR as a screening tool in 1983 and, particularly after extending its application to include all subjects with hypertension regardless of serum potassium, there was a five-fold increase in the diagnosis of PA(308). This experience has been repeated worldwide and has been summarised by Mulatero and colleagues(309) who examined rates of PA before and after introduction of the ARR in centres spanning 5 continents. This retrospective study highlights that application of the ARR to an increased cohort of patients has led to a 5- to 15-fold increase in the identification of patients affected by PA.

Hypokalaemia is no longer a prerequisite for the diagnosis of mineralocorticoid hypertension although its presence does make it more likely. Indeed, in the first clinical demonstration of the ARR, Hiramatsu et al identified adenomatous PA in 9 patients only 3 of whom were hypokalaemic (301). The experiences of investigators in the 5 Continent Study indicate that the majority of cases of proven PA had normal serum potassium levels at presentation.

Some of the high prevalence rates may reflect selection bias as many of these studies are with patients referred to secondary or even tertiary care facilities with resistant hypertension or hypokalaemia. However, some studies have been performed in unselected populations(303;306;308). Most recently, investigators in Italy randomly selected a sample of adults from the population

A. 1957-1985



B. 1999

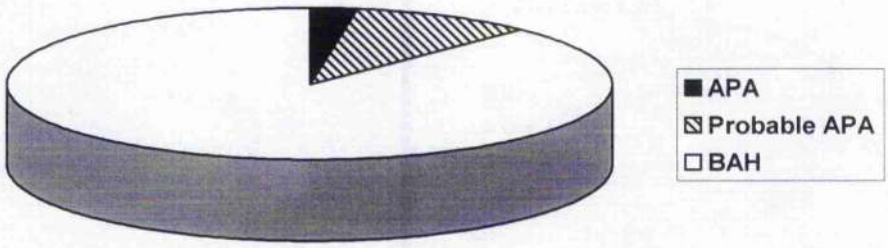


Figure 1.10a. Change in prevalence and relative proportions of PA subtypes.

A: From 1957–1985, 248 patients were diagnosed with primary aldosteronism at Mayo Clinic; 57% had surgically confirmed APA, and 11% had probable APA; the remainder (33%) had probable or confirmed bilateral adrenal hyperplasia (BAH; idiopathic aldosteronism).
B: In 1999, 120 patients were diagnosed with primary aldosteronism at Mayo Clinic; 20% had surgically confirmed APA, and 8% had probable APA; the remainder (72%) had probable or confirmed BAH.

register of a local health district(307). Of the 1348 individuals involved, 412 were found to be hypertensive. 287 of these subjects agreed to estimation of their ARR which was elevated in 93 patients, meaning that around one in three hypertensive individuals (32.4%) selected at random had evidence of aldosterone dysregulation. This rate is in excess of that reported by investigators in Dundee, Scotland who found 14% (18/125 patients) of unselected hypertensive patients selected at random from a primary care population to have an elevated ARR(303). Interestingly, this prevalence is very similar to that found in specialist referral clinics from the same population(310).

1.10.2 Causes of PA

In association with an increase in detection of PA, there has been a change in the relative proportions of PA subtypes. It had been previously accepted that adrenal adenoma was the commonest cause of PA accounting for around 60% of cases, with most of the remainder due to bilateral adrenal hyperplasia (idiopathic aldosteronism). This proportion has now effectively been reversed with two thirds of cases of PA said to be due to bilateral adrenal hyperplasia(304;311) (Figure 1.10a).

Some investigators have reported surprisingly low rates (3-10%) of adrenal adenomas in patients with PA(307). This may well reflect more widespread screening to include individuals with normal serum potassium, as bilateral adrenal hyperplasia is associated with less florid manifestations of PA than adenomatous PA.

DRUG	ALDOSTERONE LEVELS	RENIN LEVELS	EFFECT ON ARR
β-blockers	↓	↓↓	↑
ACE inhibitors	↓	↑↑	↓
ARBs	↓	↑↑	↓
Diuretics	↑	↑↑	↓
Calcium channel blockers	↓/-	↑/-	↓

Table 1.10b. Effects of anti-hypertensive medication on aldosterone-renin ratio.

1.10.3 Aldosterone-to-renin-ratio (ARR)

More recently, it has become accepted that aldosterone excess is more common in hypertension than previously thought. This has coincided with widespread use of the ARR as a screening and diagnostic test. It is well known that plasma renin activity (PRA) and aldosterone levels vary according to posture and salt status. In theory, the ratio between the two, calculated by dividing plasma aldosterone by PRA, varies to a lesser extent. This ratio capitalizes on divergence between the two neurohormones such that minor abnormalities of both parameters will be accentuated by combining the two as a ratio. In PA, the ratio should be obviously elevated as a result of suppressed PRA and elevated aldosterone levels.

However, the ARR is subject to significant problems. Concomitant drug administration affects aldosterone levels and PRA (312) (Table 1.10b). Although ideally the ARR should be measured on no anti-hypertensives, it is recognised that this can rarely be achieved. The ratio is still interpretable under these circumstances as long as drug effects are taken into consideration. The PHarst study demonstrated that beta-blockers (by suppressing renin) elevate the ratio and ACE inhibitors (by stimulating renin) suppress it (313). Thus, a high ARR in a patient on a beta-blocker should be viewed with caution but a marginally high ARR in a patient taking an ACE inhibitor is more likely to be suspicious. Calcium channel blockers and alpha-blockers do not appear to interfere with the ratio(314).

The major criticism of the ARR has been that it is overly renin-dependent. Indeed in the majority of patients identified by a raised ARR, the real explanation is that low levels of renin dominate the ratio. Subsequent analysis

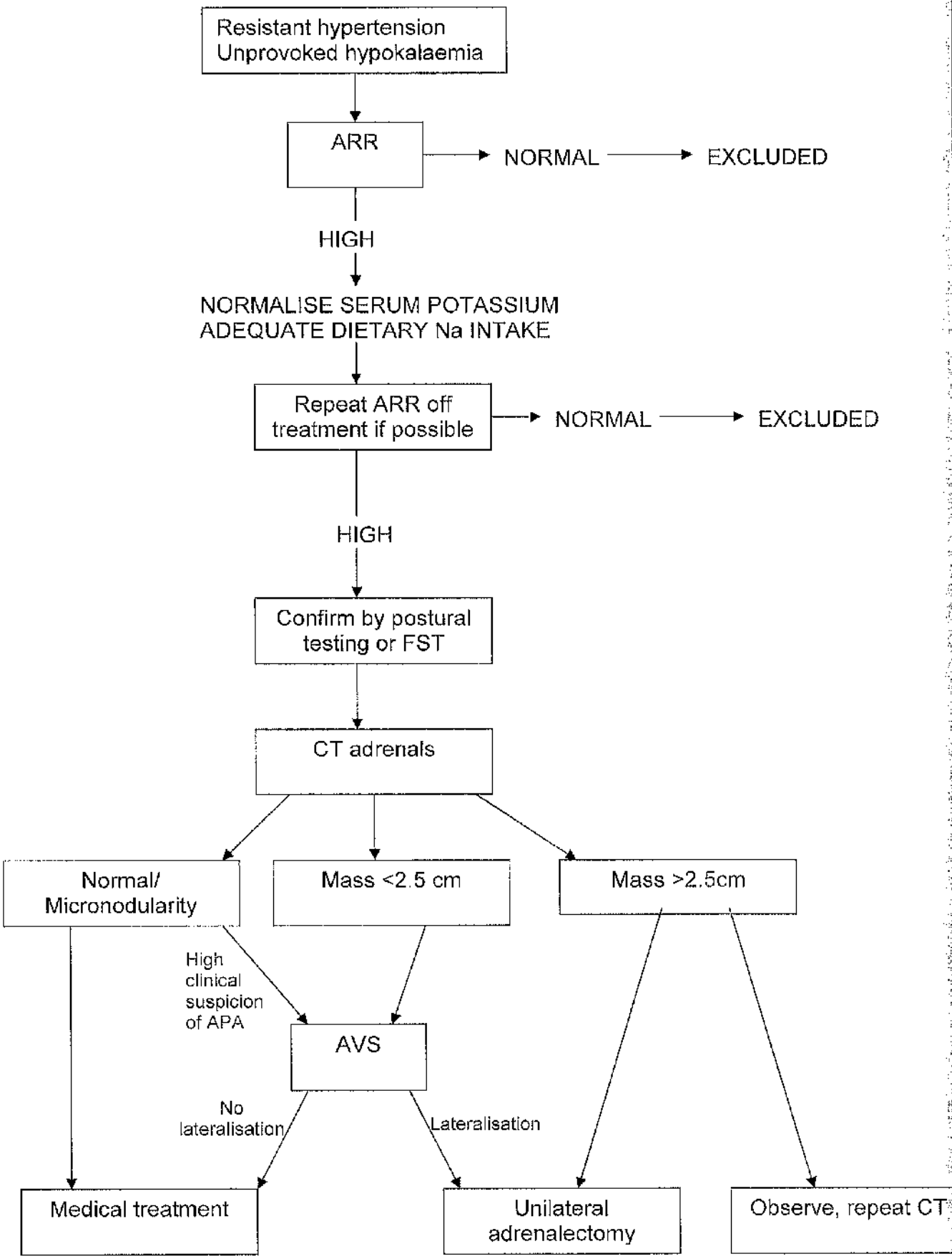


Figure 1.10b . Proposed algorithm for the screening, diagnostic confirmation and management of primary aldosteronism.
AVS-adrenal venous sampling; FST-fludrocortisone suppression testing; APA-adenomatous primary aldosteronism; ARR-aldosterone-to-renin ratio

of the mathematical derivation of the ARR, has confirmed that the renin measurement is the determinant of the ratio, so that its positive predictive power for PA is relatively low(315). This criticism is compounded by difficulties in accurately measuring PRA when it is below a minimum value of 0.3ng/ml/h. To compensate, some groups have proposed interpreting the ARR in conjunction with a plasma aldosterone threshold of > 15ng/dL(316). However, even when this more stringent of definition of PA is applied, the incidence is hardly altered(317).

1.10.4 Diagnosis of PA

The diagnosis of PA can be problematic and hampered by the lack of a single coherent diagnostic strategy. The diagnostic strategy applied in my centre is outlined in Figure 1.10b. Before performing specific diagnostic tests, serum potassium should be normalised if necessary by oral supplementation and patients should be encouraged to maintain a liberal dietary salt intake prior to ARR testing and throughout subsequent investigations for PA.

a. Detection of aldosterone excess

Although initial ARR can be performed on anti-hypertensive treatment, we advocate repeated measurement off treatment in those with an elevated ratio and those with a high clinical suspicion of PA. In particular, diuretics (including amiloride and spironolactone) should be stopped for up to 4 weeks and other medications which cause false negative (ARB or ACE inhibitors) or positive (beta-blockers) ratios ceased for at least 2 weeks prior to repeat testing(318). During this period, other agents (such as alpha-blockers or verapamil), which

have a lesser effect on the ratio, are used if necessary to maintain control of hypertension.

b. Confirmation of Aldosterone excess

Although its validity as a screening tool is becoming more widely accepted, clearly an elevated ARR indicates a need for further investigation of the RAA axis and is not sufficient to make a diagnosis of PA. Postural testing of PRA and aldosterone can help confirm aldosterone excess as well as clarify the underlying cause (319). The rationale behind this is that in normal subjects, plasma aldosterone rises with standing due to activation of the renin-angiotensin system. In the majority of aldosterone producing adenomas, renin does not rise with standing and, as a result, aldosterone is uninfluenced by posture but still responds to ACTH resulting in diurnal variation. Furthermore, aldosterone may be relatively unresponsive to Ang II in such patients. In idiopathic aldosteronism due to adrenal hyperplasia and angiotensin II-responsive adrenal adenoma, aldosterone levels may continue to rise on standing.

Further confirmation of abnormal regulation of the RAA axis can be made by attempts to suppress plasma aldosterone by the administration of fludrocortisone or saline.

c. Adrenal Imaging

Adrenal imaging should only be performed when aldosterone excess has been confirmed biochemically. Although there have been no recent comparison studies, it appears that computerised tomography (CT; using fine cuts) is the preferred imaging modality. Most authors report that CT affords better spatial resolution and so is more sensitive in detecting smaller

adenomas than magnetic resonance imaging (MRI). Nonetheless, it is estimated that CT detects only around 50 –75% of aldosterone producing adenomas(320).

d. Role of Adrenal Vein Sampling (AVS)

Although technically difficult, adrenal vein sampling (AVS) remains the gold standard for confirmation and localisation of aldosterone-producing adrenal adenomas. Given its complexity, it is generally reserved for those in whom adrenal surgery is being considered. Diagnosis of adrenal adenoma can be made if aldosterone levels are elevated in one adrenal vein compared to the other but simultaneous cortisol measurements must be made to ensure the cannula is in the adrenal vein. The Australian group advocate AVS for all patients with proven primary aldosteronism irrespective of CT findings (321); they argue that adrenal CT lacks sensitivity for adrenal adenomas. This is supported by investigators in Japan who used AVS to identify adenomatous primary aldosteronism in 43 (66%) of the 65 patients identified as having PA; only 47% of those who lateralized on AVS demonstrated adrenal tumours on CT scanning(322).

This is clearly an important issue, as lateralizing excess aldosterone production to one adrenal gland affords the possibility of surgical cure. It may well be that the apparently low prevalence of adenomatous PA compared with bilateral adrenal hyperplasia may be due, in part, to the reliance on CT imaging. Moreover, CT can be misleading by demonstrating unilateral masses in patients found to have bilateral production(323) or lateralizing to the opposite adrenal on AVS. The fact that some patients with bilateral adrenal hyperplasia may also exhibit single (focal) adrenal lesions mean that a

positive adrenal CT alone should not be an indication for surgery even in patients with unequivocal PA.

There are clear problems associated with AVS, however, which make it impossible to advocate as a diagnostic tool in all patients with PA. Technical difficulties mean only skilled, experienced operators cannulate both adrenal veins with any degree of success or frequency. This tends to limit availability of this procedure to larger centres. Controversy exists over the use of concurrent ACTH stimulation during AVS. Moreover, the procedure itself and methods used to analyse results vary across referral centres. Three recent reports highlight this lack of consensus. One Italian study of 104 patients undergoing AVS (without ACTH stimulation)(320) showed that a lateralized ratio of at least 2 was most discriminatory for the diagnosis of a unilateral adenoma. In contrast, two recent studies from large US referral centres used ACTH stimulation and a lateralized ratio of at least 4 and a contralateral ratio of <1 to define an adenoma(324;325). It would appear that ACTH stimulation does increase the sensitivity of AVS in differentiating bilateral adrenal hyperplasia from adrenal adenoma and is performed by most major investigating centres.

1.10.5 Treatment of PA

Spironolactone is an effective antihypertensive in patients with aldosterone excess due to bilateral adrenal hyperplasia. In a small observational study of hypertensive subjects with a raised ARR, Lim and colleagues demonstrated that addition of spironolactone to their existing regimen reduced the need for antihypertensive drugs by a mean of 0.5 as well as reducing mean BP by

15/8(326). Moreover, investigators in Cambridge have suggested that ARR can predict response to spironolactone treatment(313). In their study of unselected hypertensive individuals, 70% of those with a raised ARR responded favourably to 50mg of spironolactone treatment with a mean BP reduction of 32/17. In contrast, only 40% of those with a lower ARR responded adequately to spironolactone. This provides good evidence that the ARR can be used as a guide for drug therapy in hypertension. However, there has been no properly randomised clinical study demonstrating the efficacy of spironolactone in patients with a raised ARR. The use of spironolactone is limited by its side effects many of which are related to its ability to interact with sex hormone receptors as well as the mineralocorticoid receptor(327). This has led to the development of eplerenone, a selective aldosterone receptor blocker with greater specificity for the mineralocorticoid receptor, less than 1% affinity for the progesterone receptor and less than 0.1% affinity for the androgen receptor(328). A number of placebo-controlled trials have demonstrated that eplerenone is an effective antihypertensive which is better tolerated than spironolactone(329). The eplerenone-induced reduction in blood pressure is dose-dependent; response to 100mg of eplerenone is about 75% of that observed with 100mg of spironolactone. Eplerenone is as effective as ARBs in lowering blood pressure and may be more efficacious than losartan in black patients(330;331). The addition of eplerenone to treatment with ACE inhibitors or ARBs is also effective and well tolerated(332).

In patients intolerant of aldosterone receptor antagonists, amiloride may be used. This drug blocks the aldosterone-regulated epithelial sodium channel.

However, in patients with PA it may need to be given in fairly high doses to be effective.

Unilateral laparoscopic adrenalectomy is the treatment of choice for individuals with an aldosterone-producing adenoma. Although blood pressure control improves in almost all patients post-operatively, average long-term cure rates of hypertension after unilateral adrenalectomy for adenomatous PA range from 30-60%(333). Persistent hypertension after adrenalectomy correlates with having >1 first-degree relative with hypertension, use of >2 antihypertensive agents preoperatively, older age, increased serum creatinine and duration of hypertension, and is probably due to coexistent primary hypertension(334;335).

A laparascopic approach is preferable and associated with shorter in-patient stays and less long-term morbidity(327;335). Pre-operatively, patients should be treated with spironolactone to lower blood pressure and normalise plasma potassium(327). Spironolactone also reduces the risk of post-operative hypoaldosteronism because it allows recovery of the renin angiotensin aldosterone axis resulting in stimulation of the previously atrophic contralateral zona glomerulosa.

In patients who are unfit for or decline surgery medical treatment is preferred as outlined above.

1.10.6 PA or not PA?

So far, there have been in excess of 3500 patients tested for PA using the ARR as an initial step. As a result, the overall prevalence of PA has been

reported to be at least 10%, well in excess of the 1% prevalence widely quoted in most hypertension textbooks. However, whether or not these subjects have true 'classical' PA, with clear evidence of genuinely autonomous aldosterone excess, remains controversial. No gold standard definition of PA currently exists. Most centres use a rather arbitrary cut-off for a high ARR that depends upon the units used for aldosterone measurement (generally >750 if aldosterone expressed in pmol/L and >25 if expressed as ng/dL). With this approach, low renin rather than high aldosterone is the cause of an elevated ratio in the majority of subjects and aldosterone levels are often within the 'normal' range. It is uncertain whether they would meet earlier criteria for the diagnosis of PA. However, in most individuals who have been studied more intensively, dynamic tests of the RAS are abnormal, confirming an altered relationship between renin and aldosterone. How this group differs from individuals with low-renin hypertension is unclear and there is likely to be substantial overlap between the groups(83). This reprises previous arguments by Padfield et al. (336), who claimed that PA due to bilateral adrenal hyperplasia was a variant, not of classical Conn's adrenal adenoma, but of low-renin essential hypertension and that in this circumstance Ang II probably remains the major trophin for aldosterone.

The concept of 'tertiary aldosteronism' where there is sustained and prolonged stimulation of the adrenal by angiotensin II is already recognised in renovascular hypertension(337). It has been suggested that the phenotype of hypertension with a raised ARR could be redefined as a form of tertiary aldosteronism, perhaps preceded by low-renin hypertension over a much longer time. Whilst this theory focuses on a single genetic polymorphism, it is

widely accepted that hypertension is a polygenic disorder. It is likely, therefore, that other genes may interact in a synergistic manner to lead to the phenotype of hypertension with an elevated ARR.

Thus, within the heterogeneous population of essential hypertension, there may be 3 distinct subgroups which are currently classified on a hormonal or biochemical basis: low renin hypertensives, non-modulators (normal to high renin) and hypertensives with an elevated ARR. These 3 groups may form a neurohormonal spectrum which reflects differing stages of hypertension, the rate of progression of which depends on other genetic and environmental factors. Hence, the natural history of hypertension may proceed from essential (high-to-normal renin) hypertension through to low renin hypertension and finally to tertiary aldosteronism. Clearly, longitudinal studies that compare groups of patients of different ages followed up over time are needed to investigate this theory.

In summary, an elevated ARR can be demonstrated in around 10 per cent of unselected hypertensive subjects, making it the commonest recognizable phenotype in secondary hypertension. Although it is unclear how many of these individuals have 'classical' PA with truly autonomous secretion of aldosterone all clearly have demonstrable abnormalities in renin-angiotensin-aldosterone dynamics. Patients with an elevated ARR (and previously described subjects with low-renin hypertension) have an inappropriate level of aldosterone for its principal trophin Ang II and thus might be better re-designated as having 'aldosterone-associated hypertension' (338).

1.10.7 Possible genetic basis for 'Aldosterone associated hypertension'

Debate over the exact physiological differences and similarities between sub-groups is largely academic. Subjects identified by an increased ARR have an inappropriately high aldosterone concentration for its principal trophin, Ang II; whatever gives rise to this disproportionate renin for aldosterone level could clearly have pathogenic and therapeutic implications and deserves further investigation.

The corticosteroid biosynthetic pathway and the crucial role of aldosterone synthase (encoded by CYP11B2) and 11β-hydroxylase (CYP11B1) in catalysing the terminal steps of aldosterone and cortisol biosynthesis respectively have already been discussed (see section 1.6). These genes are obvious candidates that might be involved in hypertensive disorders as confirmed by monogenic forms of hypertension described earlier (Table 1.10c). Animal models, and in particular the Dahl salt sensitive rat (146) also suggest a role for this locus. Over the last few years, studies have focused on 2 common polymorphisms within the CYP11B2 gene (Figure 1.10c). One is a single nucleotide polymorphism (SNP) in the 5' promoter region at –344 (C-T) that alters a putative recognition site for the transcription factor,steroidogenic factor 1 (SF-1) (339). It is unclear whether this has any physiological significance: binding of SF1 is reduced 4-fold with the T allele and there is no detectable effect on gene transcription when studied in vitro(207). The other polymorphism involves intron 2 of CYP11B2, which is partly replaced by the corresponding intron of CYP11B1(339). These 2 polymorphisms are in close linkage disequilibrium such that the common haplotypes generated are T/conversion (38%), T/wild type (16%) and C/wild type (45%)(340).

Model	Genotype	Phenotype
Glucocorticoid Remediable Aldosteronism	CYP11B1/CYP11B2 hybrid (Figure 1.5b)	Hypertension with aldosterone excess under ACTH control
11ß-hydroxylase deficiency	Loss of function mutations in CYP11B1 (Figure 1.5c)	Hypertension with ↑ DOC and deoxycortisol
Dahl salt sensitive rat	Mutations in rat 11ß-hydroxylase gene (chromosome 7)	Salt sensitive hypertension

Table 1.10c. Summary of evidence illustrating the role of the CYP11B locus in monogenic hypertensive syndromes.

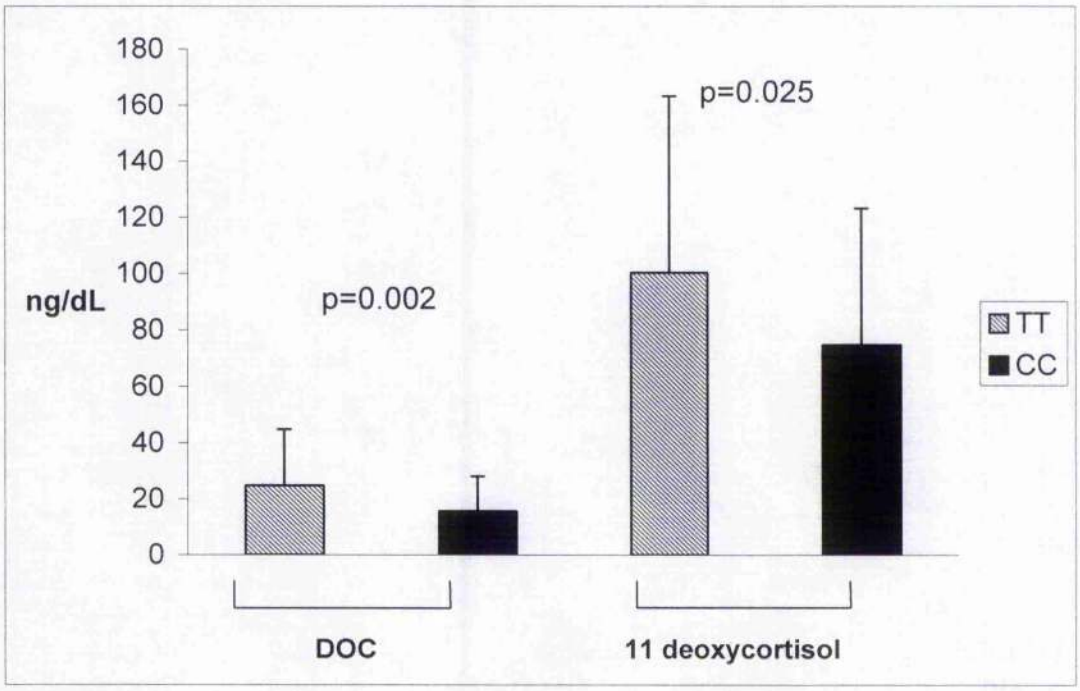
Figure 1.10c. Common polymorphisms identified in CYP11B2

In a previous local study of 138 hypertensive subjects, there was a highly significant excess of TT homozygosity compared to CC homozygosity when compared with individually matched normotensive controls(340). Other groups report similar findings(341). Moreover, subsequent assessment of aldosterone excretion (as its urinary metabolite, tetrahydroaldosterone (THAldo)) in an unrelated population demonstrated a strong association between the T allele and higher excretion rates(340). Further investigations have reported that plasma levels of aldosterone are raised in subjects with the T allele of -344(342). Additionally, an association between T allele frequency and elevated ARR has been reported within a population of hypertensive patients (Table 1.10d) (343). However, the physiological significance of these polymorphisms remains controversial with some studies suggesting either no effect of the –344C/T polymorphism on blood pressure/aldosterone(344;345) or a positive association between the C-allele and aldosterone or cardiovascular parameters(346;347). In particular, one case-control study of a Japanese population (348) there was a suggestion that the CC genotype was more common than TT in hypertensives compared to controls and that the CC genotype associated with higher ARR than TT. However, this study may have been subject to selection bias; the -344 TT homozygote rate was more than twice that observed in European populations (57% *vs.* 26%), and the T allele frequency was about 40% (0.74 *vs.* 0.53) higher than that found in other study populations (0.54–0.56(340;343). This might reflect a true genetic difference between the populations, and/or a sampling difference because a subsequent Japanese study reported lower but still relatively high T allele frequencies of 0.66 and 0.63 in hypertensive and normotensive subjects, respectively(349).

SF-1	CC	CT	TT	x^2	p-value
ARR ≥ 750	14 (0.15)	42 (0.46)	35 (0.39)	8.6	0.014
ARR < 750	52 (0.22)	129 (0.55)	53 (0.23)		
ARR ≥ 1000	8 (0.14)	26 (0.46)	23 (0.40)	6.5	0.039
ARR < 1000	58 (0.22)	145 (0.54)	65 (0.24)		
INTRON CONVERSION	**11(wild type)**	**12**	**22(conversion)**	x^2	p-value
ARR ≥ 750	26 (0.29)	40 (0.45)	23 (0.26)	3.2	0.205
ARR < 750	81 (0.35)	111 (0.48)	40 (0.17)		
ARR ≥ 1000	14 (0.25)	24 (0.43)	18 (0.32)	7.1	0.029
ARR < 1000	93 (0.35)	127 (0.48)	45 (0.17)		

Table 1.10d. Genotype distribution stratified by aldosterone to renin ratio.
375 hypertensive patients underwent genotyping and assessment of ARR. Patients with a raised ARR had a statistically significant excess of the T allele at the SF-1 site. A similar pattern was seen with the intron conversion allele although this only reached statistical significance at a higher threshold of ARR

(a)



(b)

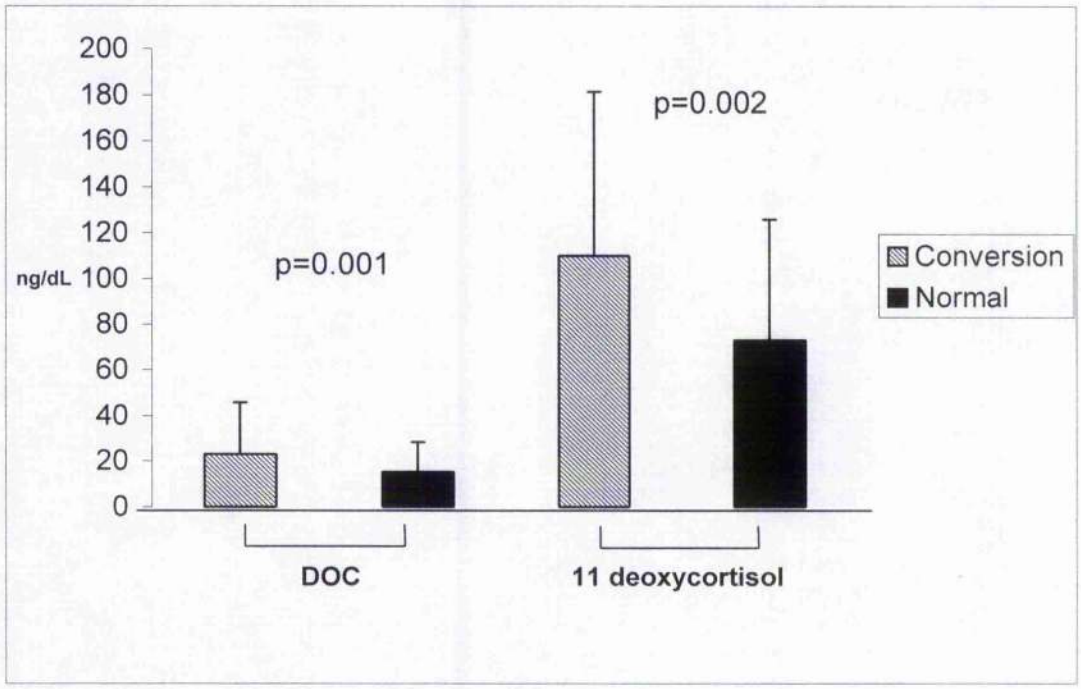


Figure 1.10d. 11-Deoxycorticosteroid responses (increase over basal concentration at 30 minutes) to ACTH (250µg i.v.).
(a) SF-1 polymorphism (CC/TT); (b) Intron conversion vs normal intron 2

Additionally, the small sample size necessitated that statistical analyses were performed by combining patients with TC and CC genotypes at position -344.

In addition to the data on aldosterone and blood pressure, several investigators have consistently shown that the –344 T allele and intron 2 conversion are also associated with raised basal and ACTH-stimulated levels of the 11-deoxysteroids, DOC and deoxycortisol (Figure 1.10d)(350). These steroids are converted to corticosterone and cortisol respectively by 11β-hydroxylase within the zona fasciculata (Figure 1.6a). This evidence, which initially appears paradoxical, suggests that the -344T allele of CYP11B2 is associated with impaired activity of 11β-hydroxylase, which is encoded by the adjacent CYP11B1. Although the exact molecular mechanism that accounts for this is not understood, we have hypothesized that the –344 T polymorphism in the promoter region of CYP11B2 is in close linkage disequilibrium with a key quantitative trait locus (QTL) in CYP11B1 adversely affecting its expression or function resulting in increased levels of 11-deoxysteroids(351).

1.11 Possible link between variation at CYP11B with hypertension and raised ARR

The theory that 11β-hydroxylation may be impaired in hypertensives is not novel (352;353). However, until now there has been no suggestion that this biochemical abnormality is related to variation at the CYP11B locus. These data also lead us to speculate on the link between a minor change in 11-hydroxylase efficiency and hypertension with aldosterone excess. It is unlikely that the minor increases in DOC and 11-deoxycortisol will have significant

biological effects. However, impaired conversion of deoxycortisol to cortisol as a consequence of reduced 11β-hydroxylase activity should result in a slight reduction in cortisol levels in response to ACTH. In turn, normal feedback regulation should result in a resetting of the hypothalamic-pituitary-adrenal axis such that cortisol levels are maintained. Consequently, there will a subtle increase in ACTH drive to the adrenal cortex (Figure 1.11a).

Thus, if this were a genetically determined phenomenon we would predict that subjects with less efficient cortisol synthesis will maintain a slightly enhanced ACTH drive to the adrenal (effectively, a minor variant of classical 11β-hydroxylase deficiency). In the long term, this is likely to cause hyperplasia of both zona fasciculata and zona glomerulosa of the adrenal cortex resulting in increased synthetic capacity for both cortisol and aldosterone. Importantly, expression of a number of genes necessary for aldosterone production, including steroidogenic acute regulatory protein, p450 side-chain cleavage (CYP11A) and p450-21-hydroxylase (CYP21), is responsive to ACTH emphasizing the potential for increased synthetic capacity of aldosterone (108).

Importantly, we do not propose that ACTH is a principal stimulator of excess aldosterone production by the adrenal. Indeed, previous studies have demonstrated the ability of pharmacological doses of ACTH to stimulate aldosterone production only in the short term with aldosterone production decreasing after a few days(354). However, these experiments concentrated on very unphysiological exposure of the adrenal to grossly excessive amounts of ACTH. In ACTH-dependent Cushing's disease, where there is chronic sustained exposure of the adrenal cortex, aldosterone concentrations are not

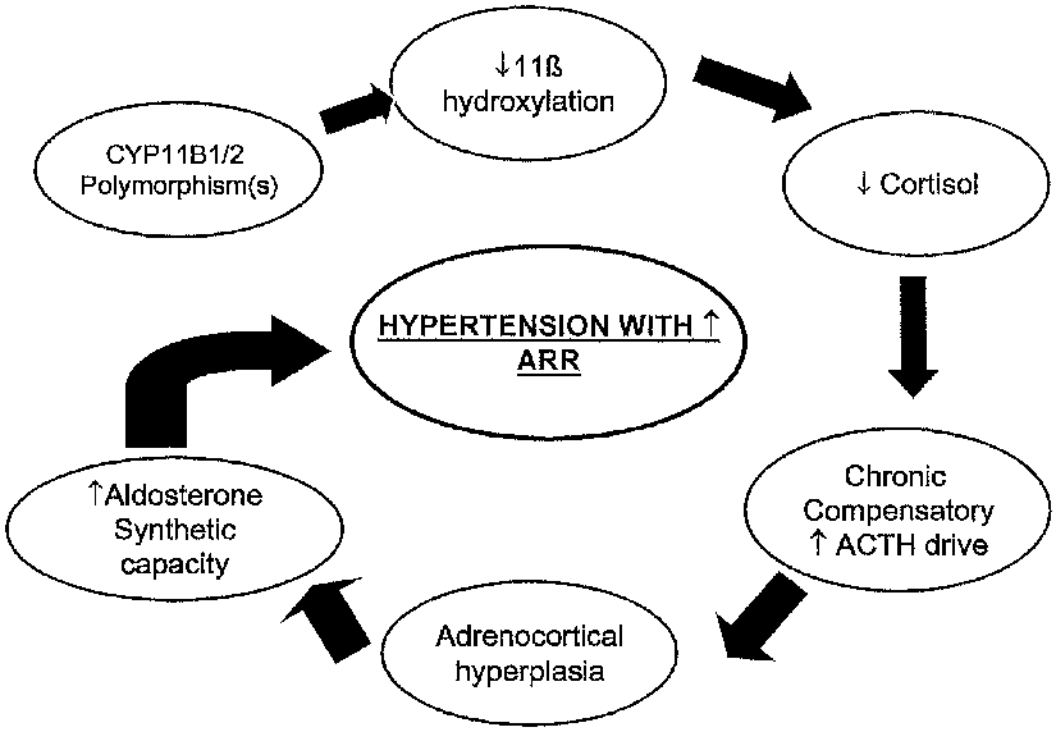


Figure 1.11a. Possible sequence linking impaired 11β hydroxylase activity and hypertension with raised ARR

diminished (184). Other POMC breakdown products such as joining peptide (JP) and β-endorphin have also been shown to stimulate aldosterone production by human adrenal cells in-vitro. Thus, we suggest that increased levels of a POMC-related product (either ACTH or a related peptide) favours zona glomerulosa hyperplasia and may enhance aldosterone secretion in response to other, more conventional, trophins such as Ang II or potassium. Thus, over a very long period, the genetic change in 11β-hydroxylation efficiency (along with an additional environmental or genetic influence) might result in ACTH-driven adrenal zonal hyperplasia, and an alteration (steepening of the dose-response relationship) of the response of aldosterone to Ang II and potassium. It is pertinent that very early studies reported that a proportion of patients with essential hypertension showed a good blood pressure lowering response to low dose dexamethasone treatment, apparently supporting the suggestion that ACTH was sustaining production of a hypertensinogenic adrenal steroid(355). Furthermore, there are reports of increased levels of DHEAS (an adrenal androgen driven by ACTH) hypertensive patients(356). Finally, it is significant that adrenal gland hyperplasia is a common histological finding in hypertensive patients at post mortem and even in patients with apparently 'solitary' adrenal adenomas (357;358).

1.11.1 Hypothesis under investigation

Thus, the principal hypothesis to be explored in this thesis is that, in susceptible subjects with hypertension, previously identified polymorphisms within CYP11B2 (-344T and intron conversion) act as markers for key variants

in CYP11B1 resulting in reduced 11ß-hydroxylase efficiency. In turn, this leads to a subtle but more pronounced ACTH drive to the adrenal cortex and enhanced synthetic capacity of the zona glomerulosa resulting in increased production of aldosterone and suppression of renin. Thus in such individuals, there should be recognisable, genotype dependent, changes in the pattern of adrenal steroid production as well as alteration in the cortisol/ACTH relationship. This will be explored in a number of different populations.

1.11.2 Aims of investigation

1. To explore the pattern of variation across the entire CYP11B1 locus, identify variants in linkage with known polymorphisms of CYP11B2 and construct common haplotypes across this locus.

2. To relate variation across the CYP11B locus with alteration in intermediate corticosteroid phenotype within hypertensive and other large populations.

3. To examine, in detail, the effect of variation across the CYP11B locus on hypothalamic-pituitary-adrenal axis activity in subjects with hypertension and normotensive controls.

4. To further explore the role of aldosterone synthase and 11ß-hydroxylase in the production of 'hybrid' corticosteroids found in certain sub-types of PA and compare patterns of production within different sub-groups (normal adrenals/ impaired adrenal function/absent adrenal function).

Chapter 2: Materials and Methods

CLINICAL

2.1 General points

All studies carried out and reported within this thesis had full approval from the local hospital ethics committee as well as the Western Infirmary Research and Development department.

All patients and volunteers within each study gave their informed, written consent. Each clinical visit was arranged and co-ordinated entirely by me according to relevant protocols.

2.2 Effect of CYP11B genotype on ACTH/cortisol relationship

Subjects with hypertension were recruited from blood pressure clinics at the Western Infirmary, local general practitioners or by advertisement in the local press. Normotensive control subjects were recruited simultaneously from advertising within the Western Infirmary.

2.2.1 Inclusion/exclusion criteria

Formal inclusion and exclusion criteria are listed in Table 2.2a. Briefly, hypertensive subjects had to satisfy British Hypertension Society Guidelines for the diagnosis of hypertension and so demonstrated at least 3 blood pressure readings of >140/90 mm/Hg at rest and each reading separated by at least a week. Resting, supine blood pressure was measured by an Omron MX3 digital device. All subjects were either on no hypertensive treatment or on medications with minimal/no effect on the RAAS (alpha-blockers or calcium channel blockers).

Inclusion criteria	Exclusion criteria
• Age 16-65 • BP>140/90 • Untreated or on treatment not affecting the RAA axis	• Pregnancy • Use of systemic steroids currently or within the preceding 6 months • Use of inhaled or topical steroids currently or in preceding 6 months • Inability to comply with study instructions • History of severe atopy or asthma

Table 2.2a. Inclusion and exclusion criteria for study examining effect of CYP11B genotype on cortisol/ACTH relationship in hypertensives

All control subjects were on no medication and had blood pressures within the normal range (<140/90 mm/Hg).

2.2.2 Initial genotyping

All volunteers who satisfied the inclusion criteria underwent genotyping for the –344 polymorphism in CYP11B2 (SF-1 polymorphism). Blood samples were obtained via direct venepuncture of a forearm vein using the vacutainer system (greiner-bio-one). Blood for DNA extraction was taken into 4.5ml bottles containing potassium EDTA (0.054ml of 0.34M EDTA K_3) as an anticoagulant. Blood was stored at -4°C until DNA was extracted. DNA extraction and details of genotyping technique are explained in sections 2.6 and 2.8 respectively.

2.2.3 Clinical protocol

On each occasion, on arrival to the day ward of ward E3/4, Western Infirmary, a 14 gauge intravenous catheter (Venflon, Helsinberg, Sweden) was inserted into the antecubital fossa and subjects lay recumbent for 30 minutes before blood was drawn for measurement of cortisol, ACTH and 11-deoxycortisol (6ml bottles containing lithium/heparin) as well as renin and aldosterone (potassium EDTA bottles). Blood was immediately spun at -4°C and 3100 rpm for 10 minutes (Allegra 21R centrifuge, Beckman Coulter, UK). Plasma was then decanted and transferred to storage at -70°C (Forma Scientific freezer, Thermo Electron Corporation) for 'snap' freezing before transfer after 24 hours for longer-term storage at -20°C (Gram UK).

Visit 1-Cortisol/ACTH relationship

Subjects attended at 10pm and between 7-8 am the following morning for baseline blood measurements. If normotensive volunteers, then no nighttime visit was required.

Visit 2-Effects of dexamethasone suppression

Four days before visit, subjects were asked to follow a standard salt diet (Appendix II) to ensure a sodium intake of approximately 120mmol/day. On the day before (midnight to midnight), a 24-hour urine collection was performed. 50mls of the final volume was aliquoted for storage at -20°C (Gram UK) for analysis of corticosteroid excretion rates whilst the rest was sent to the Biochemistry department for urinary sodium quantification.

At midnight the night before the visit, subjects took 125 micrograms of dexamethasone and attended the day ward fasted the next morning between 7-8am for blood collection as detailed above.

Visit 3-Response to ACTH

Again, subjects were asked to follow a standard salt diet and perform a 24hour urine collection as detailed above. At midnight the night before the visit, subjects took 1 milligram of dexamethasone and attended the day ward fasted the next morning between 7-8 am.

Baseline blood samples after 30 minutes supine rest were drawn in the manner described above. Subjects were then administered 1 microgram of synthetic ACTH (synacthen®, Alliance Pharmaceutical Corp USA) intravenously. After a further 30 minutes of supine rest, blood was drawn, spun and stored in the manner described above.

2.3 Analysis of genotype and intermediate corticosteroid phenotype in large study populations

2.3.1 The BRIGHT Study

The British Genetics of Hypertension (BRIGHT) Study is a large multicentre investigation into the genetic basis of hypertension. Hypertensive subjects were recruited from primary care with entry to the study being dependent on a pre-treatment blood pressure of >145/95mmHg (mean of 3 readings), which lies within the top 5% of the population blood pressure distribution in the UK. Subjects with a BMI> 30 were excluded from the study. Full details of the recruitment strategy have been published elsewhere(359). However, the primary aim of the study was to perform genetic linkage analysis, and the total study population comprised > 2000 affected sibling pairs and trios. 512 unrelated subjects were selected entirely randomly from the entire BRIGHT cohort for further analysis as detailed below. Full demographic information on the subjects included is outlined in chapter 4. Ethical approval for the study was granted by the local ethics committees of the partner institutes and fully informed written consent from all of the participants was obtained.

2.3.2 MONICA IV Cohort

The Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) project was a World Health Organisation (WHO) coordinated, observational, long-term study(360). Its main objective was to measure trends in cardiovascular mortality and morbidity and to assess the extent to which these trends were related to changes in risk factor levels and medical care, measured at the same time in different communities in different countries.

In the fourth MONICA survey, a random sample of the north Glasgow patient lists of 30 general practitioners was selected for further analysis(361). Study subjects were invited to complete a health questionnaire and attended for a structured medical examination to assess overall cardiovascular risk. Approval was obtained from the appropriate ethics committee. Of the original sample of 597 subjects in whom full urinary corticosteroid data were available, 158 were excluded from the study on the grounds that they were receiving medication likely to alter cardiovascular risk parameters (e.g. cardioactive drugs or hormone-replacement therapy) or adrenal function (e.g. inhaled or topical steroids). There remained 238 male and 201 female subjects; their demographic details are summarised in chapter 4.

2.3.3 Genotyping

DNA was extracted from all participating subjects using standard methods as described in section 2.6 and each subject was genotyped for CYP11B2 –344C/T and IC alleles as detailed in section 2.8.

2.3.4 Urinary corticosteroid excretion

The same subjects also underwent analysis of corticosteroid phenotype by measurement of urinary corticosteroid metabolites by gas chromatography/mass spectroscopy (GCMS; section 2.12). Each individual provided a 24-hour urine collection. Final volume was noted and 50 ml of urine aliquoted for storage at -20°C before further analysis.

2.4 The origin of 18-oxocortisol and 18-hydroxycortisol in man

For this clinical study of the biosynthesis of the so-called 'hybrid' corticosteroids 18-oxocortisol (18-oxoF) and 18-hydroxycortisol (18-OHF), 8 normal, healthy male volunteers were recruited by local advertisement. All were aged <40y, had a BMI<30kg/m^2, had no history of hypertension and were on no corticosteroid medications.

2.4.1 Study Protocol

The study was subdivided into three 24-hour urine collections (excretion of 18-oxoF, 18-OHF, free cortisol and free cortisone) each separated by one month (summarised in chapter 6 table 6.2a). In stage one, 24-hour urine collections were obtained without treatment. In stage two, 24-hour urine collections were obtained after treatment with dexamethasone only (1mg twice daily at 8 am and 8pm for three days). In stage three, 24-hour urine collections were obtained after treatment with dexamethasone (1mg twice daily for three days) and hydrocortisone (20mg twice daily on the 3rd day only). In addition, 24-hour urine collections were obtained from 6 patients with primary adrenocortical insufficiency. Five had autoimmune adrenal failure (Addison's disease) and one had undergone bilateral adrenalectomy for phaeochromocytoma. Five of the subjects were taking hydrocortisone (15–30mg/day) as replacement and one was on dexamethasone (0.75mg/day).

2.4.2 Sample storage and analysis

Again, 50ml aliquots of urine were stored at -20°C for analysis of steroid excretion by GCMS (performed by Dr Loai Shakerdi (362)). Urinary steroid excretion rates were measured by gas chromatography-mass spectrometry

using the methods of Shackleton (363) and Palermo et al(364) with minor modifications. The internal standard was allo-THDOC. A DB5 capillary gas chromatography column (J&W Scientific, Folsom CA), 30m, (0.25mm id, 0.25µm stationary phase) was used. The temperature programme was: 50°C for 3 minutes, 27°C/min up to 220°C, and 1.7°C/min up to 300°C. Separate Sep-pak cartridges were used for extraction of conjugates and for subsequent extraction of steroids released by hydrolysis. The source of ß-glucuronidase was the edible snail (Biosepra, Cergy-Saint-Christophe, France). A Varian Saturn GCMS was used.

2.5 Endogenous corticosteroid synthesis in patients after bilateral adrenalectomy

Ten subjects who had undergone bilateral adrenalectomy and were stable on exogenous corticosteroid replacement regimes were recruited from the endocrine clinics of the Western Infirmary. The principal exclusion criteria are listed in table 2.5a.

2.5.1 Study protocol

Subjects were studied on three occasions under different conditions in random order and at least two weeks apart. The study protocol is summarised in chapter 7 (table 7.2b). For each study period, subjects were asked to discontinue the replacement mineralocorticoid, fludrocortisone three days before the study day. On all three occasions, subjects were asked to carry out a 24-hour collection of urine commencing at 8am on day three and finishing at 8am on the study day (day 4). This was for measurement principally of 18-

Exclusion criteria
▪ Inability to follow study instructions ▪ Subjects within 6 months of bilateral adrenalectomy ▪ Any inter-current illness ▪ Hypertension ▪ Significant renal impairment (creatinine >120 mcg/L)

Table 2.5a. Exclusion criteria applied to studies of corticosteroid production patients after bilateral adrenalectomy

oxoF, 18-OHF, aldosterone and cortisol metabolites. Subjects attended the day ward at 9am on the morning of the fourth day fasted and having taken no corticosteroid replacement since the previous evening. After 30 minutes of supine rest, blood was extracted from an in-dwelling cannula for measurement of plasma steroids (Li-heparin tubes 3x 7ml), plasma electrolytes (Sep pak clot activator tubes-5ml) and renin/aldosterone (K EDTA as described previously). Blood was spun and plasma extracted and stored as detailed in section 2.2.3.

MOLECULAR

2.6 DNA extraction and quantification

Genomic DNA was extracted from peripheral blood samples using a variation of the method of Miller et al (365). Ten millilitres of EDTA-preserved whole blood was placed in a 50 ml Falcon tube (Becton Dickinson) and 40 ml of erythrocyte lysis mix (Appendix) added. Tubes were mixed by inverting five times before centrifuging (International Equipment Company PR700) at 2800 rpm for 10 minutes. The resulting pellet was resuspended in 3 ml of nuclei lysis mix (Appendix), and 200μl 10% SDS (sodium dodecyl sulphate; Sigma) and 100μl proteinase K (10mg/ml; Sigma) were added. Tubes were incubated overnight at 37°C. After incubation, 1ml of 6M (saturated) NaCl was added with vigorous shaking. 5ml of phenol:chloroform:isoamyl alcohol (25:24:1, pH aqueous phase >7.6) was added and the tubes spun (IEC) for 20 minutes at 2800rpm. The upper aqueous phase of the supernatant was then transferred to a 30ml universal container (Sterilin) and two volumes of absolute alcohol added. DNA was then spooled out with a glass rod and resuspended in 400μl

dH_2O in a 1.5ml microtube (Alpha). DNA was allowed to settle at room temperature for 48 hours before storage at 4°C.

The concentration of each DNA sample was determined by measuring the optical density of samples at 260nm. One microlitre of DNA solution was added to 1ml of dH_2O in a quartz cuvette and the OD_{260} measured in a dual beam spectrophotometer with a deuterium lamp. An optical density of 1 corresponds to 50 mg/ml of DNA.

2.7 Polymerase chain reaction of CYP11B2

The CYP11B locus is too long to be amplified in a single or even two PCR reactions. Therefore, along with my colleagues Marianne Barr and Donna Wilkinson, we amplified and sequenced this locus in segments. I concentrated on CYP11B2, subdivided into promoter and exons 1-4, exons 5-9 and 3'UTR.

2.7.1 PCR Protocol

Genomic DNA obtained from a bank of normotensive subjects was used as the reaction template. Reactions were carried out within sterile Eppendorf tubes and kept on ice at all times.

Synthetic oligonucleotides, which were HPLC-purified were designed from the published sequence of CYP11B2 and obtained from a commercial source (MWG-Biotech, Ebersberg, Germany). Primers were generally between 20-24 base pairs and had approximately 50% GC ratios (Table 2.7a). The melting temperature of the primers was around 60°C (3'UTR) or 65°C(exons 5-9).

Both fragments of CYP11B2 were amplified using an Expand High Fidelity PCR system (Roche Diagnostics, Mannheim, Germany). This contained both

Primer	Location (CYP11B2)	Sequence	Melting temperature (°C)
B2 3' UTR (sense)	3' untranslated region	5'-ATA TTG AGG CCT GGC ACG TC-3'	59.4
B2 3'UTR (anti sense)	3' untranslated region	5'-GAT GTG CTG ACT AGG GGA G-3'	60.3
T2138	Exon 5(sense)	5'-ATT TGG GTG TCG GGG CAG TCT-3'	66
T8303	Exon 1(sense)	5'-GAA TTC TGC ATC CTG TGA AAT TAT C-3'	64
M6641	Exon 9 (anti-sense)	5'-CTG TGC ACG TGG GAG AGA AGA-3'	66

Table 2.7a. Oligonucleotide sequences of primers used for PCR of CYP11B2 exons 5-9 and 3' UTR.

Taq DNA polymerase and Tgo polymerase to confer proof-reading abilities and so minimised unwanted PCR-generated mutations. This system is optimised to efficiently amplify DNA fragments up to 5kb.

A 1μl (approximately 50mng/μl) aliquot of DNA to be amplified was placed in a sterile Eppendorf tube. Two separate reaction mixes were prepared as follows:

Reaction Mix 1:		Final Concentration
dATP (10mM)	0.5μl	200μM
dGTP (10mM)	0.5μl	200μM
dTTP (10mM)	0.5μl	200μM
dCTP (10mM)	0.5μl	200μM
Sense primer (300nM)	0.75μl	300nM
Anti sense primer (300nM)	0.75μl	300nM
Distilled water	8μl	-------
Total	**11.5μl**	

Reaction Mix 2:		Final Concentration
Expand high fidelity buffer (15mM MgCl)	2.5μl	1x (1.5mM MgCl)
Expand high fidelity enzyme mix	0.75μl	2.6 U/reaction
Distilled water	9.25μl	-------
Total	**12.5μl**	

A 'blank' reagent control was prepared which contained all the necessary components for PCR but replaced template DNA with dH_20. The total reaction volume was 25 microlitres. The reaction mixes were subsequently combined on ice in single 0.2 ml thin-walled amplification tubes (ABgene, Surrey, UK) before being placed in a 96-well Alpha™ sample block powered by the PTC-225 DNA Engine Tetrad® Cycler with heated lid (MJ Research, Waltham, MA, USA). Amplification cycles and reaction conditions were as follows:

Thermal cycling protocol

1. 94°C	2 minutes	Denaturation
2. 94°C	15 seconds	
3. 60°C	30 seconds	repeated for 10 cycles
4. 72°C	1 minute	
5. 94°C	15 seconds	
6. 60°C	30 seconds	repeated for 15 cycles
6. 72°C	1 minute + 5second cycle elongation for each successive cycle	
7. 72°C	7 minutes	
8. 4°C	Indefinitely	

2.7.2 Determination of PCR products

Agarose (1g) (Invitrogen, Paisley, UK) was added to 1 x TAE buffer (100ml), mixed and heated to boiling point in a 650W microwave oven for 60 seconds. After 3 minutes of cooling, 1 microlitre of ethidium bromide (10mg/ml) was added and mixed in. The agarose was then poured into gel moulds with Teflon combs and allowed to set for 20 minutes before combs were removed. Gels were submerged in 1 x TAE buffer in standard electrophoresis tanks (Wide Minis Sub Cell Agarose gel apparatus) (Bio-Rad Laboratories, Hercules, CA, USA). 10 microlitres of PCR product was loaded with 5microlitres of loading dye onto the agarose gel. The DNA molecular weight marker Hyperladder I (Bioline, London, UK) was also loaded in a separate

well. The gel was then resolved at 95volts for 40 minutes. DNA bands were visualized under a UV light.

2.7.3 PCR clean-up

Prior to sequencing, PCR products were cleaned to remove excess dNTPs and primers. This was done using the enzymes Shrimp Alkaline Phosphatase (SAP; Amersham Biosciences, UK) and Exonuclease I (ExoI; Amersham Biosciences, UK). SAP removes the phosphate groups from the excess dNTPs left over from the PCR reaction and ExoI digests the single stranded PCR primers into dNTPs, the phosphate groups are then removed by the SAP. This was performed on ice using the following protocol:

1. 5 μl of PCR product transferred to sterile, thin walled 0.2 ml PCR tubes.
2. 1 μl SAP (1U/μl) and 0.5 μl ExoI (20U/μl) then added
3. Run on Tetrad® thermal cycler using following program:

Step 1 - 37°C for 30 minutes
Step 2 - 80°C for 10 minutes

2.7.4 Automated Cycle Sequencing

Automated sequencing was performed using the ABI Big Dye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA, USA). The technique is an adaption of the dideoxy termination method of sequencing. Each sequencing reaction could only sequence approximately 500 base pairs (bp) and so each exon and 500 bp segment of the 3'UTR was sequenced

separately using separate oligonucleotide primers (MWG-Biotech) listed in table 2.7b.

Sequencing Reaction set-up

Sequencing reactions were set up in 96-well plates containing the following:

Template (25-200ng)	1-3 µl
Sequencing Primer (3.2pmol/µl)	1 µl
Ready Reaction mix	0.5 µl
Sequencing Buffer	3.8 µl
Distilled Water (up to final vol 20µl)	11-13.7 µl

Reaction Conditions

1. 96°C	45 seconds	
2. 50°C	25 seconds	Repeat x 25
3. 60°C	4 minutes	

Sequencing reaction clean-up

Keeping the sequencing reactions in the 96-well PCR plates, Sodium acetate/Ethanol reaction purification was performed.

A solution of sodium acetate (NaOAc) in 95% ethanol (EtOH) in the ratio 2 µl 3M NaOAc: 50 µl 95% EtOH was prepared and 50 µl of this solution added to each sequencing reaction. The plate was then sealed with an adhesive paper lid and inverted a few times to mix. The samples were then incubated at room temperature for 15 minutes to precipitate the extension products. The plate was spun for 30 minutes at 6000G and the supernatant immediately discarded and the plate spun whilst inverted on a paper towel for 30 seconds

Primer	Location (CYP11B2)	Sequence
B2 3'UTR+500	3' UTR	5'-AAT ACG ATC CTG CAG GGT AG-3'
B2 3'UTR+1000	3' UTR	5'-TAG CTG ATG AGG ATC AGA GCG-3'
T2138	Exon 5(sense)	5'-ATT TGG GTG TCG GGG CAG TCT-3'
T2142	Exon 6(sense)	5'-GAC CCT GCA GAC ATG GCT TC-3'
Y6259	Exon 7(sense)	5'-TGG ATG CCC CCA CCT CCA GG-3'
F31465	Exon 8(sense)	5'-AGT GTG CAG GCT CCG CCT CT-3'
S6719	Exon 9(sense)	5'-TAC TGA CCA GCG CTG ATG GAA AC-3'
T2140	Exon 6(sense)	5'-AGG GCC ACA GGG AGG CCT CA-3'

Table 2.7b Oligonucleotide primers used in sequencing of CYP11B2

at 500G to remove all residual traces. 150µl of 70% ethanol was added to each well and the plated sealed and inverted again before being spun at 6000G for 10 minutes. Again the supernatant was removed by spinning the inverted plate for 30 seconds.

Formamide purification step

15 µl of formamide was added to each well of 96 well PCR plate. This was then denatured at 95°C for 2 minutes and placed on ice for a further 1 minute. 15µl of each sample was transferred to the corresponding well of the 96 well bar-coded sequencing plate, covered and spun at 500G for 10 seconds. Plates were then stored at -20°C until sequenced.

2.8 Determination of CYP11B2 –344 C/T genotype

This was performed in two stages. Initially, a PCR was performed using primers detailed in table 2.8 (Oswel DNA service, Southampton) and a Taq polymerase system (Promega). Subsequently, the amplified region of 228 base pairs was digested using Hae III mix. The 228 bp amplicon contains 2 HaeIII restriction enzyme sites (GG CC). The presence of a C to T transition at position –344 (GG CT) removes one of these sites. After digestion, individuals homozygous for the transition (TT) produce two bands of 175 and 53 bp, individuals homozygous for the wild-type (CC) produce bands of 104, 71 and 53 bp, and heterozygous individuals produce 4 bands.

Formal protocols for the above procedures are outlined below.

2.8.1 PCR Protocol

The reaction mix was prepared in sterile eppendorf tubes on ice.

dNTP mix (10mM)	0.5μl
Sense primer (10mM)	1.0μl
Anti-sense primer (10mM)	1.0μl
MgCl	1.0μl
Buffer	2.5μl
Taq polymerase	0.2μl
nf H_2O	17.8μl

1μl of each DNA sample was added to 24μl of reaction mix. Each sample was then placed in the Tetrad® thermal cycler.

Thermal Cycling Protocol

1. 94°C	3 minutes	
2. 94°C	1 minute	
3. 65°C	1 minute	→ Repeat 30 times
4. 72°C	90 seconds	
5. 72°C	7 minutes	
6. 4°C	indefinitely	

Determination of PCR product

10μl of PCR product were run on a 1% agarose gel as described previously.

2.8.2 HaeIII Digest reaction

Hae III	1.0μl
Buffer C (containing MgCl)	1.5μl
Distilled H20	2.5μl

This 5μl reaction mix was added to 15μl of PCR product. Samples were then incubated at 37°C for 90 minutes.

Primer	Oligonucleotide sequence
Sense	5' GTG TCA GGG CAG GGG GTA 3'
Anti-sense	5' AGG CGT GGG GTC TGG ACT 3'

Table 2.8a. Oligonucleotide primers used for –344C/T PCR

Primer	Oligonucleotide sequence
Sense	5'AGG AAC CTC TGC ACG GCC TT3'
Anti-sense	5'CAG AAA ATC CCT CCC CCC TA3'

Table 2.9a. Oligonucleotide primers used for IC PCR

Determination of genotype

After digestion reaction, the resulting DNA fragments were resolved in a 5% agarose gel (5g agarose in 100 mls of TAE buffer) as described previously.

2.9 Determination of Intron Conversion genotype

This was determined by 2 separate PCRs. Reaction 1 identified the normal gene (termed 'wild type;' WT) and reaction 2 determined the gene containing intron 2 conversion allele (termed 'intron conversion;' IC).

The PCR protocol for both reactions differed only in terms of the oligonucleotide primers used (Oswel Research Products UK; table 2.9) and is outlined below:

Template DNA (100ng)	1μl
nf H_2O	15.3μl
10X buffer	2.5μl
dATP(0.2mM)	1μl
dGTP(0.2mM)	1μl
dCTP(0.2mM)	1μl
dTTP(0.2mM)	1μl
Reaction 1 or 2 sense primer (1:10; 20pmol)	1μl
Reaction 1 or 2 anti-sense primer (1:10; 20pmol)	1μl
Taq polymerase	0.2μl

Each sample was prepared in sterile 0.5ml eppendorf tubes on ice. Each sample was then transferred to the Tetrad® thermal cycler.

Thermal Cycling Protocol (both reactions)

1. 94°C	2 minutes	
2. 94°C	1 minutes	
3. 66°C	30 seconds	X 30
4. 72°C	30 seconds	
5. 72°C	7 minutes	
6. 4°C	Indefinitely	

10 µl of PCR product were then resolved on 1% agarose gel as described previously and visualised under UV light. Detection of a 418 bp product with reaction 1 identified the WT allele and in reaction 2 identified the IC allele.

2.10 Genotyping for CYP11B1 5'UTR Polymorphisms

As discussed in chapter 3, my colleague, Marianne Barr, identified 2 novel polymorphisms within the promoter region of CYP11B1 during sequencing of this region within a normotensive cohort. These polymorphisms were subsequently identified within both hypertensive subjects and normotensive controls undergoing investigations to examine the effect of variation within CYP11B on pituitary-adrenal function (details in section 2.1).

2.10.1 Nested PCR protocol

In order to increase specificity of this technique, a nested PCR was performed. The initial reaction amplified a 2kb region specific to CYP11B1 and the second reaction used this product as a template to specifically amplify the region of interest. In both reactions, a heat-activated thermostart Taq

polymerase (Abgene, UK) was used. The sense and anti-sense primers used in both reactions are outlined in table 2.10a.

The detailed protocol is outlined below:

PCR Amplification 1

Buffer (10X)	2.5µl
MgCl	1.5µl
dNTPs	1µl x 4(200mM each)
B1 5'UTR (sense)	1µl (400mM)
B1 prom-260 (anti-sense)	1µl(400mM)
Taq polymerase	0.25µl (1.25U)
dH_2O	8.75µl

1µl (25-50 ng/µl) of each DNA sample was added to 20µl of reaction mix. Each sample was then placed in the Tetrad® thermal cycler.

Thermal Cycling Protocol

1. 95°C	15 minutes	
2. 95°C	30 seconds	x 35
3. 60°C	30 seconds	
4. 72°C	3 minutes	
5. 72°C	7 minutes	
6. 4°C	Indefinitely	

The PCR products were then diluted 1:10 in nuclease free H_20 and used as a template for a second PCR reaction as detailed below.

PCR Amplification 2

Buffer (10X)	2.5µl
MgCl	1.5µl

Reaction	Primer Sequence	
PCR 1	Sense (B15'UTR)	5'-TCC TTC GCA TCC CTT GTA AGT T-3'
	Antisense (B1prom-260)	5'-CTT GGA TTA TTC ATC TCC TTG CAA GG-3'
PCR 2	Sense (B15'7-32)	5'-GCA TCC CTT GTA AGT TGG ATT CCT AA-3'
	Antisense (B15'393-369)	5'-AAG CAT TCC CTT TGA AAA CTG GTA C-3'

Table 2.10a. Oligonucleotide sequences of primers used for nested PCR of CYP11B1 promoter SNPs.

Primer	Sequence
B1Prom-1750(as) 5'	AAG TCA AAT TGT CTC TGT TTG

Table 2.10b. Oligonucleotide primer used in sequencing of CYP11B1 promoter SNPs.

dNTPs	1μl x 4(200mM each)
B1 5' 7-32 (sense)	1μl (400mM)
B1 393-369(anti-sense)	1μl(400mM)
Taq polymerase	0.125μl (0.625U)
dH_2O	11.75μl

24 μl of reaction mix was added to the template comprising 1μl of 1:10 dilution of PCR product from reaction 1. As before, samples were transferred to the Tetrad® thermal cycler and the protocol is detailed below.

Thermal Cycling Protocol

1. 95°C	15 minutes	
2. 95°C	30 seconds	x 35
3. 60°C	30 seconds	
4. 72°C	3 minutes	
5. 72°C	7 minutes	
6. 4°C	Indefinitely	

PCR products were then cleaned using the SAP/Exo1 system outlined in section 2.7.3 prior to the determination of genotypes by sequencing (section 2.7.4. The sequencing primer used is illustrated in table 2.10b.

BIOCHEMICAL

2.11 Measurement of excretion rates of urinary steroids

24h collections of urine were performed to measure the excretion rates of a number of corticosteroid metabolites listed in Table 2.12a. The method of Shackleton (Shackleton CHL Profiling steroid hormones and urinary steroids.

	Corticosteroid	Urinary metabolite	Abbreviation
Glucocorticoids	Cortisol	Tetrahydrocortisol allotetrahydrocortisol	THF aTHF
	Cortisone	Tetrahydrocortisone	THE
	Deoxycortisol	Tetrahydrodeoxycortisol	THS
Mineralocorticoids	Corticosterone	Tetrahydrocorticosterone allotetrahydrocorticosterone	THB aTHB
	Deoxycorticosterone	Tetrahydrodeoxycorticosterone	THDOC
	Aldosterone	Tetrahydroaldosterone	THAldo
Androgens	Androsterone		
	Aetiocholanolone		
	Dehydroepiandrosterone		

Table 2.12a. Major corticosteroid hormones and their urinary metabolites

Journal of Chromatography 1986; 379: 91-156) was used with minor modifications. Briefly, steroid conjugates were extracted (Sep-Pak C-18 cartridges. Waters Chromatography Division, Millipore Corporation) and hydrolyzed with Helix pomatia juice (IBF Biotechnics). The released steroid metabolites were then extracted, also on C-18 cartridges, and their methyloxime trimethylsilyl ether derivatives were then synthesized. Two gas chromatograph – mass spectrometers (GCMS) were used. A Varian 3400 GC coupled to a Finnegan MAT ITS40 ion trap MS was used for the metabolites of cortisol, cortisone, DOC and 11-deoxycortisol. A Varian CP3000 GC coupled to a Saturn 2000 ion trap MS/MS was used for metabolites of corticosterone, aldosterone and some of the measurements of 18-oxocortisol and 18-hydroxycortisol. The internal standard was allo-tetrahydroDOC. Both GCMS machines were fitted with a fused silica capillary column (30m x 0.25 mm id, J & W Scientific) coated with a non-polar (0.25 um DB5/MS) stationary phase.

The conditions for the GCMS analysis were the same for both instruments. Mobile phase gas (helium) flow was 1ml/min. The temperature programme was as follows: 50° for 3 min, 27°/min to 220°, 1.7°/min to 300°, 3.5 min at 300°. The transfer line and ion trap temperatures were 300° and 220° respectively.

The remainder of the 18-oxo- and 18-hydoxycortisol measurements and all measurements of 18-hydroxy-tetrahydro-11-dehydrocorticosterone (18OH-THA) were made using a Thermoquest GCQ plus ion trap GCMS. Here the temperature programme was as follows: 100° for 3 min, 20°/min to 190°, 2°/min to 285°, 285° for 10min. Details of the 18OH –THA assay are given by

Shakerdi et al (J Chromatography B 2002; 784: 367-373). Importantly, a non-homologous standard, β-cortolone, was used because 18-OH-THA is not commercially available. Identification and quantitation were done using specific ion monitoring (SIM). The ions used are shown in table 2.12b.

2.12 Measurement of urinary electrolytes

Measurement of urinary sodium and potassium was performed on 24-hour urine collections by the department of biochemistry using an ion-selective electrode method.

2.13 Measurement of plasma steroid metabolites

Plasma aldosterone and cortisol were measured by radioimmunoassay utilising the 'Coat-A-Count' system (Euro/DPC Ltd, Caernarfon, Wales). The radioisotopes used were ^{125}I cortisol and ^{125}I aldosterone respectively.

Plasma ACTH measurements were performed by Professor Anne White, University of Manchester by radioimmunometric assay (IRMA). This technique uses iodinated radiolabelled monoclonal antibodies for ACTH 1-17, with another solid phase monoclonal specific for ACTH 34-39(366).

Plasma renin concentration (PRC) was measured by Dr Ian Morton with a technique based on radioimmunoassay of angiotensin I generated during incubation of plasma and excess sheep or ox renin substrate(367).

COMPOUND	ION (MASS)
aTHDOC	476
THE	579
THF	562
aTHF	562
THS	564
THDOC	476
THB	564
aTHB	564
THALDO	606
18OHF	385
18OXOF	619

Table 2.12b Ions used for selected ion monitoring (SIM) scans
18-OHF: 18-hydroxycortisol; 18-oxoF: 18-oxocortisol

Chapter 3: Genetic Variation and Linkage Disequlilbrium across the CYP11B Locus in a Normotensive Cohort

3.1 Introduction:

It has become clear that the most consistent intermediate corticosteroid phenotype seen in association with the –344 T and intron conversion (IC) alleles of CYP11B2 is increased plasma deoxysteroids (DOC and 11-deoxycortisol) basally and in response to ACTH stimulation (350). This suggests altered activity of the enzyme 11ß-hydroxylase (encoded by the adjacent and highly homologous gene, CYP11B1) in such individuals. We have therefore speculated that the –344 T ± IC alleles of CYP11B2 are in linkage with key variant(s) in CYP11B1 causing a reduction in enzyme efficiency and the phenotype of increased deoxysteroid levels.

In addition, the –344 T allele of CYP11B2 has been shown to be associated with increased plasma aldosterone(342), increased urinary excretion of the aldosterone metabolite (tetrahydroaldosterone, THAldo)(340) as well as hypertension with a raised ARR(343). However, these associations are not invariable(345;346) and in-vitro studies have shown no discernible effect of this allele on gene transcription(207). Therefore, it remains possible that this polymorphism is in linkage with others elsewhere within CYP11B2 directly resulting in changes in aldosterone production.

Thus, the aim of this study is to explore the pattern of variation across the entire CYP11B locus within a normotensive population known to be homozygous for the CYP11B2 –344C/T and IC polymorphisms. In turn, this

should allow identification of variants within CYP11B1 and CYP11B2 that are in linkage with these known polymorphisms as well as construction of common haplotypes across this locus.

3.2 Methods:

3.2.1 Samples

Genomic DNA was obtained from a bank of normotensive subjects recruited for the Scottish MONICA (Monitoring trends and determinants in cardiovascular disease) study (total of 469 subjects). These samples had already been genotyped for the -344 and IC polymorphisms. Subjects were subdivided according to haplotype, selecting those homozygous for each mutation such that 3 subgroups were identified (Table 3.2a). 10 samples from the common haplotype groups (T/conv, C/WT) and all from the less common haplotype (T/WT; n=6) underwent further sequencing analysis.

3.2.2 Sequencing

The CYP11B2 locus was amplified in segments by PCR (detailed method outlined in section 2.7.1). The presence of any genetic variants was determined by direct sequencing of the PCR products as described in section 2.7.4. This was done in collaboration with other members of the group who sequenced the CYP11B1 gene of these subjects to determine the pattern of linkage across the entire CYP11B locus.

Sequence data from PCR products was compared to the consensus sequence published by the NCBI database (D13752).

Genotype	Number of subjects
TT/ convconv	10 (from a possible 78)
CC/WTWT	10 (from a possible 88)
TT/WTWT	6

Table 3.2a Genotype subgroups from MONICA population

convconv-homozygous for intron 2 conversion allele of CYP11B2
WTWT-homozygous for wild-type allele of intron 2 in CYP11B2
Only 6 TT/WTWT samples available and no CC/convconv; this reflects population frequency of these genotypes.

3.2.3 Linkage analysis

Pairwise linkage disequilibrium (LD) between all possible pairs of loci was calculated using EMLD (expectation maximization linkage disequilibrium)(368). (http://request.mdacc.tmc.edu/~qhuang/Software/pub.htm)

This programme is specifically designed to estimate LD by first estimating pair wise haplotype frequencies and is applicable to samples from unrelated individuals. Both D' and r^2 values were measured

A Bayesian statistical method implemented in PHASE version2.1 was used to construct phased haplotypes from phase-unknown genotype data.(369)

3.3 Results:

3.3.1 Sequencing

Figures 3.3a and 3.3b show the results of a typical PCR amplification of segments of CYP11B2 on a few MONICA subjects. Sequencing reactions were subsequently carried out on PCR-amplified DNA from each of the 26 subjects studied (Figure 3.3c).

In total, 83 SNPs were identified across the CYP11B locus in linkage with –344 C/T and IC polymorphisms (Figure 3.3d &e); 64 were in CYP11B2 and the majority are previously unreported.

CYP11B2

SNPs identified in CYP11B2 are shown in Figure 3.3e. Previously identified –344C/T and IC polymorphisms are highlighted as are the three SNPs found to occur in coding regions. Of these, two (codon 169-C/T, codon 374 –A/C) do

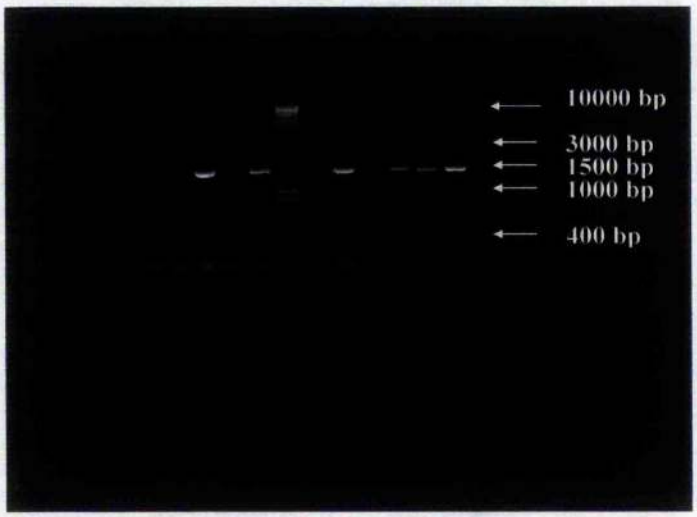


Figure 3.3a. 1500bp PCR fragment from 3' UTR of several different MONICA samples, resolved on a 1% agarose gel

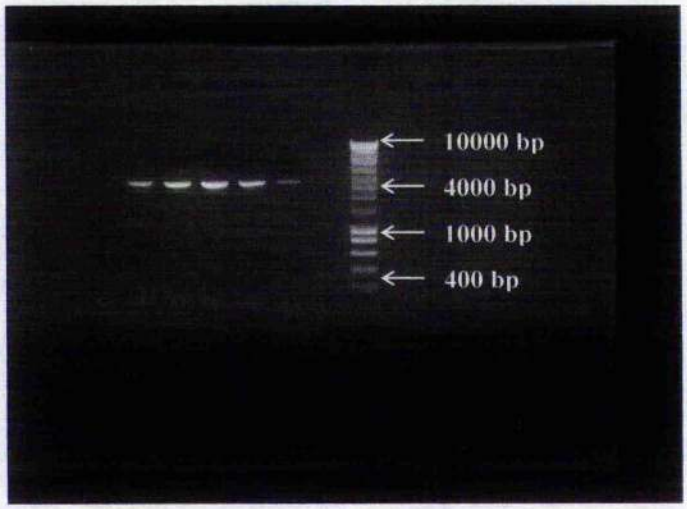


Figure 3.3b 4kb PCR fragment of exons 5-9 of MONICA samples on 1% agarose gel

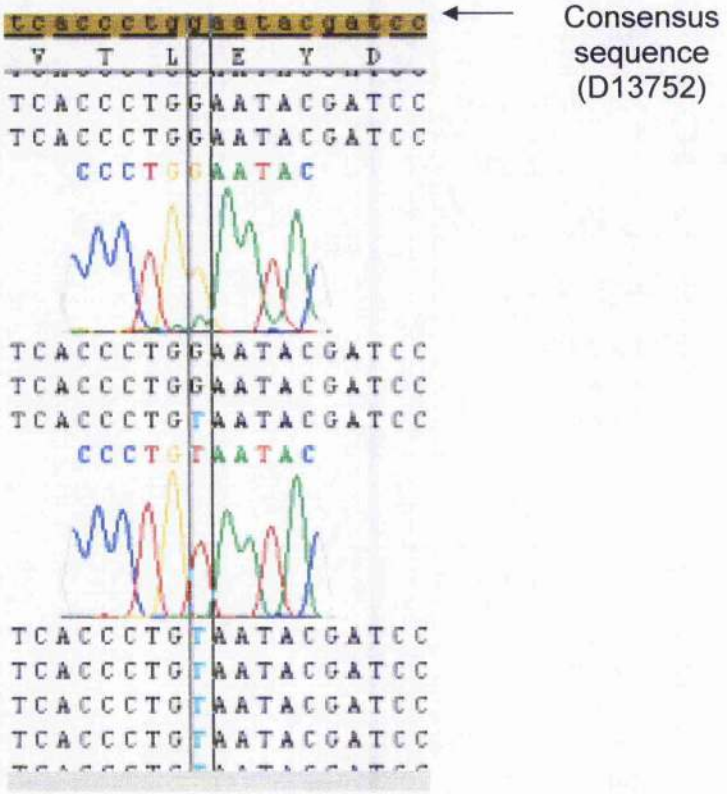


Figure 3.3c. Electropherogram of sequencing of CYP11B2 3' UTR and illustrating a base change (G/T) at position 891.

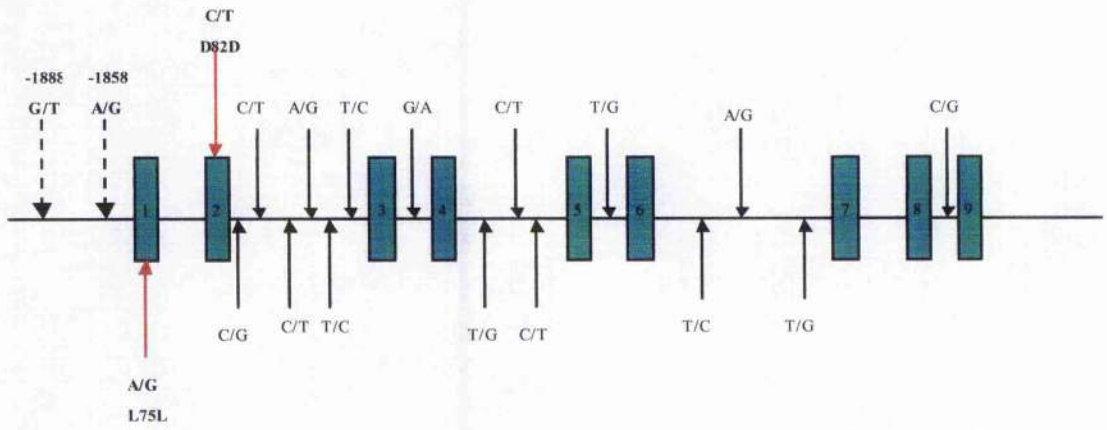


Figure 3.3d. Polymorphisms identified after sequencing of CYP11B1

– – – 5' UTR SNPs in linkage with –344 and IC of CYP11B2

——— Synonymous SNPs previously identified

——— SNPs identified within non-coding regions

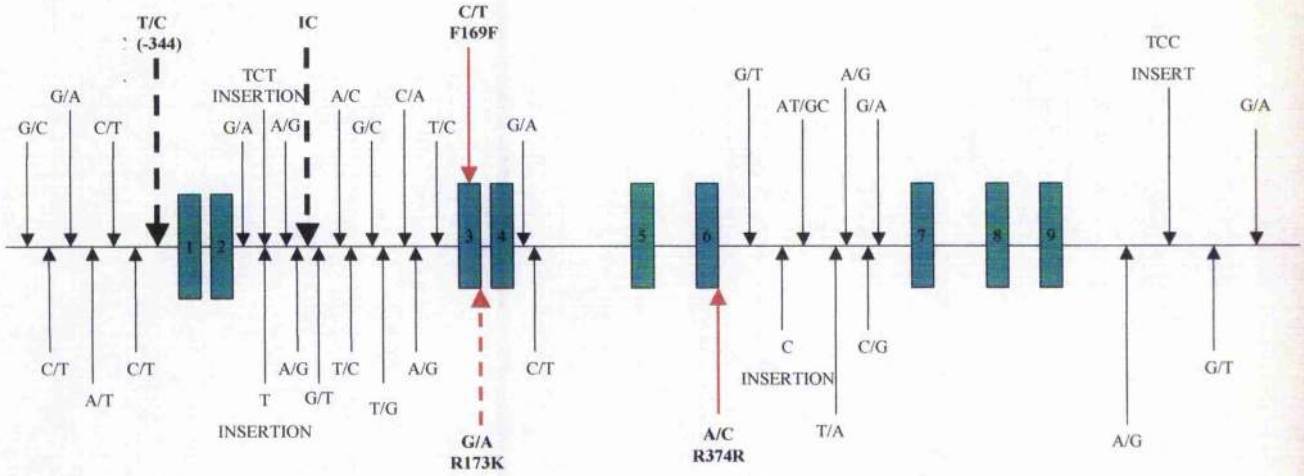


Figure 3.3e. Polymorphisms identified after sequencing of CYP11B2

– – – – Previously identified –344 and IC polymorphisms

——— Synonymous exonic polymorphisms

– – – – Non-synonymous exonic polymorphism

——— SNPs within non-coding regions

not result in an amino acid change. The A/G transition at codon 173 is a well-described variant causing a lysine-arginine transition(370).

Figure 3.3e illustrates that the remainder of identified SNPs within CYP11B2 were in untranslated regions. Whilst SNPs within the 5' untranslated regions (5' UTR) can be assessed using reporter gene constructs, SNPs within introns, splice site consensus sequences or 3' UTR are much more difficult to assess in vitro.

CYP11B1

Nineteen SNPs were identified across CYP11B1 and are illustrated in figure 3.3d. With the exception of 2 synonymous exonic SNPs (A/G-codon 75, C/T-codon 82) the remainder of these were in untranslated regions. In particular, 2 SNPs in the 5' UTR (-1888 G/T, -1858 A/G) were found to be in tight LD with the T/Conv haplotype.

3.3.2 Linkage

In general, linkage was high across the entire region although, as expected, decreased with distance. Loci within CYP11B1 were generally in greater LD with each other than with loci in CYP11B2 (figure 3.3f).

LD described by the D' metric was almost uniformly high across the entire region (Figures 3.3f1 & 3.3g1) approaching the maximum of 1 across much of the region and decreasing to approximately 0.75 between pairs of loci spanning the CYP11B1 and CYP11B2 genes.

LD described by the r^2 metric was less uniform as might be expected given that r^2 is more stringent and dependent upon identical allele frequencies. However, allele frequencies varied across the locus. There were a few pairs

of loci spanning the genes which were in strong LD. In general, r^2 ranged from approximately 0.5 to approximately 1.0 (Figures 3.3f2 & 3.3g2).

3.3.3 Haplotype analysis

The theoretical number of haplotypes across this region assuming free recombination was calculated to be 9.671×10^{24} (83 biallelic loci across 44,631 basepairs).

However, PHASE constructed 136 haplotypes most of which were low frequency (Table 3.3a). Four haplotypes had frequencies of >5% and accounted for 68% of chromosomes in the data set (Figure 3.3h). Nine haplotypes had frequencies of >1% and described 75% of chromosomes in the data set (Table 3.3a).

3.4 Discussion:

The primary purpose of this study was to identify candidate SNPs/variants in the CYP11B locus in linkage with established variants within CYP11B2 (-344 T/C and IC) accounting for the phenotype of hypertension with an elevated ARR.

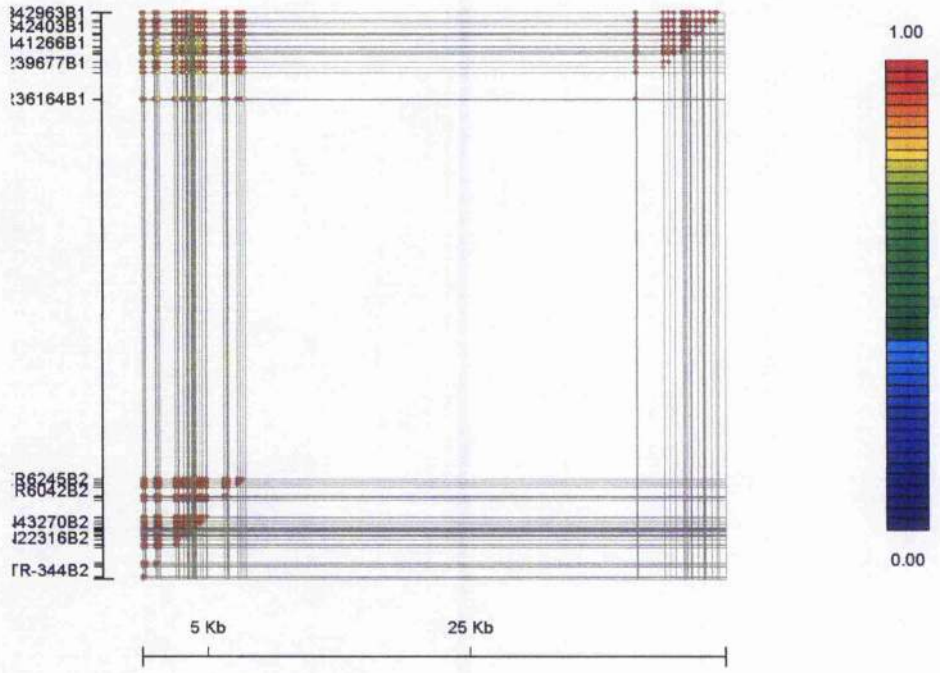
Within CYP11B2 only 3 SNPs were identified within exons. Of these, 2 were synonymous whilst the other (A/G at codon 173) resulted in the substitution of lysine (K) for arginine (R). The wild-type allele (A) is associated with the T allele of -344. This K173R polymorphism has already been described and the R variant found to be more common in hypertensive subjects with suppressed renin(371). However, it has no effect on enzymatic activity in vitro(371). More recent studies assessing levels of mRNA transcripts in adrenal tissue suggest

ndex	Haplotype	E(freq)	> 5%	>1%
1	111212121221211111211122221211112212211211221211122122211211111122211111122221122121	0.202	X	X
5	111212121221211111211122221211112212211211221211122122211211111122211111122222122121	0.014		X
9	111212121221211111211122221211112212211211221211122122211211121122211111122221122121	0.069	X	X
29	111211121221211111211122221211112212211211221211122122211211111122211111122221122121	0.112	X	X
87	212211122122211111122211112122221124122112222121112221111211222221121121221112211212	0.015		X
89	212211122122211111122211112122221121122112222121112221111211222221121122221112211212	0.011		X
132	222121212112122221222111112122221121122122112122211211222122222211211111122211211212	0.012		X
133	222121212112122221222111112122221121122122112122211211222122222211212111122211211212	0.022		X
136	222121212112122221222111112122221121122122112122211211222122222211122222211112211212	0.297	X	X

Table 3.3a 9 common haplotypes constructed after sequencing of 26 normotensive subjects from MONICA survey (136 possible haplotypes identified altogether)

83 SNPs were identified and are coded 1 or 2 in alphabetical order. In the case of insertions, no insertion is 1 insertion is 2. In the case of deletions, no deletion is 1 deletion is 2. Of the 136 possible haplotypes identified 4 had frequencies of >5% and a further 5 had frequencies of >1%

1



2

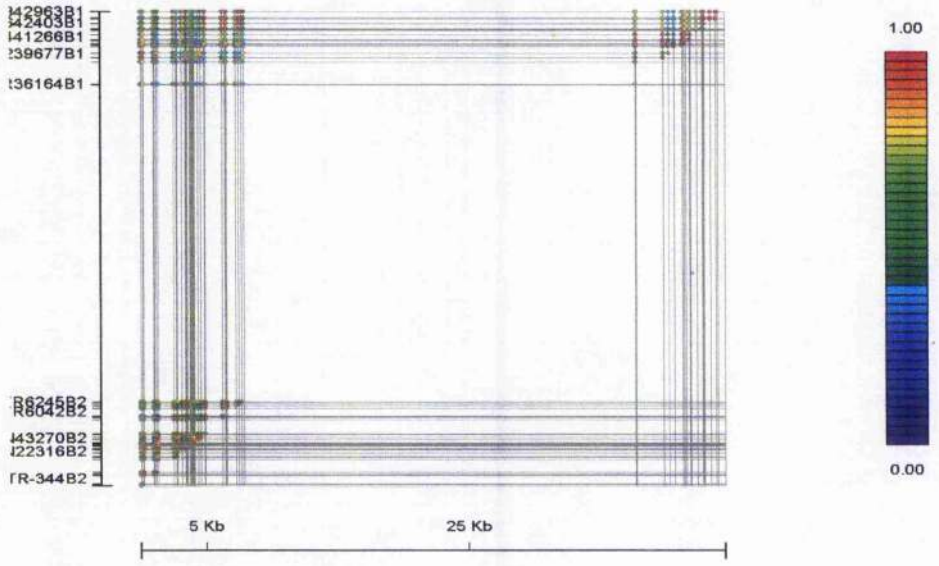
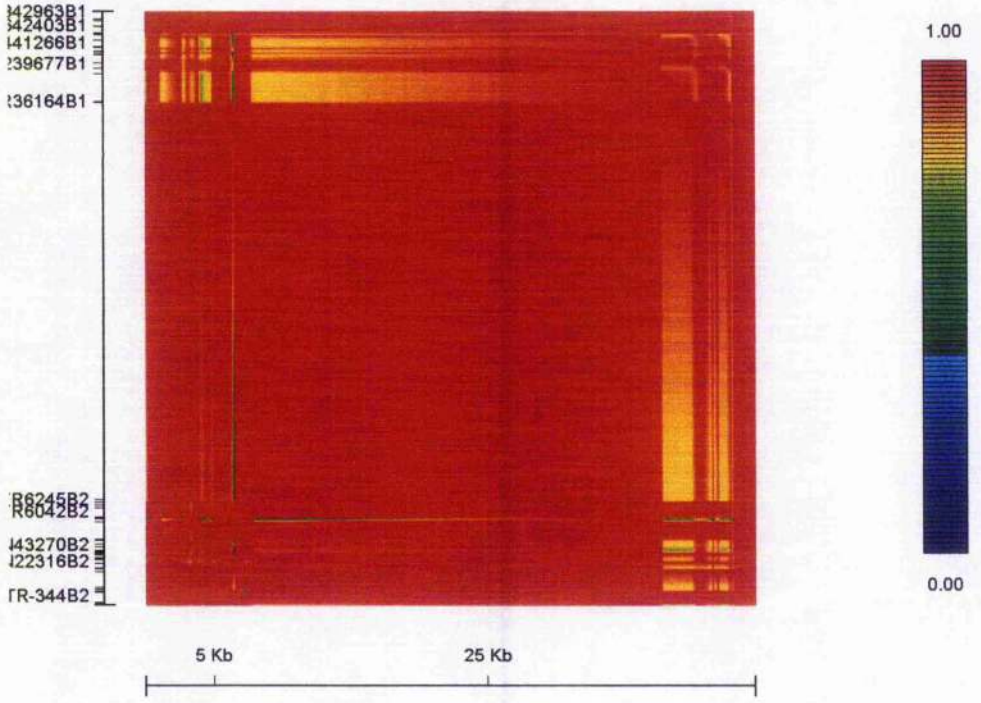


Figure 3.3f: Raw data plots showing pairwise linkage disequilibrium.
1. Plot of D'. 2. Plot of r^2.

1



2

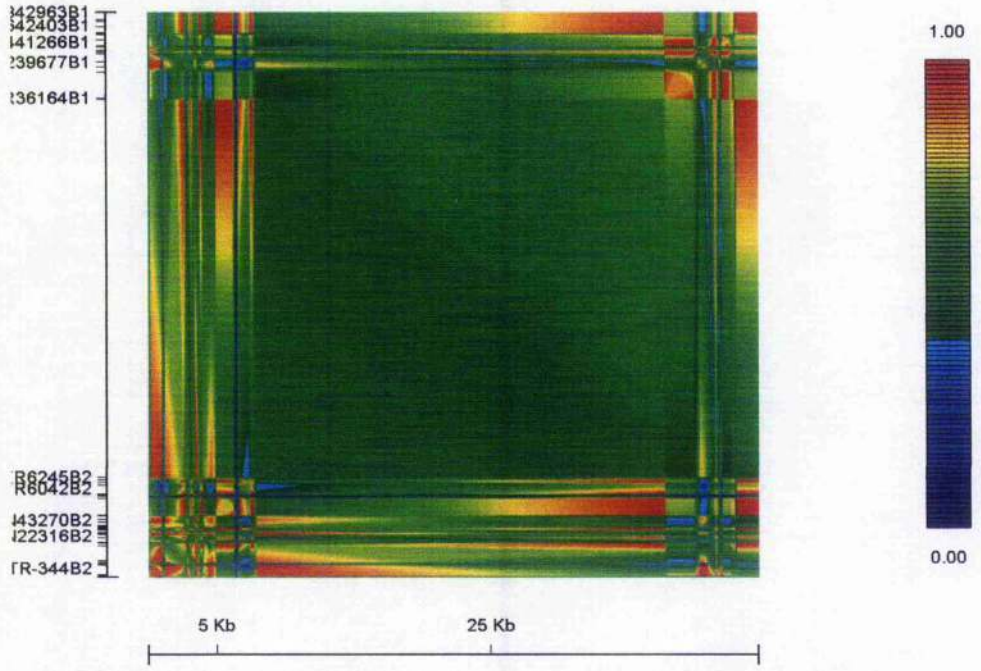


Figure 3.3g: Data plots extrapolating pairwise linkage disequilibrium across the region.
I. Plot of D'. II. Plot of r^2.

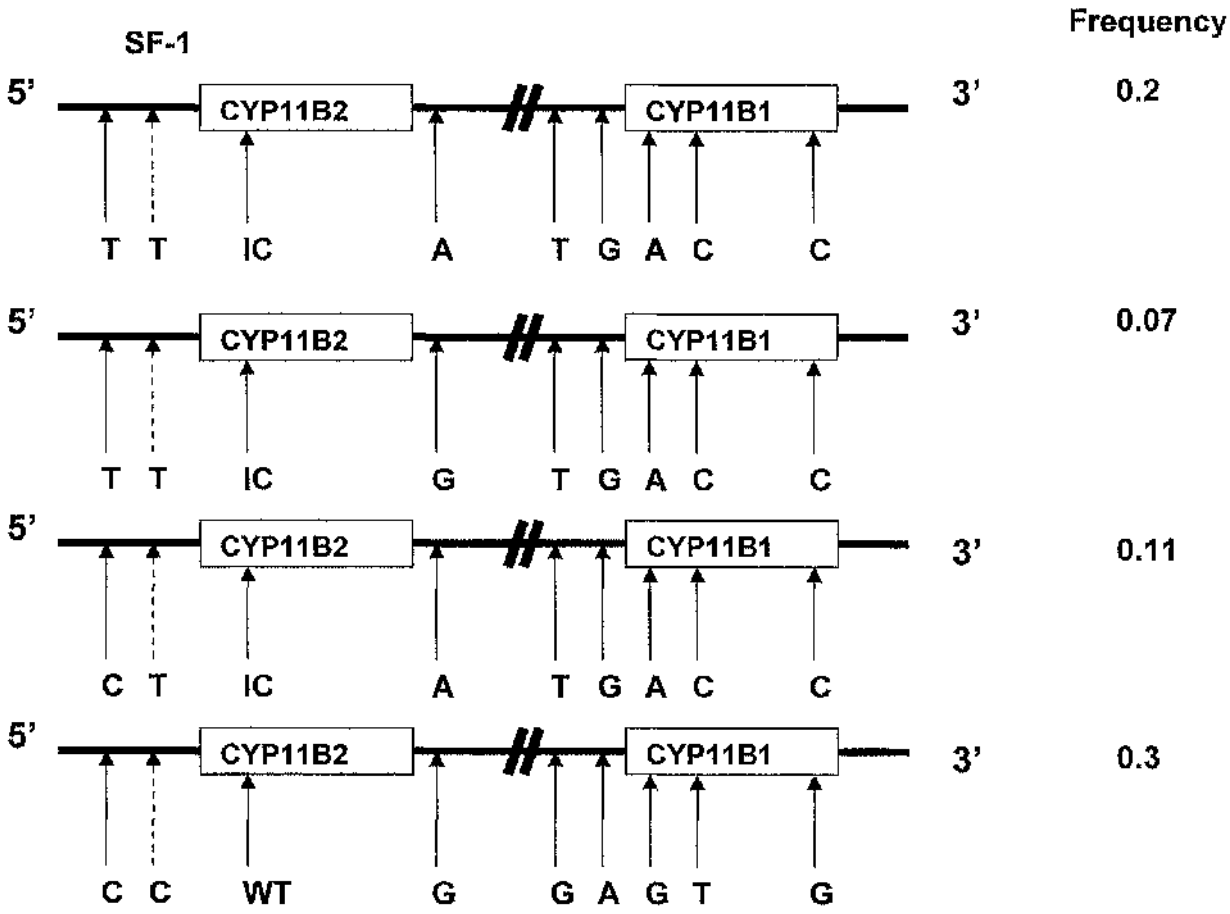


Figure 3.3h. Four commonest haplotypes across CYP11B locus and their respective frequencies.

that the –344T and K173 haplotype is associated with higher gene expression than the opposite –344C and R173 haplotype. Thus whilst this work supports previous studies linking the -344T allele with increased aldosterone production it still does not provide a causative variant within CYP11B2.

A number of SNPs were identified in linkage with the –344C/T and/or IC variants within untranslated regions of CYP11B2 (fig 3.3e). In particular, G/A (linked to –344 T allele) at the beginning of intron 2 and G/A (linked to –344 C allele) at the beginning of intron 3 are close to known splice sites and may well interfere with post transcriptional processing. Similarly, the TCC insertion site found within 3'UTR in association with IC wild type allele may also have post- transcriptional effects. In particular, the relatively long length of 3'-UTRs in human genes suggests that the human genome has evolved to make increased use of post-transcriptional control mechanisms to regulate gene transcription. In different mRNAs, 3'-UTRs have been reported to be involved in the regulation of mRNA stability, translation and localisation(372). Whilst such variants are difficult to assess in vitro their effects could be investigated in genotype-phenotype studies within different populations to look for an association with blood pressure and aldosterone status.

Thus, so far, we have failed to find an easily assessable candidate SNP within CYP11B2, linked to –344 T and/or intron conversion alleles, to account for the phenotype of aldosterone-associated hypertension. However, given that the most consistent corticosteroid phenotype in hypertensives homozygous for –344T allele is increased DOC/B and S/F ratios (implying impaired activity of 11ß-hydroxylase) then this seemingly negative finding is, in fact, hardly

surprising and places appropriate emphasis on the results of sequencing and linkage studies involving CYP11B1.

Linkage studies confirmed high LD across the entire CYP11B locus. The most stringent measure of LD (r^2) varied from 0.5-1. Thus, this provides strong evidence to support the hypothesis that variation within CYP11B2 (-344C/T and IC) is actually linked to causal variant(s) within CYP11B1.

No non-synonymous SNPs in linkage with –344C/T and/or IC alleles were identified within CYP11B1 (Fig 3.3d). However, 2 closely related SNPs in the 5' promoter region (-1888 G/T, -1858 A/G) were found to be in tight LD with the T/conversion haplotype of CYP11B2.

These SNPs have been the subject of in vitro studies using a luciferase-reporter gene construct (M Barr, personal communication) and preliminary results suggest that the variants (T and G alleles respectively) associate with reduced transcriptional activity of CYP11B1. In addition, chapter 5 details the association of these alleles and corticosteroid phenotype in hypertensive and normotensive populations.

Haplotype analysis confirms high LD across the CYP11B1 locus; 4 haplotypes account for 68% of chromosomes. In theory, this should allow selection of 'tag' SNPs to aid high-throughput genotyping to give genetic information in large study populations. However, the value of this haplotype data is limited by small study numbers and by the fact that this population was pre-selected according to genotype at –344C/T and IC. Further studies are in progress to confirm these haplotypes in larger, unselected, populations.

Thus, this study does provide a number of candidate SNPs within CYP11B2, in linkage with –344 T and/or intron conversion alleles which may account for

the phenotype of hypertension with aldosterone excess but need further evaluation. However, more importantly, this work confirms high LD across the CYP11B locus supporting the theory that –344 T and/or IC are in linkage with variant(s) within CYP11B1 accounting for the phenotype of reduced 11ß-hydroxylase efficiency found in these individuals. Finally, in addition, this study identifies 2 SNPs within the 5' promoter of CYP11B1 which appear to reduce its transcriptional activity; these could represent plausible candidate SNPs accounting for this phenotype. Further genotype/phenotype studies are reported in chapter 5 to examine the effect of these SNPs on corticosteroid phenotype in vivo.

Chapter 4: Phenotypic Consequences of Variation across the CYP11B2 Locus in large Normotensive and Hypertensive Cohorts

4.1 Introduction

The –344C/T and intron conversion (IC) variants of CYP11B2 associate consistently with an increased ratio of 11-deoxycortisol/cortisol implying reduced efficiency of 11ß-hydroxylase (encoded by CYP11B1) in subjects with the T and conversion alleles(350). This has led us to speculate that, in such individuals, cortisol levels are maintained as a consequence of a slight increase in pituitary production of ACTH (or ACTH-related peptide) at the expense of a minor increase in its precursor, 11-deoxycortisol. We have also proposed that this mild increase in ACTH will have trophic effects on the zona glomerulosa, leading to increased aldosterone production in response to its more conventional trophins, Ang II and potassium.

If altered 11ß-hydroxylase efficiency is indeed associated with these CYP11B2 polymorphisms, the resulting increase in ACTH drive required to maintain cortisol levels should leave recognisable changes in the overall pattern of adrenal steroid metabolism. This hypothesis was explored within two contrasting populations. One was a large hypertensive cohort derived from the BRItish Genetics of HyperTension (BRIGHT)(359) study and the other a group of normotensive individuals from the fourth Multinational Monitoring of Trends and Determinants of Cardiovascular Disease (MONICA) survey(360).

These cohorts were analysed to look for differences in 11ß-hydroxylase efficiency according to genotype and blood pressure as well as the influence

of genotype and other factors on indirect markers of increased ACTH drive to the adrenal gland. Relationships between these changes in corticosteroid metabolism and known cardiovascular risk factors were also examined.

4.2 Methods

4.2.1 Study Subjects

Details of recruitment of subjects for the BRIGHT and MONICA populations as well as inclusion and exclusion criteria have been outlined in chapter 2. Each subject underwent medical evaluation which included assessment of blood pressure and anthropometric indices including weight, height, body mass index (BMI; ratio of weight (kg)/height^2 (m^2)).

4.2.2 Blood Sampling

Blood was drawn using the Vacutainer system (Becton-Dickinson) for measurement of lipids and electrolytes by standard biochemical methods. DNA was also extracted from plasma and subjects were genotyped for –344 C/T and IC polymorphisms as previously described (sections 2.6, 2.8 & 2.9)).

4.2.3 Urinary Corticosteroid Metabolite Measurements

Urine samples (24 hour) were collected in plain plastic containers without preservative. Aliquots of urine were stored at -20°C. Excretion rates of steroid metabolites (listed in Table 4.3d as median and inter-quartile ranges) were measured by gas chromatography/mass spectroscopy using the method of Shackleton(373) with minor modifications (section 2.11). The ratio of 11-deoxycortisol/ total cortisol (THS/F) was used as an index of 11ß-hydroxylase activity

4.2.4 Statistical analysis

It was calculated that 180 homozygous subjects would be required to achieve 80% power to detect a difference in THS/F ratio of 0.004 with an α level of 0.05. THS/F ratios did not follow a normal distribution and so were compared by non-parametric methods (Mann-Whitney test). Steroid excretion rates (log transformed) were compared by simple correlation and then adjusted for confounders (age, sex, BMI, WHR, blood pressure) using stepwise multiple regression. All data were corrected for multiple comparisons using the Bonferroni method such that a p value of <0.001 was required for statistical significance.

Preliminary analysis comparing subjects with good blood pressure (<140/90 mm/Hg) control versus poorly controlled subjects (>140/90 mm/Hg) showed no systematic effect of the major anti-hypertensive drug groups on excretion rates of cortisol, aldosterone or androgens (Table 4.2a). Therefore, no adjustment for drug effects was required.

4.3 Results

4.3.1 Demographic data

Blood pressure and other relevant demographic data are shown in Tables 4.3a and 4.3b; the BRIGHT population was significantly hypertensive but not significantly overweight. In contrast, the MONICA population was a normotensive lean population. There were no differences in characteristics of subject groups separated according to –344C/T genotype in either of the cohorts.

Drug Group	Total F	THS/Total F	THAldo	Total Andro
A	0.072	0.402	0.254	0.336
B	0.290	0.402	0.925	0.836
C	0.142	0.490	0.846	0.278
D	0.640	0.285	0.178	0.464

Table 4.2a. Effect of anti-hypertensive drug classes on urinary corticosteroid excretion (p values shown)
BRIGHT subjects (512) were sub-divided into well controlled (BP<140/90 mm/Hg) and poorly controlled (BP>140/90 mm/Hg) groups and steroid excretion compared by Mann Whitney analysis within the major anti-hypertensive drug categories
A-ACE inhibitors, ARBs; B-beta-blockers; C-calcium channel blockers; D-diuretics

	ALL SUBJECTS n=511	TT SUBJECTS n=161	CC SUBJECTS n=73	p*
AGE (y)	64 (57-70)	63 (58-70)	63 (56-68)	0.294
MEAN SBP (mm/Hg)	180 (153-191)	157 (152-190)	183 (153-190)	0.713
MEAN DBP (mm/Hg)	103 (98-110)	103 (98-110)	104 (100-110)	0.490
BMI (kg/m^2)	27 (25-30)	27 (25-30)	27 (25-30)	0.771
WHR	0.88 (0.81-0.94)	0.88 (0.82-0.93)	0.87 (0.81-0.94)	0.315

Table 4.3a. Demographic information on BRIGHT study sub-group separated by –344C/T genotype (median and IQ range)
SBP- systolic blood pressure, DBP-diastolic blood pressure, BMI-body mass index, WHR-waist-hip-ratio
*comparison between TT and CC homozygotes

	ALL SUBJECTS n=418	TT SUBJECTS n=124	CC SUBJECTS n=69	p*
AGE (y)	49 (40-58)	49 (39-59)	48 (42-56)	0.488
MEAN SBP mm/Hg)	127 (114-141)	127 (114-143)	129 (115-142)	0.51
MEAN DBP mm/Hg)	79 (70-87)	79 (68-87)	79 (71-87)	0.695
BMI (kg/m^2)	26 (24-29)	26 (23-29)	26 (24-29)	0.481
WHR	0.85 (0.78-0.93)	0.85 (0.79-0.0.93)	0.86 (0.78-0.93)	0.928

Table 4.3b. Demographic information on MONICA IV cohort separated by –344C/T genotype (median and inter-quartile range)
SBP- systolic blood pressure, DBP-diastolic blood pressure, BMI-body mass index, WHR-waist-hip-ratio
*comparison between TT and CC homozygotes

Table 4.3c compares the key demographic variables of the 2 populations. As expected, the BRIGHT group has significantly higher blood pressures but is also significantly older, with higher BMI/WHR, than the MONICA cohort.

4.3.2 Genetic analysis of CYP11B2

The frequencies of the –344C/T and IC polymorphisms in both groups are illustrated in figure 4.3a. (BRIGHT: –344 T 0.6 C 0.4; IC: WT 0.49, Conv 0.51; MONICA: –344 T 0.566, C 0.432; IC WT 0.53, Conv 0.47). These alleles were in Hardy-Weinberg equilibrium.

There was no significant difference in genotype frequencies between the two groups (–344C/T p=0.284, IC p=0.683).

4.3.3 Urinary corticosteroid excretion rates

The major urinary corticosteroid metabolites examined are listed in table 4.3d.

BRIGHT

There was no significant difference in total cortisol metabolite excretion rate (mcg/24 hours) between –344 TT and CC genotypes (p=0.3420).

The THS/ total F ratio (index of 11ß-hydroxylase activity) was significantly higher in TT homozygotes then in CC homozygotes (p=0.001) in all subjects (Table 4.3d, Figure 4.3b) as well as in female subjects (TT 0.01175, CC 0.00794 p=0.03) and male subjects (TT 0.0081, CC 0.0058 p=0.035). Figure 4.3b summarises the ranges of THS/F ratios in the 3 genotype groups. There was no statistically significant difference between the TT and CT groups but

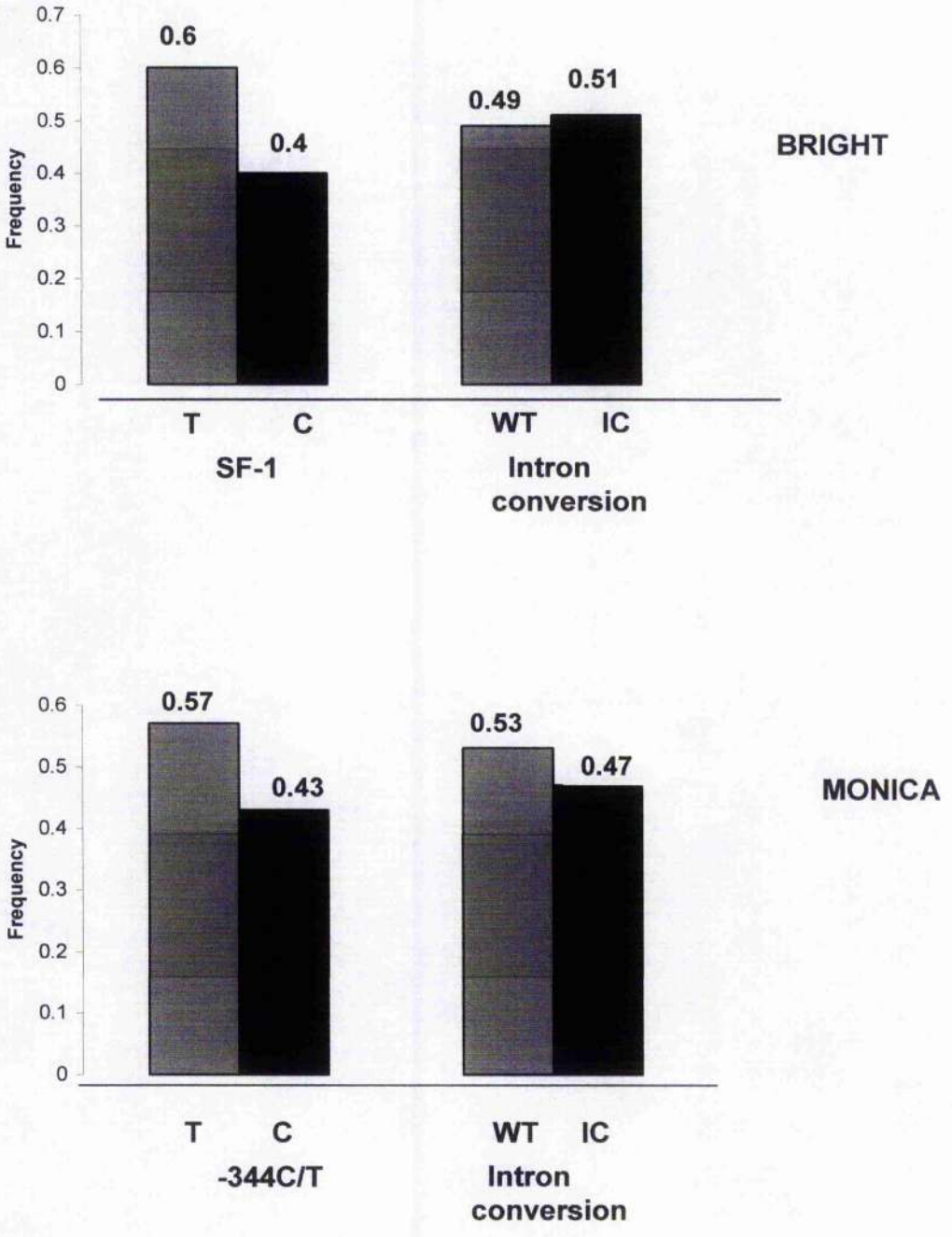


Figure 4.3a. Frequencies of –344C/T and IC polymorphisms of CYP11B2 in BRIGHT and MONICA populations

(I)

STEROID METABOLITE (mcg/24h)	ALL SUBJECTS	TT SUBJECTS	CC SUBJECTS	p*
ALDOSTERONE (THAldo)	3 (2-6)	3 (1-5)	3 (2-5)	0.95
CORTISOL METABOLITES (TOTAL F; THF+aTHF+THE)	1443 (786-2500)	1448 (731-2444)	1637 (898-2611)	0.342
ANDROGEN METABOLITES (TOTAL ANDRO; DHA+AETIO+ANDRO)	606 (318-1208)	560 (339-1150)	682 (319-1421)	0.29
11ß-HYDROXYLASE EFFICIENCY (THS/TOTAL F)	0.00856 (0.005-0.01)	0.0102 (0.006-0.015)	0.00683 (0.005-0.01)	**0.001**

(II)

STEROID METABOLITE (mcg/24h)	ALL SUBJECTS	TT SUBJECTS	CC SUBJECTS	p*
ALDOSTERONE (THAldo)	1 (0-7)	1 (0-3.5)	1 (0-2)	0.069
CORTISOL METABOLITES (TOTAL F; THF+aTHF+THE)	3652 (2250-5806)	3662 (2411-5786)	3438 (2469-5652)	0.637
ANDROGEN METABOLITES (TOTAL ANDRO; DHA+AETIO+ANDRO)	976 (559-1471)	1080 (666-1429)	1062 (628-1434)	0.971
11ß-HYDROXYLASE EFFICIENCY (THS/TOTAL F)	0.011 (0.004-0.02)	0.01 (0.004-0.02)	0.009 (0.003-0.02)	0.83

Table 4.3d. Urinary corticosteroid excretion (median and inter-quartile range) according to –344C/T genotype in (I) BRIGHT and (II) MONICA cohorts

THF: tetrahydrocortisol, (a)THE (allo) tetrahydrocortisone, DHA: dehydroepiandrosterone, Aetio: aetiocholanolone, Andro: androsterone THAldo:tetrahydroaldosterone, THS: tetrahydrodeoxycortisol

*comparison between TT and CC homozygotes

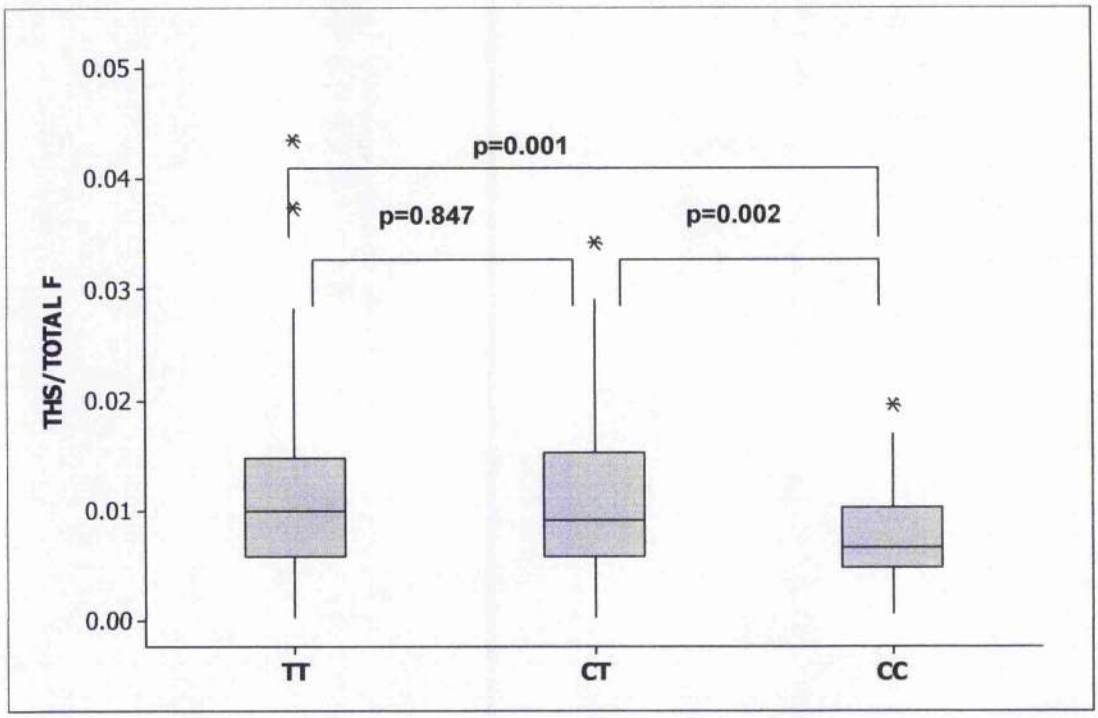


Figure 4.3b. 11ß-hydroxylase efficiency (THS/total F ratio) in all BRIGHT subjects stratified by –344C/T genotype

THS/F ratio for all 3 genotype groups compared by non-parametric testing (Mann-Whitney).

VARIABLE	BRIGHT	MONICA	p
AGE	64 (57-70)	49 (40-58)	<0.0001
SBP	180 (153-191)	127 (114-141)	<0.0001
DBP	103 (98-110)	79 (70-87)	<0.0001
BMI	27 (25-30)	26 (24-29)	<0.0001
WHR	0.88 (0.81-0.94)	0.85 (0.78-0.93)	<0.001

Table 4.3c. Comparison of demographic information summarising entire BRIGHT and MONICA study populations

the THS/F ratio remained significantly higher in the heterozygous group than the CC subjects.

The IC/WT allele had no significant effect on THS/F ratio (p=0.137) or total cortisol metabolite excretion rate (p=0.892).

MONICA

There was no significant difference in the excretion rates of any corticosteroid variable according to –344 genotype (Table 4.3d).

4.3.4 Corticosteroid excretion and demographic parameters

The relationships between urinary corticosteroid excretion rates and major demographic variables within each population, assessed by Pearson correlation co-efficients, as well as in subject groups separated by –344 C/T genotype, are shown in table 4.3e. Expected significant relationships were found (between systolic and diastolic blood pressures, systolic blood pressure and age and BMI and WHR) but there were no significant relationship patterns between these morphometric variables and any of the corticosteroid variables. In particular, the significant correlation between WHR and cortisol was lost when adjusted for sex, age and blood pressure.

4.3.5 Corticosteroid relationships

BRIGHT

As expected, given their co-dependence on ACTH drive, there was a strong correlation between excretion rates of total cortisol and total androgens ($p<0.00005$) irrespective of sex or genotype.

(a)

(i)	SBP	DBP	WHR	BMI	AGE	CHOL	THALDO	CORTISOL	ANDRO
SBP	----	**0.518**	-0.025	0.017	**0.408**	**0.15**	-0.006	-0.1	**-0.149**
DBP		----	0.032	0.005	0.097	0.077	0.07	-0.012	-0.011
WHR			----	**0.296**	0.008	-0.107	**0.209**	**0.234**	**0.276**
BMI				----	-0.021	-0.011	0.08	**0.157**	0.035
AGE					----	0.08	-0.069	-0.03	**-0.297**
CHOL						----	-0.06	-0.116	**-0.151**
(ii)									
SBP	----	**0.501**	0.1	0.137	**0.331**	0.188	0.092	-0.047	-0.082
DBP		----	0.116	0.087	0.068	0.107	0.069	0.147	0.142
WHR			----	**0.309**	0.14	**-0.233**	**0.235**	0.164	0.162
BMI				----	0.146	-0.036	0.146	0.074	-0.06
AGE					----	0.132	0.085	0.083	-0.187
CHOL						----	0.051	-0.17	-0.128
(iii)									
SBP	----	**0.614**	-0.138	0.061	**0.408**	0.102	0.05	-0.234	-0.004
DBP		----	-0.056	-0.105	0.063	0.057	0.272	-0.163	-0.102
WHR			----	**0.328**	-0.099	-0.1	0.245	**0.35**	**0.435**
BMI				----	-0.072	-0.002	-0.104	0.251	0.06
AGE					----	0.102	0.095	-0.002	**-0.308**
CHOL						----	-0.104	-0.03	-0.205

(b)

(i)	SBP	DBP	WHR	BMI	AGE	CHOL	THALDO	CORTISOL	ANDRO
SBP	----	**0.697**	**0.251**	**0.184**	**0.356**	-0.019	0.027	**0.129**	-0.049
DBP		----	**0.324**	**0.203**	**0.219**	-0.009	0.067	**0.164**	0.072
WHR			----	**0.274**	**0.289**	-0.06	0.045	**0.285**	**0.177**
BMI				----	**0.135**	0.105	0.058	**0.222**	0.065
AGE					----	0.034	-0.072	0.04	**-0.326**
CHOL						----	**0.168**	0.061	-0.015
(ii)									
SBP	----	**0.691**	0.146	**0.24**	**0.553**	**0.398**	-0.149	0.084	-0.189
DBP		----	**0.284**	0.221	**0.367**	0.229	-0.022	0.153	-0.091
WHR			----	**0.464**	**0.329**	0.194	-0.031	**0.422**	0.175
BMI				----	0.137	0.165	0.03	**0.384**	0.139
AGE					----	**0.467**	-0.235	0.101	**-0.395**
CHOL						----	0.15	0.102	-0.23
(iii)									
SBP	----	**0.627**	**0.452**	0.129	**0.458**	**0.373**	0.101	0.122	0.11
DBP		----	**0.437**	0.235	0.2	0.138	0.07	0.218	0.344
WHR			----	**0.293**	**0.348**	0.064	0.242	**0.359**	0.27
BMI				----	0.182	0.157	0.252	0.169	0.013
AGE					----	0.261	-0.033	-0.089	**-0.334**
CHOL						----	0.147	0.018	0.013

Table 4.3e Correlation analysis between cardiovascular parameters and excretion of major corticosteroid sub-groups in (a) BRIGHT and (b) MONICA populations

Pearson correlation co-efficients shown for: (i) all subjects, (ii) TT homozygotes and (iii) CC homozygotes.

Correlation relationships significant after Bonferroni correction are shown in bold ($p<0.005$)

* remains significant after adjustment for confounding factors

There was also a highly significant correlation between THAldo excretion and total cortisol ($p<0.00005$) as well as between the excretion rates of THAldo and total androgens ($p<0.00005$) in the TT group but not in the CC group ($p=0.043$, 0.085 respectively) (Figure 4.3c). There was no correlation between urinary Na:K ratio and THAldo excretion ($p=0.445$) irrespective of genotype. The correlation between THAldo and androgens and between THAldo and total F was significant in subjects possessing the IC variant as well as those with the wild type intron ($p<0.0005$ in all cases).

All these correlations remained significant when data were adjusted for age, sex, BP and BMI.

MONICA

Pearson correlation co-efficients demonstrating the relationship between excretion of corticosteroid metabolites are outlined in table 4.3f. Again, there is a robust relationship between cortisol and androgen excretion throughout the genotype groups ($p<0.001$), which is maintained when adjusted for sex, blood pressure and body mass.

In contrast to the BRIGHT cohort, no significant relationship between aldosterone excretion and cortisol or aldosterone and androgen was found in any genotype group.

4.4 Discussion

This study is the first attempt to explore the underlying genetic basis and overall consequences of an apparent alteration in adrenal 11ß-hydroxylation efficiency in hypertensive patients. Reduction in 11ß-hydroxylation efficiency

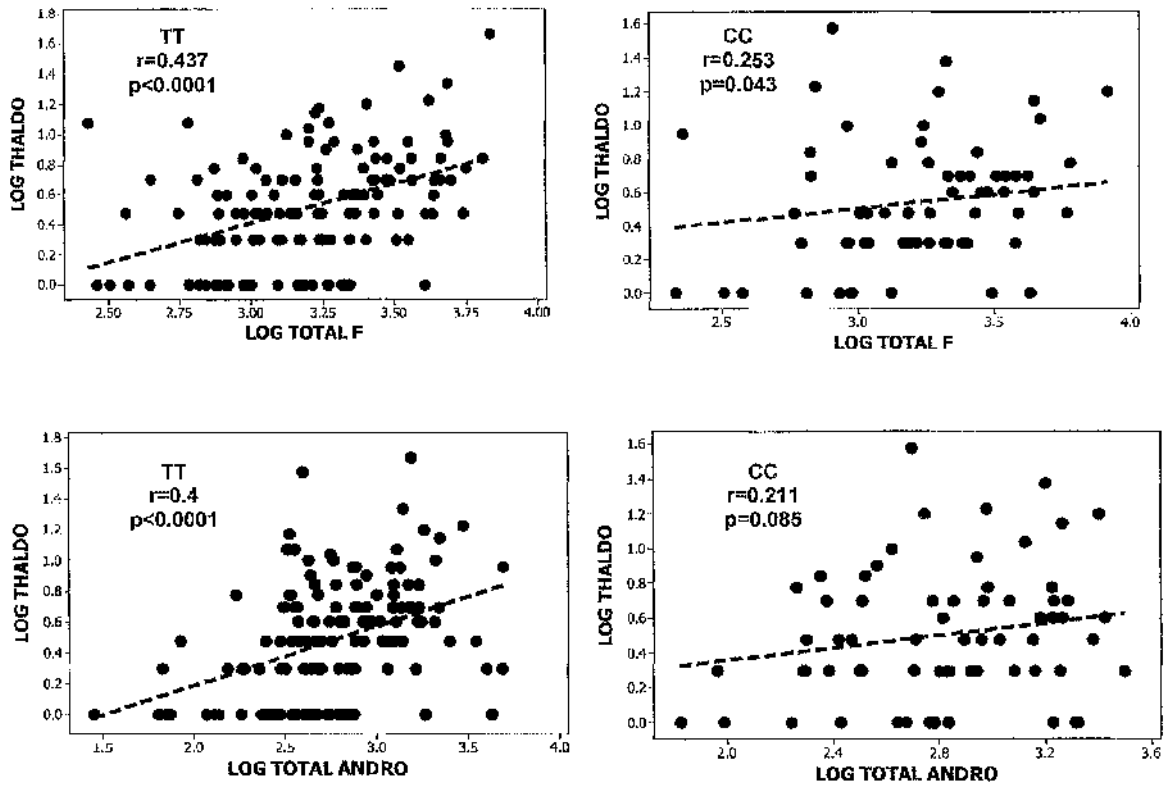


Figure 4.3c. Correlation between aldosterone excretion (THAldo) and total cortisol metabolites (Total F) as well as THAldo and total androgen metabolites (Total Andro) in BRIGHT subjects stratified by –344C/T genotype.

	TT	CC	WTWT	ConvConv
Total F/Total Andro	0.287*	0.634*	0.384*	0.364*
Total F/THAldo	0.141	0.247	0.313	0.308
THAldo/Total Andro	0.349	0.183	0.186	0.418*

Table 4.3f. Correlation data demonstrating relationships between corticosteroid excretion rates in MONICA subjects separated by –344C/T and IC genotypes (Pearson correlation co-efficients shown)

* denotes statistical significance , $p<0.001$.

in hypertensive individuals is not a novel concept. In 1985, de Simone and colleagues found a higher ratio of DOC/corticosterone and 11-deoxycortisol/cortisol in hypertensive subjects compared to normotensive controls after ACTH stimulation(353). Subsequently, colleagues made a similar observation in hypertensive subjects in whom the plasma ratio of 11-deoxycortisol/cortisol was elevated(145). More recently, in a study of normotensive subjects, Ganapathipillai and colleagues demonstrated an association between the –344 T allele and altered 11ß-hydroxylation efficiency(374). The current data are consistent with this, and with earlier observations of a similar biochemical phenotype associated with the same genetic variant in young and middle aged subjects(375).

It is of interest that in analysis of the MONICA cohort (subjects with normal blood pressure) there was no alteration in 11ß-hydroxylation efficiency (no elevation of THS/Total F ratio) according to genotype. This finding, as well as the studies mentioned above, could suggest that such an effect is confined to hypertensive individuals. However, our group has previously demonstrated impaired 11ß-hydroxylase efficiency within normotensive subjects homozygous for the -344T allele (increased plasma 11-deoxycortisol/cortisol ratios) but only after stimulation with intravenous ACTH; baseline ratios were normal(350). Given the retrospective nature of this study, we were obviously unable to look at the effect of ACTH stimulation on either cohort.

These data confirm the phenotype of altered 11ß-hydroxylation efficiency within a sub-group from a large, severely hypertensive cohort separated according to genotype at –344 of CYP11B2. Those subjects homozygous for the T allele showed lower 11ß-hydroxylation efficiency (increased urinary

THS/total F ratio) than those homozygous for the C allele. The intron conversion polymorphism did not appear to contribute to this variation, as there was no significant difference in THS/total F ratios in either genotype group (WT and IC).

Importantly, there was no difference in urinary excretion of total cortisol metabolites between the two genotype groups. These findings support the hypothesis that, in TT subjects, normal cortisol production is maintained by a subtle increase in ACTH drive to the adrenal cortex, which increases the availability of its deoxyprecursor.

Further, albeit indirect, evidence in support of this hypothesis can be found from the significant correlations seen in the BRIGHT cohort between the excretion rates of the three major corticosteroid sub-groups. Predictably, excretion of cortisol and androgen, both entirely ACTH-dependent, correlate strongly in all groups and in both study populations. However, aldosterone production is principally controlled by Ang II(227) and potassium(376) and ACTH is generally considered to have a minor influence(182). Nonetheless, within the BRIGHT subjects, the data clearly demonstrate a significant correlation between excretion rates of aldosterone with total cortisol metabolites and total androgen metabolites in the TT genotype group, where an increased ACTH drive is postulated. Thus, it is speculated that, in hypertensive subjects homozygous for the-344 T allele, there is an important common regulatory influence on adrenal corticosteroid production that could well be ACTH.

There was no such relationship seen within the normotensive MONICA population with no correlation between aldosterone and cortisol or androgens

in either –344C/T genotype group. Whilst this cohort is not a comparable control group this finding may suggest that this effect is limited only to subjects with the T allele and hypertension.

Retrospective analysis has a number of disadvantages. Ideally, when assessing mineralocorticoid activity, sodium intake should be controlled, and subjects should not be taking drugs known to affect the renin-angiotensin-aldosterone axis. However, whilst many patients were on drugs which would increase (diuretics) or decrease (beta-blockers, ACE inhibitors) aldosterone levels, we could demonstrate no systematic effect of any class of anti-hypertensive agent on corticosteroid excretion (table 4.2a). Moreover, the steroid metabolite pattern described here, indicating an alteration in 11ß-hydroxylase efficiency, is entirely consistent with previous studies using cohorts of hypertensive patients treated similarly(353) as well those on untreated subjects(350).

The MONICA cohort differed significantly from the BRIGHT population in age and BMI as well as blood pressure (Table 4.3c). Obviously, this population cannot be treated as a direct control group for BRIGHT; indeed, as a family based study, BRIGHT has no comparable control group. Thus, there can be no direct comparison between the two groups.

In conclusion, analysis of urinary steroid excretion patterns together with steroid genotype in large populations confirms genotype-dependent alteration in the efficiency of 11ß-hydroxylase in hypertensive subjects. The lack of difference in cortisol excretion between the two genotype groups, as well as genotype-dependent correlations between aldosterone, androgens and

cortisol also provides evidence in favour of increased ACTH drive in subjects with hypertension and favourable CYP11B2 genotype.

Chapter 5: Consequences of Variation in the CYP11B2 and CYP11B1 Genes on Hypothalamic-Pituitary-Adrenal Axis Activity and Aldosterone

5.1 Introduction

In chapter 3, a high degree of linkage disequilibrium (LD) across the CYP11B locus was demonstrated, supporting the hypothesis that the SF-1 –344C/T and/or IC variants are part of a haplotype block with functional variants elsewhere in CYP11B2 or in CYP11B1. Indeed, as a result of this work, two candidate SNPs in the promoter of CYP11B1 (-1888 G/T, -1858 A/G) have been identified which appear to reduce transcriptional efficacy of its enzyme product (11ß-hydroxylase)(377). In addition, chapter 4 examines the consequences of the –344C/T and IC polymorphisms on the intermediate corticosteroid phenotype within a large hypertensive cohort and confirms that the -344T allele is associated with reduced 11ß-hydroxylase efficiency as well as providing indirect evidence in favour of increased ACTH drive in hypertensive TT individuals.

However, until now, the effect of variation across the CYP11B locus on hypothalamic-pituitary-adrenal (HPA) axis activity in subjects has not been studied. It has been speculated (section 1.11) that reduced efficiency of 11ß-hydroxylation would result in a slight reduction in cortisol levels in response to ACTH. In turn, normal feedback regulation should result in a resetting of the ACTH cortisol relationship to maintain cortisol production at the expense of a slight increase in its precursor, 11-deoxycortisol. Consequently, there will a subtle increase in ACTH drive to the adrenal cortex (Figure 1.11a) resulting in hyperplasia of the zona glomerulosa and increased responsiveness to the

principal trophins of aldosterone production, angiotensin II and/or potassium. Ultimately, this could result in the final phenotype of interest, hypertension with an elevated ARR.

If this theory is correct, then there should be demonstrable differences in HPA axis activity in subjects stratified by genotype. In particular, as a result of resetting of the HPA axis, subjects might be less sensitive to suppression using exogenous glucocorticoid and more sensitive to adrenal stimulation using ACTH.

In the following study, activity of the HPA axis and the corticosteroid phenotype was examined in hypertensive patients and normotensive control subjects stratified according to genetic variation in CYP11B2 polymorphisms (-344C/T and IC) and CYP11B1 (-1888 G/T and -1858 A/G).

5.2 Methods:

5.2.1 Patient recruitment:

Recruitment of subjects with hypertension and control subjects, the study protocol and genotyping for the –344C/T polymorphism are described in detail in section 2.2. Briefly, hypertensive patients were recruited from hypertension clinics in the Western Infirmary Glasgow. Patients had blood pressure measurements consistent with BHS criteria for the diagnosis of hypertension and were either untreated or on calcium channel blockers (which should have no significant effect the on renin-angiotensin-aldosterone system)(314). Control subjects were normotensive and on no medication. Exclusion and inclusion criteria are outlined in table 2.2a.

5.2.2 Genotyping:

The major genotypic requirement for inclusion in the study was homozygosity at the CYP11B2 -344 locus (TT or CC). Subjects were subsequently genotyped for the IC polymorphism, as well as the recently identified SNPs in the 5' promoter of CYP11B1 (-1888 G/T, -1858 A/G). DNA extraction and genotyping techniques are described in chapter 2 (sections 2.6/2.8/2.10).

5.2.3 Study protocol

Subjects who satisfied the inclusion criteria and who were homozygous for the polymorphism at position –344 of CYP11B2 were asked to attend for three separate study phases (Endocrine Investigation Unit, Western Infirmary).

A summary of the protocol is detailed below and is described in greater detail in section 2.2.3.

Phase 1- Baseline and diurnal changes in corticosteroid patterns

Subjects attended at 10pm and between 7-8 am the following morning for baseline blood measurements. For normotensive volunteers, no night time visit was required. On each occasion, subjects were rested supine with a cannula in position for 30 minutes before blood sampling.

Phase 2-Sensitivity to dexamethasone

Four days before visit, subjects were asked to follow a standard salt diet (Appendix II) to ensure a sodium intake of approximately 120mmol/day. On the day before (midnight to midnight), a 24-hour urine collection was performed for urinary sodium measurement as well as measurement of corticosteroid excretion rates. At midnight the night before, subjects took 125 micrograms of dexamethasone and attended the day ward fasted the next morning between 7-8am for blood collection as detailed in section 2.2.

Phase 3- Response to ACTH

Again, subjects were asked to follow a standard salt diet and perform a 24hour urine collection as detailed above. At midnight the night before the visit, subjects took 1 milligram of dexamethasone and attended the day ward fasted the next morning between 7-8 am.

Baseline blood samples after 30 minutes supine rest were drawn in the manner described above. Subjects were then given 1 microgram of synthetic ACTH (synacthen®, Alliance Pharmaceutical Corp USA) intravenously as a bolus. After a further 30 minutes of supine rest, blood was drawn, spun and stored in the manner described in section 2.2.

5.2.4 Biochemical measurements:

Urinary sodium and potassium were measured on 24-hour urine collections by the Department of Biochemistry using an ion-selective electrode method. Plasma aldosterone and cortisol were measured by radioimmunoassay utilising the 'Coat-A-Count' system (Euro/DPC Ltd, Caernarfon, Wales). The radioisotopes used were ^{125}I cortisol and ^{125}I aldosterone respectively.

Plasma ACTH measurements were performed by Professor Anne White, University of Manchester by radioimmunometric assay (IRMA). This technique uses iodinated radiolabelled monoclonal antibodies for ACTH 1-17, with another solid phase monoclonal specific for ACTH 34-39(366). The lower limit of detection for this assay was 2.5ng/L and the coefficient of variation (CV) ranged from 0-20%.

Plasma renin concentration (PRC) was measured by radioimmunoassay based on generation of angiotensin I (section 2.17)(367). A cut off of 40

pmol/L/microunit/ml of Aldosterone/PRC ratio was used to describe an elevated ratio, as previously defined(378).

5.2.5 Data Analysis

As not all biochemical data were normally distributed, the data were log transformed (log_{10}) to allow analysis by parametric methods (Minitab, version 14). All data were corrected for multiple comparisons using the Bonferroni method such that a p value of <0.02 was required for statistical significance.

5.3 Results

5.3.1 Plasma aldosterone and PRC

Two hypertensive subjects (both TT) had an elevated baseline aldosterone/PRC ratio (57 & 65 pmol/L/microunit/ml), so that the prevalence of aldosterone-associated hypertension in this young, mildly hypertensive cohort was 5%.

There were no genotype-dependent differences in aldosterone/PRC ratios under the various study conditions (Figure 5.3a)

5.3.2 CYP11B2 Results

Genotyping

One hundred and twenty one patients were suitable for initial genotyping at the -344 locus of CYP11B2. Of these, 56 were heterozygous and so immediately excluded. Of the remainder, 42 were TT homozygotes and 23 were CC homozygotes (Figure 5.3b i). However, a number of individuals

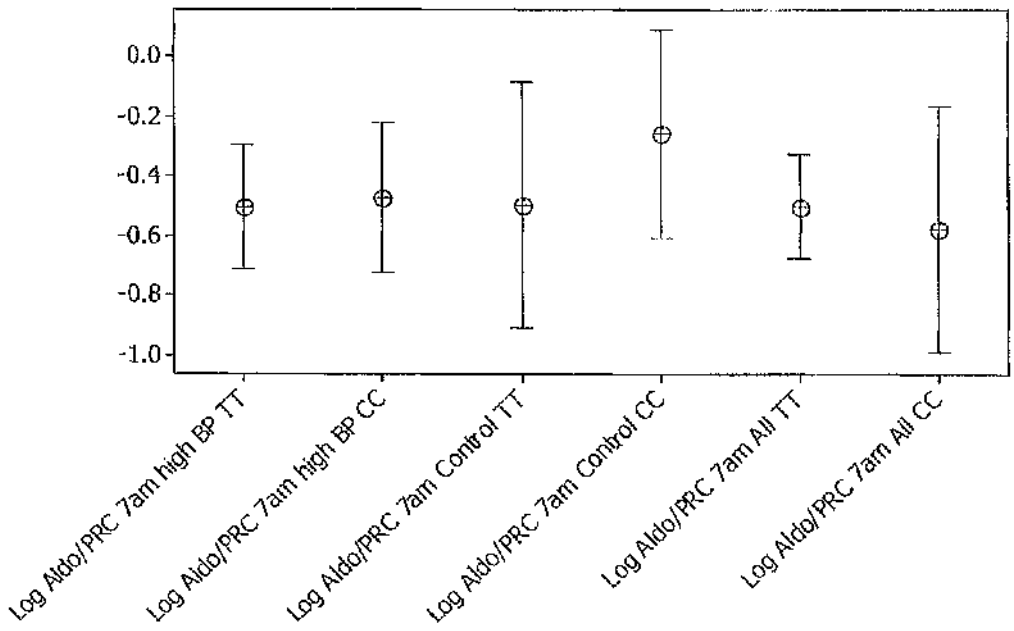


Figure 5.3a Aldosterone/Renin ratios (as Plasma Renin Concentration) in all study groups stratified by –344C/T genotype.
All data are logged

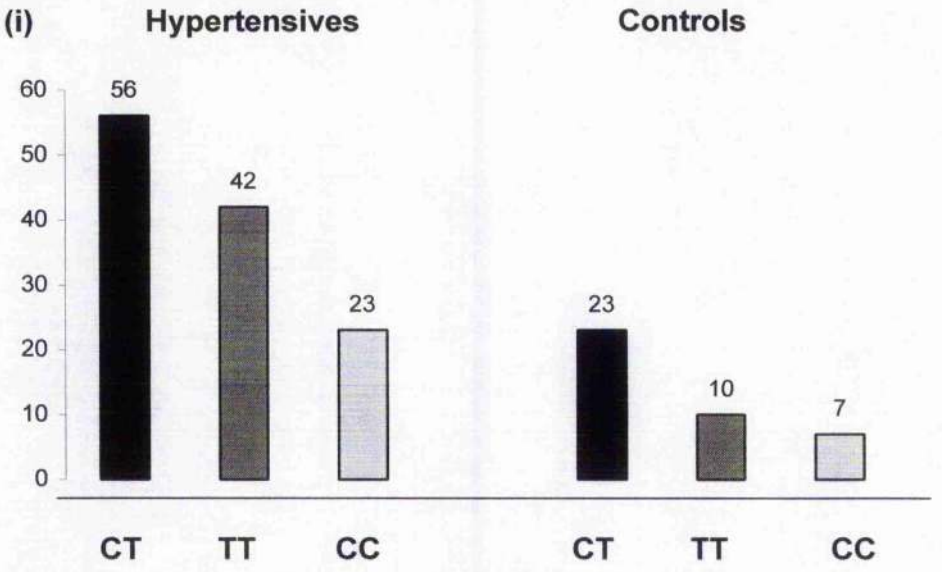
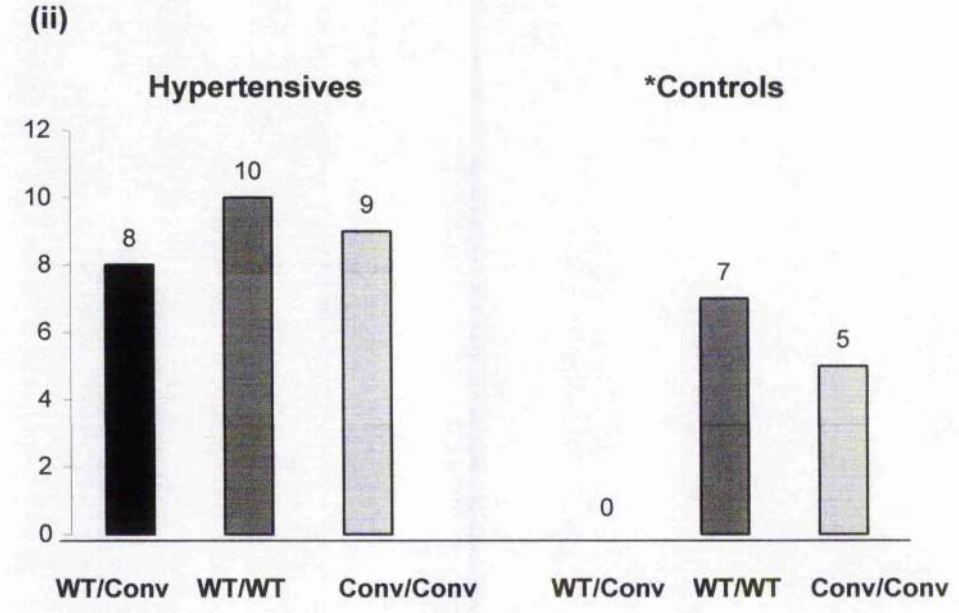


Figure 5.3b Results of genotyping at (i) –344 C/T and (ii) IC polymorphism of CYP11B2 in hypertensive subjects and normotensive controls.



*Fewer controls were genotyped for the IC polymorphism as there were technical difficulties with the assay. Moreover, only those who were homozygous or the –344C/T polymorphism were genotyped for IC.

dropped out before the study began and so in total, 27 TT and 12 CC subjects were studied in further detail. It had previously been estimated that approximately 15 patients per group would be required to give 85% power to detect a change in aldosterone concentration of 5ng/dl. Subjects included in the study underwent subsequent genotyping for the intron conversion (IC) polymorphism and these results are outlined in Figure 5.3b ii.

Forty normotensive volunteers were also recruited. Twenty-three were heterozygous and 17 homozygous (10 TT, 7CC) for the –344C/T polymorphism. Again, these subjects underwent genotyping for the IC polymorphism.

Demographic details

Blood pressure and other relevant demographic data for patients and controls are shown in Tables 5.3a & b. There was no significant difference between the two genotype groups in either patients or controls. Mean urinary sodium excretion was 117 mmol/24h (aim was 120 mmol/24h) with no genotype-dependent differences.

Effect of genotype on patterns of corticosteroid variation

The effects of diurnal variation, dexamethasone suppression and stimulation with synthetic ACTH (synacthen) on aldosterone, cortisol and ACTH are illustrated in Figure 5.3c.

As expected, cortisol and ACTH showed diurnal variation (values higher in morning, $p<0.001$ in both cases), partially suppressed with 125 micrograms of dexamethasone ($p=0.02$ cortisol, $p=0.04$ ACTH) and suppressed to a greater

(i)

	CC n=12	TT n=27	p
Age (years)	43.6 (3.74)	41.7 (2.3)	0.66
BMI (kg/m^2)	28 (1.27)	29 (0.87)	0.436
SBP (mm/Hg)	146 (3.29)	147 (1.74)	0.845
DBP (mm/Hg)	90 (1.77)	90 (1.41)	0.932
Urinary sodium excretion (nmol/24h)	112.2 (21)	106.8 (8.2)	0.817

(ii)

	CC n=7	TT n=9	p
Age (years)	33 (2.01)	34 (2.35)	0.673
BMI (kg/m^2)	27 (1.48)	25 (1.18)	0.287
SBP (mm/Hg)	122 (4.27)	121 (1.92)	0.932
DBP (mm/Hg)	76 (1.77)	74 (1.41)	0.790
Urinary sodium excretion (nmol/24h)	140.2 (23)	113.4 (23)	0.422

Table 5.3a Demographic information on (i) hypertensive subjects and (ii) normotensive controls separated according to-344C/T genotype
All figures are means (±SEM)

	Hypertension n=39	Controls n=16	p
Age (years)	42 (1.94)	34 (1.55)	0.001
BMI (kg/m^2)	29 (0.72)	26 (0.94)	0.015
SBP (mm/Hg)	147 (1.55)	121 (2.07)	<0.001
DBP (mm/Hg)	90 (1.11)	75 (2.07)	<0.001
Urinary sodium excretion (nmol/24h)	108.2 (8)	125.1 (16)	0.359

Table 5.3b. Demographic information on all study subjects separated by blood pressure status
Figures are means (± SEM)

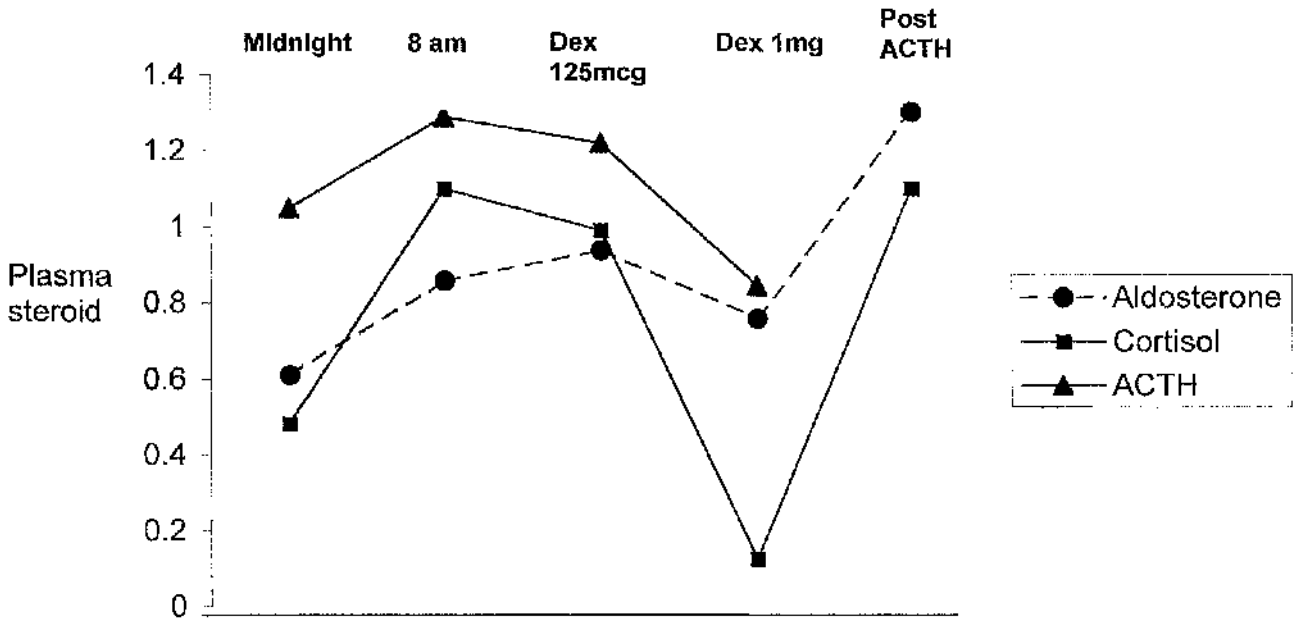


Figure 5.3c. Effect of diurnal variation, suppression with dexamethasone (125 mcg and 1mg) and stimulation with 1mcg of ACTH on plasma steroid levels in all study patients
All plasma steroid values have undergone logarithimic transformation.
Aldosterone ng/dL, Cortisol mcg/dL, ACTH ng/L.

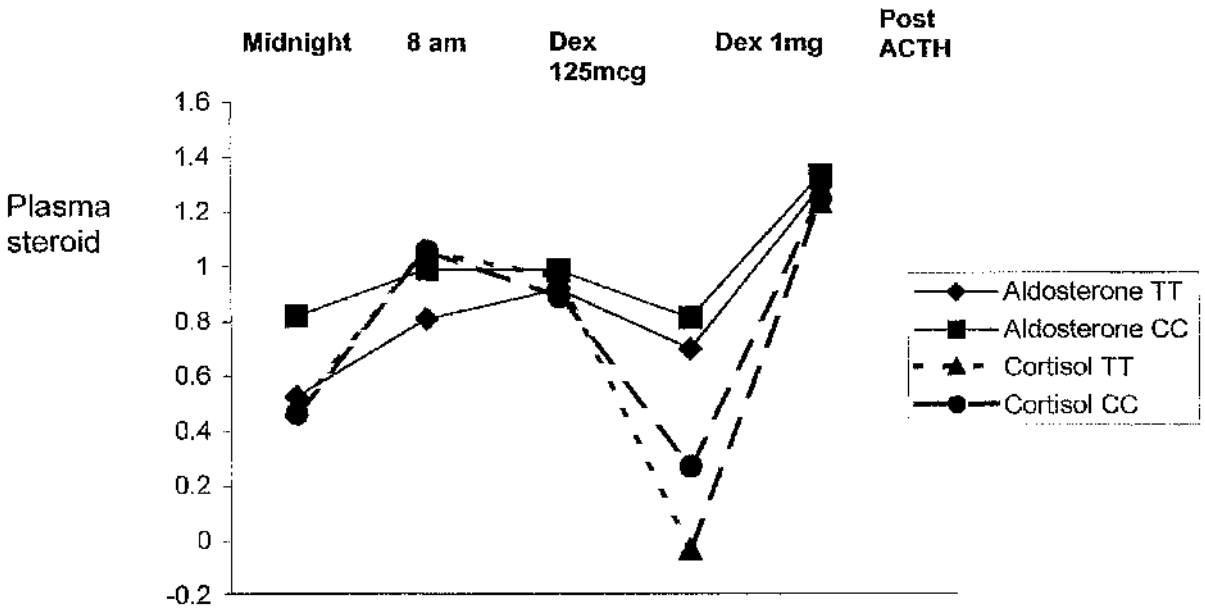


Figure 5.3d. Effect of diurnal variation, suppression with dexamethasone and stimulation with 1mcg of ACTH on mean plasma steroid levels according to –344 C/T genotype in all patients
All plasma steroid values have undergone logarithimic transformation.
Aldosterone ng/dL, Cortisol mcg/dL

extent with 1mg of dexamethasone ($p<0.001$ in both cases). Cortisol rose significantly in response to 1 microgram of intravenous ACTH ($p<0.001$). None of these results were influenced by genotype or blood pressure status.

In contrast, aldosterone failed to suppress with either dose of dexamethasone ($p=0.4$) but rose significantly after ACTH ($p<0.001$). Again, this pattern was seen when looking at the subjects as a whole group or separated according to genotype and no difference in demographic features between genotype groups was noted. However, a diurnal variation in plasma aldosterone was only seen in the subjects homozygous for the -344 T allele ($p< 0.02$).

Effect of genotype on mean corticosteroid levels

Mean levels of plasma corticosteroids under different study conditions were compared according to -344C/T genotype as a whole cohort, in hypertensives and in controls. There was no difference in cortisol or aldosterone levels in any cohort at baseline, after dexamethasone or ACTH (Figure 5.3d).

Plasma ACTH levels after dexamethasone suppression (1mg) tended to be higher in TT versus CC subjects although this did not reach statistical significance. However, the cortisol/ACTH ratio was significantly lower in TT versus CC subjects (after 1 mg dexamethasone) irrespective of blood pressure status implying a genotype-dependent alteration in their relationship (Figure 5.3e). Plasma ACTH after 1 mg of dexamethasone was also higher in subjects homozygous for the intron conversion allele when compared to wild type homozygotes ($p=0.05$). The ratio of cortisol/ACTH was also lower in this sub-group but this did not reach statistical significance ($p=0.13$).

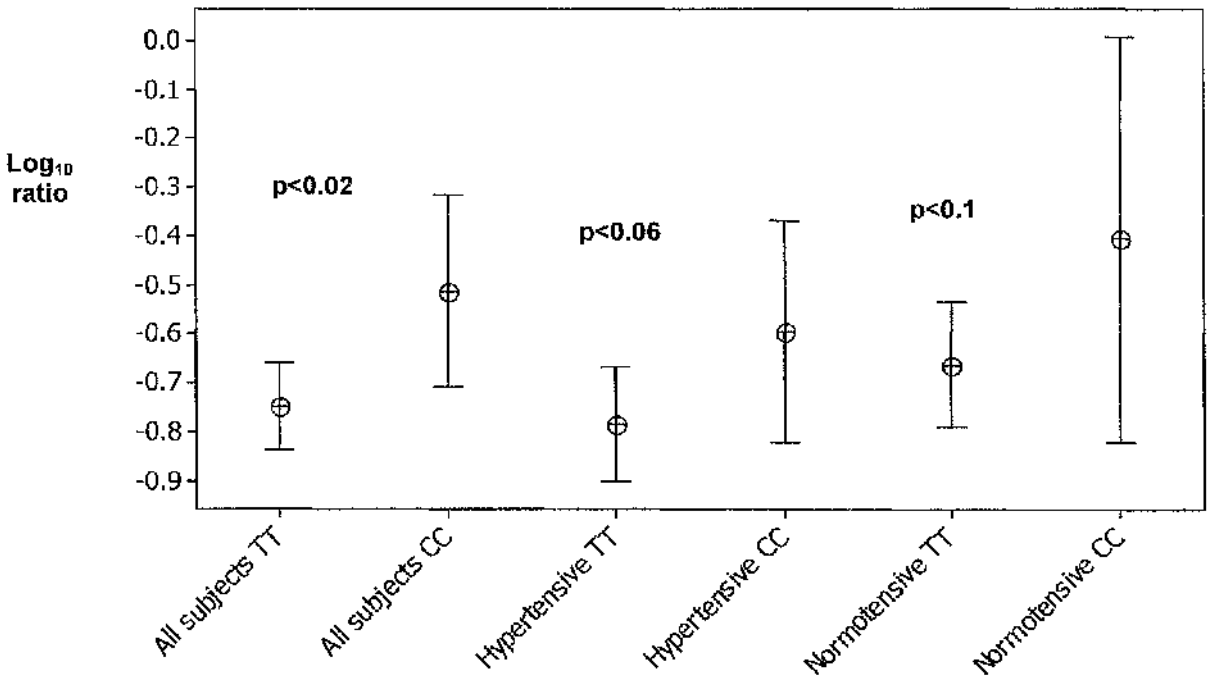


Figure 5.3e. Cortisol/ACTH ratios after 1mg dexamethasone in different patient groups separated by CYP11B2 –344C/T genotype
(data shown as means + 95% CI)

Finally, the above observations were seen when subjects were compared according to CYP11B2 haplotype. Plasma ACTH was significantly higher in T/Conv subjects (compared to C/WT; p=0.05); the cortisol/ACTH ratio was lower in subjects regardless of blood pressure status but failed to reach statistical significance (Figure 5.3f).

Correlation relationships

Pearson correlation coefficients between baseline (8am) cortisol levels and aldosterone levels in all subjects stratified by genotype are illustrated in Figure 5.3g. There was a significant correlation between cortisol and aldosterone in TT (r=0.508, p<0.004) but not CC subjects. This correlation remained significant when data were adjusted for age, BP and BMI.

There was no significant correlation between baseline (8am) aldosterone and ACTH in either genotype group (TT r=0.091, p=0.638, CC r=0.490, p=0.09).

Effect of genotype on corticosteroid response

When the mean absolute difference in plasma corticosteroid levels (according to time of day/dexamethasone suppression/ACTH stimulation) was compared between genotype groups, no significant differences were found (Table 5.3c)

5.3.3 CYP11B1 Results

Demographic data

Of the 39 hypertensive patients homozygous for the SF-1 -344 polymorphism of CYP11B2 included in the study, 20 were homozygous for the –1888 (G/T) and –1858 (A/G) polymorphisms in the promoter of CYP11B1 (9 GGAA, 11

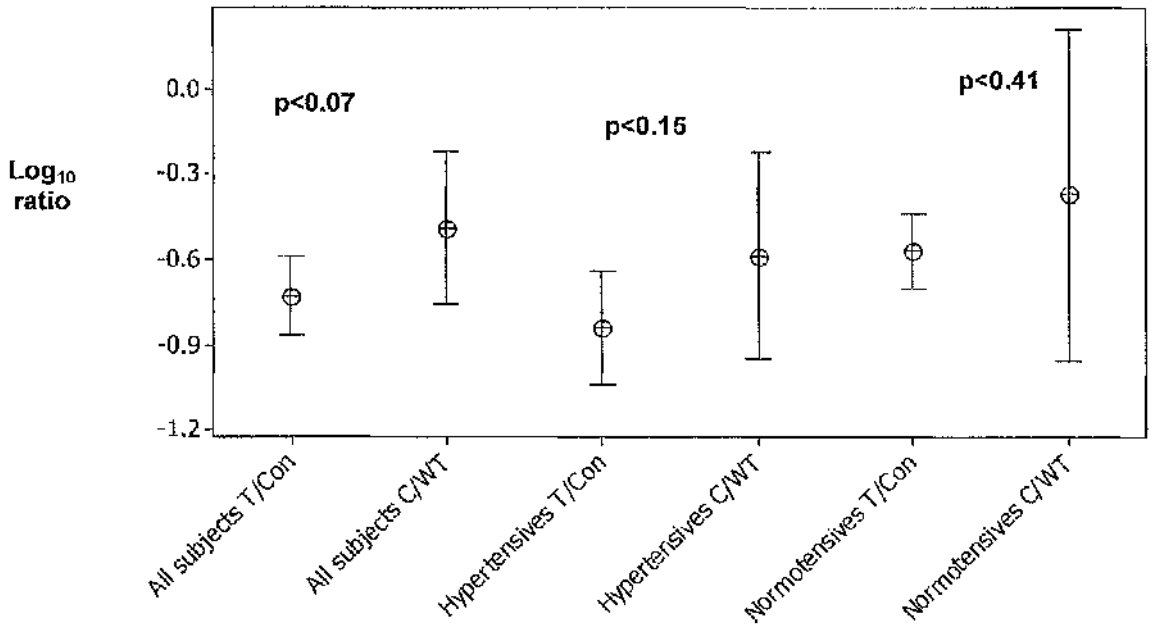


Figure 5.3f. Cortisol/ACTH ratios after 1mg dexamethasone in different patient groups separated by CYP11B2 haplotype (means + 95% CI)

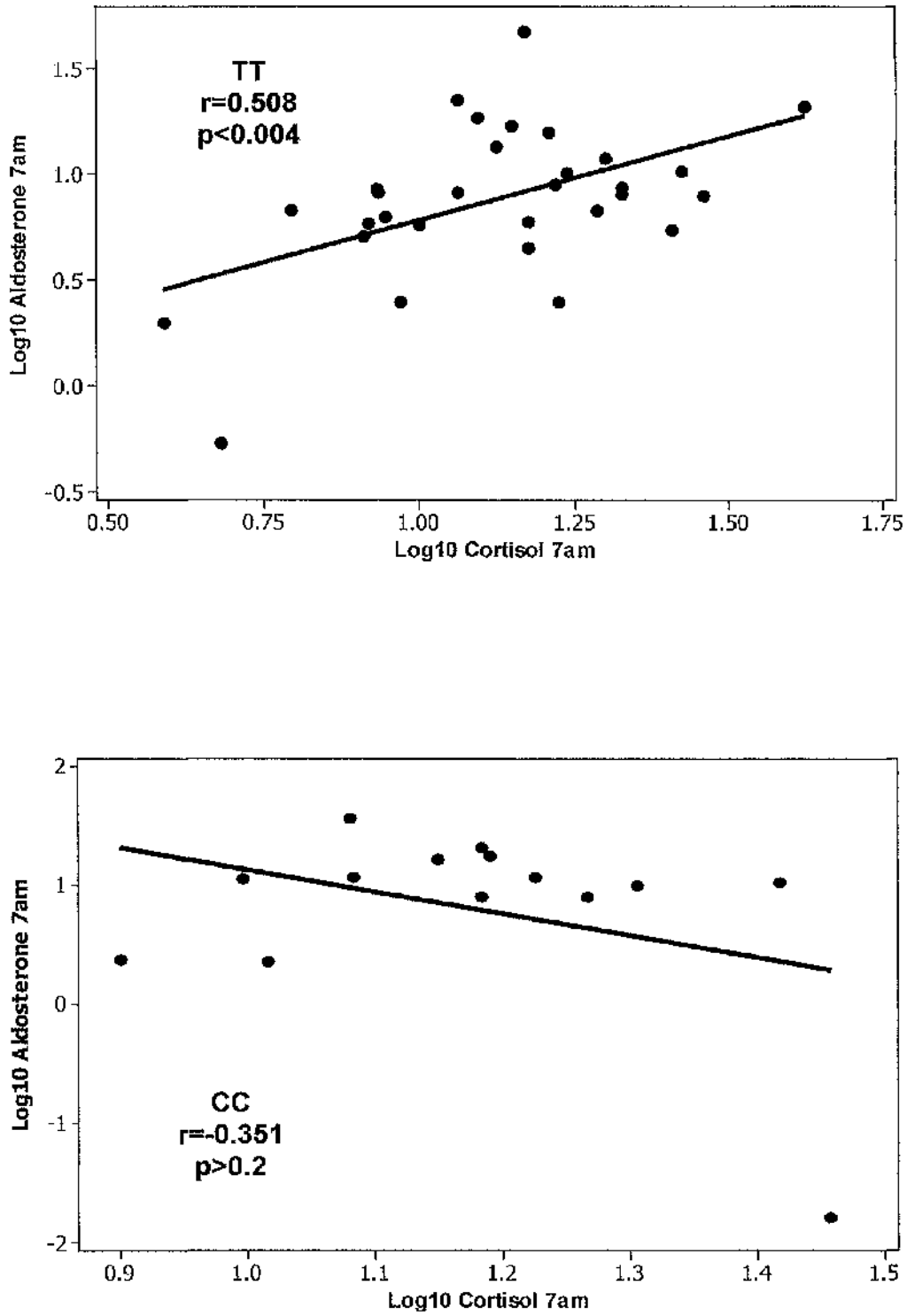


Figure 5.3g. Correlation relationships in all subjects between early morning plasma cortisol and aldosterone levels according to CYP11B2 – 344C/T genotype

		Mean absolute change TT	Mean absolute change CC	p
Aldosterone ng/dl	Diurnal	0.703	0.658	0.628
	Dex 125mcg	0.589	0.723	0.443
	Dex 1mg	0.357	0.662	0.134
	ACTH	1.068	1.131	0.506
Cortisol mcg/dl	Diurnal	0.915	0.843	0.489
	Dex 125mcg	0.457	0.525	0.638
	Dex 1mg	1.046	1.026	0.818
	ACTH	1.217	1.15	0.219
ACTH ng/l	Diurnal	0.971	1.099	0.405
	Dex 125mcg	0.530	0.734	0.345
	Dex 1mg	0.979	1.15	0.05

Table 5.3c Mean absolute change (all subjects) in plasma corticosteroid level in response to diurnal variation, suppression with dexamethasone (125mcg and 1mg) and stimulation with 1mcg of ACTH.
All data are logged

TTGG). 18 normotensive controls homozygous for these polymorphisms were also studied, (11 GGAA, 7 TTGG). Wild type alleles are GGAA whilst TTGG genotype represents the novel polymorphism.

There was no significant difference in age, BMI or blood pressure between the genotypes when looking at the entire cohort (Table 5.3d) or as hypertensive and normotensive sub-groups.

Plasma corticosteroid relationships

There was no significant effect of B1 genotype on mean absolute differences of plasma corticosteroid levels according to diurnal variation/dexamethasone/ACTH.

However, when mean levels of corticosteroids were compared according to genotype, plasma ACTH was higher in TTGG group after 1mg of dexamethasone ($p<0.05$). This also resulted in a lower F/ACTH ratio in this genotype group although this did not quite reach statistical significance (p 0.07) (Figure 5.3h).

Aldosterone and cortisol correlated closely in TTGG subjects ($r=0.563$, $p=0.02$) but not in GGAA ($r=-0.284$, $p=0.3$) whilst there was no correlation between aldosterone and ACTH in either genotype group (GGAA $p=0.08$, TTGG $p=0.4$)(Figure 5.3i).

5.4 Discussion

The –344T and IC alleles of CYP11B2 have previously been shown to associate with apparent reduced 11ßhydroxylase efficiency (increased deoxycortisol/cortisol ratio) in both normotensives(350) and in a large

	GGAA n=21	TTGG n=17	p
Age (years)	38	39.5	0.687
BMI (kg/m^2)	27	27	0.972
SBP (mm/Hg)	131	139	0.144
DBP (mm/Hg)	81	82	0.141

Table 5.3d. Demographic information on all study subjects separated by SNP identified in promoter of CYP11B1

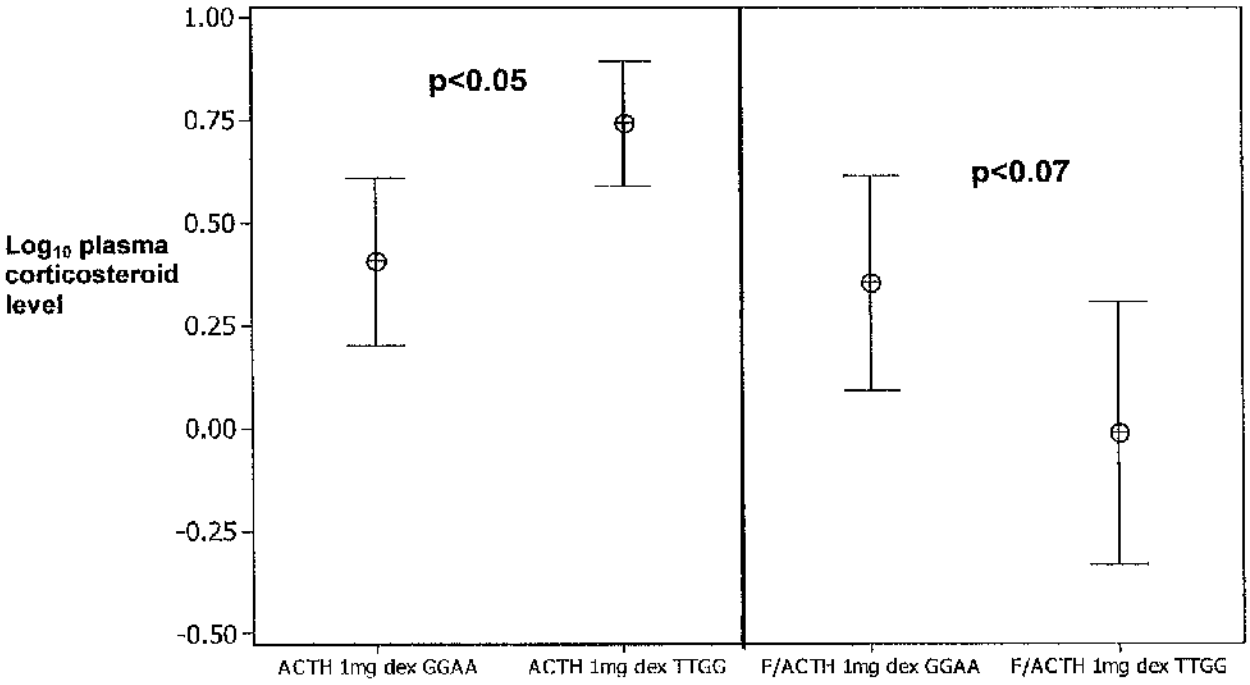


Figure 5.3h. Effect of CYP11B1 genotype on mean plasma corticosteroid levels (log transformed).

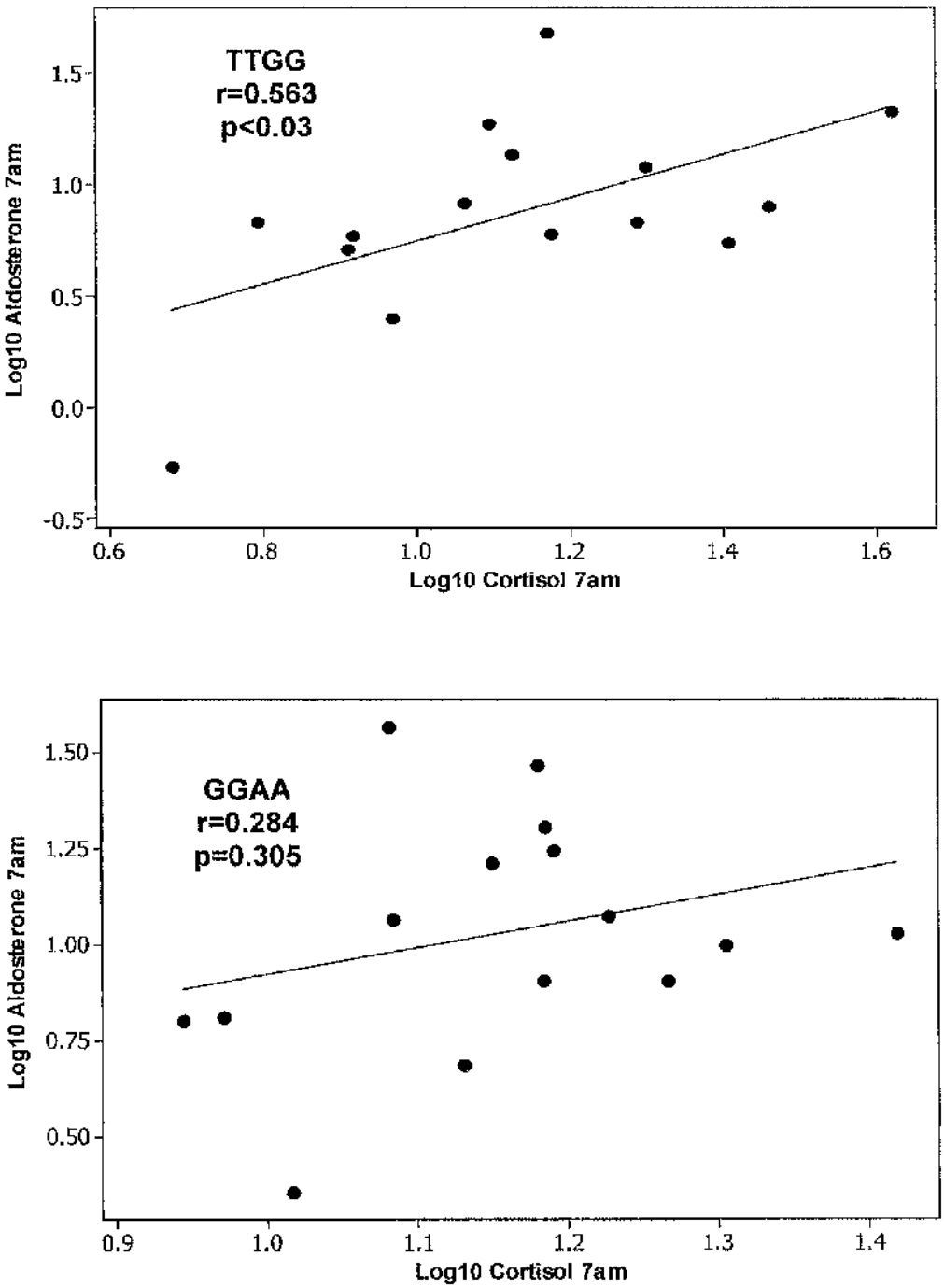


Figure 5.3i. Correlation relationships in all subjects between early morning plasma cortisol and aldosterone levels according to CYP11B1 genotype

hypertensive cohort (chapter 4). The current data indicate that these variants may be associated with altered hypothalamic-pituitary-adrenal axis activity and aldosterone regulation in subjects with hypertension and normotensive controls.

As a consequence of a tightly regulated negative feedback system, any reduction, however slight, in cortisol levels should result in an increase in adrenal stimulation by its major trophin, ACTH. In turn, this subtle increase in ACTH secretion should make it more resistant to suppression by exogenous glucocorticoid.

In support of this hypothesis, early morning plasma ACTH levels after 1mg of dexamethasone were consistently higher in individuals homozygous for the –344T or conversion alleles and subjects with the T/conv haplotype. This effect was independent of blood pressure. Moreover, there was no genotype-dependent difference in plasma cortisol levels after dexamethasone suppression, so that a significant decrease in cortisol/ACTH ratios in TT, conv/conv and T/conv subjects was seen (figures 5.3e & 5.3f). Thus, this provides evidence that the –344T ± conversion alleles are associated with an alteration in the cortisol/ACTH relationship and the feedback regulation of ACTH.

No apparent difference was noted in aldosterone or aldosterone/PRC ratio between genotype groups. However, in studying untreated hypertensives recruitment was usually limited to young patients with relatively mild hypertension. Table 5.4a highlights the significant differences in age and severity of blood pressure between this cohort and the severely hypertensive subjects studied in the BRIGHT study. Increasing age is an important risk

	BRIGHT n=511	UNTREATED n=55	p
Age (years)	63 (9)	42 (12)	<0.001
BMI (kg/m^2)	28 (3.8)	29 (4.5)	0.08
SBP (mm/Hg)	173 (24)	147 (10)	<0.001
DBP (mm/Hg)	104 (9)	90 (7)	<0.001

Table 5.4a Comparison of hypertensive cohorts studied here and in the BRIGHT study.
Values are means ±SEM

factor for hypertension and these individuals are more likely to exhibit 'low-renin' hypertension with low plasma renin activity (PRA), which does not respond normally to sodium restriction(83;336). Such subjects also respond optimally to diuretic treatment consistent with the previous findings that elderly hypertensive patients (>55 years) tend to have higher body sodium content than younger (<35 years) subjects with hypertension(379). Thus, a lack of difference in aldosterone status could be explained by the relatively young age of my cohort. Moreover, the hypothesis under investigation is that chronic subtle changes in adrenal corticosteroid production are amplified with time and other environmental factors. Thus, studies that compare groups of patients of different ages and that follow up patients over time are clearly indicated.

Despite the absence of a clear difference in aldosterone levels, these data do provide some indirect evidence of a genotype-dependent effect on the regulation of aldosterone. Specifically, diurnal variation in basal aldosterone levels was only seen in TT subjects. This is usually typical of cortisol production and is classically associated with the regulation of aldosterone production in the majority of patients with Conn's syndrome and GRA, when aldosterone production is predominantly responsive to ACTH(212;380). Furthermore, plasma cortisol and aldosterone levels correlate strongly in TT subjects implying a common regulatory factor such as ACTH. This finding is similar to data from analysis from the BRIGHT study (chapter 4). In this severely hypertensive cohort urinary excretion of THAldo correlated closely with total cortisol excretion in subjects homozygous for the –344 T allele. Although ACTH has been reported to cause only short-term stimulation of

aldosterone production(354), these results provide indirect evidence that it plays a more chronic role in the regulation of aldosterone production in TT individuals.

The concept that variation in a gene (CYP11B2) encoding one enzyme (aldosterone synthase) can affect the efficacy of another (11β-hydroxylase) initially seems paradoxical. However, section 1.6.5 illustrates that these genes are highly homologous and lie close together on chromosome 8. Thus, a key hypothesis under investigation is that the -344C/T and intron conversion alleles of CYP11B2 are in LD with functional variants in CYP11B1. Two candidate SNPs in the 5' promoter of CYP11B1(-1888 G/T and -1858 A/G) have been identified by my colleague (377). These are in linkage with the T/conv haplotype of CYP11B2 and associate with reduced 11β-hydroxylase efficiency (increased THS/Total F ratio) in the BRIGHT cohort. Moreover, in vitro studies using promoter constructs altered by site-directed mutagenesis to contain the novel alleles and transfected into murine adrenal cells have shown a significant reduction in the number of mRNA transcripts in response to ACTH or forskolin when compared to constructs containing the wild type alleles. Thus, these SNPs are plausible candidates to explain the well established but poorly understood observation of impaired 11β-hydroxylase activity in normotensives (350) and hypertensives (353) (chapter 4) seen in association with -344 T ± intron conversion alleles.

The effects of these novel SNPs on HPA axis activity and aldosterone production were studied in this population of hypertensive subjects and normotensive controls. Similar changes to those seen in -344TT subjects in the cortisol/ACTH relationship were observed in TTGG individuals.

Specifically, plasma ACTH was less suppressible by 1mg of ACTH (Figure 5.3h) leading to a decrease in the cortisol/ACTH ratio in TTGG compared to subjects with the wild type alleles. Additionally, plasma aldosterone and cortisol correlated strongly in TTGG and not GGAA individuals (figure 5.3i), again suggesting genotype-dependent co-regulation of these steroids. Testing for the phenotype associated with these novel CYP11B1 SNPs was neither the primary aim nor the major inclusion criterion of this study, and these data are limited by small patient numbers. Nonetheless the results do suggest an effect of –1888T and –1858G on HPA axis activity and aldosterone regulation.

There was no significant difference in the suppressibility of cortisol or aldosterone by dexamethasone or its responsiveness to stimulation with ACTH according to CYP11B2 or CYP11B1 genotype. This may simply be a reflection of limited patient numbers in each genotype/blood pressure group giving insufficient power to detect such subtle changes in corticosteroid responsiveness. Moreover, our hypothesis not only speculates that very subtle changes in HPA axis activity occur in response to variation in CYP11B locus but that these changes are amplified over time with increased exposure to environmental factors such as salt intake and other permissive factors to result in the final phenotype-hypertension with relative aldosterone excess. Thus, in selecting untreated hypertensives, we have been limited to a relatively young patient group where this phenotype has not been completely established.

In summary, this study has shown that variation in CYP11B2 is associated with alteration in the cortisol/ACTH relationship and provides evidence that

ACTH contributes to the regulation of aldosterone production in subjects with –344T and intron conversion alleles. In addition, a similar phenotype is found in subjects with SNPs in the promoter of CYP11B1 tightly linked to T/Conv haplotype of CYP11B2. It is variation in this gene that may lead to altered 11ß-hydroxylation efficiency and ultimately a subtle change in the cortisol/ACTH relationship demonstrated in this study. Further work, to define more closely the corticosteroid phenotype seen in association with CYP11B1 promoter variants and to examine genotype-phenotype relationships in an older, more severely hypertensive population is warranted.

Chapter 6: The Origin of 18-Oxocortisol and 18-Hydroxycortisol in Normal Human Subjects

6.1 Introduction

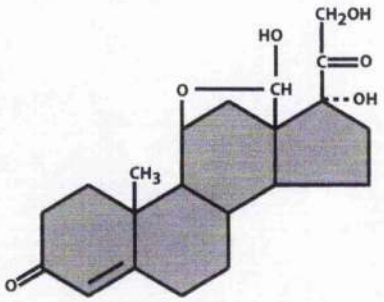
The adrenal cortex is subdivided into 3 distinct anatomical zones: zona glomerulosa, zona fasciculata and zona reticularis (described in detail in section 1.4.2). These zones are also functionally discrete due to selective expression of key enzymes in the synthesis of the principal human corticosteroids. Hence, the zona glomerulosa expresses aldosterone synthase and so is the site of aldosterone production whilst 11ß-hydroxylase is located within the zona fasciculata making this the principal location of cortisol production. Alteration in corticosteroid production by the adrenal gland is a key intermediate phenotype in a significant number of hypertensive individuals identified by a raised ARR. Thus, it is relevant to consider in more detail the precise mechanism of synthesis of adrenal corticosteroids classically found in subtypes of PA.

The major corticosteroid products of the adrenal cortex are aldosterone, cortisol and adrenal androgens. However, the adrenal gland synthesises a number of lesser-known steroid compounds. In 1982, Chu & Ulick isolated a novel compound from the urine of patients with PA, which they identified as the 20,18-hemiketal form of 11β, 17α, 18,21-tetrahydroxy-4-pregnene-3, 20-dione (18-hydroxycortisol, ≡ 17α-hydroxyaldosterone)(381). In 1983, they isolated another C-18-oxygenated steroid after incubating cortisol with bullfrog

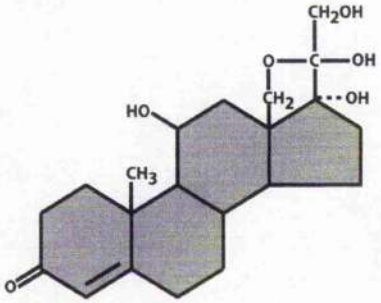
interrenal gland; this was identified as 11β, 17α, 21-trihydroxy-3, 20-diketo-4-pregnene-18-al (18-oxo-cortisol)(382).

These compounds have been subsequently termed 'hybrid' corticosteroids with both zona glomerulosa (i.e. 18-oxygenation) and zona fasciculata (i.e. cortisol-like, hydroxylation at C-17) characteristics (211;212;383) (Figure 6.1a). Their biological significance is unclear; 18-hydroxycortisol has no mineralocorticoid activity and negligible glucocorticoid activity(384), whereas 18-oxocortisol is a full mineralocorticoid agonist (with 1% of the biological activity of aldosterone)(385) with mild glucocorticoid properties.(386)

18-Oxocortisol and 18-hydroxycortisol were previously only thought to be found in hypertensive disorders with aldosterone excess, specifically Conn's syndrome and GRA where they still play a diagnostic role(387;388). However, both compounds have now been identified in normal subjects (363;389). In the normal human adrenal cortex, 18-hydroxycortisol may be produced in the zona fasciculata from cortisol by the action of 11β-hydroxylase (390). Apparent control of its levels by ACTH is further evidence of this (391). *In vitro* studies using hamster lung cells stably transfected with CYP11B1 or CYP11B2 suggest that 18-hydroxycortisol can be produced by the action of either enzyme on cortisol(392). However, 18-oxocortisol synthesis requires the additional '18-oxidation' step, the capacity for which is restricted to aldosterone synthase in the normal zona glomerulosa, and so cannot be produced by 11ß-hydroxylase. These studies confirm that 18-oxocortisol can only be produced by the action of aldosterone synthase on cortisol (Figure 6.1b).



18-Oxocortisol



18-Hydroxycortisol

Figure 6.1a Chemical structures of 18-oxocortisol and 18-hydroxycortisol

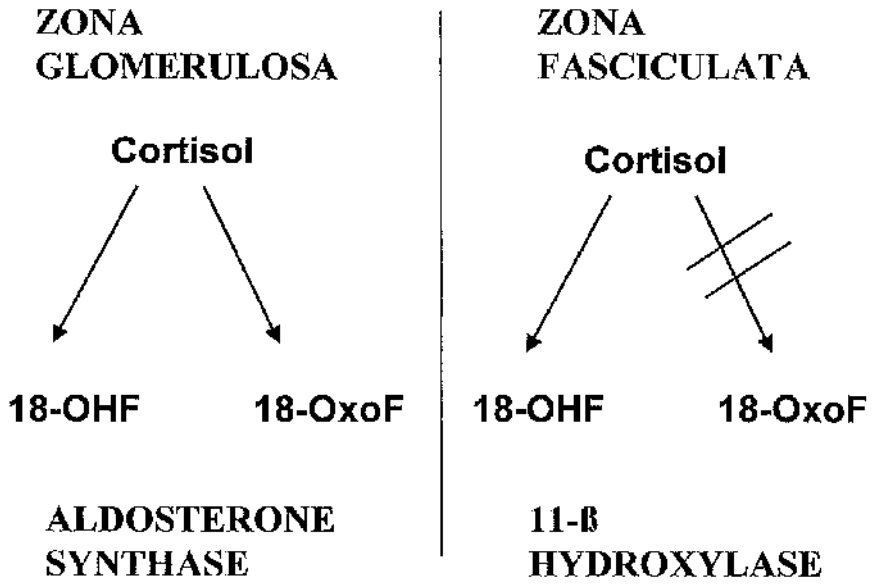


Figure 6.1b Schemata depicting production of 18-oxo and 18-hydroxycortisol within the human adrenal cortex.

However, cortisol is produced within the zonae fasciculata and reticularis and should not, under normal circumstances, gain access to the zona glomerulosa since blood flow through the adrenal cortex is centripetal towards the medulla. In PA due to GRA or Conn's adenoma, normal adrenal architecture is disrupted and so locally produced cortisol is exposed to zona glomerulosa aldosterone synthase. However, the origin of this corticosteroid in normal subjects remains obscure. One possibility is that it is produced by the action of aldosterone synthase on peripheral, recirculating, cortisol.

Thus, the aim of this study was to determine, more precisely, the origin of 18-hydroxycortisol and especially 18-oxocortisol in normal subjects; in particular, to explore the hypothesis that these steroids can be synthesised from circulating, rather than locally produced, cortisol.

6.2 Methods:

6.2.1 Study subjects:

Full details of recruitment of subjects and study protocol are found in section 2.4.1. Briefly, eight healthy men were recruited by local advertisement. There were three study phases each separated by one month (Table 6.2a) and a 24-hour urine collection was performed on day three.

In addition, 24-hour urine samples were obtained from six hypoadrenal subjects stable on glucocorticoid replacement therapy.

6.2.2 Steroid analysis:

Urinary steroid excretion rates were measured by gas chromatography-mass spectrometry using the methods of Shackleton(373) and Palermo et al(364) with minor modifications as described in chapter 2(section 2.4.2).

Phase	Protocol
1	No corticosteroid treatment
2	Dexamethasone only (1 mg twice daily for 3 days)
3	Dexamethasone (as above) plus hydrocortisone (20 mg twice daily on day 3)

Table 6.2a Study protocol for 8 healthy male volunteers.
24-hour urine collections were performed in all stages; in phases 2 and 3 this was done on day 3.

6.2.3 Statistical analysis:

Urinary steroid excretion rates were compared by one-way analysis of variance (ANOVA).

6.3 Results:

6.3.1 Normal volunteers

Average 24-hour urinary excretion rates of free cortisol, free cortisone, 18-hydroxycortisol and 18-oxocortisol are shown in Figure 6.3a. Comparisons between urinary excretion rates of these steroids are also presented in Table 6.3a. Urinary excretion rates of cortisol, cortisone, and 18-oxocortisol were significantly decreased in volunteers who were receiving dexamethasone compared with no treatment. They were significantly increased when the volunteers were taking hydrocortisone and dexamethasone together compared with dexamethasone alone. Using the sum of cortisol and cortisone excretion rates as an index of cortisol 'status', 18-oxocortisol excretion rate correlated closely with this ($r=0.914$, $p<0.01$) (Figure 6.3b) while that of 18-hydroxycortisol did not ($r=0.216$; $p=0.312$).

6.3.2 Hypoadrenal subjects

Cortisol and cortisone were found in high concentration in the urine of the 5 patients taking hydrocortisone replacement therapy (Figure 6.3c). Low concentrations of 18-hydroxycortisol, at the limit of detection, were also found

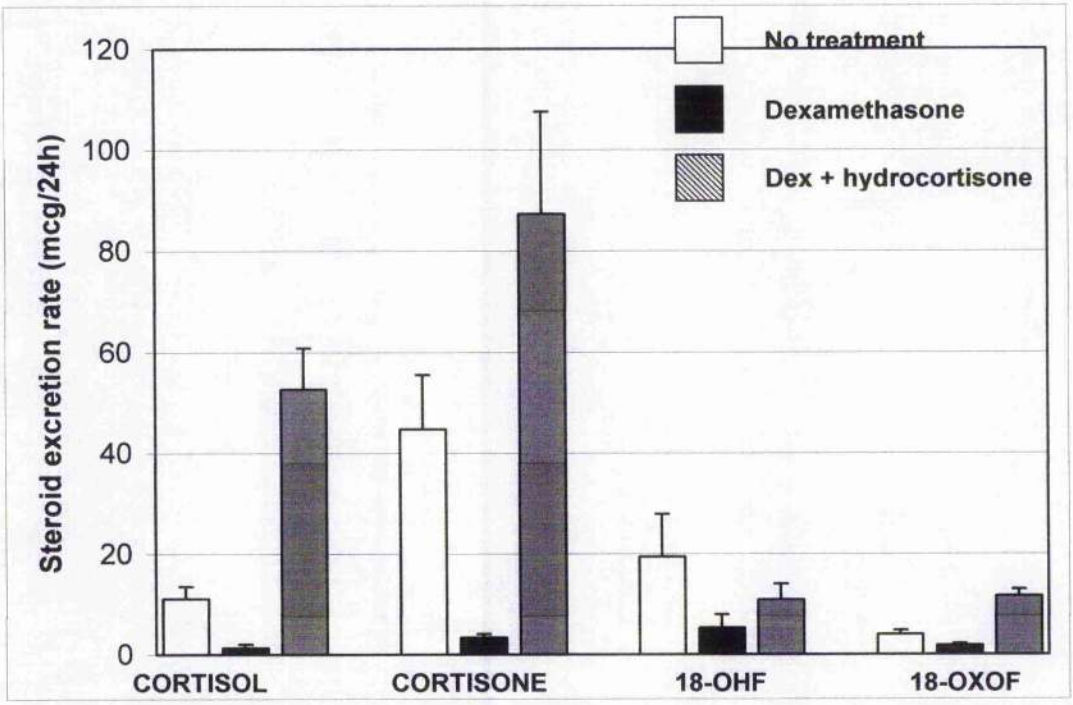


Figure 6.3a. Urinary corticosteroid excretion rates (mean +SEM) under differing conditions in 8 normal subjects

Steroid (mcg/24h)	No Treatment	Dexamethasone + Cortisol	Dexamethasone	p
Cortisol	11.2 ± 2.5	52.6 ± 8.2	1.3 ± 0.7	<0.0001
Cortisone	44.7± 10.8	87.3 ± 20.2	3.4 ± 0.7	0.001
18-OH-F	19.4 ± 8.5	10.9 ± 3.14	5.3 ± 2.7	0.205
18-Oxo-F	4.04 ± 0.8	11.6 ± 1.4	1.8 ± 0.4	<0.0001

Table 6.3a. Urinary steroid metabolite excretion rates. 8 normal male subjects were studied untreated, under dexamethasone suppression and on dexamethasone plus cortisol. The results were compared by one-way analysis of variance (ANOVA, unstacked)

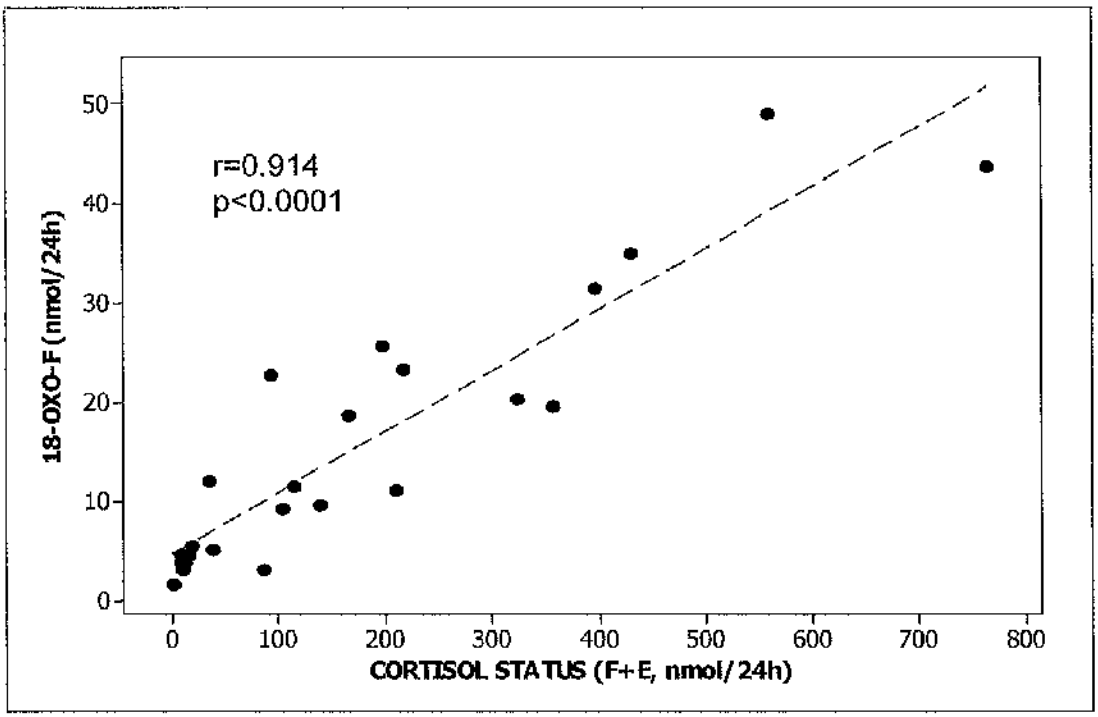


Figure 6.3b. Correlation between 'cortisol status' (sum of F +E) with 18-oxocortisol excretion in 8 healthy volunteers

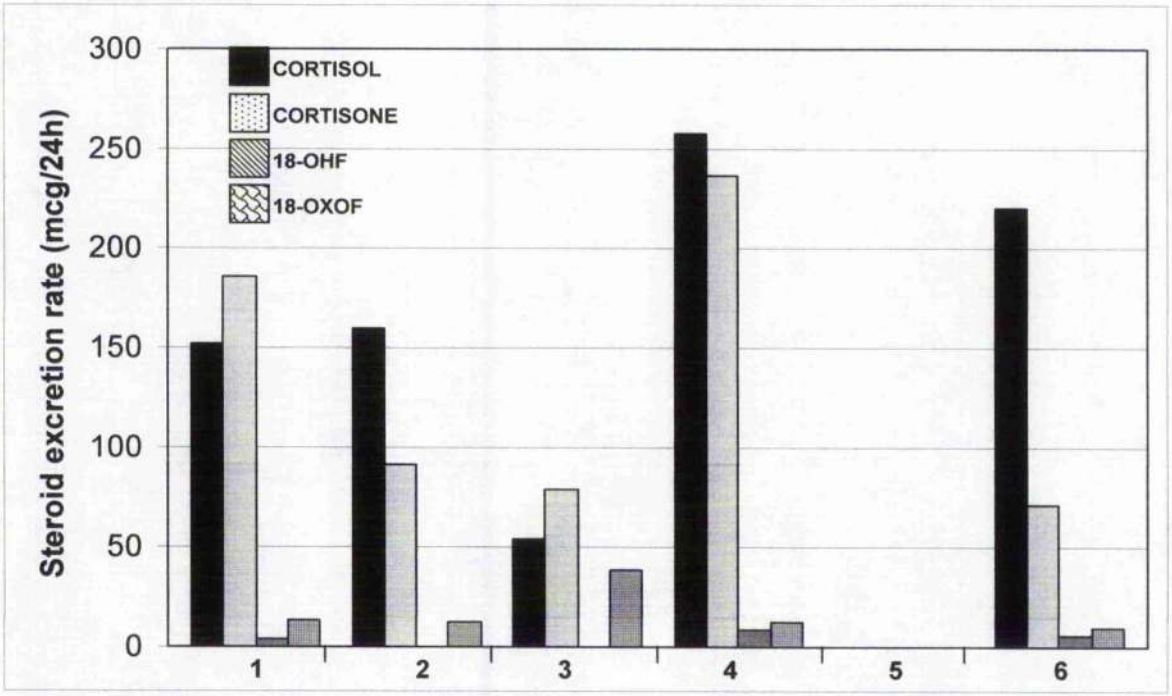


Figure 6.3c. Urinary corticosteroid patterns in 6 patients with hypoadrenalism on corticosteroid replacement therapy

in the urine of three patients who were taking hydrocortisone. 18-Oxocortisol was also easily detectable in all patients who were taking exogenous cortisol. As expected, no cortisol metabolites were detected in the patient receiving dexamethasone.

6.4 Discussion:

These data illustrate for the first time that the hybrid corticosteroids, 18-hydroxycortisol and 18-oxocortisol can be synthesised from recirculating, peripheral sources of cortisol. In normal subjects, suppressing the adrenal source of cortisol by dexamethasone reduced 18-hydroxycortisol and 18-oxo-cortisol excretion rates. Subsequent administration of hydrocortisone to subjects simultaneously with dexamethasone increased excretion rates of both compounds. In this hydrocortisone + dexamethasone phase, 18-oxo-cortisol excretion rate exceeded control (untreated) levels but that of 18-hydroxycortisol did not achieve control levels. The cortisol status achieved, of which the sum of cortisol and cortisone excretion rates is a convenient index, was higher during cortisol + dexamethasone than during the control (untreated) period. This suggests that, in this extreme situation, the zona glomerulosa used extra-adrenal cortisol to make 18-oxo-cortisol and 18-hydroxycortisol. 11β-Hydroxylase production of 18-hydroxycortisol is probably less important here because extra-adrenal cortisol is less accessible to the zona fasciculata than to the zona glomerulosa. Moreover, previous in vitro work has demonstrated that 11β-hydroxylase is less efficient than aldosterone synthase at 18-hydroxylation(393).

In patients with primary adrenal insufficiency, traces of 18-oxocortisol and 18-hydroxycortisol were found in the urine in those on hydrocortisone treatment but none was detectable in the patient on dexamethasone treatment. Since endogenous adrenal cortisol production is absent, circulating cortisol from replacement therapy must have been the substrate for the production of these steroids. In the one adrenalectomised patient, it is likely that they were synthesised from cortisol at extra-adrenal sites; in the other four patients, a proportion might also have arisen from perfusion by plasma of residual functional zona glomerulosa tissue.

In human subjects, the only source of 18-oxo-cortisol is likely to be aldosterone synthase using peripheral cortisol as a substrate. Its excretion rate was significantly increased by exogenous cortisol treatment, suggesting that cortisol reperfusing the zona glomerulosa is a significant source of this steroid. However, aldosterone synthase and 11β-hydroxylase are expressed in organs other than the adrenal glands(394-396) albeit at much lower rates; extra-adrenal production cannot be ruled out. The close correlation between urinary cortisol metabolites and 18-oxocortisol levels in the volunteer study supports the conclusion that conversion of circulating cortisol is a major source of this steroid. Other studies also support the dependence of 18-oxo- and 18-hydroxy-cortisol on cortisol availability. The plasma levels of both compounds correlate with that of cortisol and not aldosterone (389) and, although the renin-angiotensin system may have some influence(397), the major control factor is ACTH(397;398). Dexamethasone suppressed the levels of both compounds in the current study and in others (399).

In normal subjects, cortisol for 18-hydroxycortisol or 18-oxocortisol synthesis may derive either by reperfusion from the circulation or from local tissue production. Cortisol is produced in the zona fasciculata but aldosterone synthase is essential at least to 18-oxocortisol synthesis. Direct penetration of zona fasciculata-synthesised cortisol to the glomerulosa is unlikely because the blood supply of the adrenal gland is centripetal. There are, however, plausible alternative explanations. A transitional anatomical zone was identified between the fasciculata and glomerulosa in rats (400;401) but not so far humans. In the human adrenal gland, zona glomerulosa tissue may penetrate the zona fasciculata along the surfaces of small blood vessels, providing a potentially extensive interface sufficient for cortisol to enter glomerulosa cells. Finally, aldosterone might be converted to 18-oxo-cortisol by 17α-hydroxylase in the zona fasciculata. Early experiments(399;402) showed that 18-hydroxycortisol and 18-oxo-cortisol are produced in beef adrenal outer slices. The authors suggested that aldosterone synthase and 17α-hydroxylase coexist in these outer slices, allowing synthesis of these two steroids, and inferred that 17α-hydroxylase could convert aldosterone into 18-oxo-cortisol and 18-hydroxycorticosterone into 18-hydroxycortisol. Interpretation is difficult because *in vitro* incubation of mixed cell slices destroys the strict compartmentalisation of enzymes and substrates maintained *in vivo*. There is as yet no similar evidence in human tissue.

In summary, these studies demonstrate that cortisol can be converted to 18-hydroxycortisol in both the zona fasciculata and zona glomerulosa by 11β-hydroxylase and aldosterone synthase respectively. Since cortisol synthesis is restricted to the zona fasciculata *in vivo*, it is likely that most 18-

hydroxycortisol in normal subjects is produced by 11β-hydroxylase. The lack of correlation between urinary excretion rate of 18-hydroxycortisol and 'cortisol status' in normal volunteers supports this. In contrast, 18-oxo-cortisol is a unique product of aldosterone synthase. The source of cortisol in normal subjects may be circulating cortisol, zona fasciculata cortisol diffusing across the interphase between glomerulosa and fasciculata or a combination of both.

Chapter 7: Endogenous Corticosteroid Production in Subjects after Bilateral Adrenalectomy- Evidence of Extra-adrenal Aldosterone Synthase Activity?

7.1 Introduction:

Chapter 6 demonstrated that the hybrid corticosteroids, 18-oxo and 18-hydroxycortisol could be synthesized by the human adrenal cortex using recirculating (exogenous) cortisol as a substrate. This study, in normal volunteers, also concluded that 18-hydroxycortisol is normally produced by the action of 11ß-hydroxylase on locally produced cortisol (i.e. within the zona glomerulosa), whilst 18-oxocortisol is the exclusive product from the action of aldosterone synthase on cortisol (Figure 7.1a).

Given the dependence, in particular, of the production of 18-oxocortisol on aldosterone synthase, it was surprising that both steroids could be found in easily detectable amounts in patients with hypoadrenalism and probably absent adrenal function due to autoimmune adrenal cortex destruction (Addison's Disease) (Figure 6.3c). Moreover, 18-oxocortisol was readily detectable in one patient with bilateral adrenalectomy (and hence absent adrenal cortex tissue). Thus, these data have raised the possibility that extra-adrenal aldosterone synthase could contribute to the synthesis of 18-oxo and 18-hydroxycortisol as well as aldosterone. A number of studies by our group and others have already demonstrated expression of aldosterone synthase in extra-adrenal sites such as brain(258;403), blood vessels(404) and, more controversially, the heart(259;260). However, the exact functional significance and regulation of this remains unclear.

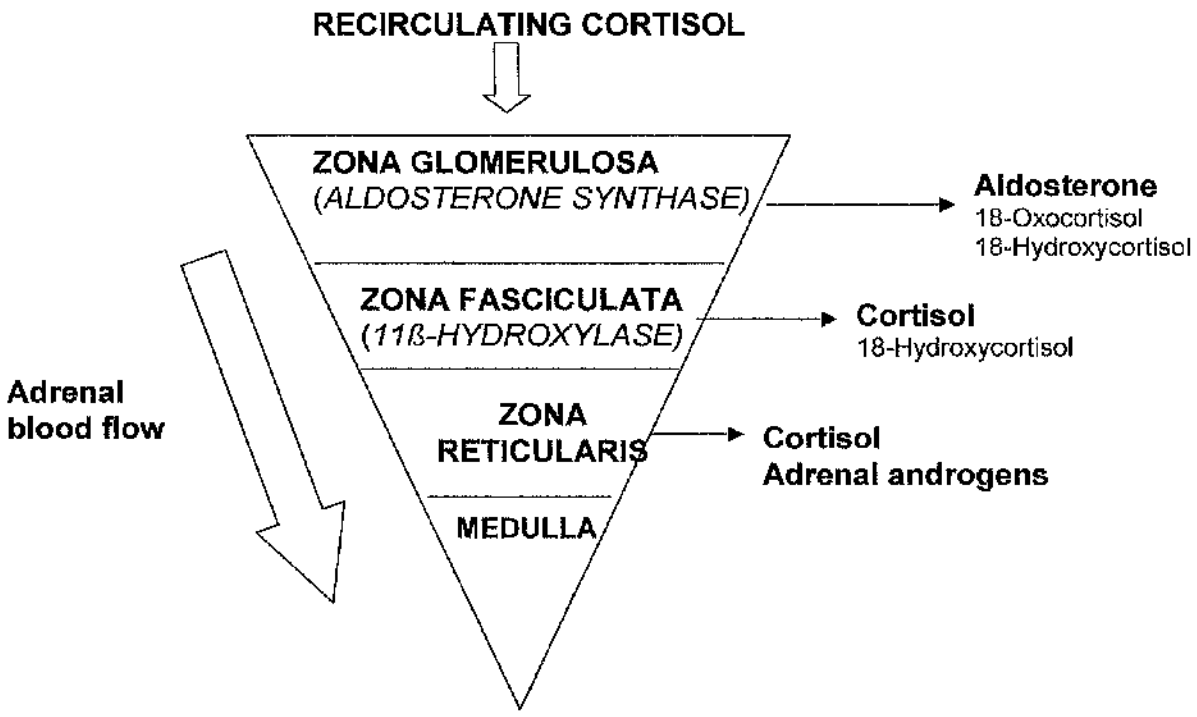


Figure 7.1a Schematic representation of functional and anatomical subdivisions of the adrenal cortex.
18-Oxocortisol is produced by the action of aldosterone synthase on recirculating cortisol whilst 18-hydroxycortisol is mainly produced by action of 11ß-hydroxylase on locally available cortisol in the zona fasciculata.

In this study, the hypothesis under investigation is that significant capacity to produce aldosterone and other adrenal steroids in a paracrine manner may exist. Using 18-oxocortisol as a marker of aldosterone synthase activity, the production of this and other adrenal steroids was studied in subjects with absent adrenal function after bilateral adrenalectomy.

7.2 Methods:

7.2.1 Study subjects:

10 patients (3 male) were recruited from the endocrine clinics of the Western Infirmary, Glasgow. All had undergone bilateral adrenalectomy at least one year previously and were stable on glucocorticoid (hydrocortisone; 10-30mg/day) and mineralocorticoid (fludrocortisone; 50-100 micrograms/day) replacement. Patient details are listed in table 7.2a. Local ethical committee approval for the study was obtained from the North Glasgow Hospitals University NHS Trust and all patients gave informed, written consent.

7.2.2 Study protocol:

This is outlined in chapter 2 (section 2.5.1) and summarized in table 7.2b.

7.2.3 Corticosteroid analysis:

24-Hour urine samples were collected into a plain container and then aliquots stored at -20°C. Urinary steroid excretion rates were measured by gas chromatography-mass spectrometry using the methods of Shackleton and Palermo et al with minor modifications(364) outlined in chapter 2 (section 2.11).

PATIENT	SEX	AGE (years)	BP (mm/Hg)	TIME SINCE SURGERY	REASON FOR SURGERY
1	F	40	95/57	11 years	Cushings disease
2	F	43	130/82	14 years	Cushings disease
3	M	47	118/66	21 years	Cushings disease
4	F	64	119/80	12 years	Cushings disease
5	F	63	141/96	3 years	Phaeochromocytoma
6	F	64	138/76	10 years	Cushings disease
7	M	52	123/68	22 years	Cushings disease
8	F	65	116/67	3 years	Adrenal hyperplasia
9	M	58	117/86	1 year	Cushings disease
10	F	57	138/76	2 years	Phaeochromocytoma

Table 7.2a Details of study patients

Phase	Protocol
1	Hydrocortisone 30mg daily (20mg mane, 10mg nocte)
2	Dexamethasone only (1 mg twice daily for 3 days)
3	Dexamethasone (as above) plus hydrocortisone (30mg in divided doses on day 3)

Table 7.2b. Study protocol for 10 adrenalectomised patients
Each phase lasted 3 days
24-hour urine collections were performed in all phases, beginning on day 3

Total cortisol was defined as the sum of tetrahydrocortisol (THF), allotetrahydrocortisol (aTHF) and tetrahydrocortisone (THE).

Urinary corticosteroid excretion rates were analysed by one-way analysis of variance (ANOVA).

7.3 Results:

Cortisol:

Total cortisol excretion rates during the three study phases are illustrated in figure 7.3a. As expected, cortisol excretion fell to almost undetectable levels when on dexamethasone replacement (phase 2) but was restored when hydrocortisone was given in conjunction with dexamethasone (phase 3).

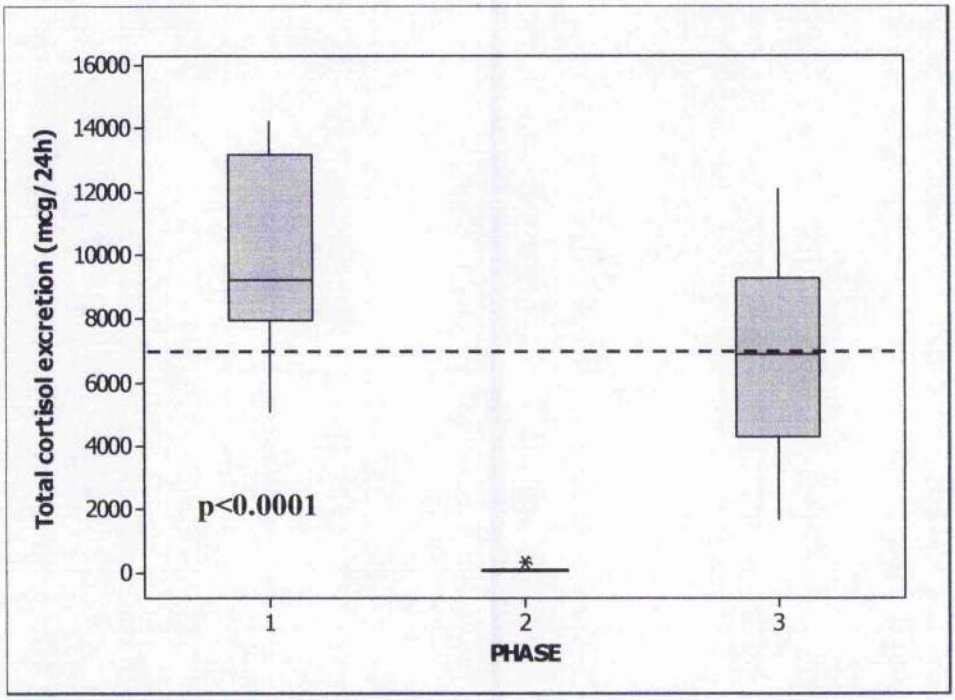
Aldosterone:

The principal urinary metabolite of aldosterone is tetrahydroaldosterone (THAldo). Figure 7.3b demonstrates that THAldo was easily detectable in all adrenalectomised patients. There was no significant difference in its urinary excretion rates during each of the study phases.

18-Hydroxycortisol/18-Oxocortisol:

18-Hydroxycortisol and 18-oxocortisol excretion rates were easily detectable in phase 1 and suppressed significantly in phase 2 illustrating their dependence upon cortisol as a substrate (Figure 7.3c & d). Excretion rates were restored in phase 3 (dexamethasone + hydrocortisone) suggesting that production of these hybrid corticosteroids was not acutely ACTH-dependent.

(a) Excretion of total cortisol metabolites



(b) Excretion of tetrahydroaldosterone:

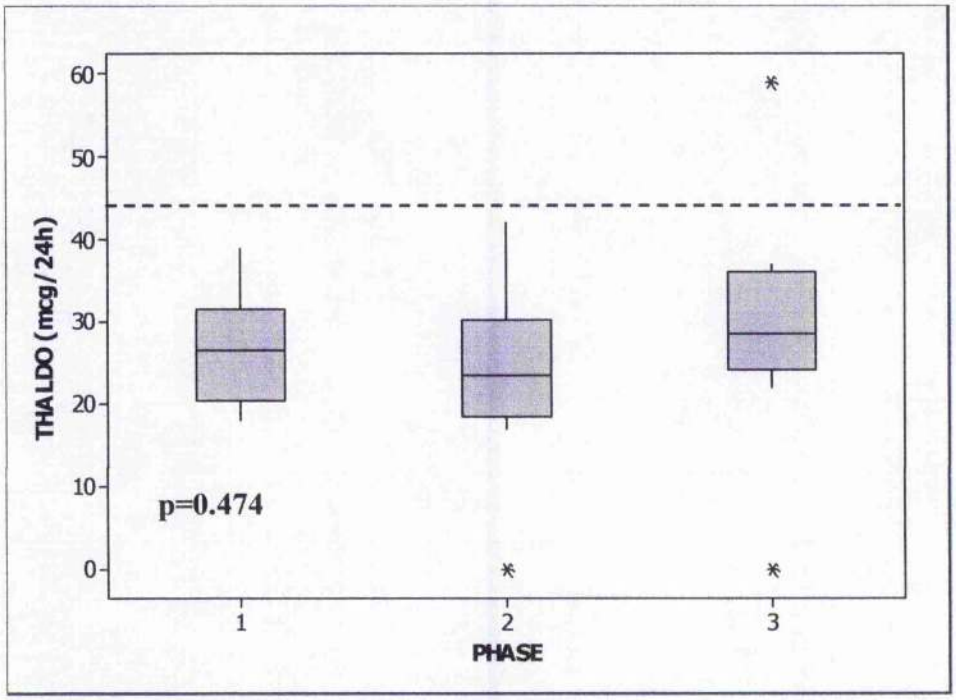
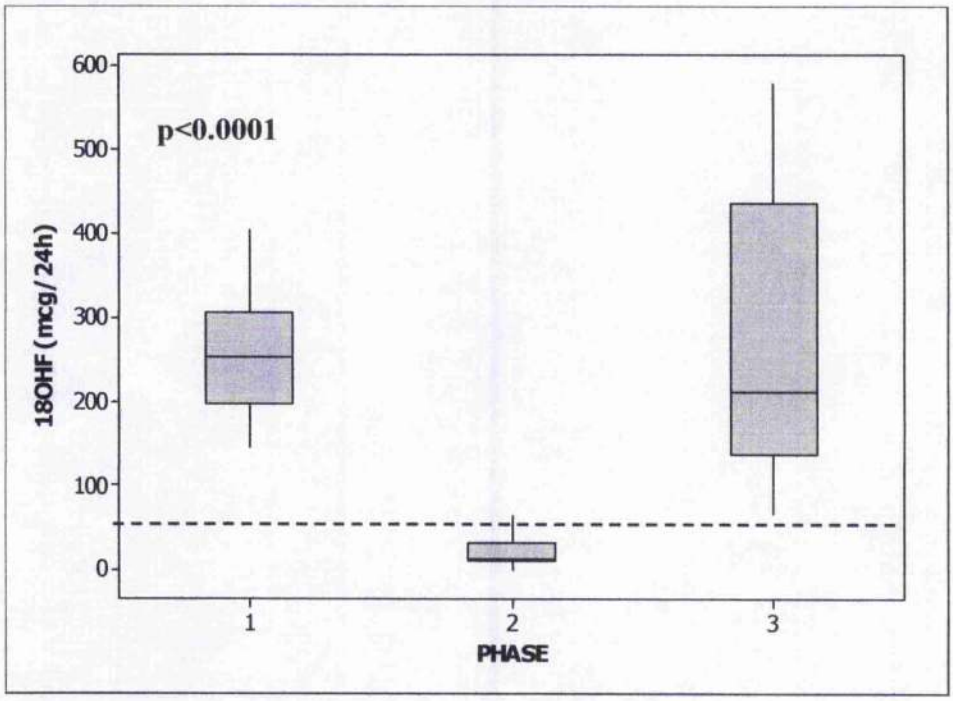


Figure 7.3(a-d) Urinary excretion rates of corticosteroids in 10 adrenalectomised subjects during all 3 study phases.

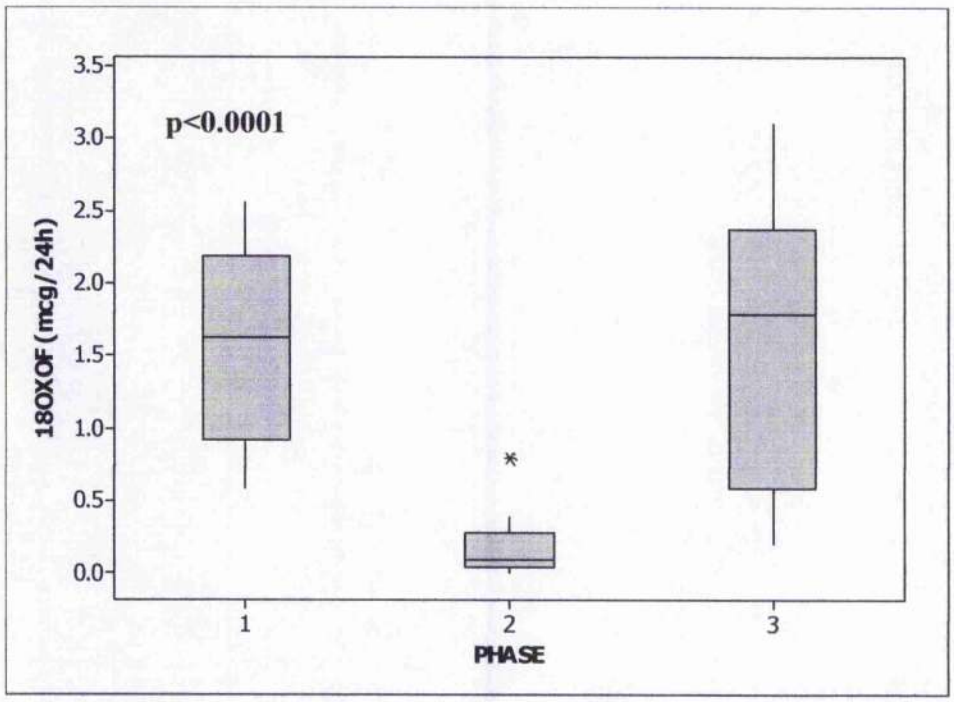
Day 1- hydrocortisone alone
Day 2-dexamethasone alone
Day 3-dexamethasone + hydrocortisone

In a, b and c, dashed line represents median normal values (from urine of 100 healthy volunteers).

(c) Excretion of 18-hydroxycortisol



(d) Excretion of 18-oxocortisol



7.4 Discussion:

These novel data demonstrate that corticosteroids usually produced by the adrenal cortex can be detected in subjects with apparently no adrenal tissue. In particular, the major urinary metabolite of aldosterone (THAldo) is clearly present, albeit at low levels, and its production is not ACTH-dependent.

18-Oxocortisol, the exclusive product of aldosterone synthase, is also easily detectable. Its production in the subjects in this study was dependent on availability of exogenous cortisol as a substrate and appeared to be independent of acute ACTH effects. The presence of aldosterone and 18-oxocortisol in adrenalectomised subjects is consistent either with significant extra-adrenal aldosterone synthase resulting in production of adrenal steroids, which may act locally in a paracrine manner, or significant re-growth of adrenocortical tissue.

Extra-adrenal conversion of cortisol to 18-oxocortisol implies that circulating cortisol is available to access aldosterone synthase within the systemic circulation. The concept that extensive modification of steroids can occur in this way is well established from the studies of cortisol to cortisone interconversion in a wide range of tissues (405). The notion that enzymes usually found within the adrenal gland might have a significant systemic distribution is also established. Expression of a number of corticosteroidogenic enzymes has been previously demonstrated in several extra-adrenal tissues, including central nervous system (CNS), vascular and renal tissue. CYP11B1 (11ß-hydroxylase) and CYP11B2 (aldosterone synthase) transcripts have been isolated from human pulmonary artery(406), aorta(407), human vascular endothelial cells (HUVEC)(408) as well as rat

mesenteric artery(409). Vascular CYP11B transcript levels also appear to be regulated in a similar manner to the adrenal cortex; in HUVEC, angiotensin II and potassium increased CYP11B2 but not CYP11B1 mRNA levels whilst ACTH raised CYP11B1 but not CYP11B2 mRNA(408). In human brain, CYP11B1 and CYP11B2 transcripts are widely distributed along with other key steroidogenic enzymes, suggesting an autocrine or paracrine model of corticosteroid action in the CNS(257).

Cardiac expression of CYP11B transcripts is more controversial. The most recent data from my group used a highly sensitive quantitative RT-PCR method to measure CYP11B1 and CYP11B2 mRNA levels in cardiac tissue of several rat models of cardiovascular pathology (congestive heart failure, genetic hypertension and cardiac hypertrophy). Cardiac CYP11B1 and CYP11B2 mRNA transcript levels from all groups were never greater than 10^2 copies per microgram of total RNA and therefore too low to be detected reproducibly(262). In human cardiac tissue, CYP11B transcripts have been reported in fetal organs(407) and in the failing heart(410) but not in normal adult human heart. Moreover, quantitative analysis of the transcript levels in heart samples estimated them, where present, to be 100-10000-fold lower than those in the adrenal gland(407).

Whilst extra-adrenal sites remain the most likely source of aldosterone synthase in these subjects, it is also possible that small amounts of functional adrenal tissue remain or re-grow after surgery. There is evidence of adrenal re-growth in salt fed adrenalectomised rats that can develop 'adrenal regeneration hypertension' with evidence of mineralocorticoid excess(411). However, in this poorly understood model, bilateral adrenalectomy was

incomplete with enucleation of the left adrenal gland (leaving an intact capsule and zona glomerulosa)(412). The presence of adrenal tissue in humans after bilateral adrenalectomy has not been studied in detail and would require precise functional imaging. Brauckhoff and colleagues described a case series of 10 subjects who underwent subtotal bilateral adrenalectomy, leaving 15-30% of the cortex intact. In each case, there was evidence of good functional recovery with no requirement for long-term steroid replacement(413). Thus it remains possible, though difficult to prove, that very small residual amounts of adrenal cortical tissue in our subjects could have been a source of low levels of hybrid corticosteroid production utilising high levels of recirculating exogenous cortisol as a substrate, although this implies that such tissue has a blood supply sufficient to deliver enough cortisol to cells expressing aldosterone synthase. Finally, ectopic adrenal tissue is present in up to 50% of neonates(414); however, such 'adrenal rests' are rare in adulthood with a prevalence of around 1%(415), and it is unlikely that this was a significant steroid source in the 10 subjects studied.

18-Hydroxycortisol was easily detectable in adrenalectomised subjects with median excretion rates far exceeding rates in normal volunteers (Figure 6.3c). This corticosteroid is produced by the action of 11ß-hydroxylase or aldosterone synthase on cortisol, although our previous work has demonstrated that aldosterone synthase is more efficient at 18-hydroxylation of cortisol than 11ß-hydroxylase(393). This fact, along with the ACTH-independence of 18-hydroxycortisol production (on phase 3), would suggest that aldosterone synthase is the likely principal source of 18-hydroxycortisol under these circumstances.

The explanation for much higher excretion rates of 18-hydroxycortisol in adrenalectomised versus normal subjects (chapter 6, figure 6.3a) remains unclear. One possibility is that extra-adrenal aldosterone synthase ± 11ß-hydroxylase enzymes are exposed to significantly higher levels of circulating cortisol than in normal subjects. It is now well accepted that many individuals on glucocorticoid replacement therapy are over-treated(416) although there is no consensus on an optimal dosing schedule. Another, less likely, possibility is that adrenalectomised subjects undergo post-operative induction of these extra-adrenal enzymes, although the mechanisms underlying this are obscure. The final possibility is variability in mass spectrometry data between the studies. Whilst this method of urinary corticosteroid quantification is well validated and robust, measurement of the hybrid corticosteroids 18-oxocortisol and 18-hydroxycortisol is less routine and it would be useful to confirm urinary levels with plasma quantification (RIA assay kits for these steroids are available commercially). Whatever the mechanism, it is unlikely that these apparent high levels of 18-hydroxycortisol are of biological significance since in vitro and in vivo bioassays have confirmed that this steroid has virtually no mineralocorticoid or glucocorticoid activity(384). The importance of its detection, as well as the detection of 18-oxocortisol (a full agonist at the mineralocorticoid receptor but with weak mineralocorticoid effects), in adrenalectomised subjects is that it demonstrates the capability of aldosterone synthase ± 11ß-hydroxylase (in extra-adrenal or residual tissue) to synthesise these corticosteroids from recirculating cortisol.

Finally, these data suggest that it may be plausible to suggest that aldosterone is produced in sites other than the adrenal cortex. This may have

important biological repercussions. As detailed in chapter one (section 1.8) aldosterone is an important cardiovascular hormone and recent studies have illustrated its role in cardiac fibrosis(240) and left ventricular hypertrophy as well as in impaired vascular reactivity(256). Moreover, many of these actions have been shown to be independent of its known effects on blood pressure(241). The risk of cardiovascular and cerebrovascular morbid events in subjects with hypertension and aldosterone excess is substantially higher than in patients with essential hypertension with similar blood pressure levels(417). In a clinical setting, the benefits of aldosterone blockade in cardiovascular disease have been adequately demonstrated in recent large, randomised clinical trials(272;274). Thus, evidence of significant extra-adrenal aldosterone production is likely to be of relevance to cardiovascular pathophysiology and provides further support for the use of aldosterone antagonists in this situation.

In summary, these results demonstrate for the first time, significant production of adrenal corticosteroids in adrenalectomised subjects. Whilst adrenal re-growth or residual adrenal tissue cannot be completely excluded as possible sources, the findings of this study provide compelling evidence for significant extra-adrenal aldosterone synthase ± 11ß-hydroxylase activities.

Chapter 8: Conclusions

2003 marked the 50th anniversary of the discovery of aldosterone by Simpson and Tait (147). This has coincided with increasing recognition of aldosterone as an important hormone in hypertension and cardiovascular disease. The evidence from almost every continent suggests that PA affects 5-13% of patients with hypertension. In addition, and as a result of increased use of the ARR as a screening tool, the prevalence of hypertension with relative aldosterone excess is much higher than previously thought. Whilst the debate continues as to how many of these individuals have classical PA, these facts highlight corticosteroid production by the adrenal gland as a key intermediate phenotype in a significant number of hypertensive individuals and study of its underlying molecular and biochemical basis forms much of the focus of this thesis.

Until now, the search for a genetic basis for aldosterone-associated hypertension has focussed on variants within the gene CYP11B2 (-344C/T, IC polymorphisms) which encodes aldosterone synthase. In particular, earlier studies have generated the hypothesis that variants within CYP11B2 (-344 C/T, IC polymorphisms) are in linkage with functional variants within CYP11B1, causing altered activity of its enzyme product, 11ß-hydroxylase. This phenotype, and its association with –344T and IC alleles, was initially identified in normotensive subjects. However, in chapter 4, it is confirmed, for the first time, in a large, severely hypertensive cohort taken from the MRC BRIGHT study.

The entire CYP11B locus was sequenced in a cohort of normotensive subjects (chapter 3). This confirmed tight LD across this locus (r^2 0.5-1) providing support for the original hypothesis. Moreover, a number of novel SNPs in linkage with –344C/T and IC polymorphisms were identified in both CYP11B1 and CYP11B2. However, as most are in non-coding regions detailed in vitro study of their functional effects is beyond the scope of this thesis. Nonetheless, 2 SNPs in the 5' promoter of CYP11B1 have emerged as plausible candidates to account for altered activity of this enzyme and their in vitro effects are described briefly elsewhere(418).

In addition, several novel polymorphisms in the 5' UTR of CYP11B2 (the gene encoding aldosterone synthase) were identified whose functional significance is unclear at this time. It may well be that these new variants also contribute to altered aldosterone regulation, possibly by changing the expression of CYP11B2 in response to angiotensin II or potassium. Thus, development of hypertension associated with inappropriate aldosterone production (ie, hypertension with a raised ARR) may be a consequence of a digenic effect mediated by variation within both CYP11B1 and CYP11B2 (analogous to the Dahl hypertensive rat, where variations across the same genes result in hypertension with altered aldosterone production and increased salt sensitivity). This is a concept that deserves further investigation.

As a result of altered efficiency of 11ß-hydroxylase, cortisol production may be maintained by a subtle and chronic increase in ACTH drive to the adrenal gland. In turn, this may also lead to increased production of aldosterone; perhaps by hyperplasia of the zona glomerulosa and an increase in its responsiveness to trophins such as potassium and Ang II. In chapter 4, this

concept was explored further in a large, severely hypertensive cohort. In this population, cortisol production was unaffected by CYP11B2 genotype but there was a significant elevation of the ratio of deoxycortisol to cortisol in subjects homozygous for the –344T allele, suggesting reduced efficiency of 11ß-hydroxylase. In addition, excretion of aldosterone was found to correlate strongly with that of ACTH-dependent steroids (total cortisol and adrenal androgens) in TT subjects. This suggests that in such subjects, there is an important common regulatory influence on adrenal corticosteroid production. This is likely to be ACTH.

Until now, there has been no report of the association between variation in the aldosterone synthase gene and activity of the HPA axis. If the above hypothesis was true, then hypertensive subjects with the –344TT genotype should demonstrate subtle changes in the ACTH/cortisol relationship as well as in aldosterone production. In chapter 5, this was investigated in hypertensive volunteers homozygous for the –344C/T polymorphism. In this study, TT homozygotes demonstrated diminished suppressibility of ACTH with dexamethasone, resulting in a decreased cortisol/ACTH ratio. These findings are in keeping with a mild increase in ACTH secretion in order to maintain cortisol production. Additionally, aldosterone exhibited diurnal variation and correlated closely with cortisol levels only in TT homozygotes. This supports further the proposal that, in TT individuals, ACTH contributes to the regulation of aldosterone production.

Clearly, the hypothesis requires that variants in CYP11B1 linked to the –344T± IC alleles of CYP11B2 lead to altered HPA axis activity as well ACTH contributing to aldosterone production. Accordingly, the relationships of novel

SNPs identified in the promoter of CYP11B1 to HPA activity were examined within the same population. The novel alleles (-1888T and –1858G) associated with similar changes in cortisol/ACTH relationship and in aldosterone regulation as the –344T± IC alleles of CYP11B2. This is in agreement with tight LD between these SNPs and CYP11B2 -344T/IC identified in chapter 3. Therefore, these alleles provide a plausible explanation for the altered efficiency of 11ß-hydroxylation described in several populations.

Finally, adrenal corticosteroids are of undoubted importance in hypertension and a better understanding of corticosteroid physiology and production is necessary. 18-Oxocortisol and 18-hydroxycortisol are corticosteroids whose levels are elevated in Conn's syndrome and GRA but are also found in normal subjects. Evidence is presented in chapter 6 that 18-oxocortisol is produced in normal subjects by the action of ZG aldosterone synthase on recirculating cortisol whilst 18-hydroxycortisol is produced by the action of ZF 11ß-hydroxylase on locally produced cortisol. Subsequently, 18-oxocortisol was used as a marker of aldosterone synthase activity and was detectable in adrenalectomised individuals (chapter 7). 18-Hydroxycortisol was also found in easily detectable amounts and its production was independent of ACTH suppression by dexamethasone again supporting the concept of extra-adrenal aldosterone synthase activity. Given the importance of aldosterone in hypertension and cardiovascular disease, evidence of significant extra-adrenal aldosterone production is of interest in cardiovascular pathophysiology and may further support the use of aldosterone antagonists in this situation.

In conclusion, I have shown that there is strong LD across the CYP11B locus. In particular, variants in the 5' UTR of CYP11B2 (–344T) are in tight LD with a number of polymorphisms across this and the adjacent CYP11B1 locus. These polymorphisms (of which -1888T and –1858G are likely candidates) result in altered expression of the enzyme product of CYP11B1, 11ß-hydroxylase. In turn, this leads to changes in HPA axis activity consistent with a subtle increase in ACTH-mediated adrenal stimulation to maintain cortisol production. Finally, the data are consistent with a contribution of ACTH to the production of aldosterone (in -344T & CYP11B1 -1888T/ –1858G), leading to the eventual phenotype of hypertension with an elevated ARR.

Conventionally, ACTH is regarded as having only a transient stimulatory effect on aldosterone production and prolonged administration of ACTH is reported to lead to a fall in aldosterone production after a few days. However, these experiments used supra-physiological amounts of ACTH in normotensive subjects (of presumably mixed genotype), often in a sodium replete state. It is possible that the suppressive effects of sodium on renin production override stimulation by ACTH to result in reduced aldosterone production in these cases. However, the effect of very subtle increases in ACTH sustained over time in individuals with hypertension has not been studied directly. It is entirely plausible that in this circumstance there is insufficient sodium retention to entirely suppress renin and so the stimulatory effect of ACTH is maintained. Accordingly, in in vitro experiments, and in humans where renin is inhibited by beta blockade, ACTH stimulation of aldosterone production is maintained, presumably because there is no secondary effect of sodium retention.

In developing this hypothesis, it is acknowledged that the development of hypertension associated with relative aldosterone excess (elevated ARR) is a chronic process and that the pathophysiological basis is present throughout life and will involve additional genes and environmental influences not studied here. However, in patients with other genetic and environmental factors that favour development of hypertension, the above proposal offers an amplification mechanism that will gradually lead to increasing mineralocorticoid effects on the vasculature with time. Given that aldosterone excess has adverse consequences on endothelial, renal, cardiac, and central nervous system tissues, early identification of subjects at risk of development of aldosterone-modulated blood pressure should be of increasing importance in reducing the worldwide cardiovascular burden.

Future work:

The studies reported in this thesis do highlight a number of areas that require further investigation:

1. Characterisation of novel polymorphisms in CYP11B2 (chapter 3)
2. Investigation of the functional significance of these polymorphisms especially in the 5' UTR.
3. Investigation of the alteration in adrenal corticosteroid production and regulation over time in hypertensive subjects by longitudinal studies.
4. Further investigation of the association with CYP11B genotype or haplotype with corticosteroid phenotype

   (In particular, to assess the effect of low dose ACTH infusion under careful conditions of salt intake (low salt and high salt states) on aldosterone production).

Appendices:

Appendix I Nucleotide sequences of human CYP11B1 and CYP11B2

Appendix II Dietary information given to all study subjects (chapter 5)

Appendix I: Nucleotide sequences of human CYP11B1 and CYP11B2

Nucleotide Sequence

The nucleotide sequences for human CYP11B1 and CYP11B2 were obtained from the Entrez Nucleotide database. The accession numbers for these sequences are as follows:

	CYP11B1	CYP11B2
5' flanking region and exon 1	D10169	D10170
Exon 1 and 2	M32863	D13752
Intron 2	X85218	D13752
Exons 3 to 8	M32878	D13752
Exon 9 and 3'UTR	M32879	M32881

Exons are shown in bold and the untranslated regions and intron in lower case. The oligonucleotides used for PCR and sequencing, listed in chapter 2, are indicated by their name above the relevant highlighted sequence. Where oligonucleotide sequences overlap the second sequence is underlined as is the primer name. Sense and antisense oligonucleotides are indicated by > and < respectively.

CYP11B1

B15'UTR > (B15'7-32 >)

```
   1 tcgttcggcat ccgttgtaag ttggattcct aagtatttta ttctctttga
  51 agcaattgtg aatgggagtt cactcatgat ttggcctctc tgtttgtctg
 101 ttaagggtgc ataagaatgc ttgtgatttt tgtacattga ttttgtatcc
 151 tagagcttgc tgaagttgct tatcagctta aggagatttt gggctgagac
                                                < B15'258-238
 201 aatggggttt tctagatata caatcatgtc cgtctgccaa acagagacaa
 251 tttgacttcc tcttttccta attgaatacc ctttatttcc ttctcctgcc
 301 taattgccct ggccagaact tccaacacta tgttgaatag gagtggtgag
                                  < B15'393-369          F81092 >
 351 agagggcatt cctgtcttgt accagctttc acagggaatg cttccagttt
 401 ttgaccattc agtatgatat tggccgtggg tttgccatag atagctctta
 451 ttattttgag atacgtccca tcaataccta atttattgag agtttttagc
 501 gtgaaggttg ttgaattttg tcaaaggcct tttctgcatc tattgagata
 551 atcatgtggt ttttgtcttt ggttctgttt atatgctgga ttacatttat
                                                F81093 >
 601 tgatttgcat atattgaacc agccttgcat ccgagggatg aagccactc
 651 gatcatggtg gataagcttt ttgatgtgct gctggattcg gttttgccagt
 701 attttattga ggatttttgc atcaatgttc atcaaggata ttggtctaaa
 751 attctctttt ttggttgtgt ctctgcccgg ctttggtatc aggatgatgc
 801 tggcctcata aaatgagtta gggaggattc cctctctttc tattgattgg
                                  F81094 >
 851 aatagtttca gaaggaatgg ttaccagttcc ttgcttgtacg tctggtataa
 901 ttcggctgtg aatccatctg gtcatggact cttttttggtt cgtaatctat
 951 tgattattgc cacaatttca gatcctgtta ttggtctatt cagagattca
1001 acttcttact ggtttagtct tgggagagtg tatgtgtcga ggaatttatc
1051 catttcttct agatttttta gtttatttgc gtagaggtgt ttgtagtatt
                F81095 >
1101 atctgatggt agttttgtatt ttcgtgggat cggtggtgat atccccttta
1151 tcatttttta ttgcatctat ttgattcttc tctcttttttt tctttattag
1201 tcttgctagc ggtctatcaa ttttgttgat cctttcaaaa aaccagctcc
1251 tggattcatt aatttttga agggtttttt gtgtcttctat ttccttcagt
```

1301 tctgcactga ttttagttat ttcttgcctt ctgctagttt tgaatgtgtt

1351 tgctcttgct tttctagttc ttttaatttgt gatgttaggg tgtcagtttt

1401 ggatctttcc tgctttctct tgtgggcact tagtgctata aatttccctc

1451 tacacactgc tttgaatgtg ttccagagat tctggtatgc tgtgtctttg

1501 ttctcgttgg tttcaagaac atctttattt ctgccttcat tttgttacgt

1551 acccagtagt cattcaggag caggttgctc agtttccatg taattgagcg

F81097 >

1601 gttttgagtg agtttcttaa ttcttgagtt tc tagtttgatt gcactaaaat

1651 ttttaaaaag taaaaaaaat acatgtggtt taatacaatt catgccaact

< B1prom-260(as)

1701 cattccctcg ttttttgcta taaatcctgc aacgacatga acaatccaag

1751 gctcttggat aagataaggg ccccatccat cttgctcctc tcagccctgg

1801 aggaggaggg agagtcctct tcccctgtct acgctcatgc acccccaatg

F31463 >

1851 agtccctgcc tccaggcctg acctctgccc tcggtcctct aggcagatcc

1901 agggccagtt ctcccatgac gtgatccctc ccgaaggcaa ggcaccaggc

1951 aagataaaag gattgcagct gaacagggtg gagggagcat tggaATGGCA Ex1

< B9865

2001 CTCAGGGCAA AGGCAGAGGT GTGCATGGCA GTGCCCTGGC TGTCCCTGCA

2051 AAGGGCACAG GCACTGGGCA CGAGAGCCGC CCGGGTCCCC AGGACAGTGC

2101 TGCCCTTTGA AGCCATGCCC CGGCGTCCAG GCAACAGGTG GCTGAGGCTG

2151 CTGCAGATCT GGAGGGAGCA GGGTTATGAG GACCTGCACC TGGAAGTACA

2201 CCAGACCTTC CAGGAACTGG GGCCCATTTT CAGgtaaagc cctccctggc

2251 cctcgctgga acacccagtg ccctgccctt gctgcccagg accctgccgg

2301 gcactcagca ctgccattcc cagcaggtcc cggcactctg catcctttgg

2351 aagagggaag atcgagcacg tgctgtctgt gcgctgcagg gcagggcatg

2401 tgcagagcaa atgggagctc ggctgcagag agggcaggac tcagaggcac

2451 tgaagttaag aggttccggg cagtcagcaa gagggcgttt agctgtgaag

2501 ccgctaatcc aggagagggg agggtggaca ggagacactt tggattggga

Y6265 >

2551 ctggcagggtg gggccagcag ggactagacc ccgtccagca gggcctcctg

2601 cttggcccca cagGTACGAT TTGGGAGGAG CAGGCATGGT GTGTGTGATG Ex2

2651 CTGCCGGAGG ACGTGGAGAA GCTGCAACAG GTGGACAGCC TGCATCCCCA

2701 CAGGATGAGC CTGGAGCCCT GGGTGGCCTA CAGACAACAT CGTGGGCACA

< Y6266

2751 **AATGTGGCGT GTTCTTGCTg** taagcggcga gctgaggagct gggagcaggg

2801 tggcagcctg gtgtaggggg gaggcgagag aggcaggacc caaaagcaca

2851 tctgccctgg gcccctgtgg tgggcagtga gggtgagcac ccgacccaga

2901 ggacggccat tccgtggggt cgtgtctgcc ctgtggttgg ggaagcaggg

2951 cgtggtggag aaatgggcac gggcacctct gcagagaaga tgcagagcaa

3001 tgagcccttc tgtgtagtga gaacccgctc tgcaccaacc ttggcggatg

3051 ctttctcttg cggtctgggg actgtcttcc ataggtcaga aaactgaggc

3101 ctgagaaggt actccactgg cccaggtcac aggctgagta ctgagcctgt

3151 ggttcgccgg ggccgcagcc tccctcaggg cgctcagggt ccctgcagtc

3201 ctggcaaacc ttcctgatgg ggacagtccg gggcaggagg caggtggggа

B1 Int2(3280) >

3251 tgcaggtggc tggtggctcc attgttctca gaaggcaaggc acgaggtggg

3301 gcggttgatg gcactgggga ggatgtttcc tggccctgcg agatgggcgg

3351 ccgttggtca ggtgggcagg gagaggctga tgcttggagt cagtcacctg

3401 caggaatgtt gtcattagga cgggcgaagg actggacgag gatgtcacag

3451 tggcgacagc ccccactcca tggcaggagg agaacgcttt tgggaatagt

3501 ggggttttagg taaaaggcca cccaagggtg ggggccttca ctgaggctgg

B1 Int2(3580) >

3551 cctacagacg gacatctggg agggagtcag gacccaggaa ggcaagtcca

3601 gaaggctggg tgcacataac ggaaggaagg ggagcgcacc tgtatgtgtg

3651 tgtgtcttgc atctgtgcac atctgtgtgt ttctctgtac ctgcatttgc

3701 acgtgttgtg tgtgcancgt gtgtgcacgt gtctgtgtgc atgtatgtgt

3751 ggtgtgtgtg cacgagtgtc tctgtgtgtg catgtgcagg tgctggcatg

3801 ggtgtagtgt ctgtgcactt gtgtacatgt gtctcttcac acatggtgtt

3851 gaggtcttgc atgggcgcat gtgagcatgt gcatcttctg cctggcatca

B1 Int2(3890) >

3901 ctgtcaacag ctcacagcca gctggacata aataaagctt tgagttttgc

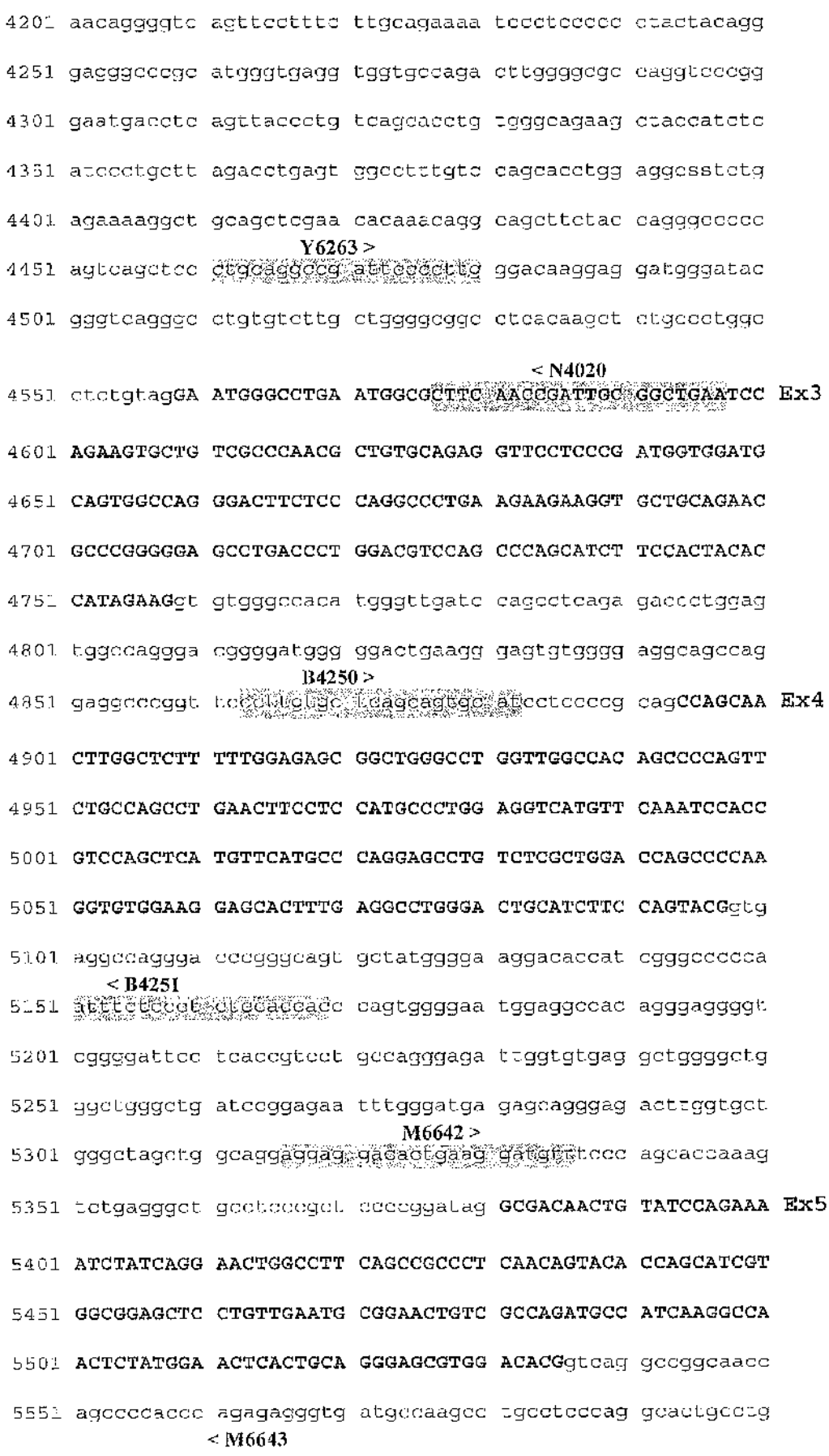
3951 aggaatgtgg ctgacagggg aaattcctcc ccaccattcc ctggggcatc

4001 catggagccc ccacgcactc tggctgtagg tgaggatggc atgaagcaca

4051 aagcttggtt tctgtcctgc agaagatgca gacacttcag tcctgctgcc

4101 ccagaggcac tgtgcccagg gcagggaagg gcggggagga gagggcagcc

4151 aggggctctc ccctcaggac actgtgtggg tgaggtgggc aaagcttgac


Y6263 >
< N4020
Ex3
B4250 >
Ex4
< B4251
M6642 >
Ex5
< M6643

5601 ccaatgtcac acggcgcccc cgtgtcccat gcccaggcta tgggccccac

5651 atttcttact tgggattgtg atgtgataaa cacgtttgca ggttgccatg

5701 gttggaatgg ggggttcctt tccttctgtg gaggactcag ggaaacgggg

5751 tttggatggg cattaggatt tgaagtcttg ggctctgtcg tgctcagggt

5801 atgcatgtct gcacccctca cagggaggtt gtcctgggag gggtgtcccg

F42117 >

5851 ggggctgagt cctcctgtgc aaggtctgac ccgtgcaggc tgtatcctgc

5901 agACGGTGTT TCCCTTGCTG ATGACGCTCT TTGAGCTGGC TCGGAACCCC Ex6

5951 AACGTGCAGC AGGCCCTGCG CCAGGAGAGC CTGGCCGCCG CAGCCAGCAT

6001 CAGTGAACAT CCCCAGAAGG CAACCACCGA GCTGCCCTTG CTGCGTGCGG

< F42299

6051 CCCTCAAGGA GACCTTGCGg tgggtgctgg ctgaggcctc cctgtgggcc

6101 tggccctgct ggagagtcag ccccccactgg gtggttgcag acagaatctg

6151 ggctataaac acctacccag cagccatcct gactgctctc tcgcgtcaag

6201 gacagggagc tctcttttcc ctggaatccc tcttcaacgc cctggggatt

6251 aacgtggggg catgtccttc tgcgctcggg gctgcttaag ttaggggagg

6301 cttggccggg ctcagcaggt gcaaggaagc acttcctaca cctgggcttc

6351 ccatggatct gggacctctg cggggtcttc ggtaggaagg gtgcagagag

Y6259 >

6401 cacaggaacc ccatcccagc tgagacccct tctatcggatg ccccgacctc

6451 cagGCTCTAC CCTGTGGGTC TGTTTCTGGA GCGAGTGGCG AGCTCAGACT Ex7

6501 TGGTGCTTCA GAACTACCAC ATCCCAGCTG GGgtgagtga gccccacacc

< Y6260

6551 ctcgagctga gaacctccct cccccagtcat tccctgatcc ccgctctgct

6601 ccgtccgcag ACATTGGTGC GCGTGTTCCT CTACTCTCTG GGTCGCAACC Ex8

6651 CCGCCTTGTT CCCGAGGCCT GAGCGCTATA ACCCCCAGCG CTGGCTAGAC

6701 ATCAGGGGCT CCGGCAGGAA CTTCTACCAC GTGCCCTTTG GCTTTGGCAT

6751 GCGCCAGTGC CTTGGGCGGC GCCTGGCAGA GGCAGAGATG CTGCTGCTGC

6801 TGCACCATgt gagcaggccc gggctgggga ggggcctggg cggggtctgg

6851 gcagcatggg cggggcttga gcaatgtggg actggcctcg gcagagtggg

6901 agtggcctgc atgtttcctg gactgggcag agcgggtact gggagaacct

6951 gggccaggtt gaggctgtgt aggtcctggg caggagttgg tatggtgagg

7001 agcgtaccat ctgggtgagg ttgctgctaa accgggtcag gtgggaactg

7051 ggggaagtcg ggtggagcct gtacaggata gtggggcttg ggcaatacct

7101 gggctggatg aattctgggc ctgggctgta aggtggggct ggtcaggaat

M6635 >

7151 gaaacaggtt ggaggccagg ctgctgttcc ccctcaagga taatctctgc

7201 aactttgagg gtctgagaag gctgcaccac gtgcatgggc tgcggaccaa

7251 gccagatgga aaccccggct ctgtcctagG **TGCTGAAACA CCTCCAGGTG** Ex9

7301 **GAGACACTAA CCCAAGAGGA CATAAAGATG GTCTACAGCT TCATATTGAG**

< B1Exon9(as)

7351 **GCCCAGCATG TGCCCCCTCC TCACCTTCAG AGCCATCAAG TAA**tcacgtc

7401 tctgcaccca gggtcccagc ctggcaccag cctcccattc tgcctgaccc

7451 caggccaccc ctcttctctc ccacatgcac agcttcctga gtcacccctc

7501 tgtctaacca gccccagcac aaatggaact cccgagggcc tctaggacca

7551 gggtttgcca ggctaagcag caatgccagg gcacagctgg ggaagatctt

7601 gctgaccttg tccccagccc cacctggccc tttctccagc aagcactgtc

7651 ctctggcagt ttgccccccat cccteccagt gctggctcca ggctcctcgt

7701 gtggccatgc aagggtgctg tggttttgtc ccttgccttc ctgcctctag

7751 tctcacatgt cccigttccc cttccccigc cagggccccc gcgcagactg

7801 tcagagtcat taagcgggat cccagcatct agagtccagt caagttccct

7851 cctgcagcct gccccctagg cagctcgagc atgccctgag ctctctgaaa

< B13'UTR+500

7901 gtcgtcgccc tggaataggg tcctgcaggg tagaataaaa aggccccigt

7951 ggtcacttgt cctgacatcc ccatttttcaa gtgatacaac tgagtctcga

8001 gggacgtgtg ttccccagct gatcgtgtca gcctcatgcc cctggcctca

8051 tctttcatgg accaggcctt gttccaggag tgggcgttgg gtcctctgct

8101 tcctgtgctg tcccctgggg aaggtcccaa ggatgctgtc aggagatgga

8151 agagtcatgt ggggtgggaa cctggggtgt ggttccagaa atgtttttgg

8201 caacaggaga gacaggattg ggccaacaag gactcagacg agttttattg

8251 actattcict gaca

CYP11B2

T8303D >

```
   1 gaattctgca tcctgtgaaa ttatccttca aaagtgaaca taaatatttt
  51 ctcaggtaaa taaaaaattga ggggattcgc tgccaataga actgacttgc
 101 cagaaatgtt ttttaaaagt tctgcagaga gaaagaaaat gatacaggtc
 151 agcaaccctg agctacataa agaaaggaag agcatttcag aaggaatcag
 201 taaagagaaa atgaagtctt ttatttttte ttaatcttaa ttgatctaag
 251 agtttgctaa aacaaaacaa caacaataaa aataggccgg gcgcggtgac
 301 tcaccacacc tgtaatccca gcactttggg aggccgaggt gggcagatca
 351 cctgaggtca ggagttcgag actagcctgg ccaacacggt gaaaccccgt
 401 ctctactaaa aatacaaaaa ttagctgggc gtggtggtgg cacatgcctg
 451 taatcccagc tacttgggag gctgaggcgg gagaattgct tgaacccggg
 501 agacagaggt cgcagtgagc cgagatcaca ccattgcact ctagcctggg
 551 cgacagagtg agactctgtc tcaaaaaaaa ataaaaaaat aaataaataa
 601 ataataaaaa taaataaata aaagccagaa agtgtatttg atgatcatag
 651 ttatgtatat gtgaaatgaa ggacagcaat gatgcaaggg atgggtgagt
 701 ggaattaaaa atatcttatt atttatttat tttgagatgg agtcttgctt
 751 tgctgcccag gttggagtgc agtgggatga tctcaactca ctgcaacctc
 801 cgcctccttg attcaagcat tcatcttgac tcagcctgct gagaagccga
 851 gattacaggc atgcgccacc acacctggct aattttgtat ttttagtaga
 901 gacagggttt tgccatgttg gccaggctgg tctcgaactc ctgacctcag
 951 gtgatccacc tgcatcagcc tcccaaagtg ctgggatgac agacatgagc
1001 cactatgccc agcctaagaa tatctgatga ttataaagtg cttgcattac
1051 ctctgaagct gtatagtgtt atatgaaggt ggagttggag agatgagttt
1101 taagcgtata ttgcaaactc tagggcaacc actaaagaag tgagacccag
1151 cctctagaaa aaaaaaaaaa aaaggaaatt agctatcaag ccacgaaaag
1201 aaatggagga accttaaacg catattacta actgagatac gtcactttga
1251 aaaggctaca aacggtgtca ttccaactat acaacatttt ggaaaaggcc
1301 aaagcatggt gatgataaaa aagatcggag atgtcaggga ctggggcagg
1351 agggatgagc aggcagagca caggttttct tttcctcttt ttaagacagt
1401 gaaaatactc ctaggatcct gcaaggaggg atacaaatta catacatttg
```

1451 tcaaaaccca cagcatgttg accaccagga ggagaccaca tgtgactcca

1501 ggaccctggt tgataacaac gtatcgagat tcctcacatg gaaccagtgc

1551 gctcctgtgg tcgagggtgt acctgtgtca gggcaggggg tacgtggaca

1601 ttttctgcag ttttttgatca attttgcaat gaactaaatc tgtggtataa

1651 aaataaagtc tattaaaaga atccaaggct ccctctcatc tcacgataag

1701 ataaagtccc catccatttt actcctctca gccctggaga aaggagaggc

1751 caggtcccac cacctttcac cagcatggac ccccagtcca gaccccacgc

1801 ctttttctcag catcctcaga ccagcaggac ttgcagcaat ggggaattag

1851 gcacctgact tctcctttcat ctaccttttgg ctgggggcct ccagccttga

1901 ccttcgctct gagagtctca ggcaggtcca gagccagttc tcccatgacg

1951 tgatatgttt ccagagcagg ttcctgggtg agataaaagg atttgggctg

2001 aacagggtgg agggagcatt ggaATGGCAC TCAGGGCAAA GGCAGAGGTG Ex1

2051 TGCGTGGCAG CGCCCTGGCT GTCCCTGCAA AGGGCACGGG CACTGGGCAC

2101 TAGAGCCGCT CGGGCCCCTA GGACGGTGCT GCCGTTTGAA GCCATGCCCC

2151 AGCATCCAGG CAACAGGTGG CTGAGGCTGC TGCAGATCTG GAGGGAGCAG

2201 GGTTATGAGC ACCTGCACCT GGAGATGCAC CAGACCTTCC AGGAGCTGGG

2251 GCCCATTTTC AGgtaaagcc ctccctggcc ctcgctggga acacccagat

2301 ccctgccccc gctgcccagg accctgccag gcactcagca ctgccattcc

2351 cagcaggtcc cggcactctg catcctttgg aggatgggga aggagtgcag

2401 cacatgctgg tctgtggtgc tgccagggca ggggatagtg cagagaaaac

2451 ccnagctcac tgcagagagg gcaggactca gaagcactaa agttgaaagg

2501 tncccagggag ccagcaggag ggctttagct gtgaagccgc taatccagga

2551 gcagggaggg tggacaggag acactttgga ttgggactgc agggtggggc

2601 cacgagggac atgacccctt ccagcagggc ctcctgcttg gccccacagG

2651 TACAACTTGG GAGGACCACG CATGGTGTGT GTGATGCTGC CGGAGGATGT Ex2

2701 GGAGAAGCTG CAACAGGTGG ACAGCCTGCA TCCCTGCAGG ATGATCCTGG

2751 AGCCCTGGGT GGCCTACAGA CAACATCGTG GGCACAAATG TGGCGTGTTC

2801 TTGTTgtaag cggcgagttg ggagctgaga gctgggagca gggtgggcag

2851 cctgggtgta gggggggaggc gagagaggta ggacccaaaa gcacatctgc

2901 cctgggcccc tgtggtgggc agtgagggtg agcacccggc ccagaggacg

2951 gccatcctgt ggggtcgcgt ctgcactgtg ggttggggaa gcagggcggt

3001 ggtggagaaa tgggcacggg cacctctgca gagaagacgc agagcaatga

3051 gcccttctgt gtagtgagaa cccgctctgc accaacctcg gcggctgctt

3101 tctcttgcgg tctggggact gtccttccca taggtcagaa aactgaggcc

3151 ctgagaaggg gacttccact ggcccaggtc acaggctgag tgctgagcct

3201 ggtgttcgcc ggggccgcag cctccctcag ggcgctcagg gtccctgcag

3251 tcctggcaaa ccttcctgat ggggacagtc cggggcagga ggcaggtggg

3301 gacgcaggtg gctggtggtt ccgttgttct cagaagcaag gcacaaggtg

3351 gggcggttga tggcactggg gaggatgttt cctggcccgt ggagagggtg

3401 gcgcctggtc aggtgggcag ggagaggctg atgcttggag tcggtcacct

3451 gcagggatgt tgtcattagg acgggggaag gactggatga ggatgtcaca

3501 gtggtgacag cccccactcc atggtaggaa gggaacgcta ttgggaatag

3551 tggggtttag gtaaaagggc acccgtgggt cggggccttc actgaggctg

3601 gcctatagat gacatctggg agagagtcag gacccaggaa ggcaggtcca

3651 ggaggctggg tgcgcataat ggaaggaagg ggagcgctcc tgtctgtgtg

3701 tgtgtcttgc atctgtgcac atgctgtgtg tttctctgta cctgcattgc

3751 acatgtgtag tgtgtgcacg tgtcgtgtgt gaatgtatgt gtggtgtgtg

3801 tgcacaagtg tctgtgtgtg tgcatgtgca ggtgccggca tgggtgtagt

3851 gtttgtgcac acatgcacat gcgtctcttc acacatggtg ttgaggtctt

3901 gcatgggcgc acgtgtgcat gtgcatcttc tgcctgtcat cactgtcaac

3951 agctcacagc agccagctgg acataaataa aggagttttg caggaatgtg

4001 gctgacaggg gaaattcctc cccaccattc cctgggggca tccatggagc

4051 ccccacgcac tctggctgtg ggtaggatgg catgaagcac aaagcttggt

4101 ttctgtcctg cagaagatat agatgcttca cagagacagc agagcagatg

4151 ccccagaggc actgtgccca gggcgggggaa gggtggggag gagagggcag

4201 ccaggggctc tcccctcagg acactgtgtg ggtgaggtgg gcaaagcttg

4251 acaacagggg tcacctcctt tcttggagaa aagccctacc ctgttactac

4301 agggagggcc cgcatgggtg aggtggtgcc agacttgggt cgccaggtcc

4351 cgggaatgac ctcagttacc ctgtcagcac ctgtgggcag aagctaccat

4401 ctcatccctg cttagacctg agtggccttt gcccagcacc tggaggccgc

4451 tctgagaaaa ggctgcagct cgaacacaaa caggcagctt ctaccagggc

4501 ccccagtcag ctccctgcag gccgattccc cttggggaca aggaggatgg

4551 gatacgggtc agggcctgtg tcttgctggg gcggcctcac aagctctgcc

4601 ctggcctctg tag**GAATGGG CCTGAATGGC GCTTCAACCG ATTGCGGCTG** Ex3

4651 AACCCAGATG TGCTGTCGCC CAAGGCCGTG CAGAGGTTCC TCCCGATGGT

4701 GGATGCAGTG GCCAGGGACT TCTCCCAGGC CCTGAAGAAG AAGGTGCTGC

4751 AGAACGCCCG GGGGAGCCTG ACCCTGGACG TCCAGCCCAG CATCTTCCAC

4801 TACACCATAG AAGgtgtggg ccatgcgcga aggtccagcc ccagagaccc

4851 tggagtggcc agggatgggg atggaggact gaagggagtg tggggaggca

4901 gccaggaggc ctggggctgc cttgtgctca gcagtgcatc ctccccgcag

4951 **CCAGCAACTT AGCTCTTTTT GGAGAGCGGC TGGGCCTGGT TGGCCACAGC** Ex4

5001 CCCAGTTCTG CCAGCCTGAA CTTCCTCCAT GCCCTGGAGG TCATGTTCAA

5051 ATCCACCGTC CAGCTCATGT TCATGCCCAG GAGCCTGTCT CGCTGGATCA

5101 GCCCCAAGGT GTGGAAGGAG CACTTTGAGG CCTGGGACTG CATCTTCCAG

5151 TACGgtgagg ccagggaccc gggcagtgct atggggaagg gacaccatgg

< F33915

5201 gggcccaatt tgtccttctc caccagccag tggggaatgg aggccacagg

5251 gaggggtcgg ggattcctca ccttcctgcc ggggagattg gtgcgaggct

5301 ggggctgggc tgggctgatc cggagaattt gggatgagag cagggagatt

T2138 >

5351 tgggtgtcgg ccgagtctgg gcaggaggag gacactgaag gatgcttccc

5401 agcaccaaga tctgagggct gtcccctgct cctggacag **GTGACAACTG** Ex5

5451 TATCCAGAAA ATCTACCAGG AACTGGCCTT CAACCGCCCT CAACACTACA

5501 CAGGCATCGT GGCGGAGCTC CTGTTGAAGG CGGAACTGTC ACTAGAAGCC

5551 ATCAAGGCCA ACTCTATGGA ACTCACTGCA GGGAGCGTGG ACACGgtcag

5601 gccagcaacc agccccaccc agagagggtg atgccaagcc tgcctcccag

5651 gcactgcctg ccaatgccac acggcaccca cgttccccat ccccaggcta

5701 caggccccac atttctgttg ccctcagcct tcccctcct ttgttaaggg

5751 atgagatttg caggggaggg gaaatgtgag ctccccctca catgagactg

5801 agtttgcagt tacctgtgtg gggatccatg ctccaggctg gaagaaagtt

5851 ggatgaggcc ctggacacac agcagctctg tccccactgg aaagctctgg

5901 gtgtacaagg agaaggaggg ttgagaggca gctggaggac tccactgggc

5951 acccttccca gtgtgccccgg tcaccttggg ccagaaatgt agatgcatgg

6001 gagggcaggg ttgtggggaa gacagcagca caggctccag ccagtgcaga

6051 ggggcctgtg ggtgcacagt ggggagaact caatggaagc agagggagct

6101 ggggctccag aactccctgg atgatgctga ggtgtggccc cctgccctaa

6151 tggtggctgt gagaacccgc cctgaagagg ctgcagggga cctgggcctt

6201 ggtggagatg ggggtcacct ttcccctgaag aagtcaggga atctggccca

6251 agtggtcatc aagggtttcag atccggggtc ccagggctct gtttttgctc

6301 agggcatgga tgtctccacc cctcagaggg aggttgtcct gggaggggtg

6351 tcccgggggc tgagtcctcc tgtgcaaggt ctgaccctgc agacatggct

6401 tctgtag**ACA GCGTTTCCCT TGCTGATGAC GCTCTTTGAG CTGGCTCGGA Ex6**

6451 ACCCCGACGT GCAGCAGATC CTGCGCCAGG AGAGCCTGGC CGCCGCAGCC

6501 AGCATCAGTG AACATCCCCA GAAGGCAACC ACCGAGCTGC CCTTGCTGCG

6551 GGCGGCCCTC AAGGAGACCT TGAGgtgggt gctggctgag gcctccctgt

6601 ggccctggcc ccctgctgga gagcagcccc cactgggtgg tggcagacag

6651 aatctggggc tgataaacag cgtcacccag cagcccattc ccctgcacct

6701 gctcttcctc cccctcaagg acagggagct cttcttcctc tggaatcccct

6751 cttcaacgcc ctggggatta acgtgggcca tgtcctctg cgctcggggc

6801 tgcttaagtt aggggaggtt tggctgggct cagcaggtgc aaggaagcac

6851 ttcctacgac ctgggcttcc catggatctg ggacctctgc ggggtcttcg

6901 gtaggaaggg tgctgagagc acagggagcc ccatccagct gaggaccctt

6951 tctgtggatg ccccccacctc cag**GCTCTAC CCTGTGGGTC TGTTTTTGGA Ex7**

7001 GCGAGTGGTG AGCTCAGACT TGGTGCTTCA GAACTACCAC ATCCCAGCTG

7051 **GG**gtgagtga gccccacacc cctcgagctg agaacctccc tccccagtca

7101 ttccctgatc cctgctctgc accgtccgca g**ACATTGGTA CAGGTTTTCC Ex8**

7151 TCTACTCGCT GGGTCGCAAT GCCGCCTTGT TCCCGAGGCC TGAGCGGTAT

7201 AATCCCCAGC GCTGGCTAGA CATCAGGGGC TCCGGCAGGA ACTTCCACCA

7251 CGTGCCCTTT GGCTTTGGCA TGCGCCAGTG CCTCGGGCGG CGCCTGGCAG

7301 AGGCAGAGAT GCTGCTGCTG CTGCACCACG TAAGCAGGCC TGGGGGCGGG

7351 GGCGGGACCT GGGCAGCAGA GGCGGGACCT GCACACTGGG GGCGGGGCTT

7401 GCATGGTGTg attgacacct gggaacagtg gatggggcct tggttggttg

7451 aggtcggcgt gaccagggag gatctgtgct gagcaagaca gggtaggatc

7501 tgggtgaggt tgcttctaaa cattgaaatg gggactaggg gagtggggtg

7551 gagcctgtac agaataatgg ggcttgggca agacctgggc aggattcagt

7601 ctgggcctgg tccgcaaggt ggggctggtc agaaatggga taggttgggg

7651 cccaggctgc tgctcccccc tcagcataat tgttgcacct gggacgatgg

7701 gaggaagctg ccccaggtcc atgggctact gaccaggcca gatggaaacc

7751 cagcctctgt cctag**GTGCT GAAGCACTTC CTGGTGGAGA CACTAACTCA** Ex9

B2 3'UTR >

7801 AGAGGACATA AAGATGGTCT ACAGCTTCAT ATTGAGGCCT GGCACGTGCC

7851 CCCTCCTCAC TTTCAGAGCG ATTAACTAGt cttgcatctg cacccagggt

7901 cccagcctgg ccaccagctt ccctctgcct gaccccaggc cacctgtgtt

< M6641

7951 ctctcccacg tgcacagctt cctgagtcac cccctctgtc agccagctcc

8001 tgcacaaatg gaactcccca gggcctccag gactgggggt tgccaggctt

8051 gtcaaatagc aaggccaggg cacagctgga gacgatcttg ctggcagggc

8101 ctggccttgt ccccagcccc acctggcccc ttctccagca agcagtgccc

8151 tctggacagc ttgactctac tcctcccagc gctggctcca ggctcctcat

8201 gaggccatgc aagggtgctg tgattttgtc cttgccttc ctgcctagtc

8251 tcacatgtcc ctgtccctct cgccctggcc agggcctctg tgcagacagt

8301 gtcagagtca ttaagcggga tcccagcatc tcagagtcca gtcaagttcc

8351 ctcctgcagc ctgaccccag gcagctcgag catgccctga gctctctgaa

8401 agttgtcacc cagaaatacg atcctgcagg gtagactaaa aaggcccctg

8451 tggtcactta tactgacaca ttttaagtga tacaactgag tctcgagggg

8501 cgtgtgttcc ccagctgatc atgtcagcct catgccccag gcctcgtctt

8551 tcatggacca ggtcttgttc aagcagcgag tgttgggtcc tctgcttcct

```
8601 gagctgtccc ctggaaaagg tcccgaggat gctgtcagga gatggaagag

8651 tcatgtgggg tgggaacctg gggtgtggtt ccagaaatgt ttttggcaac

8701 aggagagaca ggattgggcc aacaaggact cagatgagtt tattgactca
                                                    < B2 3'UTR
8751 ttcctctgga agatacgcag c.....................ctcccct

9251 agtcagcaca tc
```

Appendix II: Dietary information given to all study patients (chapter 5)

Food Group	Salty Foods to Avoid	Suitable Alternatives
Breakfast Cereals	All others	Weetabix, Shredded Wheat
Bread, Cakes and Biscuits	All others	Bread (Wholemeal/white max 4 slices/day), sweet cakes or sweet biscuits only.
Starchy Carbohydrates	All others	Potatoes, rice, pasta.
Eggs	Scotch Eggs	Boiled, poached, scrambled or fried

Milk	Salted Lassi	½ pint semi-skimmed milk daily
Cheese		Cottage/cream cheese, maximum of 50g daily
Meats and Poultry	Bacon, ham, gammon. All smoked meats. Salami, sausages, beefburgers. Tinned meats, e.g. corned beef, luncheon meat. Meat pies and manufactured meat dishes. Dehydrated packed meals. Meat paste and pate.	All fresh and frozen meats e.g. beef, lamb, pork, turkey, chicken etc.
Fish	All smoked, cured, salted, tinned or pickled fish. Fish paste and pate, taramasalata. Shellfish e.g. prawns, shrimps. Manufactured fish dishes.	All fresh or frozen fish. Fish in batter or breadcrumbs.
Soups	Packet and tinned.	Home made soup with no added salt or stock
Drinks		Fruit juice, reduced sugar fruit squash, diet fizzy drinks, mineral water, tea, coffee, tonic water.
Fruit	Olives, salted nuts.	Fresh, frozen or tinned in natural juice. Unsalted nuts.
Vegetables	Tinned unless labelled "no added salt". Baked beans.	Fresh, frozen or tinned in natural juice.
Spreads	All others.	Polyunsaturated margarine.
Miscellaneous	Meat or yeast extract e.g. Bovril, Marmite, Oxo. Gravy granules, stock cubes, garlic salt. Pickles, chutneys, tomato ketchup, Worcester sauce, tomato juice, chilli sauce.	Use black pepper, herbs and spices to flavour food. Use unsalted snacks or fresh or dried fruit as snacks

Reference List

(1) HARVEY W. [Anatomical treatise on the movements of the heart and the blood in animals.]. [Undetermined]. Imprensa Medica 26(436):57-107, 1950.

(2) Hales S. Statical essays: containing haemastaticks; or an account of some hydraulic and hydrocstatical experiments made on blood and blood-vessels of some animals. London: Innys & Manby, 1733.

(3) Korotkoff N. K voprosu o metodoach eezldovania krovyanovo davlenia. Izv Imperatoor Vorenno Med Akad 1905; 11:365-367.

(4) Brown MJ. Science, medicine, and the future. Hypertension. [Review] [27 refs]. BMJ 314(7089):1258-61, 1997.

(5) Carretero OA, Oparil S. Essential hypertension. Part I: definition and etiology. [Review] [34 refs]. Circulation 101(3):329-35, 2000.

(6) PLATT R. The nature of essential hypertension. Lancet 2(7091):55-7, 1959.

(7) OLDHAM PD, PICKERING G, ROBERTS JA, SOWRY GS. The nature of essential hypertension. Lancet 1:1085-93, 1960.

(8) Cutler JA. High blood pressure and end-organ damage. [Review] [24 refs]. Journal of Hypertension - Supplement 14(6):S3-6, 1996.

(9) Williams B, Poulter NR, Brown MJ, Davis M, McInnes GT, Potter JF et al. British Hypertension Society guidelines for hypertension management 2004 (BHS-IV): summary.[see comment][erratum appears in BMJ. 2004 Apr 17;328(7445):926]. BMJ 328(7440):634-40, 2004.

(10) 1999 World Health Organization--International Society of Hypertension Guidelines for the Management of Hypertension. Guidelines Sub-Committee. Blood Pressure Supplement 1:9-43, 1999.

(11) Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr. et al. The Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure: the JNC 7 report.[see comment][erratum appears in JAMA. 2003 Jul 9;290(2):197]. JAMA 289(19):2560-72, 2003.

(12) Wolf-Maier K, Cooper RS, Banegas JR, Giampaoli S, Hense HW, Joffres M et al. Hypertension prevalence and blood pressure levels in 6 European countries, Canada, and the United States.[see comment]. JAMA 289(18):2363-9, 2003.

(13) Hajjar I, Kotchen TA. Trends in prevalence, awareness, treatment, and control of hypertension in the United States, 1988-2000.[see comment]. JAMA 290(2):199-206, 2003.

(14) Dawber TR, Kannel WB. The Framingham study. An epidemiological approach to coronary heart disease. Circulation 34(4):553-5, 1966.

(15) Kannel WB. Fifty years of Framingham Study contributions to understanding hypertension. [Review] [49 refs]. Journal of Human Hypertension 14(2):83-90, 2000.

(16) Dannenberg AL, Garrison RJ, Kannel WB. Incidence of hypertension in the Framingham Study. American Journal of Public Health 78(6):676-9, 1988.

(17) Lloyd-Jones DM, Evans JC, Levy D. Hypertension in adults across the age spectrum: current outcomes and control in the community. JAMA 294(4):466-72, 2005.

(18) Gus M, Fuchs SC, Moreira LB, Moraes RS, Wiehe M, Silva AF et al. Association between different measurements of obesity and the incidence of hypertension. American Journal of Hypertension 17(1):50-3, 2004.

(19) Fuchs FD, Chambless LE, Whelton PK, Nieto FJ, Heiss G. Alcohol consumption and the incidence of hypertension: The Atherosclerosis Risk in Communities Study. Hypertension 37(5):1242-50, 2001.

(20) Lee DH, Ha MH, Kim JR, Jacobs DR, Jr. Effects of smoking cessation on changes in blood pressure and incidence of hypertension: a 4-year follow-up study. Hypertension 37(2):194-8, 2001.

(21) Fagard RH. Physical activity, physical fitness and the incidence of hypertension.[comment]. Journal of Hypertension 23(2):265-7, 2005.

(22) Laurent S. Guidelines from the British Hypertension Society.[see comment][comment]. BMJ 328(7440):593-4, 2004.

(23) Chalmers J, MacMahon S, Mancia G, Whitworth J, Beilin L, Hansson L et al. 1999 World Health Organization-International Society of Hypertension Guidelines for the management of hypertension. Guidelines sub-committee of the World Health Organization. Clinical & Experimental Hypertension (New York) 21(5-6):1009-60, 1999;-Aug.

(24) MacMahon S, Peto R, Cutler J, Collins R, Sorlie P, Neaton J et al. Blood pressure, stroke, and coronary heart disease. Part 1, Prolonged differences in blood pressure: prospective observational studies corrected for the regression dilution bias.[see comment]. Lancet 335(8692):765-74, 1990.

(25) Almgren T, Persson B, Wilhelmsen L, Rosengren A, Andersson OK. Stroke and coronary heart disease in treated hypertension -- a prospective cohort study over three decades. Journal of Internal Medicine 257(6):496-502, 2005.

(26) Kannel WB, Castelli WP, McNamara PM, McKee PA, Feinleib M. Role of blood pressure in the development of congestive heart failure. The Framingham study. New England Journal of Medicine 287(16):781-7, 1972.

(27) Klag MJ, Whelton PK, Randall BL, Neaton JD, Brancati FL, Ford CE et al. Blood pressure and end-stage renal disease in men. New England Journal of Medicine 334(1):13-8, 1996.

(28) Neal B, MacMahon S, Chapman N, Blood Pressure Lowering Treatment Trialists' Collaboration. Effects of ACE inhibitors, calcium antagonists, and other blood-pressure-lowering drugs: results of prospectively designed overviews of randomised trials. Blood

Pressure Lowering Treatment Trialists' Collaboration.[see comment]. Lancet 356(9246):1955-64, 2000.

(29) Turnbull F, Blood Pressure Lowering Treatment Trialists' Collaboration. Effects of different blood-pressure-lowering regimens on major cardiovascular events: results of prospectively-designed overviews of randomised trials.[see comment]. Lancet 362(9395):1527-35, 2003.

(30) Dahlof B, Sever PS, Poulter NR, Wedel H, Beevers DG, Caulfield M et al. Prevention of cardiovascular events with an antihypertensive regimen of amlodipine adding perindopril as required versus atenolol adding bendroflumethiazide as required, in the Anglo-Scandinavian Cardiac Outcomes Trial-Blood Pressure Lowering Arm (ASCOT-BPLA): a multicentre randomised controlled trial.[see comment]. Lancet 366(9489):895-906, 2005.

(31) Hansson L, Zanchetti A, Carruthers SG, Dahlof B, Elmfeldt D, Julius S et al. Effects of intensive blood-pressure lowering and low-dose aspirin in patients with hypertension: principal results of the Hypertension Optimal Treatment (HOT) randomised trial. HOT Study Group.[see comment]. Lancet 351(9118):1755-62, 1998.

(32) Wolf-Maier K, Cooper RS, Kramer H, Banegas JR, Giampaoli S, Joffres MR et al. Hypertension treatment and control in five European countries, Canada, and the United States. Hypertension 43(1):10-7, 2004.

(33) Berglund G, Andersson O, Wilhelmsen L. Prevalence of primary and secondary hypertension: studies in a random population sample. British Medical Journal 2(6035):554-6, 1976.

(34) Gifford J. Evaluation of the hypertensive patient with emphasis on detecting curable causes. Millbank Memorial Foundation Quarterly 1969; 47:170-175.

(35) Lewin A, Blaufox MD, Castle H, Entwisle G, Langford H. Apparent prevalence of curable hypertension in the Hypertension Detection and Follow-up Program. Archives of Internal Medicine 145(3):424-7, 1985.

(36) Sinclair AM, Isles CG, Brown I, Cameron H, Murray GD, Robertson JW. Secondary hypertension in a blood pressure clinic. Archives of Internal Medicine 147(7):1289-93, 1987.

(37) Swales J. Textbook of Hypertension. 1st ed. Oxford: Blackwell Scientific, 1994.

(38) Beilin LJ, Puddey IB, Burke V. Lifestyle and hypertension. [Review] [85 refs]. American Journal of Hypertension 12(9 Pt 1):934-45, 1999.

(39) Lindquist TL, Beilin LJ, Knuiman MW. Influence of lifestyle, coping, and job stress on blood pressure in men and women. Hypertension 29(1 Pt 1):1-7, 1997.

(40) Cook NR, Cohen J, Hebert PR, Taylor JO, Hennekens CH. Implications of small reductions in diastolic blood pressure for primary prevention. Archives of Internal Medicine 155(7):701-9, 1995.

(41) Oparil S, Zaman MA, Calhoun DA. Pathogenesis of hypertension.[see comment]. [Review] [118 refs]. Annals of Internal Medicine 139(9):761-76, 2003.

(42) McConnaughey MM, McConnaughey JS, Ingenito AJ. Practical considerations of the pharmacology of angiotensin receptor blockers. [Review] [170 refs]. Journal of Clinical Pharmacology 39(6):547-59, 1999.

(43) Tea BS, Der SS, Touyz RM, Hamet P, deBlois D. Proapoptotic and growth-inhibitory role of angiotensin II type 2 receptor in vascular smooth muscle cells of spontaneously hypertensive rats in vivo. Hypertension 35(5):1069-73, 2000.

(44) Feinleib M, Garrison RJ, Fabsitz R, Christian JC, Hrubec Z, Borhani NO et al. The NHLBI twin study of cardiovascular disease risk factors: methodology and summary of results. American Journal of Epidemiology 106(4):284-5, 1977.

(45) Longini IM, Jr., Higgins MW, Hinton PC, Moll PP, Keller JB. Environmental and genetic sources of familial aggregation of blood pressure in Tecumseh, Michigan. American Journal of Epidemiology 120(1):131-44, 1984.

(46) Biron P, Mongeau JG, Bertrand D. Familial aggregation of blood pressure in 558 adopted children. Canadian Medical Association Journal 115(8):773-4, 1976.

(47) Samani NJ. Genome scans for hypertension and blood pressure regulation. [Review] [21 refs]. American Journal of Hypertension 16(2):167-71, 2003.

(48) Morris BJ, Benjafield AV, Lin RC. Essential hypertension: genes and dreams. [Review] [65 refs]. Clinical Chemistry & Laboratory Medicine 41(7):834-44, 2003.

(49) Agarwal A, Williams GH, Fisher ND. Genetics of human hypertension. [Review] [134 refs]. Trends in Endocrinology & Metabolism 16(3):127-33, 2005.

(50) Samani NJ, Brammar WJ, Swales JD. A major structural abnormality in the renin gene of the spontaneously hypertensive rat. Journal of Hypertension 7(4):249-54, 1989.

(51) Watt GC, Harrap SB, Foy CJ, Holton DW, Edwards HV, Davidson HR et al. Abnormalities of glucocorticoid metabolism and the renin-angiotensin system: a four-corners approach to the identification of genetic determinants of blood pressure. Journal of Hypertension 10(5):473-82, 1992.

(52) Kimura S, Mullins JJ, Bunnemann B, Metzger R, Hilgenfeldt U, Zimmermann F et al. High blood pressure in transgenic mice carrying the rat angiotensinogen gene. EMBO Journal 11(3):821-7, 1992.

(53) Jeunemaitre X, Charru A, Chatellier G, Dumont C, Sassano P, Soubrier F et al. M235T variant of the human angiotensinogen gene in unselected hypertensive patients. Journal of Hypertension - Supplement 11(5):S80-1, 1993.

(54) Corvol P, Jeunemaitre X, Charru A, Soubrier F. Can the genetic factors influence the treatment of systemic hypertension? The case of the renin-angiotensin-aldosterone system. [Review] [38 refs]. American Journal of Cardiology 70(12):14D-20D, 1992.

(55) Ward K, Hata A, Jeunemaitre X, Helin C, Nelson L, Namikawa C et al. A molecular variant of angiotensinogen associated with preeclampsia.[see comment]. Nature Genetics 4(1):59-61, 1993.

(56) Hegele RA, Brunt JH, Connelly PW. A polymorphism of the angiotensinogen gene associated with variation in blood pressure in a genetic isolate. Circulation 90(5):2207-12, 1994.

(57) Tiret L, Ricard S, Poirier O, Arveiler D, Cambou JP, Luc G et al. Genetic variation at the angiotensinogen locus in relation to high blood pressure and myocardial infarction: the ECTIM Study. Journal of Hypertension 13(3):311-7, 1995.

(58) Jeunemaitre X, Inoue I, Williams C, Charru A, Tichet J, Powers M et al. Haplotypes of angiotensinogen in essential hypertension. American Journal of Human Genetics 60(6):1448-60, 1997.

(59) Hubert C, Houot AM, Corvol P, Soubrier F. Structure of the angiotensin I-converting enzyme gene. Two alternate promoters correspond to evolutionary steps of a duplicated gene. Journal of Biological Chemistry 266(23):15377-83, 1991.

(60) Rigat B, Hubert C, Alhenc-Gelas F, Cambien F, Corvol P, Soubrier F. An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. Journal of Clinical Investigation 86(4):1343-6, 1990.

(61) Jeunemaitre X, Lifton RP, Hunt SC, Williams RR, Lalouel JM. Absence of linkage between the angiotensin converting enzyme locus and human essential hypertension. Nature Genetics 1(1):72-5, 1992.

(62) Harrap SB, Davidson HR, Connor JM, Soubrier F, Corvol P, Fraser R et al. The angiotensin I converting enzyme gene and predisposition to high blood pressure. Hypertension 21(4):455-60, 1993.

(63) Fornage M, Amos CI, Kardia S, Sing CF, Turner ST, Boerwinkle E. Variation in the region of the angiotensin-converting enzyme gene influences interindividual differences in blood pressure levels in young white males.[see comment][comment]. Circulation 97(18):1773-9, 1998.

(64) O'Donnell CJ, Lindpaintner K, Larson MG, Rao VS, Ordovas JM, Schaefer EJ et al. Evidence for association and genetic linkage of the angiotensin-converting enzyme locus with hypertension and blood pressure in men but not women in the Framingham Heart Study.[see comment]. Circulation 97(18):1766-72, 1998.

(65) Staessen JA, Wang JG, Ginocchio G, Petrov V, Saavedra AP, Soubrier F et al. The deletion/insertion polymorphism of the angiotensin converting enzyme gene and cardiovascular-renal risk. Journal of Hypertension 15(12 Pt 2):1579-92, 1997.

(66) Ferrandi M, Salardi S, Tripodi G, Barassi P, Rivera R, Manunta P et al. Evidence for an interaction between adducin and Na(+)-K(+)-ATPase: relation to genetic hypertension. American Journal of Physiology 277(4 Pt 2):H1338-49, 1999.

(67) Casari G, Barlassina C, Cusi D, Zagato L, Muirhead R, Righetti M et al. Association of the alpha-adducin locus with essential hypertension. Hypertension 25(3):320-6, 1995.

(68) Cusi D, Barlassina C, Azzani T, Casari G, Citterio L, Devoto M et al. Polymorphisms of alpha-adducin and salt sensitivity in patients with essential hypertension.[see comment][erratum appears in Lancet 1997 Aug 16;350(9076):524]. Lancet 349(9062):1353-7, 1997.

(69) Province MA, Arnett DK, Hunt SC, Leiendecker-Foster C, Eckfeldt JH, Oberman A et al. Association between the alpha-adducin gene and hypertension in the HyperGEN Study.[see comment][comment]. American Journal of Hypertension 13(6 Pt 1):710-8, 2000.

(70) Hopkins PN, Hunt SC. Genetics of hypertension. [Review] [288 refs]. Genetics in Medicine 5(6):413-29, 2003;-Dec.

(71) Lanzani C, Citterio L, Jankaricova M, Sciarrone MT, Barlassina C, Fattori S et al. Role of the adducin family genes in human essential hypertension.[see comment]. Journal of Hypertension 23(3):543-9, 2005.

(72) Cope G, Golbang A, O'Shaughnessy KM. WNK kinases and the control of blood pressure. [Review] [51 refs]. Pharmacology & Therapeutics 106(2):221-31, 2005.

(73) Gordon RD, Ravenscroft PJ, Klemm SA, Tunny TJ, Hamlet SM. A new Australian kindred with the syndrome of hypertension and hyperkalaemia has dysregulation of atrial natriuretic factor. Journal of Hypertension - Supplement 6(4):S323-6, 1988.

(74) Jacob HJ, Lindpaintner K, Lincoln SE, Kusumi K, Bunker RK, Mao YP et al. Genetic mapping of a gene causing hypertension in the stroke-prone spontaneously hypertensive rat. Cell 67(1):213-24, 1991.

(75) Levy D, DeStefano AL, Larson MG, O'Donnell CJ, Lifton RP, Gavras H et al. Evidence for a gene influencing blood pressure on chromosome 17. Genome scan linkage results for longitudinal blood pressure phenotypes in subjects from the framingham heart study.[see comment]. Hypertension 36(4):477-83, 2000.

(76) Erlich PM, Cui J, Chazaro I, Farrer LA, Baldwin CT, Gavras H et al. Genetic variants of WNK4 in whites and African Americans with hypertension. Hypertension 41(6):1191-5, 2003.

(77) Speirs HJ, Morris BJ. WNK4 intron 10 polymorphism is not associated with hypertension. Hypertension 43(4):766-8, 2004.

(78) Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. [Review] [101 refs]. Cell 104(4):545-56, 2001.

(79) Shoback DM, Williams GH, Moore TJ, Dluhy RG, Podolsky S, Hollenberg NK. Defect in the sodium-modulated tissue responsiveness to angiotensin II in essential hypertension. Journal of Clinical Investigation 72(6):2115-24, 1983.

(80) Lifton RP, Hopkins PN, Williams RR, Hollenberg NK, Williams GH, Dluhy RG. Evidence for heritability of non-modulating essential hypertension. Hypertension 13(6 Pt 2):884-9, 1989.

(81) Dluhy RG, Hopkins P, Hollenberg NK, Williams GH, Williams RR. Heritable abnormalities of the renin-angiotensin-aldosterone system in essential hypertension. Journal of Cardiovascular Pharmacology 12 Suppl 3:S149-54, 1988.

(82) Williams JS, Williams GH. 50th anniversary of aldosterone.[see comment]. Journal of Clinical Endocrinology & Metabolism 88(6):2364-72, 2003.

(83) Niarchos AP, Weinstein DL, Laragh JH. Comparison of the effects of diuretic therapy and low sodium intake in isolated systolic hypertension. American Journal of Medicine 77(6):1061-8, 1984.

(84) Fisher ND, Hurwitz S, Ferri C, Jeunemaitre X, Hollenberg NK, Williams GH. Altered adrenal sensitivity to angiotensin II in low-renin essential hypertension.[see comment]. Hypertension 34(3):388-94, 1999.

(85) Griffing GT, Wilson TE, Melby JC. Alterations in aldosterone secretion and metabolism in low renin hypertension. Journal of Clinical Endocrinology & Metabolism 71(6):1454-60, 1990.

(86) Fisher ND, Hurwitz S, Jeunemaitre X, Hopkins PN, Hollenberg NK, Williams GH. Familial aggregation of low-renin hypertension. Hypertension 39(4):914-8, 2002.

(87) Tabulae Anatomicae. Amsterdam: 1774.

(88) Arnold J. Ein Beitrag zu der feineren Structur und dem Chemismus der Nebennieren. Arch Pathol Anat Physiol Klin Med 1866; 35:64-107.

(89) Addison T. On anaemia: disease of the suprarenal capsules. London Medical Gazette 1849;517-518.

(90) Addison T. On the Constitutional and Local Effects of Disease of the Supra-renal Capsules. London: Highley, 1855.

(91) Brown-Sequard C. Recherches experimentales sur la physiologie et la pathologie des capsules surrenales. Archives of General Medicine 1856; 5(8):385-401.

(92) Swingle W, Pfiffner J. The effects of a lipid fraction upon the life-span of adrenalectomised cats. American Journal of Physiology 96, 153-163. 1931.
Ref Type: Journal (Full)

(93) Rowntree L, Greene C. The treatment of patients with Addison's disease with the 'cortical hormone' of Swingle and Pfiffner. Science 72, 482-483. 1930.
Ref Type: Journal (Full)

(94) Orth D, Kovacs W, Debold C. The Adrenal Cortex. In: Williams R, Wilson J, Foster D, editors. Williams Textbook of Endocrinology. Philadelphia: W.B Saunders, 1992: 489-619.

(95) Gwynne JT, Strauss JF, III. The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. [Review] [219 refs]. Endocrine Reviews 3(3):299-329, 1982.

(96) Goldstein JL, Anderson RG, Brown MS. Coated pits, coated vesicles, and receptor-mediated endocytosis. Nature 279(5715):679-85, 1979.

(97) Kimura T, Suzuki K. Components of the electron transport system in adrenal steroid hydroxylase. Isolation and properties of non-heme iron protein (adrenodoxin). Journal of Biological Chemistry 242(3):485-91, 1967.

(98) Kominami S, Ochi H, Kobayashi Y, Takemori S. Studies on the steroid hydroxylation system in adrenal cortex microsomes.

Purification and characterization of cytochrome P-450 specific for steroid C-21 hydroxylation. Journal of Biological Chemistry 255(8):3386-94, 1980.

(99) Yanagibashi K, Hall PF. Role of electron transport in the regulation of the lyase activity of C21 side-chain cleavage P-450 from porcine adrenal and testicular microsomes. Journal of Biological Chemistry 261(18):8429-33, 1986.

(100) Strott CA. The search for the elusive adrenal steroidogenic 'regulatory' protein. Trends in Endocrinology & Metabolism, 1990; 1:312-314.

(101) Chung BC, Matteson KJ, Voutilainen R, Mohandas TK, Miller WL. Human cholesterol side-chain cleavage enzyme, P450scc: cDNA cloning, assignment of the gene to chromosome 15, and expression in the placenta. Proceedings of the National Academy of Sciences of the United States of America 83(23):8962-6, 1986.

(102) Stocco DM. StARTing to understand cholesterol transfer.[comment]. Nature Structural Biology 7(6):445-7, 2000.

(103) Stocco DM. Intramitochondrial cholesterol transfer. [Review] [92 refs]. Biochimica et Biophysica Acta 1486(1):184-97, 2000.

(104) Bose HS, Lingappa VR, Miller WL. The steroidogenic acute regulatory protein, StAR, works only at the outer mitochondrial membrane. [Review] [47 refs]. Endocrine Research 28(4):295-308, 2002.

(105) Berube D, Luu T, V, Lachance Y, Gagne R, Labrie F. Assignment of the human 3 beta-hydroxysteroid dehydrogenase gene (HSDB3) to the p13 band of chromosome 1. Cytogenetics & Cell Genetics 52(3-4):199-200, 1989.

(106) Lachance Y, Luu-The V, Verreault H, Dumont M, Rheaume E, Leblanc G et al. Structure of the human type II 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase (3 beta-HSD) gene: adrenal and gonadal specificity. DNA & Cell Biology 10(10):701-11, 1991.

(107) Matteson KJ, Picado-Leonard J, Chung BC, Mohandas TK, Miller WL. Assignment of the gene for adrenal P450c17 (steroid 17 alpha-hydroxylase/17,20 lyase) to human chromosome 10. Journal of Clinical Endocrinology & Metabolism 63(3):789-91, 1986.

(108) Miller WL. Molecular biology of steroid hormone synthesis. [Review] [274 refs]. Endocrine Reviews 9(3):295-318, 1988.

(109) White PC, Chaplin DD, Weis JH, Dupont B, New MI, Seidman JG. Two steroid 21-hydroxylase genes are located in the murine S region. Nature 312(5993):465-7, 1984;-Dec.

(110) Mornet E, Dupont J, Vitek A, White PC. Characterization of two genes encoding human steroid 11 beta-hydroxylase (P-450(11) beta). Journal of Biological Chemistry 264(35):20961-7, 1989.

(111) Shizuta Y, Kawamoto T, Mitsuuchi Y, Toda K, Miyahara K, Ichikawa K et al. Molecular genetic studies on the biosynthesis of aldosterone in humans. Journal of Steroid Biochemistry & Molecular Biology 1992; 43:981-987.

(112) Owerbach D, Rutter WJ, Roberts JL, Whitfeld P, Shine J, Seeburg PH et al. The proopiocortin (adrenocorticotropin/beta-lipoprotein) gene is located on chromosome 2 in humans. Somatic Cell Genetics 7(3):359-69, 1981.

(113) Whitfield P, Seeburg P, Shine J. The human pro-opiomelanocortin gene: organisation, sequence, and interspersion with repetitive DNA. DNA 1982; 1:133-143.

(114) Smith I, Funder J. Proopiomelanocortin processing in the pituitary, central nervous system and peripheral tissues. Endocrine Reviews 1988; 9:159-179.

(115) Veldhuis JD, Iranmanesh A, Johnson ML, Lizarralde G. Amplitude, but not frequency, modulation of adrenocorticotropin secretory bursts gives rise to the nyctohemeral rhythm of the corticotropic axis in man. Journal of Clinical Endocrinology & Metabolism 71(2):452-63, 1990.

(116) Krieger DT, Allen W, Rizzo F, Krieger HP. Characterization of the normal temporal pattern of plasma corticosteroid levels. Journal of Clinical Endocrinology & Metabolism 32(2):266-84, 1971.

(117) Dickerman Z, Grant DR, Faiman C, Winter JS. Intraadrenal steroid concentrations in man: zonal differences and developmental changes. Journal of Clinical Endocrinology & Metabolism 59(6):1031-6, 1984.

(118) Hall PF. Trophic stimulation of steroidogenesis: in search of the elusive trigger. [Review] [71 refs]. Recent Progress in Hormone Research 41:1-39, 1985.

(119) Long C. Relations of cholesterol and ascorbic acid to secretion of adrenal cortex. Recent Progress in Hormone Research 1985; 1:99-122.

(120) Simpson ER, Waterman MR. Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. [Review] [75 refs]. Annual Review of Physiology 50:427-40, 1988.

(121) John ME, John MC, Boggaram V, Simpson ER, Waterman MR. Transcriptional regulation of steroid hydroxylase genes by corticotropin. Proceedings of the National Academy of Sciences of the United States of America 83(13):4715-9, 1986.

(122) Beato M. Gene regulation by steroid hormones. [Review] [122 refs]. Cell 56(3):335-44, 1989.

(123) Bodine PV, Litwack G. Purification and structural analysis of the modulator of the glucocorticoid-receptor complex. Evidence that modulator is a novel phosphoglyceride. Journal of Biological Chemistry 263(7):3501-12, 1988.

(124) Freedman LP. Anatomy of the steroid receptor zinc finger region. [Review] [132 refs]. Endocrine Reviews 13(2):129-45, 1992.

(125) Carson-Jurica MA, Schrader WT, O'Malley BW. Steroid receptor family: structure and functions. [Review] [310 refs]. Endocrine Reviews 11(2):201-20, 1990.

(126) Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS et al. Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. Lancet 2(8618):986-9, 1988.

(127) Walker BR, Best R, Shackleton CH, Padfield PL, Edwards CR. Increased vasoconstrictor sensitivity to glucocorticoids in essential hypertension. Hypertension 27(2):190-6, 1996.

(128) Williamson PM, Kelly JJ, Whitworth JA. Dose-response relationships and mineralocorticoid activity in cortisol-induced hypertension in humans. Journal of Hypertension - Supplement 14(5):S37-41, 1996.

(129) Clore JN, Estep H, Ross-Clunis H, Watlington CO. Adrenocorticotropin and cortisol-induced changes in urinary sodium and potassium excretion in man: effects of spironolactone and RU486. Journal of Clinical Endocrinology & Metabolism 67(4):824-31, 1988.

(130) Pirpiris M, Yeung S, Dewar E, Jennings GL, Whitworth JA. Hydrocortisone-induced hypertension in men. The role of cardiac output. American Journal of Hypertension 6(4):287-94, 1993.

(131) Macefield VG, Williamson PM, Wilson LR, Kelly JJ, Gandevia SC, Whitworth JA. Muscle sympathetic vasoconstrictor activity in hydrocortisone-induced hypertension in humans. Blood Pressure 7(4):215-22, 1998.

(132) Tam SH, Kelly JJ, Williamson PM, Whitworth JA. Reflex sympathetic function in cortisol-induced hypertension in humans. Clinical & Experimental Hypertension (New York) 1919;(4):479-493.

(133) Tam SH, Williamson PM, Kelly JJ, Whitworth JA. Autonomic blockade amplifies cortisol-induced hypertension in man. Clinical & Experimental Pharmacology & Physiology 24(1):31-3, 1997.

(134) Genest J, Lemieux G, Davignon A, Nowaczynski W, Steyermark P. Human arterial hypertension: a state of mild chronic hyperaldosteronism? Science 123, 503. 1956.
Ref Type: Journal (Full)

(135) Symington T. The adrenal in hypertension. Functional pathology of the adrenal gland. Edinburgh: E & S Livingstone Limited, 2002.

(136) Komiya I, Yamada T, Aizawa T, Takasu N, Niwa A, Maruyama Y et al. Inappropriate elevation of the aldosterone/plasma renin activity ratio in hypertensive patients with increases of 11-deoxycorticosterone and 18-hydroxy-11-deoxycorticosterone: a subtype of essential hypertension? Cardiology 78(2):99-110, 1991.

(137) Brown JJ, Fraser R, Love DR, Ferriss JB, Lever AF, Robertson JI et al. Apparently isolated excess deoxycorticosterone in hypertension. A variant of the mineralocorticoid-excess syndrome. Lancet 2(7771):243-7, 1972.

(138) Genest J, Nowaczynski W, Kuchel O, Boucher R, Rojo-Ortega JM. The role of the adrenal cortex in human essential hypertension: keynote address. Mayo Clinic Proceedings 52(5):291-307, 1977.

(139) Genest J, Nowaczynski W, Kuchel O, Boucher R, Rojo-Ortega JM, Constantopoulos G et al. The adrenal cortex and essential hypertension. [Review] [151 refs]. Recent Progress in Hormone Research 32:377-427, 1976.

(140) Honda M, Nowaczynski W, Messerli FH, Kuchel O, Genest J. Plasma deoxycorticosterone and aldosterone in essential hypertension. Journal of Steroid Biochemistry 7:565-9, 1976.

(141) Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S et al. A chimaeric 11 beta-hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. Nature 355(6357):262-5, 1992.

(142) White PC. Steroid 11 beta-hydroxylase deficiency and related disorders. [Review] [60 refs]. Endocrinology & Metabolism Clinics of North America 30(1):61-79, vi, 2001.

(143) de Simone G, Tommaselli AP, Rossi R, Valentino R, Lauria R, Scopacasa F et al. Partial deficiency of adrenal 11-hydroxylase. A possible cause of primary hypertension. Hypertension 7(2):204-10, 1985;-Apr.

(144) Honda M, Nowaczynski W, Guthrie GP, Jr., Messerli FH, Tolis G, Kuchel O et al. Response of several adrenal steroids to ACTH stimulation in essential hypertension. Journal of Clinical Endocrinology & Metabolism 44(2):264-72, 1977.

(145) Connell JM, Jamieson AJ, Davies E, Ingram M, Soro A, Fraser R. 11 beta-Hydroxylase activity in glucocorticoid suppressible hyperaldosteronism: lessons for essential hypertension? Endocrine Research 22(4):691-700, 1996.

(146) Cicila GT, Rapp JP, Wang JM, St Lezin E, Ng SC, Kurtz TW. Linkage of 11 beta-hydroxylase mutations with altered steroid biosynthesis and blood pressure in the Dahl rat. Nature Genetics 3(4):346-53, 1993.

(147) SIMPSON SA, Tait JF, WETTSTEIN A, NEHER R, VON EUW J, REICHSTEIN T. [Isolation from the adrenals of a new crystalline hormone with especially high effectiveness on mineral metabolism.]. [Undetermined]. Experientia 9(9):333-5, 1953.

(148) SIMPSON SA, Tait JF, WETTSTEIN A, NEHER R, VON EUW J, SCHINDLER O et al. [Constitution of aldosterone, a new mineralocorticoid.]. [Undetermined]. Experientia 10(3):132-3, 1954.

(149) KLIMAN B, PETERSON RE. Double isotope derivative assay of aldosterone in biological extracts. Journal of Biological Chemistry 235:1639-48, 1960.

(150) Conn JW. Primary aldosteronism: a new clinical syndrome. J Lab Clin Med 45, 3-17. 1955.
Ref Type: Journal (Full)

(151) Conn J. Plasma renin activity in primary aldosteronism. Importance in differential diagnosis and in research of essential hypertension. Journal of the American Medical Association 190, 222-225. 1964.
Ref Type: Journal (Full)

(152) Kaplan N. Clinical Hypertension. 6 ed. Baltimore: Williams and Wilkins, 1994.

(153) Curnow KM, Tusie-Luna MT, Pascoe L, Natarajan R, Gu JL, Nadler JL et al. The product of the CYP11B2 gene is required for aldosterone biosynthesis in the human adrenal cortex. Molecular Endocrinology 5(10):1513-22, 1991.

(154) White PC, Curnow KM, Pascoe L. Disorders of steroid 11 beta-hydroxylase isozymes. [Review] [209 refs]. Endocrine Reviews 15(4):421-38, 1994.

(155) Wagner MJ, Ge Y, Siciliano M, Wells DE. A hybrid cell mapping panel for regional localization of probes to human chromosome 8. Genomics 10(1):114-25, 1991.

(156) Kawamoto T, Mitsuuchi Y, Toda K, Yokoyama Y, Miyahara K, Miura S et al. Role of steroid 11 beta-hydroxylase and steroid 18-hydroxylase in the biosynthesis of glucocorticoids and

mineralocorticoids in humans. Proceedings of the National Academy of Sciences of the United States of America 89(4):1458-62, 1992.

(157) Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T, Sraer JD. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin.[see comment]. Journal of Clinical Investigation 109(11):1417-27, 2002.

(158) Farese RV, Larson RE, Davis JS. Rapid effects of angiotensin-II on polyphosphoinositide metabolism in the rat adrenal glomerulosa. Endocrinology 114(1):302-4, 1984.

(159) Spat A, Hunyady L. Control of aldosterone secretion: a model for convergence in cellular signaling pathways. [Review] [602 refs]. Physiological Reviews 84(2):489-539, 2004.

(160) Bassett MH, Suzuki T, Sasano H, White PC, Rainey WE. The orphan nuclear receptors NURR1 and NGFIB regulate adrenal aldosterone production. Molecular Endocrinology 18(2):279-90, 2004.

(161) Kenyon CJ, Shepherd RM, Fraser R, Pediani JD, Elder HY. The role of potassium and other ions in the control of aldosterone synthesis. Endocrine Research 17(1-2):225-36, 1991.

(162) Braley LM, Menachery AI, Brown EM, Williams GH. Comparative effect of angiotensin II, potassium, adrenocorticotropin, and cyclic adenosine 3',5'-monophosphate on cytosolic calcium in rat adrenal cells. Endocrinology 119(3):1010-9, 1986.

(163) Aguilera G, Catt KJ. Regulation of aldosterone secretion by the renin-angiotensin system during sodium restriction in rats. Proceedings of the National Academy of Sciences of the United States of America 75(8):4057-61, 1978.

(164) Oelkers W, Brown JJ, Fraser R, Lever AF, Morton JJ, Robertson JI. Sensitization of the adrenal cortex to angiotensin II in sodium-deplete man. Circulation Research 40(4):69-77, 1974.

(165) Hollenberg NK, Chenitz WR, Adams DF, Williams GH. Reciprocal influence of salt intake on adrenal glomerulosa and renal vascular responses to angiotensin II in normal man. Journal of Clinical Investigation 54(1):34-42, 1974.

(166) Hollenberg NK, Williams G, Burger B, Hooshmand I. The influence of potassium on the renal vasculature and the adrenal gland, and their responsiveness to angiotensin II in normal man. Clinical Science & Molecular Medicine 49(6):527-34, 1975.

(167) Cannon PJ, Ames RP, Laragh JH. Relation between potassium balance and aldosterone secretion in normal subjects and in patients with hypertensive or renal tubular disease. Journal of Clinical Investigation 45(6):865-79, 1966.

(168) Carey RM, Wang ZQ, Siragy HM. Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. [Review] [92 refs]. Hypertension 35(1 Pt 2):155-63, 2000.

(169) Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S et al. Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation.[see comment]. Journal of Clinical Investigation 104(7):925-35, 1999.

(170) Batenburg WW, Garrelds IM, Bernasconi CC, Juillerat-Jeanneret L, van Kats JP, Saxena PR et al. Angiotensin II type 2 receptor-mediated vasodilation in human coronary microarteries. Circulation 109(19):2296-301, 2004.

(171) Hollenberg NK, Fisher ND, Price DA. Pathways for angiotensin II generation in intact human tissue: evidence from comparative pharmacological interruption of the renin system. [Review] [36 refs]. Hypertension 32(3):387-92, 1998.

(172) Shiota N, Okunishi H, Takai S, Mikoshiba I, Sakonjo H, Shibata N et al. Tranilast suppresses vascular chymase expression and neointima formation in balloon-injured dog carotid artery. Circulation 99(8):1084-90, 1999.

(173) Urata H, Nishimura H, Ganten D, Arakawa K. Angiotensin-converting enzyme-independent pathways of angiotensin II formation in human tissues and cardiovascular diseases. [Review] [49 refs]. Blood Pressure Supplement 2:22-8, 1996.

(174) Urata H, Nishimura H, Ganten D. Chymase-dependent angiotensin II forming systems in humans. [Review] [63 refs]. American Journal of Hypertension 9(3):277-84, 1996.

(175) Spat A. Glomerulosa cell--a unique sensor of extracellular K+ concentration. [Review] [31 refs]. Molecular & Cellular Endocrinology 217(1-2):23-6, 2004.

(176) Dluhy RG, Axelrod L, Underwood RH, Williams GH. Studies of the control of plasma aldosterone concentration in normal man. II. Effect of dietary potassium and acute potassium infusion. Journal of Clinical Investigation 51(8):1950-7, 1972.

(177) Kojima I, Kojima K, Rasmussen H. Intracellular calcium and adenosine 3',5'-cyclic monophosphate as mediators of potassium-induced aldosterone secretion. Biochemical Journal 228(1):69-76, 1985.

(178) Clyne CD, Zhang Y, Slutsker L, Mathis JM, White PC, Rainey WE. Angiotensin II and potassium regulate human CYP11B2 transcription through common cis-elements. Molecular Endocrinology 11(5):638-49, 1997.

(179) Arvat E, Di Vito L, Lanfranco F, MacCario M, Baffoni C, Rossetto R et al. Stimulatory effect of adrenocorticotropin on cortisol, aldosterone, and dehydroepiandrosterone secretion in normal humans: dose-response study. Journal of Clinical Endocrinology & Metabolism 85(9):3141-6, 2000.

(180) Ramirez G, Ganguly A, Brueggemeyer CD. Acute effect of captopril on aldosterone secretory responses to endogenous or exogenous adrenocorticotropin. Journal of Clinical Endocrinology & Metabolism 66(1):46-50, 1988.

(181) Giordano R, Di Vito L, Lanfranco F, Broglio F, Benso A, Gianotti L et al. Elderly subjects show severe impairment of dehydroepiandrosterone sulphate and reduced sensitivity of cortisol and aldosterone response to the stimulatory effect of ACTH(1-24). Clinical Endocrinology 55(2):259-65, 2001.

(182) Connell JM, Whitworth JA, Davies DL, Lever AF, Richards AM, Fraser R. Effects of ACTH and cortisol administration on blood pressure, electrolyte metabolism, atrial natriuretic peptide and renal function in normal man. Journal of Hypertension 5(4):425-33, 1987.

(183) Connell JM, Whitworth JA, Davies DL, Richards AM, Fraser R. Haemodynamic, hormonal and renal effects of adrenocorticotrophic hormone in sodium-restricted man. Journal of Hypertension 6(1):17-23, 1988.

(184) Whitworth JA, Mangos GJ, Kelly JJ. Cushing, cortisol, and cardiovascular disease. [Review] [56 refs]. Hypertension 36(5):912-6, 2000.

(185) Coll AP, Challis BG, Yeo GS, Snell K, Piper SJ, Halsall D et al. The effects of proopiomelanocortin deficiency on murine adrenal development and responsiveness to adrenocorticotropin. Endocrinology 145(10):4721-7, 2004.

(186) Johnston CI, Hodsman PG, Kohzuki M, Casley DJ, Fabris B, Phillips PA. Interaction between atrial natriuretic peptide and the renin angiotensin aldosterone system. Endogenous antagonists. [Review] [67 refs]. American Journal of Medicine 87(6B):24S-28S, 1989.

(187) Kawamoto T, Mitsuuchi Y, Ohnishi Y, Ichikawa Y, Sumimoto H, Toda K et al. Cloning and expression of a cDNA for human cytochrome P-450aldo as related to primary aldosteronism. Biochemical & Biophysical Research Communications 1990; 173:309-316.

(188) Ogishima T, Shibata H, Shimada H, Mitani F, Suzuki H, Saruta T et al. Aldosterone synthase cytochrome P-450 expressed in the adrenals of patients with primary aldosteronism. Journal of Biological Chemistry 266(17):10731-4, 1991.

(189) Zuber MX, Mason JI, Simpson ER, Waterman MR. Simultaneous transfection of COS-1 cells with mitochondrial and microsomal steroid hydroxylases: incorporation of a steroidogenic pathway into nonsteroidogenic cells. Proceedings of the National Academy of Sciences of the United States of America 85(3):699-703, 1988.

(190) Chua SC, Szabo P, Vitek A, Grzeschik KH, John M, White PC. Cloning of cDNA encoding steroid 11 beta-hydroxylase (P450c11). Proceedings of the National Academy of Sciences of the United States of America 84(20):7193-7, 1987.

(191) Morohashi K, Sogawa K, Omura T, Fujii-Kuriyama Y. Gene structure of human cytochrome P-450(SCC), cholesterol desmolase. Journal of Biochemistry 101(4):879-87, 1987.

(192) Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R et al. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. [Review] [605 refs]. DNA & Cell Biology 12(1):1-51, 1993;-Feb.

(193) Kao CC, Lieberman PM, Schmidt MC, Zhou Q, Pei R, Berk AJ. Cloning of a transcriptionally active human TATA binding factor. Science 248(4963):1646-50, 1990.

(194) Meyer TE, Habener JF. Cyclic adenosine 3',5'-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. [Review] [170 refs]. Endocrine Reviews 14(3):269-90, 1993.

(195) Kawamoto T, Mitsuuchi Y, Toda K, Miyahara K, Yokoyama Y, Nakao K et al. Cloning of cDNA and genomic DNA for human cytochrome P-45011 beta. FEBS Letters 269(2):345-9, 1990.

(196) Rice DA, Mouw AR, Bogerd AM, Parker KL. A shared promoter element regulates the expression of three steroidogenic enzymes. Molecular Endocrinology 5(10):1552-61, 1991.

(197) Morohashi K, Honda S, Inomata Y, Handa H, Omura T. A common trans-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. Journal of Biological Chemistry 267(25):17913-9, 1992.

(198) Tsukiyama T, Ueda H, Hirose S, Niwa O. Embryonal long terminal repeat-binding protein is a murine homolog of FTZ-F1, a member of the steroid receptor superfamily. Molecular & Cellular Biology 12(3):1286-91, 1992.

(199) Wilson TE, Fahrner TJ, Milbrandt J. The orphan receptors NGFI-B and steroidogenic factor 1 establish monomer binding as a third

paradigm of nuclear receptor-DNA interaction. Molecular & Cellular Biology 13(9):5794-804, 1993.

(200) Parker KL, Schimmer BP. Steroidogenic factor 1: a key determinant of endocrine development and function. [Review] [123 refs]. Endocrine Reviews 18(3):361-77, 1997.

(201) Luo X, Ikeda Y, Parker KL. A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell 77(4):481-90, 1994.

(202) Sadovsky Y, Crawford PA, Woodson KG, Polish JA, Clements MA, Tourtellotte LM et al. Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. Proceedings of the National Academy of Sciences of the United States of America 92(24):10939-43, 1995.

(203) Marchal R, Naville D, Durand P, Begeot M, Penhoat A. A steroidogenic factor-1 binding element is essential for basal human ACTH receptor gene transcription. Biochemical & Biophysical Research Communications 247(1):28-32, 1998.

(204) Sugawara T, Holt JA, Kiriakidou M, Strauss JF, III. Steroidogenic factor 1-dependent promoter activity of the human steroidogenic acute regulatory protein (StAR) gene. Biochemistry 35(28):9052-9, 1996.

(205) Sugawara T, Kiriakidou M, McAllister JM, Holt JA, Arakane F, Strauss JF, III. Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: a central role for steroidogenic factor 1.[erratum appears in Steroids 1997 Apr;62(4):395]. [Review] [12 refs]. Steroids 62(1):5-9, 1997.

(206) LeHoux JG, Fleury A, Ducharme L. The acute and chronic effects of adrenocorticotropin on the levels of messenger ribonucleic acid and protein of steroidogenic enzymes in rat adrenal in vivo. Endocrinology 139(9):3913-22, 1998.

(207) Bassett MH, Zhang Y, Clyne C, White PC, Rainey WE. Differential regulation of aldosterone synthase and 11beta-hydroxylase transcription by steroidogenic factor-1. Journal of Molecular Endocrinology 28(2):125-35, 2002.

(208) Clyne CD, White PC, Rainey WE. Calcium regulates human CYP11B2 transcription. Endocrine Research 22(4):485-92, 1996.

(209) Domalik LJ, Chaplin DD, Kirkman MS, Wu RC, Liu WW, Howard TA et al. Different isozymes of mouse 11 beta-hydroxylase produce

mineralocorticoids and glucocorticoids. Molecular Endocrinology 5(12):1853-61, 1991.

(210) Pascoe L, Curnow KM, Slutsker L, Rosler A, White PC. Mutations in the human CYP11B2 (aldosterone synthase) gene causing corticosterone methyloxidase II deficiency. Proceedings of the National Academy of Sciences of the United States of America 89(11):4996-5000, 1992.

(211) Pascoe L, Curnow KM, Slutsker L, Connell JM, Speiser PW, New MI et al. Glucocorticoid-suppressible hyperaldosteronism results from hybrid genes created by unequal crossovers between CYP11B1 and CYP11B2. Proceedings of the National Academy of Sciences of the United States of America 89(17):8327-31, 1992.

(212) Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S et al. A chimaeric 11 beta-hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and human hypertension. Nature 355(6357):262-5, 1992.

(213) Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE et al. Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237(4812):268-75, 1987.

(214) Komesaroff P, Funder J, Fuller P. Mineralocorticoid Resistance. Ballieres Clinical Endocrinology and Metabolism. London: Balliere Tindall, 1994: 333-355.

(215) Webster MK, Goya L, Ge Y, Maiyar AC, Firestone GL. Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. Molecular & Cellular Biology 13(4):2031-40, 1993.

(216) Naray-Fejes-Toth A, Canessa C, Cleaveland ES, Aldrich G, Fejes-Toth G. sgk is an aldosterone-induced kinase in the renal collecting duct. Effects on epithelial na+ channels. Journal of Biological Chemistry 274(24):16973-8, 1999.

(217) Rotin D. Regulation of the epithelial sodium channel (ENaC) by accessory proteins. [Review] [72 refs]. Current Opinion in Nephrology & Hypertension 9(5):529-34, 2000.

(218) Stockand JD, Meszaros JG. Aldosterone stimulates proliferation of cardiac fibroblasts by activating Ki-RasA and MAPK1/2 signaling. American Journal of Physiology - Heart & Circulatory Physiology 284(1):H176-84, 2003.

(219) Stockand JD. New ideas about aldosterone signaling in epithelia. [Review] [202 refs]. American Journal of Physiology - Renal Physiology 282(4):F559-76, 2002.

(220) Schulz R, Nava E, Moncada S. Induction and potential biological relevance of a Ca(2+)-independent nitric oxide synthase in the myocardium. British Journal of Pharmacology 105(3):575-80, 1992.

(221) Winter C, Schulz N, Giebisch G, Geibel JP, Wagner CA. Nongenomic stimulation of vacuolar H+-ATPases in intercalated renal tubule cells by aldosterone. Proceedings of the National Academy of Sciences of the United States of America 101(8):2636-41, 2004.

(222) Mihailidou AS, Mardini M, Funder JW. Rapid, nongenomic effects of aldosterone in the heart mediated by epsilon protein kinase C. Endocrinology 145(2):773-80, 2004.

(223) Krozowski ZS, Funder JW. Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. Proceedings of the National Academy of Sciences of the United States of America 80(19):6056-60, 1983.

(224) Agarwal AK, Monder C, Eckstein B, White PC. Cloning and expression of rat cDNA encoding corticosteroid 11 beta-dehydrogenase. Journal of Biological Chemistry 264(32):18939-43, 1989.

(225) Jamieson PM, Chapman KE, Edwards CR, Seckl JR. 11 beta-hydroxysteroid dehydrogenase is an exclusive 11 beta- reductase in primary cultures of rat hepatocytes: effect of physicochemical and hormonal manipulations. Endocrinology 136(11):4754-61, 1995.

(226) Wake DJ, Walker BR. 11 beta-hydroxysteroid dehydrogenase type 1 in obesity and the metabolic syndrome. [Review] [84 refs]. Molecular & Cellular Endocrinology 215(1-2):45-54, 2004.

(227) White PC. Disorders of aldosterone biosynthesis and action. [Review] [63 refs]. New England Journal of Medicine 331(4):250-8, 1994.

(228) Horisberger JD. Amiloride-sensitive Na channels. [Review] [43 refs]. Current Opinion in Cell Biology 10(4):443-9, 1998.

(229) Garty H, Palmer LG. Epithelial sodium channels: function, structure, and regulation. [Review] [451 refs]. Physiological Reviews 77(2):359-96, 1997.

(230) Hays SR. Mineralocorticoid modulation of apical and basolateral membrane H+/OH-/HCO3- transport processes in the rabbit inner stripe of outer medullary collecting duct. Journal of Clinical Investigation 90(1):180-7, 1992.

(231) Kremer D, Boddy K, Brown JJ, Davies DL, Fraser R, Lever AF et al. Amiloride in the treatment of primary hyperaldosteronism and essential hypertension. Clinical Endocrinology 7(2):151-7, 1977.

(232) Agarwal MK, Mirshahi F, Mirshahi M, Rostene W. Immunochemical detection of the mineralocorticoid receptor in rat brain. Neuroendocrinology 58(5):575-80, 1993.

(233) Diaz R, Brown RW, Seckl JR. Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase types I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions. Journal of Neuroscience 18(7):2570-80, 1998.

(234) Robson AC, Leckie CM, Seckl JR, Holmes MC. 11 Beta-hydroxysteroid dehydrogenase type 2 in the postnatal and adult rat brain. Brain Research Molecular Brain Research 61(1-2):1-10, 1998.

(235) Seckl JR. 11beta-Hydroxysteroid dehydrogenase in the brain: a novel regulator of glucocorticoid action?. [Review] [265 refs]. Frontiers in Neuroendocrinology 18(1):49-99, 1997.

(236) Sakai RR, Ma LY, Zhang DM, McEwen BS, Fluharty SJ. Intracerebral administration of mineralocorticoid receptor antisense oligonucleotides attenuate adrenal steroid-induced salt appetite in rats. Neuroendocrinology 64(6):425-9, 1996.

(237) Tan LB, Schlosshan D, Barker D. Fiftieth anniversary of aldosterone: from discovery to cardiovascular therapy. [Review] [151 refs]. International Journal of Cardiology 96(3):321-33, 2004.

(238) van Zwieten PA. Neuroendocrine effects of diuretics in heart failure. [Review] [21 refs]. British Heart Journal 72(2 Suppl):S51-3, 1994.

(239) Bayliss J, Norell M, Canepa-Anson R, Sutton G, Poole-Wilson P. Untreated heart failure: clinical and neuroendocrine effects of introducing diuretics. British Heart Journal 57(1):17-22, 1987.

(240) Weber KT, Janicki JS, Pick R, Capasso J, Anversa P. Myocardial fibrosis and pathologic hypertrophy in the rat with renovascular hypertension. [Review] [66 refs]. American Journal of Cardiology 65(14):1G-7G, 1990.

(241) Weber KT, Brilla CG. Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. [Review] [109 refs]. Circulation 83(6):1849-65, 1991.

(242) Young M, Fullerton M, Dilley R, Funder J. Mineralocorticoids, hypertension, and cardiac fibrosis. Journal of Clinical Investigation 93(6):2578-83, 1994.

(243) Brilla CG, Weber KT. Reactive and reparative myocardial fibrosis in arterial hypertension in the rat. Cardiovascular Research 26(7):671-7, 1992.

(244) Funder JW. Is aldosterone bad for the heart?. [Review] [20 refs]. Trends in Endocrinology & Metabolism 15(4):139-42, 2004;-Jun.

(245) Funder JW. Aldosterone, mineralocorticoid receptors and vascular inflammation. [Review] [41 refs]. Molecular & Cellular Endocrinology 217(1-2):263-9, 2004.

(246) White PC. Aldosterone: direct effects on and production by the heart. [Review] [80 refs]. Journal of Clinical Endocrinology & Metabolism 88(6):2376-83, 2003.

(247) Sun Y, Zhang J, Lu L, Chen SS, Quinn MT, Weber KT. Aldosterone-induced inflammation in the rat heart : role of oxidative stress. American Journal of Pathology 161(5):1773-81, 2002.

(248) Ammarguellat F, Larouche I, Schiffrin EL. Myocardial fibrosis in DOCA-salt hypertensive rats: effect of endothelin ET(A) receptor antagonism. Circulation 103(2):319-24, 2001.

(249) Brilla CG, Zhou G, Matsubara L, Weber KT. Collagen metabolism in cultured adult rat cardiac fibroblasts: response to angiotensin II and aldosterone. Journal of Molecular & Cellular Cardiology 26(7):809-20, 1994.

(250) Sheppard KE, Autelitano DJ. 11Beta-hydroxysteroid dehydrogenase 1 transforms 11-dehydrocorticosterone into transcriptionally active glucocorticoid in neonatal rat heart. Endocrinology 143(1):198-204, 2002.

(251) Cai TQ, Wong B, Mundt SS, Thieringer R, Wright SD, Hermanowski-Vosatka A. Induction of 11beta-hydroxysteroid dehydrogenase type 1 but not -2 in human aortic smooth muscle cells by inflammatory stimuli. Journal of Steroid Biochemistry & Molecular Biology 77(2-3):117-22, 2001.

(252) Keidar S, Kaplan M, Pavlotzky E, Coleman R, Hayek T, Hamoud S et al. Aldosterone administration to mice stimulates macrophage NADPH oxidase and increases atherosclerosis development: a possible role for angiotensin-converting enzyme and the receptors for angiotensin II and aldosterone. Circulation 109(18):2213-20, 2004.

(253) Ikeda U, Kanbe T, Nakayama I, Kawahara Y, Yokoyama M, Shimada K. Aldosterone inhibits nitric oxide synthesis in rat vascular smooth muscle cells induced by interleukin-1 beta. European Journal of Pharmacology 290(2):69-73, 1995.

(254) Farquharson CA, Struthers AD. Spironolactone increases nitric oxide bioactivity, improves endothelial vasodilator dysfunction, and

suppresses vascular angiotensin I/angiotensin II conversion in patients with chronic heart failure. Circulation 101(6):594-7, 2000.

(255) Duprez D, De Buyzere M, Rietzschel ER, Clement DL. Aldosterone and vascular damage. [Review] [48 refs]. Current Hypertension Reports 2(3):327-34, 2000.

(256) Duprez DA, De Buyzere ML, Rietzschel ER, Taes Y, Clement DL, Morgan D et al. Inverse relationship between aldosterone and large artery compliance in chronically treated heart failure patients.[see comment]. European Heart Journal 1919;(9):1371-1376.

(257) Davies E, MacKenzie SM. Extra-adrenal production of corticosteroids. [Review] [77 refs]. Clinical & Experimental Pharmacology & Physiology 30(7):437-45, 2003.

(258) Ye P, Kenyon CJ, MacKenzie SM, Seckl JR, Fraser R, Connell JM et al. Regulation of aldosterone synthase gene expression in the rat adrenal gland and central nervous system by sodium and angiotensin II. Endocrinology 144(8):3321-8, 2003.

(259) Silvestre JS, Robert V, Heymes C, Aupetit-Faisant B, Mouas C, Moalic JM et al. Myocardial production of aldosterone and corticosterone in the rat. Physiological regulation. Journal of Biological Chemistry 273(9):4883-91, 1998.

(260) Gomez-Sanchez EP, Ahmad N, Romero DG, Gomez-Sanchez CE. Origin of aldosterone in the rat heart.[see comment]. Endocrinology 145(11):4796-802, 2004.

(261) Yoshimura M, Nakamura S, Ito T, Nakayama M, Harada E, Mizuno Y et al. Expression of aldosterone synthase gene in failing human heart: quantitative analysis using modified real-time polymerase chain reaction. Journal of Clinical Endocrinology & Metabolism 87(8):3936-40, 2002.

(262) Ye P, Kenyon CJ, MacKenzie SM, Jong AS, Miller C, Gray GA et al. The aldosterone synthase (CYP11B2) and 11beta-hydroxylase (CYP11B1) genes are not expressed in the rat heart. Endocrinology 146(12):5287-93, 2005.

(263) Fiebeler A, Nussberger J, Shagdarsuren E, Rong S, Hilfenhaus G, Al Saadi N et al. Aldosterone synthase inhibitor ameliorates angiotensin II-induced organ damage. Circulation 111(23):3087-94, 2005.

(264) Borghi C, Boschi S, Ambrosioni E, Melandri G, Branzi A, Magnani B. Evidence of a partial escape of renin-angiotensin-aldosterone blockade in patients with acute myocardial infarction treated with ACE inhibitors. Journal of Clinical Pharmacology 33(1):40-5, 1993.

(265) McKelvie RS, Yusuf S, Pericak D, Avezum A, Burns RJ, Probstfield J et al. Comparison of candesartan, enalapril, and their combination in

congestive heart failure: randomized evaluation of strategies for left ventricular dysfunction (RESOLVD) pilot study. The RESOLVD Pilot Study Investigators.[see comment]. Circulation 100(10):1056-64, 1999.

(266) Umemura S, Kihara M, Sumida Y, Yabana M, Ishigami T, Tamura K et al. Endocrinological abnormalities in angiotensinogen-gene knockout mice: studies of hormonal responses to dietary salt loading. Journal of Hypertension 16(3):285-9, 1998.

(267) Vantrimpont P, Rouleau JL, Ciampi A, Harel F, de Champlain J, Bichet D et al. Two-year time course and significance of neurohumoral activation in the Survival and Ventricular Enlargement (SAVE) Study.[see comment]. European Heart Journal 1919;(10):1552-1563.

(268) Swedberg K, Eneroth P, Kjekshus J, Snapinn S. Effects of enalapril and neuroendocrine activation on prognosis in severe congestive heart failure (follow-up of the CONSENSUS trial). CONSENSUS Trial Study Group. American Journal of Cardiology 66(11):40D-44D; discussion 44D-45D, 1990.

(269) Cicoira M, Zanolla L, Franceschini L, Rossi A, Golia G, Zeni P et al. Relation of aldosterone "escape" despite angiotensin-converting enzyme inhibitor administration to impaired exercise capacity in chronic congestive heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. American Journal of Cardiology 89(4):403-7, 2002.

(270) Blasi ER, Rocha R, Rudolph AE, Blomme EA, Polly ML, McMahon EG. Aldosterone/salt induces renal inflammation and fibrosis in hypertensive rats. Kidney International 63(5):1791-800, 2003.

(271) Sato A, Hayashi K, Naruse M, Saruta T. Effectiveness of aldosterone blockade in patients with diabetic nephropathy. Hypertension 41(1):64-8, 2003.

(272) Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A et al. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized Aldactone Evaluation Study Investigators.[see comment]. New England Journal of Medicine 341(10):709-17, 1999.

(273) Zannad F, Alla F, Dousset B, Perez A, Pitt B. Limitation of excessive extracellular matrix turnover may contribute to survival benefit of spironolactone therapy in patients with congestive heart failure: insights from the randomized aldactone evaluation study (RALES). Rales Investigators.[erratum appears in Circulation 2001 Jan 23;103(3):476]. Circulation 102(22):2700-6, 2000.

(274) Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B et al. Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction.[see comment][erratum appears in N Engl J Med. 2003 May 29;348(22):2271]. New England Journal of Medicine 348(14):1309-21, 2003.

(275) SHEPARD TH, CLAUSEN SW. Case of adrenogenital syndrome with hypertension treated with cortisone. Pediatrics 8(6):805-11, 1951.

(276) Levine LS, Rauh W, Gottesdiener K, Chow D, Gunczler P, Rapaport R et al. New studies of the 11 beta-hydroxylase and 18-hydroxylase enzymes in the hypertensive form of congenital adrenal hyperplasia. Journal of Clinical Endocrinology & Metabolism 50(2):258-63, 1980.

(277) Zachmann M, Tassinari D, Prader A. Clinical and biochemical variability of congenital adrenal hyperplasia due to 11 beta-hydroxylase deficiency. A study of 25 patients. Journal of Clinical Endocrinology & Metabolism 56(2):222-9, 1983.

(278) Rosler A, Leiberman E, Cohen T. High frequency of congenital adrenal hyperplasia (classic 11 beta-hydroxylase deficiency) among Jews from Morocco. American Journal of Medical Genetics 42(6):827-34, 1992.

(279) Lee HH, Won GS, Chao HT, Lee YJ, Chung BC. Novel missense mutations, GCC [Ala306]- > GTC [Val] and ACG [Thr318]- > CCG [Pro], in the CYP11B1 gene cause steroid 11beta-hydroxylase deficiency in the Chinese. Clinical Endocrinology 62(4):418-22, 2005.

(280) White PC, Dupont J, New MI, Leiberman E, Hochberg Z, Rosler A. A mutation in CYP11B1 (Arg-448----His) associated with steroid 11 beta-hydroxylase deficiency in Jews of Moroccan origin. Journal of Clinical Investigation 87(5):1664-7, 1991.

(281) Curnow KM, Slutsker L, Vitek J, Cole T, Speiser PW, New MI et al. Mutations in the CYP11B1 gene causing congenital adrenal hyperplasia and hypertension cluster in exons 6, 7, and 8. Proceedings of the National Academy of Sciences of the United States of America 90(10):4552-6, 1993.

(282) Biglieri EG, Herron MA, Brust N. 17-hydroxylation deficiency in man. Journal of Clinical Investigation 45(12):1946-54, 1966.

(283) Fraser R, Brown JJ, Mason PA, Morton JJ, Lever AF, Robertson JI et al. Severe hypertension with absent secondary sex characteristics due to partial deficiency of steroid 17 alpha-hydroxylase activity. Journal of Human Hypertension 1(1):53-8, 1987.

(284) Hahm JR, Kim DR, Jeong DK, Chung JH, Lee MS, Min YK et al. A novel compound heterozygous mutation in the CYP17 (P450

17alpha-hydroxylase) gene leading to 17alpha-hydroxylase/17,20-lyase deficiency. Metabolism: Clinical & Experimental 52(4):488-92, 2003.

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

(286) Hahm JR, Jung TS, Byun SY, Lee YN, Lee KH, Kim DR. Functional characterization of mutant CYP17 genes isolated from a 17 alpha-hydroxylase/17,20-lyase-deficient patient. Metabolism: Clinical & Experimental 53(12):1527-31, 2004.

(287) Liddle GW. A familial renal disorder simulating prilary aldosteronism but with negligible aldosterone secretion. Transactions of the Associations of American Physicians 1963; 76:199-213.

(288) Shimkets RA, Warnock DG, Bositis CM, Nelson-Williams C, Hansson JH, Schambelan M et al. Liddle's syndrome: heritable human hypertension caused by mutations in the beta subunit of the epithelial sodium channel. Cell 79(3):407-14, 1994.

(289) Hiltunen TP, Hannila-Handelberg T, Petajaniemi N, Kantola I, Tikkanen I, Virtamo J et al. Liddle's syndrome associated with a point mutation in the extracellular domain of the epithelial sodium channel gamma subunit.[see comment]. Journal of Hypertension 1920;(12):2383-2390.

(290) Persu A, Coscoy S, Houot AM, Corvol P, Barbry P, Jeunemaitre X. Polymorphisms of the gamma subunit of the epithelial Na+ channel in essential hypertension. Journal of Hypertension 17(5):639-45, 1999.

(291) Botero-Velez M, Curtis JJ, Warnock DG. Brief report: Liddle's syndrome revisited--a disorder of sodium reabsorption in the distal tubule. New England Journal of Medicine 330(3):178-81, 1994.

(292) Ulick S, Levine LS, Gunczler P, Zanconato G, Ramirez LC, Rauh W et al. A syndrome of apparent mineralocorticoid excess associated with defects in the peripheral metabolism of cortisol. Journal of Clinical Endocrinology & Metabolism 49(5):757-64, 1979.

(293) Agarwal AK, Rogerson FM, Mune T, White PC. Analysis of the human gene encoding the kidney isozyme of 11 beta-hydroxysteroid dehydrogenase. Journal of Steroid Biochemistry & Molecular Biology 55(5-6):473-9, 1995.

(294) Quinkler M, Bappal B, Draper N, Atterbury AJ, Lavery GG, Walker EA et al. Molecular basis for the apparent mineralocorticoid excess

syndrome in the Oman population. Molecular & Cellular Endocrinology 217(1-2):143-9, 2004.

(295) Stewart PM, Corrie JE, Shackleton CH, Edwards CR. Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. Journal of Clinical Investigation 82(1):340-9, 1988.

(296) Stewart PM, Wallace AM, Valentino R, Burt D, Shackleton CH, Edwards CR. Mineralocorticoid activity of liquorice: 11-beta-hydroxysteroid dehydrogenase deficiency comes of age. Lancet 2(8563):821-4, 1987.

(297) Funder JW, Pearce PT, Smith R, Smith AI. Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science 242(4878):583-5, 1988.

(298) Ulick S. Diagnosis and nomenclature of the disorders of the terminal portion of the aldosterone biosynthetic pathway. Journal of Clinical Endocrinology & Metabolism 43(1):92-6, 1976.

(299) White PC. Aldosterone synthase deficiency and related disorders. Molecular & Cellular Endocrinology 217(1-2):81-7, 2004.

(300) Rosler A. The natural history of salt-wasting disorders of adrenal and renal origin. Journal of Clinical Endocrinology & Metabolism 59(4):689-700, 1984.

(301) Hiramatsu K, Yamada T, Yukimura Y, Komiya I, Ichikawa K, Ishihara M et al. A screening test to identify aldosterone-producing adenoma by measuring plasma renin activity. Results in hypertensive patients. Archives of Internal Medicine 141(12):1589-93, 1981.

(302) Gordon RD, Stowasser M, Tunny TJ, Klemm SA, Rutherford JC. High incidence of primary aldosteronism in 199 patients referred with hypertension. Clinical & Experimental Pharmacology & Physiology 21(4):315-8, 1994.

(303) Lim PO, Rodgers P, Cardale K, Watson AD, Macdonald TM. Potentially high prevalence of primary aldosteronism in a primary-care population.[see comment]. Lancet 353(9146):40, 1999.

(304) Fardella CE, Mosso L, Gomez-Sanchez C, Cortes P, Soto J, Gomez L et al. Primary hyperaldosteronism in essential hypertensives: prevalence, biochemical profile, and molecular biology.[see comment]. Journal of Clinical Endocrinology & Metabolism 85(5):1863-7, 2000.

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

(306) Schwartz GL, Chapman AB, Boerwinkle E, Kisabeth RM, Turner ST. Screening for primary aldosteronism: implications of an increased plasma aldosterone/renin ratio. Clinical Chemistry 48(11):1919-23, 2002.

(307) Olivieri O, Ciacciarelli A, Signorelli D, Pizzolo F, Guarini P, Pavan C et al. Aldosterone to Renin ratio in a primary care setting: the Bussolengo study.[see comment]. Journal of Clinical Endocrinology & Metabolism 89(9):4221-6, 2004.

(308) Stowasser M, Gordon RD. Primary aldosteronism--careful investigation is essential and rewarding. Molecular & Cellular Endocrinology 217(1-2):33-9, 2004.

(309) Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L et al. Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. Journal of Clinical Endocrinology & Metabolism 89(3):1045-50, 2004.

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

(311) Stowasser M. How common is adrenal-based mineralocorticoid hypertension? Current Opinion in Enocrinology and Diabetes 7, 143-150. 2000.
Ref Type: Journal (Full)

(312) Mulatero P, Rabbia F, Milan A, Paglieri C, Morello F, Chiandussi L et al. Drug effects on aldosterone/plasma renin activity ratio in primary aldosteronism. Hypertension 40(6):897-902, 2002.

(313) Hood S, Cannon J, Foo R, Brown M. Prevalence of primary hyperaldosteronism assessed by aldosterone/renin ratio and spironolactone testing. Clinical Medicine 5(1):55-60, 2005;-Feb.

(314) Lim PO, Jung RT, Macdonald TM. Is aldosterone the missing link in refractory hypertension?: aldosterone-to-renin ratio as a marker of inappropriate aldosterone activity.[see comment]. [Review] [65 refs]. Journal of Human Hypertension 16(3):153-8, 2002.

(315) Montori VM, Young WF, Jr. Use of plasma aldosterone concentration-to-plasma renin activity ratio as a screening test for primary aldosteronism. A systematic review of the literature. [Review] [66 refs]. Endocrinology & Metabolism Clinics of North America 31(3):619-32, xi, 2002.

(316) Young WF, Jr. Primary aldosteronism: A common and curable form of hypertension. [Review] [40 refs]. Cardiology in Review 7(4):207-14, 1999;-Aug.

(317) Young WF, Jr. Primary aldosteronism: management issues. [Review] [71 refs]. Annals of the New York Academy of Sciences 970:61-76, 2002.

(318) Stowasser M, Gordon RD. The aldosterone to renin ratio in screening for primary aldosteronism. The Endocrinologist 2004; 14(5):267-276.

(319) Connell J, Fraser R. Primary Aldosteronism. In: Wass J, Shalet S, editors. Oxford Textbook of Endocrinology and Diabetes. Oxford: Oxford University Press, 2002: 791-799.

(320) Rossi GP, Sacchetto A, Chiesura-Corona M, De Toni R, Gallina M, Feltrin GP et al. Identification of the etiology of primary aldosteronism with adrenal vein sampling in patients with equivocal computed tomography and magnetic resonance findings: results in 104 consecutive cases. Journal of Clinical Endocrinology & Metabolism 86(3):1083-90, 2001.

(321) Stowasser M, Gordon RD, Gunasekera TG, Cowley DC, Ward G, Archibald C et al. High rate of detection of primary aldosteronism, including surgically treatable forms, after 'non-selective' screening of hypertensive patients.[see comment]. Journal of Hypertension 21(11):2149-57, 2003.

(322) Nishikawa T, Omura M, Ito H, Saito J. Prevalence of primary aldosteronism among Japanese hypertensive patients. Journal of Hypertension - Supplement 2002; 20:S172.

(323) McAlister FA, Lewanczuk RZ. Primary hyperaldosteronism and adrenal incidentaloma: an argument for physiologic testing before adrenalectomy. Canadian Journal of Surgery 41(4):299-305, 1998.

(324) Magill SB, Raff H, Shaker JL, Brickner RC, Knechtges TE, Kehoe ME et al. Comparison of adrenal vein sampling and computed tomography in the differentiation of primary aldosteronism. Journal of Clinical Endocrinology & Metabolism 86(3):1066-71, 2001.

(325) Phillips JL, Walther MM, Pezzullo JC, Rayford W, Choyke PL, Berman AA et al. Predictive value of preoperative tests in discriminating bilateral adrenal hyperplasia from an aldosterone-producing adrenal adenoma.[see comment]. Journal of Clinical Endocrinology & Metabolism 85(12):4526-33, 2000.

(326) Lim PO, Jung RT, Macdonald TM. Raised aldosterone to renin ratio predicts antihypertensive efficacy of spironolactone: a prospective cohort follow-up study. British Journal of Clinical Pharmacology 48(5):756-60, 1999.

(327) Young WF, Jr. Minireview: primary aldosteronism--changing concepts in diagnosis and treatment. [Review] [82 refs]. Endocrinology 144(6):2208-13, 2003.

(328) de Gasparo M, Joss U, Ramjoue HP, Whitebread SE, Haenni H, Schenkel L et al. Three new epoxy-spirolactone derivatives: characterization in vivo and in vitro. Journal of Pharmacology & Experimental Therapeutics 240(2):650-6, 1987.

(329) Weinberger MH, Roniker B, Krause SL, Weiss RJ. Eplerenone, a selective aldosterone blocker, in mild-to-moderate hypertension. American Journal of Hypertension 15(8):709-16, 2002.

(330) Struthers AD. Aldosterone blockade in heart failure. [Review] [22 refs]. Journal of the Renin-Angiotensin-Aldosterone System 5 Suppl 1:S23-7, 2004.

(331) Struthers AD. Aldosterone blockade in cardiovascular disease. [Review] [21 refs]. Heart (British Cardiac Society) 90(10):1229-34, 2004.

(332) Krum H, Nolly H, Workman D, He W, Roniker B, Krause S et al. Efficacy of eplerenone added to renin-angiotensin blockade in hypertensive patients. Hypertension 40(2):117-23, 2002.

(333) Sawka AM, Young WF, Thompson GB, Grant CS, Farley DR, Leibson C et al. Primary aldosteronism: factors associated with normalization of blood pressure after surgery.[see comment]. Annals of Internal Medicine 135(4):258-61, 2001.

(334) Celen O, O'Brien MJ, Melby JC, Beazley RM. Factors influencing outcome of surgery for primary aldosteronism. Archives of Surgery 131(6):646-50, 1996.

(335) Meria P, Kempf BF, Hermieu JF, Plouin PF, Duclos JM. Laparoscopic management of primary hyperaldosteronism: clinical experience with 212 cases. Journal of Urology 169(1):32-5, 2003.

(336) Padfield PL, Brown JJ, Davies D, Fraser R, Lever AF, Morton JJ et al. The myth of idiopathic hyperaldosteronism. Lancet 2(8237):83-4, 1981.

(337) Lim PO, Struthers AD, Macdonald TM. The neurohormonal natural history of essential hypertension: towards primary or tertiary aldosteronism?[see comment]. [Review] [78 refs]. Journal of Hypertension 1920;(1):11-15.

(338) FREEL EM, Connell JMC. Mechanisms of Hypertension: The Expanding Role of Aldosterone. [Miscellaneous Article]. Journal of the American Society of Nephrology 2004; 15(8):1993-2001.

(339) White PC, Slutsker L. Haplotype analysis of CYP11B2. Endocrine Research 21(1-2):437-42, 1995;-May.

(340) Davies E, Holloway CD, Ingram MC, Inglis GC, Friel EC, Morrison C et al. Aldosterone excretion rate and blood pressure in essential

hypertension are related to polymorphic differences in the aldosterone synthase gene CYP11B2. Hypertension 33(2):703-7, 1999.

(341) Brand E, Chatelain N, Mulatero P, Fery I, Curnow K, Jeunemaitre X et al. Structural analysis and evaluation of the aldosterone synthase gene in hypertension. Hypertension 32(2):198-204, 1998.

(342) Paillard F, Chansel D, Brand E, Benetos A, Thomas F, Czekalski S et al. Genotype-phenotype relationships for the renin-angiotensin-aldosterone system in a normal population. Hypertension 34(3):423-9, 1999.

(343) Lim PO, Macdonald TM, Holloway C, Friel E, Anderson NH, Dow E et al. Variation at the aldosterone synthase (CYP11B2) locus contributes to hypertension in subjects with a raised aldosterone-to-renin ratio. Journal of Clinical Endocrinology & Metabolism 87(9):4398-402, 2002.

(344) Schunkert H, Hengstenberg C, Holmer SR, Broeckel U, Luchner A, Muscholl MW et al. Lack of association between a polymorphism of the aldosterone synthase gene and left ventricular structure. Circulation 99(17):2255-60, 1999.

(345) Brand E, Schorr U, Ringel J, Beige J, Distler A, Sharma AM. Aldosterone synthase gene (CYP11B2) C-344T polymorphism in Caucasians from the Berlin Salt-Sensitivity Trial (BeSST). Journal of Hypertension 17(11):1563-7, 1999.

(346) Pojoga L, Gautier S, Blanc H, Guyene TT, Poirier O, Cambien F et al. Genetic determination of plasma aldosterone levels in essential hypertension. American Journal of Hypertension 11(7):856-60, 1998.

(347) Wojciechowska W, Staessen JA, Stolarz K, Nawrot T, Filipovsky J, Ticha M et al. Association of peripheral and central arterial wave reflections with the CYP11B2 -344C allele and sodium excretion. Journal of Hypertension 22(12):2311-9, 2004.

(348) Tamaki S, Iwai N, Tsujita Y, Kinoshita M. Genetic polymorphism of CYP11B2 gene and hypertension in Japanese. Hypertension 33(1 Pt 2):266-70, 1999.

(349) Komiya I, Yamada T, Takara M, Asawa T, Shimabukuro M, Nishimori T et al. Lys(173)Arg and -344T/C variants of CYP11B2 in Japanese patients with low-renin hypertension. Hypertension 35(3):699-703, 2000.

(350) Davies E, Holloway CD, Ingram MC, Friel EC, Inglis GC, Swan L et al. An influence of variation in the aldosterone synthase gene (CYP11B2) on corticosteroid responses to ACTH in normal human subjects. Clinical Endocrinology 54(6):813-7, 2001.

(351) Connell JM, Fraser R, MacKenzie S, Davies E. Is altered adrenal steroid biosynthesis a key intermediate phenotype in hypertension?. [Review] [50 refs]. Hypertension 41(5):993-9, 2003.

(352) Honda M, Nowaczynski W, Guthrie GP, Jr., Messerli FH, Tolis G, Kuchel O et al. Response of several adrenal steroids to ACTH stimulation in essential hypertension. Journal of Clinical Endocrinology & Metabolism 44(2):264-72, 1977.

(353) de Simone G, Tommaselli AP, Rossi R, Valentino R, Lauria R, Scopacasa F et al. Partial deficiency of adrenal 11-hydroxylase. A possible cause of primary hypertension. Hypertension 7(2):204-10, 1985;-Apr.

(354) Oelkers W. Prolonged ACTH infusion suppresses aldosterone secretion in spite of high renin activity. Acta Endocrinologica 108(1):91-7, 1985.

(355) Hamilton BP, Zadik Z, Edwin CM, Hamilton JH, Kowarski AA. Effect of adrenal suppression with dexamethasone in essential hypertension. Journal of Clinical Endocrinology & Metabolism 48(5):848-53, 1979.

(356) Schunkert H, Hense HW, Andus T, Riegger GA, Straub RH. Relation between dehydroepiandrosterone sulfate and blood pressure levels in a population-based sample. American Journal of Hypertension 12(11 Pt 1):1140-3, 1999.

(357) Komiya I, Yamada T, Aizawa T, Takasu N, Niwa A, Maruyama Y et al. Inappropriate elevation of the aldosterone/plasma renin activity ratio in hypertensive patients with increases of 11-deoxycorticosterone and 18-hydroxy-11-deoxycorticosterone: a subtype of essential hypertension? Cardiology 78(2):99-110, 1991.

(358) Brown JJ, Fraser R, Love DR, Ferriss JB, Lever AF, Robertson JI et al. Apparently isolated excess deoxycorticosterone in hypertension. A variant of the mineralocorticoid-excess syndrome. Lancet 2(7771):243-7, 1972.

(359) Caulfield M, Munroe P, Pembroke J, Samani N, Dominiczak A, Brown M et al. Genome-wide mapping of human loci for essential hypertension. Lancet 361(9375):2118-23, 2003.

(360) Tunstall-Pedoe H. Monitoring trends in cardiovascular disease and risk factors: the WHO "Monica" project. WHO Chronicle 39(1):3-5, 1985.

(361) Fraser R, Ingram MC, Anderson NH, Morrison C, Davies E, Connell JM. Cortisol effects on body mass, blood pressure, and cholesterol in the general population. Hypertension 33(6):1364-8, 1999.

(362) Shakerdi L. Biochemistry of Mineralocorticoids in Cardiovascular Disease: G/MS analysis of urinary steroid patterns in human hypertension. University of Glasgow, 2004.

(363) Shackleton CH, Honour JW. Identification and measurement of 18-hydroxycorticosterone metabolites by gas chromatography-mass spectrometry. Journal of Steroid Biochemistry 8(3):199-203, 1977.

(364) Palermo M, Gomez-Sanchez C, Roitman E, Shackleton CH. Quantitation of cortisol and related 3-oxo-4-ene steroids in urine using gas chromatography/mass spectrometry with stable isotope-labeled internal standards. Steroids 61(10):583-9, 1996.

(365) Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Research 16(3):1215, 1988.

(366) Dobson SH, Gibson S, White A. Assessment of the potency of different standards in the immunoradiometric assay of ACTH. Annals of Clinical Biochemistry 26 (Pt 1):96-101, 1989.

(367) Millar JA, Leckie BJ, Morton JJ, Jordan J, Tree M. A microassay for active and total renin concentration in human plasma based on antibody trapping. Clinica Chimica Acta 101(1):5-15, 1980.

(368) Huang Q, Fu YX, Boerwinkle E. Comparison of strategies for selecting single nucleotide polymorphisms for case/control association studies. Human Genetics 113(3):253-7, 2003.

(369) Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data.[see comment]. American Journal of Human Genetics 68(4):978-89, 2001.

(370) Tanahashi H, Mune T, Takahashi Y, Isaji M, Suwa T, Morita H et al. Association of Lys173Arg polymorphism with CYP11B2 expression in normal adrenal glands and aldosterone-producing adenomas. Journal of Clinical Endocrinology & Metabolism 90(11):6226-31, 2005.

(371) Fardella CE, Rodriguez H, Montero J, Zhang G, Vignolo P, Rojas A et al. Genetic variation in P450c11AS in Chilean patients with low renin hypertension. Journal of Clinical Endocrinology & Metabolism 81(12):4347-51, 1996.

(372) Hesketh J. 3'-Untranslated regions are important in mRNA localization and translation: lessons from selenium and metallothionein. [Review] [28 refs]. Biochemical Society Transactions 32(Pt 6):990-3, 2004.

(373) Shackleton CH. Mass spectrometry in the diagnosis of steroid-related disorders and in hypertension research. Journal of Steroid Biochemistry & Molecular Biology 45(1-3):127-40, 1993.

(374) Ganapathipillai S, Laval G, Hoffmann IS, Castejon AM, Nicod J, Dick B et al. CYP11B2-CYP11B1 haplotypes associated with decreased 11 beta-hydroxylase activity. Journal of Clinical Endocrinology & Metabolism 90(2):1220-5, 2005.

(375) Inglis GC, Ingram MC, Holloway CD, Swan L, Birnie D, Hillis WS et al. Familial pattern of corticosteroids and their metabolism in adult human subjects--the Scottish Adult Twin Study. Journal of Clinical Endocrinology & Metabolism 84(11):4132-7, 1999.

(376) Hollenberg NK, Williams G, Burger B, Hooshmand I. The influence of potassium on the renal vasculature and the adrenal gland, and their responsiveness to angiotensin II in normal man. Clinical Science & Molecular Medicine 49(6):527-34, 1975.

(377) Barr M, Freel EM, Wilkinson D, Friel E, Ingram M, Brain N et al. Functional impact of polymorphic variation in the gene encoding 11beta-hydroxylase (CYP11B1): reduced adrenal 11-hydroxylase efficiency identifies a key intermediate phenotype in hypertension. 2006. 4-4-0006.
Ref Type: Unpublished Work

(378) Al-Hashmi K, Wallace A, Connell J. The aldosterone/renin ratio using renin concentration compared to renin activity in a healthy population. Endocrine Abstracts 9, P145. 2005.
Ref Type: Abstract

(379) Beretta-Piccoli C, Davies D, Boddy K, Brown JJ, Cumming A, East B et al. Relation of arterial pressure with body sodium, body potassium and plasma potassium in essential hypertension. Clinical Science 63, 257-270. 1982.
Ref Type: Journal (Full)

(380) Vallotton MB. Primary aldosteronism. Part I. Diagnosis of primary hyperaldosteronism. [Review] [31 refs]. Clinical Endocrinology 45(1):47-52, 1996.

(381) Chu MD, Ulick S. Isolation and identification of 18-hydroxycortisol from the urine of patients with primary aldosteronism. Journal of Biological Chemistry 257(5):2218-24, 1982.

(382) Ulick S, Chu MD, Land M. Biosynthesis of 18-oxocortisol by aldosterone-producing adrenal tissue. Journal of Biological Chemistry 258(9):5498-502, 1983.

(383) Lifton RP, Dluhy RG, Powers M, Rich GM, Gutkin M, Fallo F et al. Hereditary hypertension caused by chimaeric gene duplications and ectopic expression of aldosterone synthase. Nature Genetics 2(1):66-74, 1992.

(384) Gomez-Sanchez EP, Gomez-Sanchez CE, Smith JS, Ferris MW, Foecking M. Receptor binding and biological activity of 18-hydroxycortisol. Endocrinology 115(2):462-6, 1984.

(385) Ulick S, Land M, Chu MD. 18-oxocortisol, a naturally occurring mineralocorticoid agonist. Endocrinology 113(6):2320-2, 1983.

(386) Gomez-Sanchez CE, Gomez-Sanchez EP, Smith JS, Ferris MW, Foecking MF. Receptor binding and biological activity of 18-oxocortisol. Endocrinology 116(1):6-10, 1985.

(387) Fraser R, Connell JM, Budd PS, Corrie JE, Kenyon CJ. The origin and significance of 18-hydroxycortisol: studies in hyperaldosteronism and in bovine adrenocortical cells in vitro. Journal of Steroid Biochemistry & Molecular Biology 39(5B):839-50, 1991.

(388) Ulick S, Chu MD. Hypersecretion of a new corticosteroid, 18-hydroxycortisol in two types of adrenocortical hypertension. Clinical & Experimental Hypertension - Part A, Theory & Practice 4(9-10):1771-7, 1982.

(389) Yamakita N, Gomez-Sanchez CE, Mune T, Morita H, Yoshida H, Miyazaki S et al. Simultaneous measurement of plasma 18-oxocortisol and 18-hydroxycortisol levels in normal man. European Journal of Endocrinology 131(1):74-9, 1994.

(390) Ohta M, Fujii S, Miura R, Nonaka Y, Okamoto M. Bovine adrenal cytochrome P-450(11 beta)-mediated conversion of 11- deoxycortisol to 18- and 19-hydroxy derivatives; structural analysis by 1H-NMR. J Steroid Biochem Mol Biol 1991; 39(6):911-920.

(391) Fraser R, Connell JM, Budd PS, Corrie JE, Kenyon CJ. The origin and significance of 18-hydroxycortisol: studies in hyperaldosteronism and in bovine adrenocortical cells in vitro. J Steroid Biochem Mol Biol 1991; 39(5B):839-850.

(392) Freel EM, Shakerdi LA, Friel EC, Wallace AM, Davies E, Fraser R et al. Studies on the Origin of Circulating 18-Hydroxycortisol and 18-Oxocortisol in Normal Human Subjects. [Article]. Journal of Clinical Endocrinology & Metabolism 2004; 89(9):4628-4633.

(393) Freel EM, Shakerdi LA, Friel EC, Wallace AM, Davies E, Fraser R et al. Studies on the origin of circulating 18-hydroxycortisol and 18-oxocortisol in normal human subjects. Journal of Clinical Endocrinology & Metabolism 89(9):4628-33, 2004.

(394) Hatakeyama H, Miyamori I, Fujita T, Takeda Y, Takeda R, Yamamoto H. Vascular aldosterone. Biosynthesis and a link to angiotensin II-induced hypertrophy of vascular smooth muscle cells. Journal of Biological Chemistry 269(39):24316-20, 1994.

(395) Gomez-Sanchez CE, Zhou MY, Cozza EN, Morita H, Foecking MF, Gomez-Sanchez EP. Aldosterone biosynthesis in the rat brain. Endocrinology 138(8):3369-73, 1997.

(396) MacKenzie SM, Clark CJ, Ingram MC, Lai M, Seckl J, Gomez-Sanchez CE et al. Corticosteroid production by fetal rat hippocampal neurons. Endocrine Research 26(4):531-5, 2000.

(397) Miyamori I, Takeda Y, Takasaki H, Itoh Y, Iki K, Takeda R. Determination of urinary 18-hydroxycortisol in the diagnosis of primary aldosteronism. Journal of Endocrinological Investigation 15(1):19-24, 1992.

(398) Yamakita N, Gomez-Sanchez CE, Mune T, Yoshida H, Miyazaki S, Yasuda K et al. Regulation of 18-oxocortisol and 18-hydroxycortisol by the renin-angiotensin system and ACTH in man. Journal of Steroid Biochemistry & Molecular Biology 46(3):395-9, 1993.

(399) Gomez-Sanchez CE, Upcavage RJ, Zager PG, Foecking MF, Holland OB, Ganguly A. Urinary 18-hydroxycortisol and its relationship to the excretion of other adrenal steroids. Journal of Clinical Endocrinology & Metabolism 65(2):310-4, 1987.

(400) Gomez-Sanchez CE. 18-Hydroxycortisol and 18-oxocortisol, steroids from the transitional zone. Endocrine Research 10(3-4):609-15, 1984;-85.

(401) Shigematsu K, Kawai K, Tsuchiyama H. Functional morphology of the adrenocortical glomerular zone by incomplete ligation of bilateral ureters of rats. An experimental model for secondary aldosteronism. Acta Pathologica Japonica 35(6):1435-44, 1985.

(402) Cozza EN, Chavarri MR, Foecking MF, Gomez-Sanchez CE. Synthesis of 18-hydroxycortisol and 18-oxocortisol in bovine adrenal zona glomerulosa mitochondria. Proceedings of the Society for Experimental Biology & Medicine 203;(3):317-322.

(403) MacKenzie SM, Clark CJ, Fraser R, Gomez-Sanchez CE, Connell JM, Davies E. Expression of 11beta-hydroxylase and aldosterone synthase genes in the rat brain. Journal of Molecular Endocrinology 24(3):321-8, 2000.

(404) Takeda R, Hatakeyama H, Takeda Y, Iki K, Miyamori I, Sheng WP et al. Aldosterone biosynthesis and action in vascular cells.[erratum appears in Steroids 1995 Aug;60(8):540]. Steroids 60(1):120-4, 1995.

(405) Stewart PM. 11 beta-Hydroxysteroid dehydrogenase: implications for clinical medicine. [Review] [55 refs]. Clinical Endocrinology 44(5):493-9, 1996.

(406) Hatakeyama H, Miyamori I, Takeda Y, Yamamoto H, Mabuchi H. The expression of steroidogenic enzyme genes in human vascular cells. Biochemistry & Molecular Biology International 40(3):639-45, 1996.

(407) Kayes-Wandover KM, White PC. Steroidogenic enzyme gene expression in the human heart. Journal of Clinical Endocrinology & Metabolism 85(7):2519-25, 2000.

(408) Takeda Y, Miyamori I, Yoneda T, Hatakeyama H, Inaba S, Furukawa K et al. Regulation of aldosterone synthase in human vascular endothelial cells by angiotensin II and adrenocorticotropin. Journal of Clinical Endocrinology & Metabolism 81(8):2797-800, 1996.

(409) Takeda Y, Miyamori I, Yoneda T, Iki K, Hatakeyama H, Blair IA et al. Production of aldosterone in isolated rat blood vessels. Hypertension 25(2):170-3, 1995.

(410) Young MJ, Clyne CD, Cole TJ, Funder JW. Cardiac steroidogenesis in the normal and failing heart.[see comment]. Journal of Clinical Endocrinology & Metabolism 86(11):5121-6, 2001.

(411) SKELTON FR. Development of hypertension and cardiovascular-renal lesions during adrenal regeneration in the rat. Proceedings of the Society for Experimental Biology & Medicine 90(2):342-6, 1955.

(412) Gomez-Sanchez CE, Gomez-Sanchez EP, Upcavage RJ, Hall EB. Urinary free and serum 19-nor-deoxycorticosterone in adrenal regeneration hypertension. Hypertension 5(2 Pt 2):I32-4, 1983;-Apr.

(413) Brauckhoff M, Gimm O, Thanh PN, Bar A, Ukkat J, Brauckhoff K et al. Critical size of residual adrenal tissue and recovery from impaired early postoperative adrenocortical function after subtotal bilateral adrenalectomy. Surgery 134(6):1020-7; discussion 1027-8, 2003.

(414) Anderson JR, Ross AH. Ectopic adrenal tissue in adults. Postgraduate Medical Journal 56(661):806-8, 1980.

(415) Souverijns G, Peene P, Keuleers H, Vanbockrijck M. Ectopic localisation of adrenal cortex. European Radiology 10(7):1165-8, 2000.

(416) Peacey SR, Guo CY, Robinson AM, Price A, Giles MA, Eastell R et al. Glucocorticoid replacement therapy: are patients over treated and does it matter?[see comment]. Clinical Endocrinology 46(3):255-61, 1997.

(417) Milliez P, Girerd X, Plouin PF, Blacher J, Safar ME, Mourad JJ. Evidence for an increased rate of cardiovascular events in patients with primary aldosteronism.[see comment]. Journal of the American College of Cardiology 45(8):1243-8, 2005.

(418) Barr M. A study of genetic variability at the CYP11B1/CYP11B2 locus and its importance in human hypertension. University of Glasgow, 2006.