Studies of aldosterone renin ratio and genetic variation at the *CYP11B1/CYP11B2* locus in human essential hypertension

By

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Abstract

Essential hypertension is seen as a contemporary public health challenge not only because of its high prevalence and variable treatment response but also because it represents a major risk factor for cardiovascular disease. Both genetic and environmental factors contribute to the regulation of blood pressure, thus making the study of hypertension difficult and complex. Over recent years, with the advent of new molecular technologies, there has been an increasing interest in its genetic component.

Alterations in the rate and pattern of adrenal steroid biosynthesis can contribute to blood pressure changes and its heritable component. In humans, mutations in the genes encoding aldosterone synthase (*CYP11B2)* and 11β-hydroxylase (*CYP11B1),* responsible for the final stages of aldosterone and cortisol production respectively, lead to rare monogenic disorders. Both, glucocorticoid remediable aldosteronism and 11β-hydroxylase deficiency are associated with mineralocorticoid hypertension. More subtle but much more common genetic variations, such as single nucleotide polymorphisms (SNPs), are associated with more common intermediate phenotypes of essential hypertension such as the aldosterone to renin ratio (ARR). Up to 15 % of hypertensive patients may have an altered aldosterone biosynthesis as indicated by an elevated ARR. However, many studies using the ARR have widely variable results, as there is no standardization of the assays used and because many factors may influence the measurement of renin and aldosterone levels.

Previous studies have reported associations between a variation in the promoter region (-344T/C) and intron 2 (intron conversion) in the *CYP11B2* gene with inappropriate aldosterone production and hypertension. However, the findings have not been consistent. Interestingly, associations have also been reported between these polymorphisms and raised basal and ACTH-stimulated levels of the 11-deoxysteroids (DOC and 11-deoxycortisol), indicating a reduced 11β-hydroxylase efficiency, encoded by *CYP11B1*. This is not entirely surprising considering that the polymorphisms within both genes are known to be in linkage disequilibrium. Recent findings indicate that two polymorphisms in the promoter region of *CYP11B1* (-1889G/T and –1859A/G) are associated with altered efficiency of 11β-hydroxylase. Using this evidence, a hypothesis linking reduced 11β-hydroxylation efficiency to an elevated ARR and hypertension, involving a small but chronic increase in adrenocorticotrophic hormone (ACTH) stimulation of the adrenal gland, has been proposed to explain the undetermined interaction between the *CYP11B*

genes. This hypothesis requires further testing. Thus, identification of the specific SNPs or group of SNPs along the *CYP11B1/CYP11B2* locus responsible for the observed elevation in ARR and hypertension requires further investigation. In this thesis, the factors that influence the measurement of the ARR are examined and the relationship between ARR, blood pressure and genetic variation at the *CYP11B1/CYP11B2* locus is studied in several large population samples.

Since minor changes in renin measurement may lead to significant changes in the ARR, an evaluation of methods to measure it in population studies was of fundamental importance. In Chapter 3, the performance of a novel automated plasma renin concentration (PRC) assay was evaluated in normotensive subjects and compared with the routinely used plasma renin activity (PRA) assay. The analytical and functional sensitivity, precision and stability of the new PRC assay were excellent. Overall, the correlation between the PRC and PRA assays was good. The only limitation of the PRC assay was a small decrease in sensitivity at lower concentrations, possibly due to cross-reactivity with prorenin. Although this limitation might be a drawback for its use in clinical diagnosis, for large scale population studies, practical issues as well as performance were relevant. The PRC assay offers several advantages over the traditional PRA assay; it allows efficient processing of more samples at a lower cost in less time. The assay has high precision, detecting small differences with greater certainty. In addition, the storage and handling of a radioactive reagent are eliminated. Thus, the new PRC assay was preferred as the method of choice for the family study in Chapter 4.

The study in Chapter 4 examined the significance of a raised ARR in normotensive and hypertensive subjects and the phenotypic and familial factors affecting it. The distribution of ARR and its heritability was estimated in 1172 individuals from 248 Caucasian families. The associations between ARR and blood pressure with polymorphic variations at the *CYP11B1/CYP11B2* locus were also tested. Unadjusted and adjusted ARR values, for age, gender, body mass index, ACE inhibitors and beta-blocker use, were continuously distributed in normal and hypertensive cohorts; there was no evidence of a cut-off that would identify a separate population with primary aldosteronism. The median ARR was 4.19 ng/L per mIU/L (range 0.04-253.16). The majority of subjects (> 80%) had an ARR < 10ng/L per mIU/L; ARR levels were higher in females, and associated with age, body mass index and potassium. Antihypertensive agents had significant predictable effects on the ARR. Renin was negatively and ARR positively associated with ambulatory blood pressure readings ($p<0.001$) in subjects not taking antihypertensives. The heritability of

the ARR was 38.1% ($p<10^{-8}$), and the heritability of renin and aldosterone was 27.4% and 28.7% respectively with $p<10^{-8}$. Plasma aldosterone, but not ARR, was influenced by intron 2 conversion genotype in the *CYP11B2* gene (beta = -0.07, p=0.04).

There is substantial genetic determination of the ARR and its components. The effects of gender and other confounders indicate that care must be taken when interpreting the ARR as a screening test for primary aldosteronism. The ratio is not a marker of a distinct pathological abnormality but subjects with a high ratio tend to have higher blood pressure readings, possibly reflecting the long term influence of aldosterone on cardiovascular homeostasis.

The study in Chapter 5 sought to identify the most informative SNPs in the *CYP11B1/CYP11B2* locus in Caucasians. There is tight linkage disequilibrium across the entire locus, facilitating the selection of the SNPs. For the study, the selection of the first four SNPs chosen for genotyping was based on previous indications of their association with hypertension. These SNPs were the -344T/C and the intron 2 conversion in the *CYP11B2* and the -1889G/T and -1859A/G in the promoter region of *CYP11B1*. Using the HapMap data for Caucasians together with information from previous genotyping of this locus in 26 normotensive subjects from the MONItoring of trends and determinants in CArdiovascular disease Study (MONICA), a further eighteen SNPs were chosen for genotyping. A 90% coverage of this locus was achieved. The eighteen SNPs were located in the exon 3 and intron 6 of the *CYP11B2*, exon 1, intron 2, intron 5 and the 3'UTR of *CYP11B1*, and the intergenic region. The 22 SNPs thus selected were genotyped in 79 unrelated individuals from the British Genetics of Hypertension Study (BRIGHT). After evaluating the minor allele frequencies, Hardy-Weinberg equilibrium and percentage of genotyping, 15 polymorphisms were chosen for haplotype tagSNP analysis. The final set of 7 SNPs, which according to this tagSNP analysis maximize haplotype diversity in Caucasians and minimize genotyping redundancy, comprised: 3 SNPs in *CYP11B1* (rs5301, rs6410, rs4313136 (-1889G/T)), 3 SNPs in *CYP11B2* (rs4546, the intron conversion (IC) and rs1799998 (-344T/C)) and one in the intergenic region (rs4736354). This set of SNPs was used in Chapter 7 in a large case-control study to test for association with hypertension.

The study in Chapter 6 analyzed the allele frequency and linkage disequilibrium differences in the *CYP11B1/CYP11B2* locus in 35 founders of families with African ancestry, 149 founders of families with Caucasian ancestry and the 79 Caucasian unrelated

individuals used in Chapter 5. In addition, the genotyping results from the 35 Afro-Caribbean founders, the 79 Caucasians and the results available from eleven populations in HapMap for the *CYP11B1/CYP11B2* locus were combined to generate phylogenetic trees.

From the 15 SNPs selected in the pilot study in Chapter 5, 12 SNPs were chosen. Considerable allelic variation was observed in these 12 SNPs genotyped in the Afro-Caribbean and the 79 subjects from the BRIGHT with Caucasian ancestry. Most significant differences were in the following SNP: rs4313136 (-1889G/T) and rs6471580, $p<0.05$; rs4736354, rs4546 and rs1799998 (-344T/C), p.<0.01 and rs5283 and intron conversion, $p<0.0001$. These differences are located at the 5' end of the genes and the intergenic sections possibly because SNPs in regions crucial for the synthesis would result in deleterious effects in the enzymes. The comparison of allele frequencies of common SNPs between the Afro-Caribbean and Caucasian founders from the family study showed the same trend as with the subjects from the BRIGHT. The variation in linkage disequilibrium (LD) length was also compared between these groups. Subjects with African ancestry had shorter LD patterns in the *CYP11B1/CYP11B2* locus when compared with individuals of Caucasian ancestry. The high levels of LD across these regions in Caucasians, while advantageous in designing efficient association studies, do not allow identification of causal SNPs. African ancestry is more conducive to the identification of such variants. It is clearly not recommended that individuals from different ethnic origin are combined in an association study as there is an increased risk of type 1 errors with population stratification.

The distribution of the Afro-Caribbean subjects and the 79 Caucasians from the BRIGHT in the branches of the phylogenetic trees was different, suggesting different patterns of evolution.

In Chapter 7, association with hypertension and the *CYP11B1/CYP11B2* locus in a Caucasian case-control population was tested and replicated in an independent case-control population. In the discovery phase, the seven SNPs selected in Chapter 5 and rs4471016 (-1859A/G) were genotyped in 3340 unrelated individuals of the BRIGHT Case Control cohort. The SNPs significantly associated with hypertension (rs6410, rs4471016 (-1859A/G), rs43131369 (-1889G/T), rs6471581, rs4546, intron conversion and rs1799998(-344T/C)) were tested for replication in 2929 unrelated individuals of the Swedish study. Only the intron conversion showed significant association with hypertension in the single SNP analysis. Subjects with the conversion allele in the intron 2

of *CYP11B2* had a higher risk of hypertension (OR=1.19 [1.10-1.29], p value=1.06 x 10^{-5}). In the haplotype association analysis, three haplotypes showed significant association with hypertension in the BRIGHT Study and were replicated in the Swedish Study. The first haplotype was T/A/G comprising rs6410, rs4471016 and 4313136 in the *CYP11B1* gene and this had an OR=3.14 [2.27-4.32], p=2.99x10^{-12}. The second haplotype was A/G/G comprising SNPs from both genes (rs4471016, rs4313136 and rs6414) and this had an OR=2.64 [1.87-3.74], p=3.90x10^{-8}. The third haplotype was A/T/Conv comprising rs6414, rs4546 and the intron conversion in the *CYP11B2* and this had an OR=4.64 [2.63-8.18], p=1.11x10^{-7}. These three haplotypes were present in approximately 2% of the populations studied. Thus, the regions most strongly associated with hypertension in Caucasians were narrowed to a region between intron 2 and intron 3 in the *CYP11B2,* and to another region between the promoter and exon 1 in the *CYP11B1* gene. However, functional studies are required to elucidate the mechanism by which these genetic variations lead to alterations in aldosterone and cortisol production and subsequent hypertension.

In summary, the results show that the PRC assay is an entirely acceptable option for measuring renin levels in population studies. The ARR is continuously distributed, influenced by genetic factors and is not a marker of a distinct pathological abnormality. There is significant disparity in allele frequencies and linkage disequilibrium structure at the *CYP11B* locus between ethnic groups. These differences should allow for the design of efficient association studies and the identification of causal variants. Using sophisticated statistical analyses, the studies have allowed a more precise identification of the key regions in this locus associated with hypertension in Caucasians. Although the identification of causal variants remains to be elucidated, the findings of the studies presented in this thesis are of considerable interest. This is the first time a positive association of a locus that is known to be involved in a rodent model of hypertension, rare autosomal human disorders, and functional changes that lead to an altered biochemical intermediate phenotype has been confirmed. Additionally, the evidence in this thesis shows that differences in genotype at the *CYP11B1/CYP11B2* locus might play an important role in modulating the adverse effects of lifelong exposure to inappropriate aldosterone levels. In the future, screening susceptible subjects by combining the use of the ARR and genotyping at the *CYP11B1/CYP11B2* locus will enable early lifestyle interventions or a personalised therapy to slow the progression towards aldosterone modulated hypertension and cardiovascular disease.

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Publications

Original article

Alvarez-Madrazo S, Padmanabhan S, Mayosi BM, Watkins H, Avery P, Wallace AM, Fraser R, Davies E, Keavney B & Connell JC 2009 Familial and Phenotypic Associations of the Aldosterone Renin Ratio. *J. Clin. Endocrinol. Metab* (accepted for publication on August 18, 2009 and is Chapter 4 in this thesis)

Dorrian CA, Toole BJ, Alvarez-Madrazo S, Kelly A, Connell JMC & Wallace A.M. 2009 A screening procedure for primary aldosteronism based on the Diasorin Liaison ® automated chemiluminescent immunoassay for direct renin. *Ann.Clin.Biochem.* (recommended for publication on December 02, 2009)

Review article

Davies E, MacKenzie SM, Freel EM, Alvarez-Madrazo S, Fraser R & Connell JM 2009 Altered corticosteroid biosynthesis in essential hypertension: A digenic phenomenon. *Mol Cell Endocrinol*. **300** 185-91.

Published abstracts

Alvarez-Madrazo S, Padmanabhan S, Wallace M, Campbell M, Friel E, Keavney B, Davies E & Connell JC 2008 Heritability of plasma aldosterone levels and genetic variation association with the aldosterone synthase (*CYP11B2*) and 11beta-hydroxylase (*CYP11B1*) genes. Endocrine Abstracts 15 P325

Alvarez-Madrazo S, Padmanabhan S, Wallace M, Campbell M, Smith J, Friel E, Dorrian C, Keavney B, Davies E & Connell JC 2009 Familial and Phenotypic Associations of the Aldosterone Renin Ratio in the General Population Endocrine Abstracts 19 OC11

Alvarez-Madrazo S, Padmanabhan S, Friel E, McKenzie C, Keavney B, Davies E & Connell JC 2009 Differences in aldosterone synthase and 11β-hydroxylase genes between Caucasian and Afro-Caribbean hypertensive families Endocrine Abstracts 19 P305

Alvarez-Madrazo S, Padmanabhan S, Friel E, MacKenzie SM, Brown MJ, Caulfield MJ, Munroe PB, Farrall M, Webster J, Samani NJ, Dominiczak AF, Davies E & Connell JM 2009 Association of narrower regions in *CYP11B1/B2* locus with hypertension in Caucasians. Endocrine Society's 91st Annual Meeting P3-571.

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Author's Declaration

I declare that the work presented in this thesis is, to the best of my knowledge and belief, original and my own work, unless specified otherwise in the text. This work has not been submitted previously for a higher degree. It was carried out under the supervision of Professor John Connell and Professor Eleanor Davies in the MRC Blood Pressure Group at the British Heart Foundation Glasgow Cardiovascular Research Centre.

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Abbreviations

11β-HSD1/2	11beta-hydroxysteroid dehydrogenase type 1 or type 2
12-HETE	12-hydroxyeicosatetraenoic acid
16K	N-terminal fragment 16K peptide
18-OHDOC	18-hydroxy-11deoxycorticosterone
19-OHDOC	19-hydroxy-11-deoxycorticosterone
3β-HSD	3β Hydroxysteroid dehydrogenase
95% CI	95% confidence interval
ACE	Angiotensin converting enzyme
ACE	Gene encoding angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACE2	Gene encoding angiotensin converting enzyme 2
ACTH	Adrenocorticotrophin hormone
Ad^0	Adrenodoxin
Ad4BP	Adrenal 4 binding protein (transcription factor)
ADD1	Gene encoding the cytoskeleton protein adducing
ADMTS1	A desintegrin and metalloproteinase with thrombospondin-like motifs 1
Ad^r	Adrenodoxin reductase
AGT	Gene encoding angiotensinogen
AGTR1	Gene encoding angiotensin II receptor, type 1
AIP	Aldosterone-induced proteins
Ang	Angiotensinogen
AngI	Angiotensin I
AngII	Angiotensin II
ANOVA	Analysis of variance
AP-1	Activator protein-1
APA	Aldosterone-producing adenoma
ARBs	Angiotensin receptor blockers
ARR	Aldosterone renin ratio
ASW	African ancestry population in Southwest USA
AT1 or AT2	Angiotensin II receptor 1 or 2
ATF-1	Activating transcription factor 1
ATP1B1	Gene encoding the β subunit of the Na^+/K^+ ATPase pump
B	Corticosterone
B0	Maximum binding
bFGF	Basic fibroblast growth factor
BMI	Body mass index
bp	Base pairs
BP	Blood pressure
BRIGHT/MRC BRIGHT	Medical Research Council BRItish Genetics of HyperTension
c10orf107	Gene encoding chromosome 10 open reading frame 107
Ca^{2+}	Calcium ions
CAH	Congenital adrenal hyperplasia

CCT	Cortical collecting duct
CEU	Utah residents with Northern and Western European ancestry from the Centre d'Etude du Polymorphisme Humain (CEPH) collection
cGK-II	cGMP-dependent protein kinase II
CHB	Han Chinese in Beijing, China
CHD	Chinese in Metropolitan Denver, Colorado
CHIF	Corticosteroid hormone-induced factor
CHISQ	Chi square test
Chr	Chromosome
Cl^-	Chloride ions
CLCNKA	Gene encoding the chloride channel Ka
CLCNKB	Gene encoding chloride channel Kb
CLIA	Chemiluminescent immunoassay
cM	CentiMorgan
CMO-I/II	Corticosteroid methyloxidase type I or II
CNKSR3	Connector enhancer of kinase suppressor of ras 3
CNS	Central nervous system
CNV	Copy number variation
COL1α1	Procollagen 1α1
Conv	Conversion
COX-2	Cyclooxygenase-2
CREB	cAMP response element binding
CRH	Corticotrophin-releasing hormone
CV	Coefficient of variation
CYP11A1	Gene encoding cholesterol side-chain cleavage enzyme
CYP11A1	Cholesterol side-chain cleavage enzyme
CYP11B1	11β-hydroxylase
CYP11B1	Gene encoding 11β-hydroxylase
CYP11B2	Aldosterone synthase
CYP11B2	Gene encoding aldosterone synthase
CYP17A1	17α-hydroxylase
CYP17A1	Gene encoding 17α-hydroxylase
CYP1A2	Gene encoding cytochrome P450, family 1, subfamily A, polypeptide 2
CYP21B or CYP21A2	Gene encoding 21-hydroxylase
CYP21B	21-hydroxylase
CYP450	Cytochrome P450 enzymes
DAG	1,2 Diacylglycerol
DASH	Dietary Approaches to Stop Hypertension
DAX-1	Gene encoding dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, 1
DBP	Diastolic blood pressure
DCT	Distal convoluted tubule
DHEA	Dehydroepiandrosterone
DHEAS	Androgen dehydroepiandrosterone sulfate
DME	Diabetes mellitus insulin dependant

DNA	Deoxyribonucleic acid
DOC	11-Deoxycorticosterone
DOC:B	Deoxycorticosterone to corticosterone ratio
E	Cortisone
EDTA	Ethylenediaminetetraacetic acid
EGF-R	Epidermal growth factor receptor
ENaC	Epithelial sodium channel
EPIC-NORFOLK	European Prospective Investigation into Cancer-the Norfolk cohort
ET-1	Endothelin-1
F	Cortisol
FAD	Flavin adenine dinucleotide
FGF5	Gene encoding fibroblast growth factor 5.
FH-I/II/III	Familial hyperaldosteronism type I or II or III
FMN	Flavin mononucleotide
G6PD	Glucose-6-phosphate dehydrogenase
G6PD	Gene encoding glucose-6-phosphate dehydrogenase
Gamma 3MSH	Gamma 3-melanocyte stimulating hormone
GML	Gene encoding glycosylphosphatidylinositol-anchored molecule-like protein
GFR	Glomerular filtration rate
GIH	Gujarati Indians in Houston, Texas
GILZ	Glucocorticoid-induced leucine zipper
GR	Glucocorticoid receptor
GRA	Glucocorticoid remediable aldosteronism
GWA	Genome wide association
h^2	Heritability
HBB	Gene encoding beta globin
HC	Hepatic cirrhosis
HDL	High-density lipoprotein
Het	Heterozygosity
HF	Heart failure
hGR	Gene encoding glucocorticoid receptor
HO	Hypotension
HPA	Hypothalamic-pituitary-adrenal axis
HPLC-MS/MS	High-performance liquid chromatography-tandem mass spectrometry
HRE	Hormone responsive elements
HRT	Hormone replacement therapy
HSD11B2	Gene encoding hydroxysteroid (11-beta) dehydrogenase 2
HSE	Health Survey for England
HTN	Hypertension or hypertensive subjects
htSNP	Haplotype tagging single nucleotide polymorphism
HWE p	Hardy-Weinberg equilibrium p-value
IC	Intron conversion
IHA	Bilateral idiopathic hyperplasia
IL-1	Interleukin 1
IL-6	Interleukin 6

IP3	1,4,5 Inositol triphosphate
IRMA	Immunoradiometric
JAM	Jamaican Study
JNC	Joint National Committee
JPT	Japanese population in Tokyo, Japan
K^+	Potassium ions
KCNJ1	Gene encoding potassium inwardly-rectifying channel, subfamily J, member 1
Ki-RasA	Kirsten Ras GTP-binding protein 2A
LD	Linkage disequilibrium
LDL	Low-density lipoprotein
LOD	Logarithm of the odds to the base 10
LPA	Apolipoprotein A gene
LRH	Low renin hypertension
LVmass	Left ventricular mass
LWK	Luhya population in Webuye, Kenya
M	Major allele
m	minor allele
M:F	Male to female ratio
MAF	Minor allele frequency
MBP	Mean blood pressure
MCM6	Gene encoding minichromosome maintenance deficient 6
MCP-1	Macrophage chemoattractant protein 1
MDC	Malmo Diet and Cancer study
MDM2	Murine double minute gene 2
MEX	Mexican ancestry population in Los Angeles, California
MKK	Maasai population in Kinyawa, Kenya
MONICA	MONitoring of trends and determinants in CArdiovascular disease
MR/MCR	Mineralocorticoid receptor
MTHFR	Gene encoding 5,10-methylenetetrahydrofolate reductase
MT-TI	Gene encoding mitochondrially encoded tRNA isoleucine
Na^+-K^+-ATPase	Sodium-potassium adenosin triphosphatase pump
Na^+	Sodium ions
Na-Cl cotransporter	Sodium chloride cotransporter
NaCl	Sodium chloride/salt
NAD^+	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAHR	Nonallelic homologous recombination
NCCLS	National Committee on Clinical Laboratory Standards
NCCT	Sodium chloride cotransporter
NCI-NHGRI	National Cancer Institute-National Human Genome Research Institute
NDRG2	N-Myc downstream regulated gene 2
Nedd4-2	Neuronal precursor cell-expressed developmentally down regulated protein 4-2
NEDD4L	Gene encoding the neural precursor cell expressed, developmentally down-regulated 4-like

NEP	Gene encoding Discoidin receptor tyrosine kinase
NF water	Nuclease free water
NGF1B	Nerve growth factor-1B
NHANES III	National Health and Nutrition Examination Survey III
NHE3	Na+/H+-exchanger
NORDIL	Nordic Diltiazem study
NPPA	Gene encoding natriuretic peptide A
NPPB	Gene encoding natriuretic peptide B
NR3C2	Gene encoding Nuclear receptor subfamily 3 group C member 2
NSB	Non-specific binding
NT	Nonhypertensive subjects
Nurr1	Nuclear receptor related 1 protein
Nurr77	Nuclear receptor related 77 protein
Obs Het	Observed heterozygosity
OCP	Oral contraceptives
OR	Odds ratio
Orm-1	Orosomucoid-1
PA	Primary aldosteronism
PAI-1	Plasminogen activator inhibitor-1
PAPY	Primary Aldosteronism Prevalence in Hypertensives
PBR	Peripheral benzodiazepine receptor
PCR	Polymerase chain reaction
PCR-SEQ	Polymerase chain reaction followed by sequencing
PGI2	Prostacyclin or prostaglandin I2
PKC	Protein kinase C
PLC	Phospholipase C
PLCD3	Gene encoding phospholipase C, delta 3
PMS2	Gene encoding postmeiotic segregation increased 2
PMSF	Phenilmethylsulfonyl fluoride
POMC	Proopiomelanocortin
PRA	Plasma renin activity
PRC	Plasma renin concentration
PREECL	Preeclamptic women
PREGN	Pregnant women
PRKG2	Gene encoding cGMP-dependent protein kinase II
PT	Proximal tubule
QC	Quality control
QFAM	Quantitative family based association test
RAAS	Renin-angiotensin-aldosterone system
RAS	Renal artery stenosis
RBaK	Gene encoding retinoblastoma-associated Kruppel-associated box gene
RED	Restriction enzyme digestion
REN	Gene encoding renin
RGS2	Regulator of G-protein signaling 2
RGS5	Gene encoding regulator of G-protein signaling 5
RIA	Radioimmunoassay

RKHD1	Gene encoding for RING finger and KH domain-containing protein 1
RLU	Relative light units
RNA	Ribonucleic acid
ROMK	Renal outer medullary potassium channel
S	11-Deoxycortisol
S:F	11-Deoxycortisol to cortisol ratio
SAME	Syndrome of apparent mineralocorticoid excess
SBP	Systolic blood pressure
SCNN1B	Gene encoding sodium channel, nonvoltage-gated 1, beta
SCNN1G	Gene encoding sodium channel, nonvoltage-gated 1, gamma
SD	Standard deviation
SE	Standard error
SELE	Gene encoding selectin E
SF-1	Steroidogenic factor-1
Sgk1	Serum- and glucocorticoid-regulated kinase 1
SH2B3	Gene encoding SH2B adaptor protein 3
SLC12A1	Gene encoding solute carrier family 12 (sodium/potassium/chloride transporters), member 1
SLC12A3	Gene encoding solute carrier family 12 (sodium/chloride transporters), member 3
SLC24A5	Gene encoding solute carrier family 24, member 5
SNP	Single nucleotide polymorphism
SPRI	Solid phase reversible immobilization
StAR	Steroidogenic acute regulatory protein
T	Total radioactivity
tagSNP	Tagging single nucleotide polymorphism
TASK-1	Two-pore domain acid-sensitive potassium channel 1
TASK-3	Two-pore domain acid-sensitive potassium channel 3
TBE	Tris-borate-EDTA
TDT	Transmission disequilibrium test
THDOC	Tetrahydrodeoxycorticosterone
THE	Tetrahydrocortisone
THF	Tetrahydrocortisol
THS	Tetrahydrodeoxycortisol
TNF alpha	Tumor necrosis factor alpha
TNX	Tenascin-X
tRNA	Transfer RNA
TSI	Tuscans in Italy
UCP1	Uncoupling protein 1
UCP3	Uncoupling protein 3
UPAR	Urokinase-type plasminogen activator receptor
UPGMA	Unweighted Pair-Group Method using arithmetic Averages
Usp2-45	Ubiquitin-specific protease 2-45
WNK	Serine-threonine kinase
WNK1	Gene encoding WNK lysine deficient protein kinase 1
WNK4	Gene encoding WNK lysine deficient protein kinase 4
Wt	Wild-type

WTCCC	Wellcome Trust Case-Control Consortium
YRI	Yoruban population in Ibadan, Nigeria
ZNF652	Gene encoding zinc finger protein 652

1 Introduction

1.1 Hypertension

Hypertension is the most common risk factor for the development of cardiovascular diseases (i.e. stroke and myocardial infarction) which are among the major causes of global morbidity and mortality. Despite this, it is inadequately controlled worldwide. For this reason, great effort is being made to identify its causes, to improve its management and to prevent it (Kaplan & Opie 2006).

Blood pressure (BP) is a highly variable quantitative trait and therefore it has been difficult and somewhat arbitrary to define specific levels at which high blood pressure becomes too high, i.e. hypertension. As there is a strong correlation between cardiovascular and renal complications and hypertension, a practical definition and classification of high blood pressure to assess patients and provide treatment has been agreed upon and revised (Carretero & Oparil 2000). The classification of hypertension is based on the average of two or more properly measured blood pressure readings on each of two or more visits. The classification of hypertension for adults > 18 years is based on the recommendations of the Seventh Report of the Joint National Committee of Prevention, Detection, Evaluation and Treatment of High Blood Pressure-JNC VII (Table 1-1) (Chobanian *et al.* 2003).

Table 1-1. JNC VII Classification of hypertension

BP Classification	Systolic BP (mmHg)	Diastolic BP (mmHg)
Normal	< 120	And < 80
Prehypertension	120-139	Or 80-89
Stage 1 hypertension	140-159	Or 90-99
Stage 2 hypertension	≥ 160	Or ≥ 100

The term “prehypertension” has been included to be able to identify individuals in whom early intervention by adoption of healthy lifestyles could reduce blood pressure and/or reduce its rate of progression. Patients with readings in stage 1 or 2 should be considered for treatment for hypertension.

The following few sections survey the gender-ethnic-sociological distribution of hypertension and briefly discuss the influences on which it seems to depend.

1.1.1 Epidemiology-Prevalence

The prevalence of hypertension is high in developed and developing countries, ranging from 20% to 50%, and is an increasing trend. In 2000, 26.4% of the adult world population had hypertension and it is estimated that, by 2025, at least 29% of the population will be affected (Kearney *et al.* 2005).

In agreement with the JNC definitions, hypertension has been defined in health surveys as blood pressure ≥ 140/90 mmHg or on medication prescribed to treat hypertension. The Health Survey for England 2006 (HSE 2006) and the Scottish Health Survey (2003) reported that prevalence of hypertension was similar in men of both populations (31% and 33%, respectively) but significantly higher in Scottish than English women (31.7% and 28%, respectively). In England as well as in Scotland, the prevalence increases considerably with age in both sexes. At the time of the surveys, 42% of men and 54% of women in England were being treated to reduce their blood pressure, while in Scotland 37.2% of men and 49.3% of women were being treated. In each case, only about half of the patients taking antihypertensive medication successfully achieved control of blood pressure. This highlights the importance of improving its management (Bromley *et al.* 2005; Chaudhury 2008).

Although it has been suggested that Caucasian-origin populations are less susceptible than black populations to hypertension, a comparative study of hypertension prevalence in 8 white and 3 black populations shows a wide variation in whites (27-55%) and blacks (14-44%). A gradient among black populations is consistent with a hypertensive effect of an industrialized lifestyle, as Nigerians had a lower prevalence compared with Jamaicans and US blacks (13.5%, 28.6% and 44.0%, respectively). The trend in white populations is less apparent as Canadians and US whites have a lower prevalence than the Europeans included in this study (Figure 1-1) (Cooper *et al.* 2005). Other known or unknown environmental factors might make an important contribution to the development of hypertension independent of the ethnic background (Cooper *et al.* 2003). Some of these are discussed in section 1.1.3.

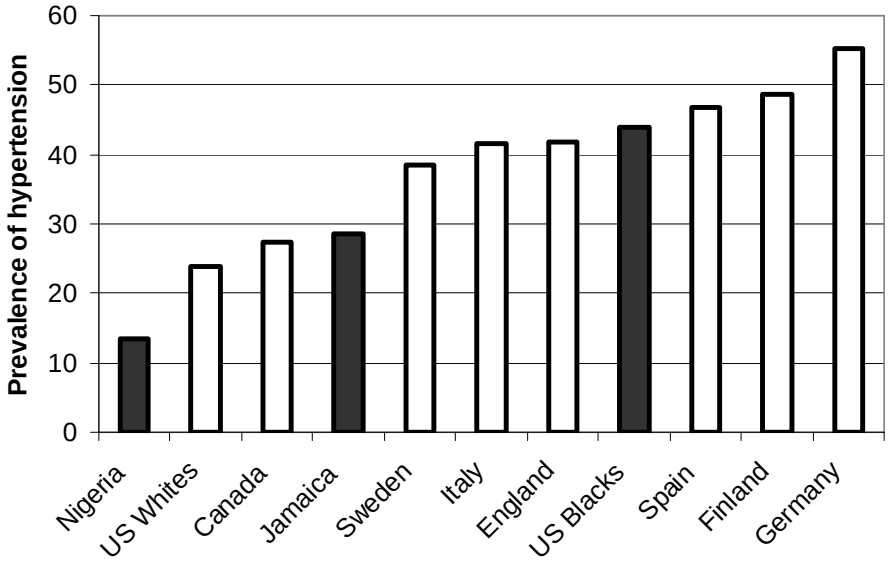


Figure 1-1. Hypertension prevalence (%) among persons 35-64 years, age adjusted, in populations of African (in black) and European- (in white) origin. Adapted from Cooper *et al*. 2005.

1.1.2 Pathophysiology

An increase in blood pressure can be triggered by myriad factors whose actions are reflected in increased cardiac output and/or peripheral resistance. These two elements are mainly regulated by neural, endocrine and paracrine factors that, in turn, may be affected by the environmental, demographic and genetic factors described in the following sections, that influence the severity of blood pressure elevation and the timing of its onset.

Cardiac output is a function of heart rate and stroke volume, while total peripheral resistance is determined by the structure and function of the vasculature and by local autoregulatory mechanisms. Cardiac output increases with augmented fluid volume (preload) or heart contractility. In turn, peripheral resistance is affected by constriction or structural hypertrophy of blood vessels (Kaplan 1994).

Derangement of the neural, endocrine and paracrine factors is complex and affects cardiac output, peripheral resistance or both by influencing kidney and vascular function. Although homeostatic negative feedback is important in controlling the above mentioned factors to regulate blood pressure, this mechanism may be altered in hypertension.

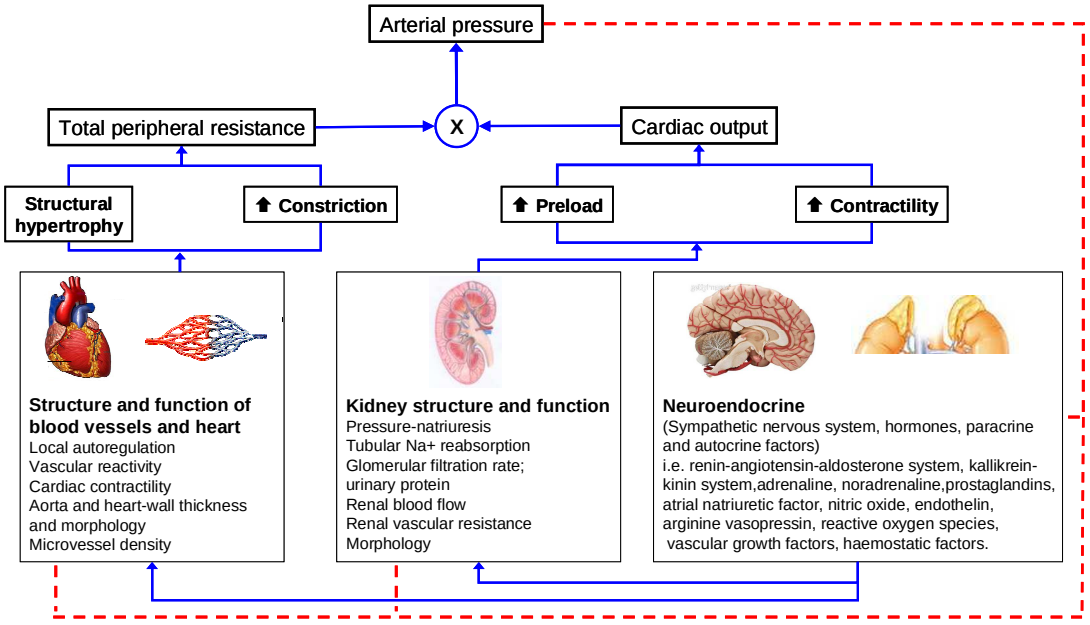


Figure 1-2. Factors involved in regulating blood pressure. Solid lines show the interactions between the different factors and dashed lines show the negative feedback. Adapted from Cowley A.W. Jr. 2006 .

Of all the factors mentioned, Guyton and colleagues suggested that renal mechanisms have a primary part in the regulation of fluid and electrolyte balance and thus a determining role in blood pressure. Further clinical and experimental data have reinforced this hypothesis (Cowley, Jr. 1992; Guyton *et al.* 1972). The pressor effects of the kidneys can be modified by other mechanisms, creating the dynamic network illustrated in Figure 1-2.

The renin-angiotensin-aldosterone system (RAAS) plays an important role in regulating body fluid balance and arterial pressure. Aldosterone, one of the major active components of this system, is considered a prime candidate in the development and maintenance of hypertension, affecting electrolyte metabolism profoundly but also, newer research suggests, acting on the heart and vasculature independently of changes in blood pressure (Slight *et al.* 1999). The evidence for this will be addressed in the next sections.

1.1.3 Aetiological factors

Most patients with arterial hypertension are said to have essential hypertension as no cause is identifiable. Only 10-15% of patients are classified as having secondary hypertension with an identifiable cause, for example a defective gene (see sections 1.1.3 and 1.3.1) or an underlying and generally correctable cause, mainly attributed to renal, endocrine and neurological disorders, as well as pregnancy, sleep apnea, acute stress and coarctation of the aorta (Kaplan 1994; Moneva & Gomez-Sanchez 2002).

Primary or essential hypertension is a multifactorial disease affected by genetic and environmental factors. These will be discussed in detail in the following sections.

Environmental and demographical factors

From the environmental point of view, a sedentary lifestyle, stress, low potassium intake and low calcium intake can have an impact on blood pressure levels. Several factors, including obesity, insulin resistance, high alcohol intake, high salt intake and aging can also increase blood pressure (Kannel 1990; Sever & Poulter 1989). In addition, the evidence of intrauterine influence on birth weight and consequent blood pressure in adulthood is increasing (Victora *et al.* 2008). The influences of many of these factors are additive.

Among these factors, the link between dietary sodium intake and blood pressure has been widely studied given the relationship between salt and vascular volume homeostasis. Epidemiological and clinical research in humans and animals has provided insight into the relationships between dietary salt (NaCl), renal salt handling and blood pressure. Evidence suggests a chronic increase in dietary salt leads to the development of hypertension due to a decline in the kidneys' ability to excrete salt, and animal studies show that blood pressure rises with increases in dietary salt in a dose-dependent fashion (Penner *et al.* 2007). Many large observational epidemiological studies also link high salt diet with hypertension. One major study was INTERSALT which involved 52 centres worldwide (1988). Despite the criticism of its data and statistical methods (Hanneman 1996; Smith & Phillips 1996), it showed a significant association between 24 hour urinary sodium and blood pressure and an increase of blood pressure with age. In addition, a decrease in sodium intake was associated with a reduction in systolic blood pressure after adjustment for age, sex, body mass index (BMI) and alcohol intake (Elliott *et al.* 1996; Stamler *et al.* 1989). Recent studies such as the EPIC-NORFOLK (European Prospective Investigation into Cancer-the

Norfolk cohort) and NHANES III (National Health and Nutrition Examination Survey III) have confirmed these findings (Hajjar & Kotchen 2003; Khaw *et al.* 2004). Major intervention trials like Dietary Approaches to Stop Hypertension (DASH) (Vollmer *et al.* 2001), as well as meta-analysis (He & Macgregor 2004; Hooper *et al.* 2002), have also provided strong evidence that sodium reduction lowers blood pressure in normotensive and hypertensive subjects. Despite these compelling findings of a relationship of sodium intake to blood pressure in the population, a heterogeneity in individual blood pressure responses to acute or chronic salt depletion or repletion and extracellular fluid volume manipulation has been reported (Weinberger & Fineberg 1991), thus leading to the concepts of salt sensitivity and resistance in normotensive and hypertensive individuals as well as animal models like the Dahl salt sensitive rat (Cicila *et al.* 1993; Miller *et al.* 1983; Weinberger *et al.* 1986). Salt sensitivity is defined as the tendency of blood pressure to fall during salt restriction and to rise during salt supplementation (Weinberger *et al.* 1986). Salt sensitivity and resistance are in turn influenced by genetic factors, race, age, body mass and diet. With age, salt sensitivity increases and is more common in patients of African ancestry (Campese 1994; Falkner & Kushner 1990). There is accumulating evidence to suggest that those individuals with an increase in blood pressure for a given salt intake have a reduced ability to excrete sodium; this result in a mild rise in body sodium and, therefore a tendency towards an elevated extracellular volume (de Wardener *et al.* 2004). Advancing age may influence the susceptibility to salt sensitivity and the development of hypertension as there are changes in renal function that lead to sodium retention and volume expansion (Luft *et al.* 1987), possibly as a consequence of aldosterone action (Beevers *et al.* 1975).

The onset of essential hypertension usually occurs in middle age and its main haemodynamic feature is renal and systemic vasoconstriction. This vasoconstriction persists as hypertension progresses with age but is sustained by structural changes in the vessel walls. These changes may induce oxidative stress and further injury (Touyz 2004). The progress to end stage renal disease in hypertension varies according to population. Thus, African or Asian ancestry can confer a more rapid disease progression compared with European ancestry (Cooper *et al.* 2005; Jones 2003; Kotchen *et al.* 2000c).

Worldwide there are efforts to define genes responsible for altering blood pressure at different stages. This thesis is mainly focused on the evidence of the influence of two genes which contribute to sodium homeostasis and the development of hypertension. In the next section various approaches to dissect the genetic basis of hypertension will be described.

Genetic factors

Genetic variation in many physiological systems have been associated with hypertension or increased susceptibility to hypertension. This variation can interact with the environmental factors previously mentioned to produce a spectrum of intermediate phenotypes involving sympathetic nerve activity, the renin-angiotensin-aldosterone system, renal kallikrein-kinin system, sodium balance, vascular reactivity and cardiac contractility. The search continues for genetic factors causing hypertension in each individual so that control of their blood pressure can be precisely focused.

It has been estimated that, within a population, 30-60% of blood pressure depends on genetic variation (Ward 1990). Population studies have shown that a shared environment is not the sole cause of familial trends as there is greater similarity in blood pressure within families than between families (Longini, Jr. *et al.* 1984), and between biological than adopted relatives, with more concordance between biological than adopted siblings (Biron *et al.* 1976; Havlik *et al.* 1979; Mongeau *et al.* 1986; Rice *et al.* 1989). Moreover, monozygotic twins have higher correlation of blood pressure than dizygotic twins (Fagard *et al.* 1995; Hunt *et al.* 1989; Slattery *et al.* 1988).

Heritability is the parameter used to quantify how much of the total phenotypic variation in a population is attributable to variation among individual genotypes compared with variation in their environment. A high heritability means that most of the variation in the population being studied can be attributed to variation in genotype. The heritability of clinic systolic blood pressure (SBP) and diastolic blood pressure (DBP) has been estimated at between 15-40% and 15-30% respectively. The heritability values for ambulatory night-time measurements tend to be higher but more variable (SBP 32-70% and DBP 32-50%) (Bochud *et al.* 2005; Fagard *et al.* 1995; Fava *et al.* 2004; Kotchen *et al.* 2000b; Kupper *et al.* 2005). A comprehensive review of heritability based on family studies has yielded an estimate for blood pressure of approximately 35% (Ward 1990). In addition to cardiovascular traits, physiological/haemodynamic (Seasholtz *et al.* 2006),

autonomic/sympathetic (Kennedy *et al.* 2005), metabolic (Wessel *et al.* 2007), inflammatory, oxidative (Kennedy *et al.* 2005), endothelial (Lillie *et al.* 2007) and renal (Rao *et al.* 2007) traits also display significant heritability. These estimates can be put in context by comparison with heritability of physical traits like height (70-80%) and body mass index (~ 45%). Thus, the fraction of variance of cardiovascular and other "intermediate phenotypes" accounted for by genetic variance is considerable. It has been suggested that "intermediate phenotypes" can help to elucidate hypertension-predisposition loci as variations in the genome might provide more insight than those in the ultimate disease trait (Lillie & O'Connor 2006). A recent example of this approach has been used by Newton-Cheh and colleagues. They used the intermediate phenotype of natriuretic peptide concentration to select common single nucleotide polymorphisms (SNPs) in the *NPPA-NPPB* locus and then extended the association analysis to include circulating natriuretic peptide concentration, blood pressure variation and hypertension in several populations of European ancestry. The approach proved to be successful as a positive and reproducible association was found between variants in the *NPPA-NPPB* locus, leading to higher concentrations of natriuretic peptides and lower blood pressure (Newton-Cheh *et al.* 2009b).

The question therefore arises as to which genetic loci contribute to this considerable heritability and which techniques might be suitable both to discern and to quantify these effects. There is agreement that heritability of essential hypertension depends upon small effects at many loci. Some categories of secondary hypertension can also be the result of large changes at a few loci and these may provide clues to candidate loci in essential hypertension. These are briefly summarised below.

Monogenic disorders of blood pressure variation

Mendelian or single gene disorders have been identified in more than 10 genes known to cause hypertension or hypotension. These primarily affect the renal tubular electrolyte transport functions or the synthesis or activity of mineralocorticoid hormones (described in Table 1-2). Although these disorders are rare, they highlight the influence of genetic components in blood pressure control and may help to elucidate mechanisms predisposing to essential hypertension.

Table 1-2. Causative mutations in monogenic disorders affecting blood pressure

Disorder	CV component	Gene	Type of mutation	Functional consequences	Mode of inheritance
Glucocorticoid-remediable aldosteronism or Familial hyperaldosteronism Type I (FH-I) (OMIM #103900) (Gong & Hubner 2006; Lifton *et al.* 1992a; Lifton *et al.* 1992b; Lifton *et al.* 2001)	Mineralocorticoid hypertension	*CYP11B1 & CYP11B2*	Hybrid gene mutation (5' regulatory region of *CYP11B1* fused to *CYP11B2* coding region)	Ectopic expression of aldosterone synthase activity in adrenal fasciculata controlled by ACTH.	Autosomal dominant
11β-hydroxylase deficiency (OMIM #202010) (Geley *et al.* 1996; Krone *et al.* 2005; Krone *et al.* 2006; New 2003; White & Speiser 1994)	Mineralocorticoid hypertension	*CYP11B1*	Missense and nonsense point mutations, insertions and deletions.	Decrease production of cortisol and corticosterone, with accumulation of 11-deoxycortisol and DOC. The production of androgens is increased.	Autosomal recessive
17α-hydroxylase deficiency (OMIM #202110) (Kater & Biglieri 1994; Rumsby *et al.* 1993; Yanase 1995)	Mineralocorticoid hypertension	*CYP17A1*	Point mutations, deletions and insertions causing missense, nonsense and splice site mutations.	Impaired cortisol biosynthesis, leading to increased ACTH secretion. Inability to secrete androgens.	Autosomal recessive
Mutations in mineralocorticoid receptor (Geller *et al.* 2000)	Early-onset hypertension	*NR3C2*	S810L missense mutations in ligand-binding domain	Converts receptor antagonists to agonists, pregnancy exacerbates hypertension. Constitutive activation	Autosomal dominant
Primary glucocorticoid resistance (OMIM +138040) (Charmandari *et al.* 2008)	Mineralocorticoid hypertension	Glucocorticoid receptor gene *(hGR).*	Non-synonymous single point mutations and a deletion causing a splice site mutation.	Impaired receptor function and impaired signal transduction resulting in reduced sensitivity to glucocorticoids. There is excess cortisol and ACTH.	Sporadic, autosomal dominant or autosomal recessive
Syndrome of apparent mineralocorticoid excess (SAME) (Li *et al.* 1998; Mune & White 1996)	Mineralocorticoid hypertension	*HSD11B2*	Missense loss of function mutations.	Excess stimulation of the mineralocorticoid receptor (MR); hypertension mediated by increased ENaC activity	Autosomal recessive
Liddle's syndrome (OMIM #177200) (Warnock 2001; Shimkets *et al.* 1994; Hansson *et al.* 1995a; Hansson *et al.* 1995b)	Severe hypertension	*SCNN1B* and *SCNN1G (β* and *γ* subunits of the ENaC channel*)*	Missense or frameshift mutations	Truncation mutations in C-terminal region and missense mutations resulting in activation of ENaC and excess sodium retention.	Autosomal dominant
Pseudohypoaldosteronism type II (OMIM 145260) (San Cristobal *et al.* 2008)	Hypertension	*WNK1* and *WNK4*	*PRKWNK1:* intronic deletion, *PRKWNK4*: missense mutations	A gain of function mutation in *WNK1* results in hyperactive Na-Cl cotransporter. A loss of function mutation in *WNK4* has same effect. Less sodium is available leading to volume expansion, hypertension and hyperkalemia.	Autosomal dominant
Gitelman syndrome (OMIM #263800) (Kurtz 1998)	Hypotension	*SLC12A3*	Missense, splice site mutations, deletions and insertions.	Inactivation of thiazide-sensitive Na-Cl cotransporter. Decrease in salt reabsorption resulting in dehydration and hypotension.	Autosomal recessive
Antenatal Bartter's syndrome type 1 (OMIM #601678) (Kurtz 1998)	Hypotension	*SLC12A1*	Frameshifts, missense and nonsense mutations	Salt wasting in ascending loop of Henle leading to activation of the RAAS and the mineralocorticoid receptor, increased ENaC activity and relative salt homeostasis.	Autosomal recessive
Antenatal Bartter's syndrome type 2 (OMIM #241200) (Kurtz 1998)	Hypotension	Potassium channel ROMK gene *(KCNJ1)*	Nonsense, missense, insertion, premature termination codon, and frameshift mutations.	Impaired salt reabsorption due to inability of recycling K^+.	Autosomal recessive
Bartter's syndrome type 3 (OMIM #607364) (Kurtz 1998)	Hypotension	Kidney chloride channel B gene *(CLCNKB)*	Deletions, missense and nonsense mutations.	Defective salt reabsorption due to a defect in Cl^- transport.	Autosomal recessive
Syndrome of hypertension, hypercholesterolaemia and hypomagnesaemia (Ashraf *et al.* 1999; Wilson *et al.* 2004)	Hypertension	*MT-TI*	Missense mutation	Maternal inheritance of the mutation causes a substitution of cytidine for uridine in mitochondrial tRNA impairing ribosome binding.	Mitochondrial inheritance

FH-1: Familial hyperaldosteronism type I; ACTH: adrenocorticotrophin hormone; DOC: 11-deoxycorticosterone; SAME: syndrome of apparent mineralocorticoid excess; MR:mineralocorticoid receptor; ENaC: epithelial sodium channel; Na-Cl cotransporter: sodium-chloride cotransporter; RAAS: renin-angiotensin-aldosterone-system; ROMK: renal outer medullary potassium channel; K^+: potassium; Cl^-:chloride; tRNA: transfer RNA.

It is common in these disorders that hypertension develops as a consequence of increased sodium, chloride and water reabsorption followed by volume expansion (Figure 1-3). The increased volume can lead to low plasma renin activity, a common feature in the monogenic forms of hypertension. In addition, the aldosterone and potassium plasma levels can vary according to the functional consequences of the mutations. The main mechanisms involved in the development of monogenic hypertension are an abnormal level of hormone-induced sodium retention (i.e. mineralocorticoid activity in FH-I, hydroxylase deficiencies and SAME) or an abnormal sensitivity of hormone receptor (mutations in MR and primary glucocorticoid resistance) or post-receptor mechanisms (i.e. ENaC and Na-Cl cotransporters). It is the mineralocorticoid systems that are a principal concern of this thesis; they are described in detail in sections 1.2 and 1.3.

Figure 1-3 has been removed due to Copyright restrictions. See Figure 3 from Garovic VD, Hilliard AA & Turner ST 2006 Monogenic forms of low-renin hypertension. *Nat.Clin.Pract.Nephrol.* **2** 624-630.

Figure 1-3. Single gene disorders affecting nephron and causing hypertension. (A) Mutations in mineralocorticoid receptor (MCR), (B) Deficiency of 11β-HSD2 caused by syndrome of apparent mineralocorticoid excess (AME) or licorice consumption, (C) Liddle's syndrome and (D) Gordon's syndrome. Abbreviations: CCT, cortical collecting tubule; DCT, distal convoluted tubule; ENaC, amiloride-sensitive sodium channel; MCR, mineralocorticoid receptor; NCCT, Na–Cl co-transporter; PT, proximal tubule; ROMK, renal outer medullary potassium channel; WNK, serine–threonine kinase. From (Garovic *et al.* 2006).

While gene mapping of monogenic disorders has been highly rewarding, the major challenge is to map a complex trait, such as hypertension, which is affected by multiple genes. The three most common approaches for gene mapping are linkage, association and genome wide association studies.

Linkage analysis

Linkage analysis aims to identify a small chromosome region containing the gene of interest that cosegregates in families with a disease phenotype by using polymorphic markers. The availability of microsatellites as markers and the technology to genotype them has made genome-scanning linkage studies feasible. The main advantage of this method is that it is not driven by a biological hypothesis.

There is evidence of regions being linked to blood pressure and essential hypertension in almost all chromosomes. Nevertheless, it is unlikely that any single region has a large effect on blood pressure as more than 100 hypertension-related quantitative trait loci across the human genome have now been identified. Many of these associations have not been reproduced. The main reasons for this lack of replication may be due to differences in ethnic origin, stratification, inadequate power, pleiotropic variations of low penetrance, epistasis, limited phenotype-genotype assessment and environmental factors. Multiple linkage loci with overlapping confidence intervals in human chromosomes 1,2,3,17 and 18 indicate the likelihood of several regions being involved in the predisposition to hypertension (Cowley, Jr. 2006). This is further supported by subsequent studies following linkage analysis that reported genes with a plausible (*ATP1B1*, *RGS5* and *SELE*) (Chang *et al.* 2007) or unknown *(RKHD1*) role in hypertension (2002; Chang *et al.* 2007) (Table 1-3).

Table 1-3 Examples of linkage studies in hypertension

Population	Scan	Lod score	Chromosome	Phenotype	Genes
Australian & Dutch 922 normotensive sibling-pairs (Hottenga *et al.* 2007)	5-10cM with 400-800 microsatellites	2-3 (suggestive)	5p13,14q12,17p12	DBP	No genes
Family Blood Pressure Programme (2002) Sibling-pairs (>10 000 indiv.) Subseq, candidate assoc. study (Chang *et al.* 2007)	10cM with 391 microsatellites	>3 (significant)	1,7,17,19,20,21 1q	Pulse pressure & BP	No genes *ATP1B1*, *RGS5* & *SELE*

Sibling-pairs (probands with HTN) (Guzman *et al.* 2006) Follow-up Case-controls	Selected 27 microsatellites in chr.9,17 & 18	>3	18q21.1	Hypertension (HTN)	*RKHD1*
BRIGHT (Caulfield *et al.* 2003) 2010 hypertensive sibling-pairs Subsequent sib-pairs and TDT analysis (Munroe *et al.* 2006)	10 cM	>3 >1.5	6q 2q,5q,9q 5q13	HTN HTN	No genes

Candidate genes

An alternative and complementary approach to linkage analysis is association analysis, which aims to identify correlations between specific genetic variations and a given trait. Usually the genetic variations of choice are the single nucleotide polymorphisms (SNPs). SNPs are single nucleotides at specific locations in the genome with alternative alleles, wherein the least frequent allele is found in ≥ 1% of the population (Brookes 1999). In an association study, it is expected that the frequency of the variations being studied will significantly increase or decrease in the disease trait compared to the normal state, being a deviation from the random frequency of alleles.

Candidate gene studies are usually carried out in cases and controls selected under certain criteria; comparison is conducted of the prevalence of hypertension or level of blood pressure among individuals of contrasting genotypes or haplotypes in genes selected on the basis of known mechanisms of hypertension. The main categories of genes selected are those related to the RAAS, the adrenergic system, metabolism-related genes, growth factors, oxidative stress and inflammatory response. For the purpose of this thesis, only the promising findings for the genes involved in RAAS are described in Table 1-4. An overview of this system is provided in section 1.2.5. In addition to showing association, between blood pressure and the levels of proteins, such as angiotensinogen or aldosterone, there is also evidence of altered transcriptional regulation. Moreover, it is suggested that gene-gene and gene-gender interactions such as the ones shown between the *AGT* and *ACE* genes can account for more blood pressure variation than if they are considered individually.

Table 1-4 Most studied candidate genes in the Renin-angiotensin-aldosterone system

Gene	Variant	Association with
AGT	-20 and -217 (promoter)	Cell-specific regulation of *AGT* transcription (Dickson & Sigmund 2006)
AGT	Met235Thr	BP, angiotensinogen plasma levels, coronary events, mortality (Dickson & Sigmund 2006; Goldenberg *et al.* 2006; Pilbrow *et al.* 2007) Gene-gene interaction with *ACE*,gene-gender interaction (Ge *et al.* 2007)
ACE	A-239T (promoter)	BP, gene-gene interaction with *AGT*, gene-gender interaction (Ge *et al.* 2007)
CYP11B2	T-344C Intron conversion (IC)	Plasma aldosterone levels (Iwai *et al.* 2007), HTN (Sookoian *et al.* 2007), plasma renin activity (Sookoian *et al.* 2007; Staessen *et al.* 2001),11-deoxycortisol & 11-deoxycorticosterone (Davies *et al.* 2001) 11-deoxycortisol & 11-deoxycorticosterone (Davies *et al.* 2001)
CYP11B1	G-1889C, A-1859G	Decreased 11β-hydroxylase efficiency. Altered transcriptional response to ACTH (Barr *et al.* 2007)
AGTR1	A1166C(rs5186, in 3'UTR)	1166C associated with HTN, expression possibly not regulated by miRNA hsa-miR-155 (Sethupathy *et al.* 2007)
ACE, ACE2 and NEP		SBP and DBP, only *ACE* association prevails after adjustment (Rice *et al.* 2006)

Several genes associated with blood pressure or salt-sensitive hypertension have also been reported to influence sodium and fluid balance. These include the gene encoding the β subunit of the Na^+/K^+ ATPase pump (*ATP1B1*) (Chang *et al.* 2007), the cytoskeleton protein adducin (*ADD1*) (Bianchi *et al.* 2005), the chlorine channel (*CLCNKA*) (Barlassina *et al.* 2007), the ubiquitin ligase controlling the endocytosis of the epithelial Na^+ channel (*NEDD4L*) (Russo *et al.* 2005) and the $NaHCO_3$ cotransporter (*SLC4A5*) (Hunt *et al.* 2006).

The candidate gene approach has several disadvantages. Most of the association studies have a weak level of significance, present conflicting results or have been difficult to replicate. As the candidate gene approach is hypothesis driven, unknown pathways are not explored. An inappropriate choice of the candidate gene within a system might lead to lack of consideration of genes coding for other components of the system. The SNPs selected for the association study might not provide a complete coverage of other SNPs or genetic variants within the gene, as the pattern of linkage disequilibrium (LD) may vary widely among and within populations. For example, Ji *et al*. showed heterozygous, rare variants in the *SLC12A3, SCL12A1* and *KCNJ1* genes reduce blood pressure in carriers through their effects on renal salt reabsorption, and protect them from the development of hypertension (Ji *et al.* 2008). Most studies have been underpowered due to population stratification, or environmental, phenotypic and locus heterogeneity. Candidate genes, at best, can identify only a fraction of genetic risk factors considering the polygenic nature of hypertension.

To broaden the search for candidate genes without prior assumptions, genome wide association studies have been suggested as an alternative approach.

Genome wide association (GWA)

This approach aims to cover variations throughout the entire genome in order to detect association. The basis of the GWA is the common disease/common variant hypothesis that suggests that SNPs with a minor allele frequency (MAF) > 1%, which account for more than 90% of the genetic differences between individuals, will have a great influence on disease variations. It is likely that common variations will contribute significantly to hypertension as susceptibility alleles might not be under strongly negative selection pressure. To test this hypothesis, a large number of SNPs are selected and their frequencies compared between hypertensives and controls. No assumptions regarding genomic location or functional causal variant are required, thus allowing the exploration of many common variants in the human genome. There are two main approaches in GWA: the direct or

sequence-based design where the variants chosen have known biological function within the genes; and the indirect or map-based design which considers LD in order to select a set of tagSNPs which captures as many of the SNPs as possible. (TagSNPs are defined as a subset of SNPs chosen for genotyping that represent the common variations.) The direct design requires less genotyping than the indirect design to achieve adequate power, but the indirect design can provide coverage outside the targeted genes if the tagSNPs are in LD with variations in the intergenic region or contiguous genes. Therefore, it has been suggested that the advantages of both designs be combined to provide an enriched GWA for hypertension. To maximize coverage, the recommendation is to include tagSNPs provided by HapMap, nonsynonymous SNPs and SNPs in evolutionarily conserved regions (Padmanabhan *et al.* 2008).

Recent GWA studies have been conducted using gene chips with an intermarker density of 6-40kbp, as opposed to the microsatellite markers used in the genome-wide linkage scanning that were spaced every 4-8Mbp across the genome (Shih & O'Connor 2008). One GWA study for hypertension was conducted by the UK Wellcome Trust on 2000 hypertensives and 3000 common population controls using the Affymetrix 500K panel (2007). No significant association was found at the significance level established for this study ($p<5x10^{-7}$). A moderate association was found in regions on chromosomes 1q43, 8q24, 12p12, 12q23, 13q21 and 15q26, but no genes from physiological pathways previously implicated with hypertension were found in these regions. Several reasons have been proposed for the lack of detectable associations. First, there was poor coverage of many genes by the Affymetrix chip. Second, the use of common controls was detrimental in the power to detect association with hypertension. Third, hypertension may have fewer common risk alleles of larger effect size than other complex diseases and larger cohorts of more than 6000 cases and controls might be required to detect significant association (Padmanabhan *et al.* 2008).

Another GWA study was conducted in the Framingham Heart Study with phenotypic data of SBP, DBP and arterial stiffness in more than 1300 subjects followed over more than 25 years (Levy *et al.* 2007). A 100K Affymetrix chip was used for genotyping. No significant association was found at a level of $p< 4.4 \times 10^{-8}$; with a more moderate stringency ($p<10^{-5}$), 7 associations were found for SBP and DBP, and 5 associations for arterial stiffness traits. From the 6 candidate genes chosen in the RAAS (*ACE, AGT, AGTR1,CYP11B2,NR3C2,REN*), only a weak association ($p<0.05$) was found with SBP and DBP for *AGT* and *NR3C2*.

Recently, a GWA study in 34,433 subjects of European ancestry from the Global BPgen consortium tested 2.5 million genotyped and imputed SNPs for association with SBP and DBP. The results were confirmed by direct genotyping and *in silico*. Significant associations were found in common variants in eight regions near the following genes: *CYP17A1, CYP1A2, FGF5, SH2B3, MTHFR, c10orf107, ZNF652* and *PLCD3*. These associations were also found for hypertension, thus establishing a better relationship between genetic variations and blood pressure regulation (Newton-Cheh *et al.* 2009a).

Worldwide, there are efforts to define genes responsible for altering BP at different stages using these three strategies. This thesis is mainly focused on the study of two candidate genes; the *CYP11B1* and *CYP11B2* genes participating in corticosteroid biosynthesis and implicated not only in the rare Mendelian disorders but also in the more common phenotype of essential hypertension. This will be discussed in detail in sections 1.3 and 1.4. As explained previously, the selection of candidate genes is based on the physiological mechanisms altered in hypertension. For this reason, the pathophysiology will be addressed in the following section.

1.2 Aldosterone

To evaluate aldosterone concentration as an intermediate phenotype in hypertension studies, an informative index of *in vivo* status must be devised. This is discussed in section 1.3.3. The next sections describe its biosynthesis, actions and catabolism.

1.2.1 Biosynthesis of corticosteroids

The adrenal glands, located above each kidney, have an inner medulla and a surrounding cortex. The cortex has three histologically and enzymatically distinct zones. The zona glomerulosa, with concentrically arranged cells, secretes the mineralocorticoid aldosterone, essential in electrolyte and fluid homeostasis; the zona fasciculata, with polygonal cells arranged in columns, and the zona reticularis, constituted by cells arranged in a network surrounded by capillaries, produce the glucocorticoid cortisol and the sex steroids, mainly the androgen precursors dehydroepiandrosterone (DHEA) and androstenedione (Figure 1-4).

Figure 1-4. Zones of the adrenal gland (adapted from the Thyroid Institute http://www.thyroidinstitute.org/images/adrenal_gland.jpg and http://www.mc.vanderbilt.edu/histology/labmanual2002/labsection3/AdrenalGlands03_files/image004.jpg)

Although the main focus of this thesis is aldosterone, the concomitant synthesis of cortisol has a number of features in common with aldosterone and factors which disrupt it have repercussions for aldosterone production. Therefore, information on the biosynthesis and regulation of cortisol is also included.

All of the adrenal steroids are derived from a common substrate cholesterol (see Figure 1-5). In the human, cholesterol is taken up from the circulation, generally as low-density lipoproteins (LDL) (Bolte *et al.* 1974; Borkowski *et al.* 1967; Gwynne & Strauss, III 1982). When this mechanism is impaired, as in patients with abetalipoproteinemia or familial hypercholesterolemia, high-density lipoproteins (HDL) provide cholesterol (Illingworth *et al.* 1982). Moreover, there is also evidence of a small contribution of cholesterol synthesised *de novo* from acetate within the adrenal cortex (Borkowski *et al.* 1967). Cholesterol is transported to the inner mitochondrial membrane and, although several proteins have been proposed to play a role, the steroidogenic acute regulatory protein (StAR) and the peripheral benzodiazepine receptor (PBR) are the most important (Clark *et al.* 1994; Lin *et al.* 1995; Stocco 2001; Waterman 1995). This cholesterol translocation, regulated by StAR, is the first rate-limiting step of aldosterone and cortisol production.

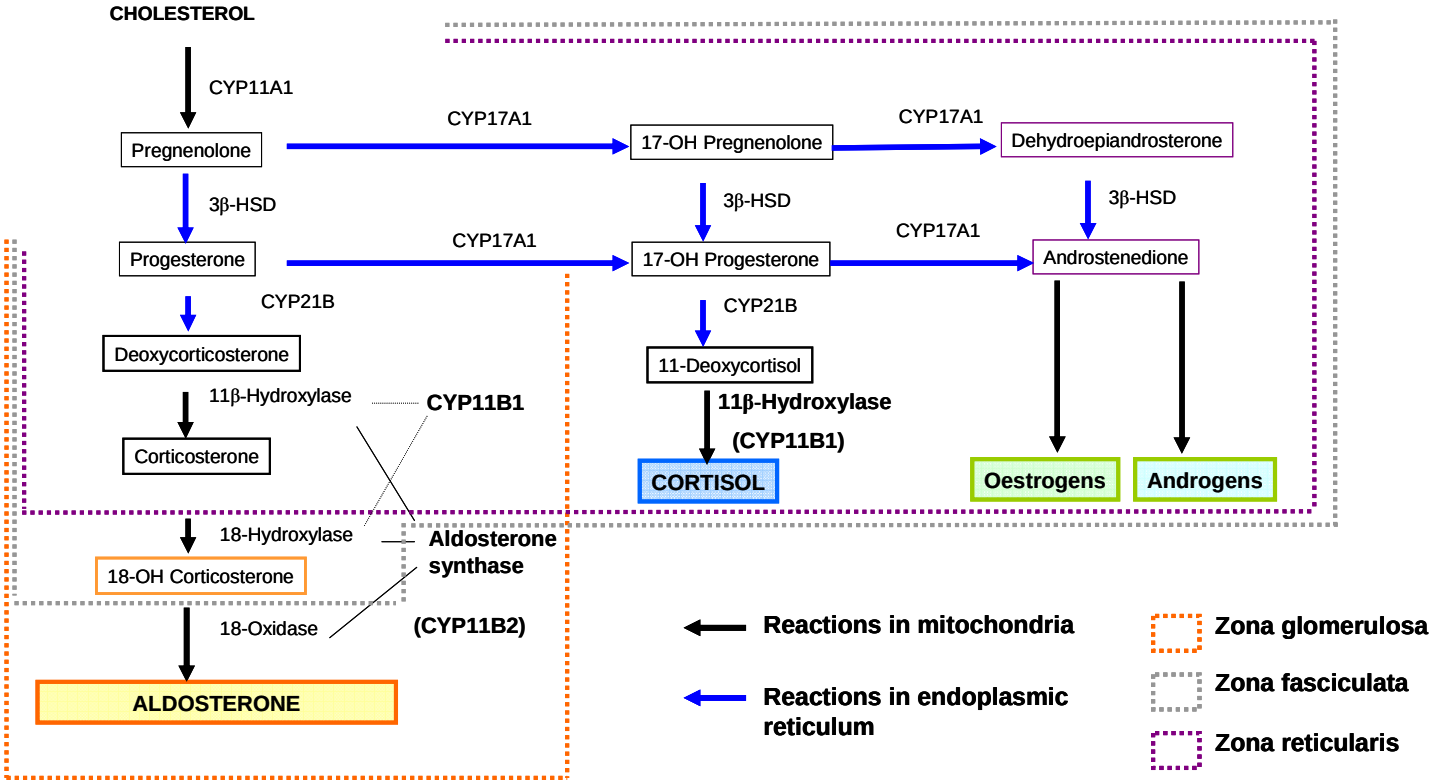


Figure 1-5. Human Corticosteroid Biosynthetic Pathway. 3β-HSD/isomerase: 3β-Hydroxysteroid dehydrogenase; -OH:hydroxy

Cholesterol then undergoes a series of reactions to generate aldosterone and cortisol. These reactions are catalysed by two types of enzymes: cytochrome P450 containing mixed function oxidases and hydroxysteroid dehydrogenases.

Cytochrome P450 (CYP450) enzymes

These enzymes form a large superfamily of haem-containing proteins. They are present in humans, mammals, insects and bacteria, catalyzing a wide range of substrates. In the human, 57 putatively-functional genes have been identified (Nelson *et al.* 2004). Advances in molecular biology and genomics revealed that these enzymes not only play a crucial role in drug detoxification but are also involved in a myriad of enzymatic reactions important in life processes. They act on endogenous compounds, including fatty acids, eicosanoids, sterols, steroids, bile acids, vitamin D and retinoids, and metabolise exogenous substrates such as drugs and natural plant products (Nebert & Russell 2002). In the adrenal glands and the gonads CYP450 enzymes are essential for the biosynthesis of steroids catalyzing the hydroxylation and cleavage of steroid substrate. Five CYP450 enzymes participate in steroid biosynthesis: three are associated with mitochondrial membranes (CYP11A1, CYP11B1 and CYP11B2) and two are associated with the endoplasmic reticulum (microsomal-CYP17A1 and CYP21B).

The function of CYP450 enzymes as monooxygenases allows them to introduce into the substrate RH an oxygen atom as a hydroxyl group and to reduce the other oxygen atom with water in the presence of the nicotinamide adenine dinucleotide phosphate (NADPH) acting as an electron donor. The general reaction is:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

The haem prosthetic group in these enzymes is crucial to control the oxygen and electrons. The electron transfer from the NADPH to the substrate is controlled by two different systems. In the enzymes located in the mitochondrial membranes, first, two high-energy electrons are transferred from NADPH to a flavoprotein, adrenodoxin reductase; then to adrenodoxin, a nonhaem iron-sulfur protein; then to the haem iron of the CYP450 and finally to the substrate. (Figure 1-6) (Bernhardt *et al.* 1998). In contrast, in the microsomal enzymes, the transfer takes place in only one protein, CYP450 oxidoreductase, with two flavins (flavinadenine dinucleotide-FAD and flavin mononucleotide-FMN) as shown in Figure 1-6.

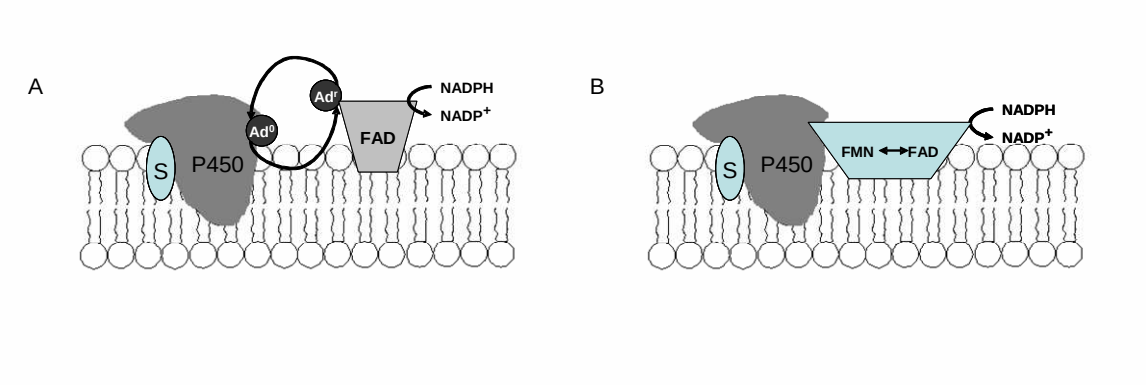


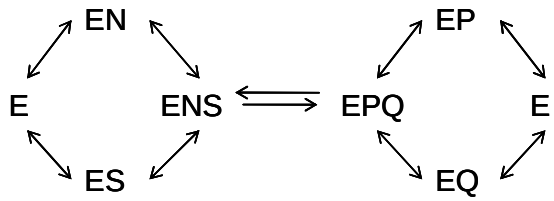
Figure 1-6. Systems of electron transfer from NADPH to the substrate. Panel A shows the mitochondrial system and panel B the microsomal system. Adapted from Payne AH and Hales DB 2004 (Payne & Hales 2004). FAD: Flavin adenine dinucleotide; FMN: flavin mononucleotide; Ad^r, adrenodoxin reductase; Ad^0, adrenodoxin; S, substrate.

The ability of the CYP450 enzymes to undergo conformational changes allows them to bind substrates with different structures.

Hydroxysteroid dehydrogenenases

These non-metalloenzymes have two essential roles. In steroidogenic tissues they participate in the biosynthesis of steroid hormones and in peripheral tissues they convert the steroids to inactive metabolites, regulating binding to members of the nuclear receptor superfamily and, ultimately, gene expression. Like P450 enzymes, the dehydrogenases are present in a wide variety of life forms. However, while each P450 enzyme is the product of a single gene, the dehydrogenases have several isoforms each encoded by a different gene. The isoforms differ in tissue distribution, catalytic activity (reductase or dehydrogenase), substrate and cofactor specificity and subcellular location. The enzymes relevant to this thesis belong to the short-chain alcohol dehydrogenase reductase superfamily. 3β-hydroxysteroid dehydrogenase is involved in steroidogenesis and 11β-hydroxysteroid dehydrogenase in metabolism. These enzymes catalyse oxidoreduction of steroids using NAD^+/$NADP^+$ as a cofactor. The reactions can follow a random kinetic mechanism, in which either the steroid or the cofactor can bind first, or an ordered kinetic mechanism, in which a sequence for binding is followed (Figure 1-7) (Payne & Hales 2004; Penning 1997).

(a) Random mechanism



(b) Ordered mechanism

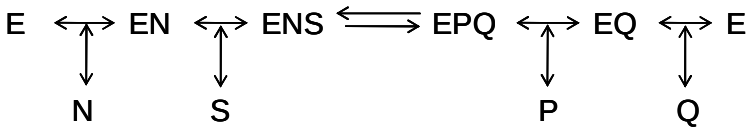
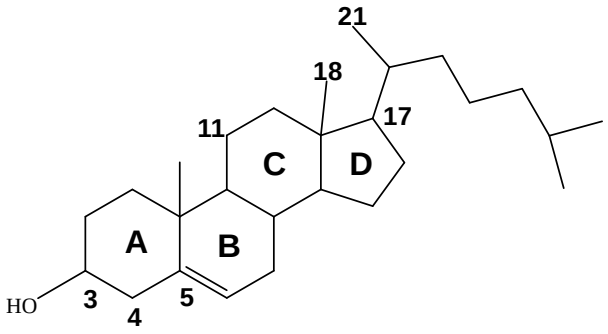


Figure 1-7. Kinetic mechanisms for hydroxysteroid dehydrogenases taken from Penning TM and Ricigliano JW (1991). Panel (a) shows the order of ligand binding in a random kinetic mechanism; panel (b) shows an ordered kinetic mechanism. E, enzyme; N, NADP+; S, steroid substrate; P, steroid products and Q, NADPH.

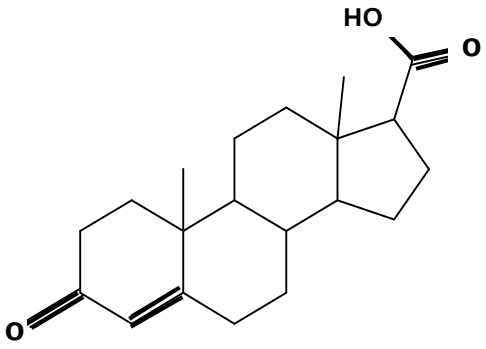
Cholesterol and principal steroids

Before each reaction is described, it is important to know the structure of the steroids, as shown in Figure 1-8.

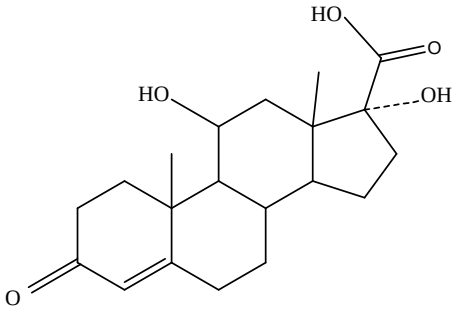
(a) cholesterol

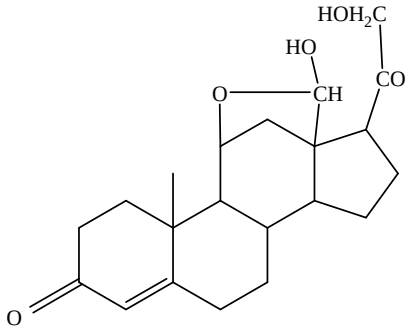


(b) Features of a corticosteroid hormone



(c) **cortisol** **aldosterone**





hemi-acetal form

Figure 1-8. Structure of cholesterol and principal steroids

Cholesterol side-chain cleavage enzyme (CYP11A1)

The cholesterol side-chain cleavage enzyme, also known as desmolase, is encoded by the *CYP11A1* gene which is located on chromosome 15. This enzyme catalyses three reactions: 20α-hydroxylation, 22-hydroxylation and the removal of the side chain at C21 of cholesterol to produce pregnenolone (Lieberman & Lin 2001).

Pregnenolone is released into the cytosol where the generation of progesterone takes place.

3β-Hydroxysteroid dehydrogenase (3β-HSD)

The enzyme responsible for the conversion of pregnenolone to progesterone is the only non-P450-containing enzyme in the pathway as it is a member of the microsomal short chain dehydrogenase family. It has two isoforms: 3β-HSD1 and 3β-HSD2. Their genes lie in tandem on human chromosome 1 (Berube *et al.* 1989). The isoform 3β-HSD2 expressed in the adrenal cortex is responsible for the oxidation and isomerization of the Δ^5-3β-hydroxysteroids pregnenolone, 17-hydroxypregnenolone, previously generated by *CYP17*, and DHEA into the Δ^4-3-ketosteroids, progesterone, 17-hydroxyprogesterone and androstenedione, respectively. The enzyme first catalyzes a dehydrogenation followed by the isomerization of the Δ^5-3-keto-steroid to the Δ^4-3-ketosteroid, requiring the NAD^+ cofactor in both steps (Payne & Hales 2004; Simard *et al.* 1996).

17α-Hydroxylase (CYP17A1)

This enzyme is encoded by the *CYP17A1* gene on human chromosome 10 (Carey *et al.* 1994). It has two activities. It can catalyze hydroxylation at C17 of either progesterone or pregnenolone, generating 17α-hydroxy-progesterone and 17α-hydroxy-pregnenolone, respectively (Sparkes *et al.* 1991). When 17-hydroxylation takes place, the products become cortisol precursors. When, in addition to this hydroxylation, there is a cleavage of the residual 2-carbon side chain at C17 (17,20-lyase activity), the products are directed towards androgen and oestrogen synthesis (Yanase *et al.* 1991). There is no expression of CYP17A1 in the zona glomerulosa, where aldosterone is produced. Thus, this enzyme plays an important role in the natural delimitation of steroid production in the adrenal cortex.

21-Hydroxylase (CYP21B)

This enzyme is expressed in all three zones of the adrenal cortex (Shinzawa *et al.* 1988). In the zona glomerulosa, progesterone is 21-hydroxylated to produce 11-deoxycorticosterone (DOC), while in the zona fasciculata 17-hydroxy progesterone is hydroxylated to produce 11-deoxycortisol (S). CYP21 is related to two genes (*CYP21A* and *CYP21B*) located in chromosome 6 but only *CYP21B* is active (White *et al.* 1986).

Aldosterone synthase (CYP11B2)

In the human, the last three steps from DOC to aldosterone are catalysed by the same mitochondrial P450 enzyme, aldosterone synthase. Its expression is unique to the zona glomerulosa where it performs the following reactions in the inner mitochondria: first it performs 11β-hydroxylation from DOC to produce corticosterone (B), then an 18-hydroxylation takes place generating 18-hydroxycorticosterone (18-OHB) which finally is 18 -methyl oxidated to aldosterone (Denner *et al.* 1995).

11β-Hydroxylase (CYP11B1)

The mitochondrial enzyme 11β-hydroxylase, encoded by the *CYP11B1* gene on chromosome 8, catalyses the last step in the conversion of 11-deoxycortisol to cortisol (Mornet *et al.* 1989). This reaction is restricted to the zona fasciculata-reticularis (Ogishima *et al.* 1992). In addition, 11β-hydroxylase, as with aldosterone synthase, can convert DOC to corticosterone (B); DOC to 18-hydroxy-11deoxycorticosterone (18-OHDOC) and 19-hydroxy-11-deoxycorticosterone (19-OHDOC) and corticosterone to 18-hydroxycorticosterone (18-OHB) (Okamoto & Nonaka 1992).

Aldosterone synthase is encoded by the *CYP11B2* gene and is very similar to the *CYP11B1* gene, which encodes 11β-hydroxylase (see section 1.4.1). These genes are highly homologous and lie in tandem in human chromosome 8 (Chua *et al.* 1987; Mornet *et al.* 1989; Wagner *et al.* 1991). It is hypothesised that both genes originated from a common ancestral gene (Colombo *et al.* 2006) and that, before duplication, the last steps in mineralocorticoid and glucocorticoid synthesis were catalysed by these ancestral enzyme (Miller 1987). Cows, pigs, sheep and frog continue to rely on a single enzyme (Bulow & Bernhardt 2002; Nonaka *et al.* 1995) but the human, rat, mouse and hamster have two proteins to accomplish these final stages.

1.2.2 Extra-adrenal synthesis of aldosterone

Aldosterone production has been studied in extra-adrenal tissues such as the central nervous system, the cardiovascular system and the adipose tissue. However, the presence of the enzymes required for corticosteroid synthesis has only been demonstrated in the brain, heart and vasculature (Davies & MacKenzie 2003; MacKenzie *et al.* 2008). Although the evidence of local aldosterone production in the brain is strong in rodents and *CYP11B2* transcription is subject to regulation by dietary sodium, studies in human brain suggest there is no *CYP11B1* and *CYP11B2* transcription. Thus, the possibility of the synthesis of the mineralocorticoid DOC has been proposed (Sharma *et al.* 2006; Yu *et al.* 2002). The evidence from studies concerning the local synthesis of aldosterone in the heart or peripheral vasculature remains controversial (Ahmad *et al.* 2004; Takeda *et al.* 1996; Kayes-Wandover & White 2000; Pezzi *et al.* 2003; Yamamoto *et al.* 2002; Mizuno *et al.* 2001; Tsutamoto *et al.* 2000). Nevertheless, the emerging evidence suggests some or all the steroid conversions can take place in extra-adrenal tissue. This local production might make only a small contribution to circulating aldosterone levels and it is debatable whether or not it is physiologically relevant. However, it has been speculated that locally-produced aldosterone might act in a paracrine or autocrine mode by occupying the mineralocorticoid receptor (Davies & MacKenzie 2003).

Hormone levels in the body depend not only on the rate of biosynthesis and secretion but also on the rate of catabolism and clearance. Target organ metabolism has also been shown to modulate activities. This is now addressed briefly.

1.2.3 Metabolism of corticosteroids

Corticosteroids are mainly metabolised in the liver. The major metabolites are tetrahydro-compounds which are then conjugated with a glucuronide molecule. These water-soluble compounds are excreted by the kidney. In addition, small amounts of other aldosterone and cortisol derivatives are also excreted (Gower 1975).

For cortisol, there are two main metabolites, tetrahydrocortisol (THF) and cortisone. The tetrahydro compound is formed by a reduction of the A-ring (5α and 5β reductase) and the 3-oxo group (3α and 3β dehydrogenase), as shown in Figure 1-9. Cortisol and inactive cortisone are interconverted by two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD), as depicted in Figure 1-10. In man, the type 1 enzyme (11β-HSD1), is largely found

in the liver and adipose tissue. Its main function is to convert cortisone to cortisol. The type 2 enzyme (11β-HSD2) is found predominantly in mineralocorticoid target tissues (kidney, colon and salivary gland) and its function is to convert cortisol to cortisone (Figure 1-10). Similar to other steroids, cortisone is also excreted as tetrahydrocortisone (THE). The efficiency of the 11β-hydroxysteroid dehydrogenases can be measured by the ratio of cortisol:cortisone metabolites in the urine. The ratio of the free urinary excretion rates of cortisol to cortisone is an index of 11β-HSD2 activity in the kidney. The importance of this will become apparent in sections 1.2.4 and 1.3.1.

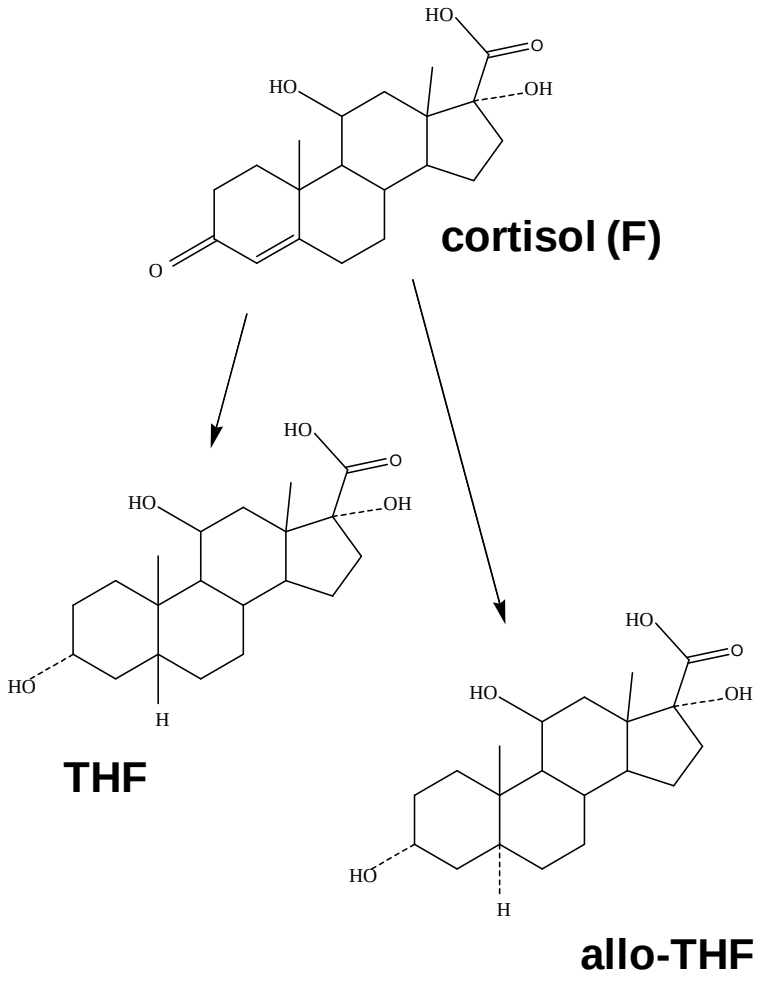


Figure 1-9. Formation of tetrahydrocortisol (THF)

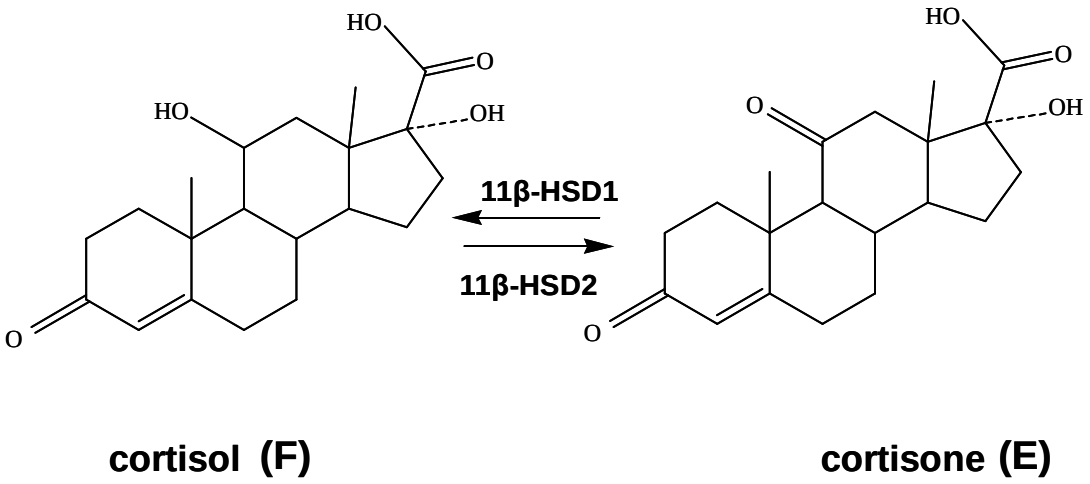


Figure 1-10. Interconversion of cortisol and cortisone by the 11β-hydroxysteroid dehydrogenases (11β-HSDs).

Aldosterone is catabolized similarly to cortisol forming tetrahydro-aldosterone (THaldo). In addition, aldosterone is also excreted as aldosterone-18-glucuronide, possibly synthesised in the kidney, and a very small proportion of free aldosterone (Gower 1975).

1.2.4 Aldosterone Actions

Aldosterone's main role is in the regulation of electrolyte homeostasis and blood pressure. Originally the kidney was recognised as its site of action but subsequent research showed important effects in the colon, the sweat and salivary glands, heart, brain, vascular smooth muscle, liver and peripheral blood leukocytes (Freel & Connell 2004). Aldosterone's actions have been classified as genomic acting via the MR and non-genomic via an as yet unidentified cell membrane receptor.

Epithelial actions of aldosterone

These actions have been described in epithelial cells in the kidney, colon and salivary gland. Aldosterone exerts its classical genomic action by binding to the type I or mineralocorticoid receptor (MR), a member of the steroid/thyroid/retinoid nuclear receptor family. The receptor is kept inactive but receptive by associating with a complex of chaperone proteins. Once the receptor is activated in the presence of a ligand, the chaperones dissociate and the ligand-receptor complex translocates to the nucleus where it dimerizes and binds to hormone-responsive elements (HRE) in the DNA. The dimer acts

as a transcription factor of aldosterone-responsive genes, the expression of which will be activated or repressed (Rogerson *et al.* 2004). As the MR is non-specific, unable to distinguish between aldosterone and cortisol, aldosterone specificity is maintained by the coexpression of the 11β-hydroxysteroid dehydrogenase type 2 enzyme (11β-HSD2) which converts cortisol and corticosterone to cortisone and 11-dehydrocorticosterone respectively (see section 1.2.3) (Edwards *et al.* 1988). 11β-HSD2 plays a crucial role as it protects the MR from glucocorticoid excess. MR has affinity for both cortisol and aldosterone *in vitro* (Arriza *et al.* 1987), thus it has been proposed that the inactivation of cortisol by 11β-HSD2 allows the binding of aldosterone *in vivo* (Edwards *et al.* 1988; Funder *et al.* 1988). Recently, it has been argued that this inactivation of cortisol is not itself sufficient to confer aldosterone selectivity on a tissue and that the truly effective part of 11β-HSD2s action is not to prevent glucocorticoid binding of the MR, but to prevent its activation of the receptor (Funder 2009). It is important to mention the deficiency in 11β-HSD2 leads to hypertension and this will be discussed in section 1.3.1.

There are also findings that suggest a transcriptional effect of activated steroid receptors independent of DNA binding. This alternative mechanism is proposed to occur by direct protein-protein interactions with other factors (Karin 1998; Reichardt *et al.* 1998).

Aldosterone's most distinct effect is to increase sodium reabsorption in the distal nephron and colon, while increasing potassium and hydrogen excretion, thus helping to maintain electrolyte homeostasis and regulate intravascular volume (White 1994). Aldosterone-induced sodium (Na^+) and potassium (K^+) transport within the kidney mainly takes place in the luminal cells of the cortical collecting tubules and the distal convoluted tubule which have high MR expression. The transport of electrolytes is mediated by several channels. The epithelial sodium channel (ENaC) is the major determinant of renal sodium reabsorption (Horisberger 1998). Evidence suggests that aldosterone increases its activity by altering the open probability of channels and inserting new ones in the apical membrane (Butterworth *et al.* 2009; Garty & Palmer 1997; Kemendy *et al.* 1992). The reabsorption of sodium is coupled to an influx of water. Aldosterone also increases the activity of the sodium-potassium ATPase pump (Na^+- K^+-ATPase) (Horisberger *et al.* 1991). This pump returns the sodium to systemic circulation, creating a negative potential difference. To achieve electroneutrality in the cortical collecting tubule, either Cl^- can be reabsorbed via the thiazide-sensitive Na^+/Cl^- cotransporter, K^+ secreted via the K^+ channel (Kim *et al.* 1998; Palmer & Frindt 2000) and/or H^+ secreted into the lumen. Moreover, there is a direct increase in H^+ secretion by a stimulation of the H^+-ATPase activity in the collecting duct. This H^+ release affects acid-base homeostasis (Harvey *et al.* 2008). In addition to these

targets, aldosterone can also stimulate the activity and surface expression of Na^+/H^+ exchanger isoform 3 (NHE3) in human proximal tubule epithelial cells (Drumm *et al.* 2006).

These responses must be mediated directly or indirectly by aldosterone-induced proteins (AIPs). The AIPs act on the apical membrane, on cellular energy production, and/or the basolateral Na^+- K^+-ATPase pump or other biochemical systems. Thus, much research has been devoted to identifying AIPs. The first AIP characterized with convincing supporting evidence was the serum- and glucocorticoid-regulated kinase 1 (Sgk1) which contributes to activation of Na^+-K^+-ATPase and subsequent expression of NHE3 (Musch *et al.* 2008), and increases ENaC activity (Webster *et al.* 1993). Although it has been suggested that Sgk1 might phosphorylate the ENaC α-subunit (Boyd & Naray-Fejes-Toth 2005; Diakov & Korbmacher 2004), recent work shows that the main mechanism might be the phosphorylation of ENaC modulatory protein, Nedd4-2 (neuronal precursor cell-expressed, developmentally down-regulated protein 4-2). The importance of Nedd4-2 is illustrated by Liddle's syndrome (see Table 1-2). Nedd4-2 is a ubiquitin protein ligase which allows ENaC degradation. Its phosphorylation hampers its interaction with ENaC which leads to an elevation in channel density and increased capacity to retain sodium (Naray-Fejes-Toth *et al.* 1999; Rotin 2000). Another recently discovered AIP involved in the early regulation of ENaC ubiquitylation is the de-ubiquitylating enzyme, Usp2-45, the expression of which can stimulate ENaC-mediated sodium transport (Verrey *et al.* 2008).

Three other AIPs under investigation are the glucocorticoid-induced leucine zipper (GILZ), the corticosteroid hormone-induced factor (CHIF) and the kirsten Ras GTP-binding protein 2A (Ki-RasA). GILZ interaction with aldosterone blocks the ERK signaling cascade, thus inhibiting its usual action of limiting sodium reabsorption by facilitating interaction between ENaC and Nedd4 proteins (Bhalla *et al.* 2006; Soundararajan *et al.* 2005). CHIF is expressed constitutively in the kidney and induced in the colon (Wald *et al.* 1996). Its expression is increased in the distal colon by aldosterone, dexamethasone, Na^+ restriction and K^+ loading (Brennan & Fuller 1999; Capurro *et al.* 1996). It has been proposed that its function is associated with Na^+- K^+-ATPase and the enhancement of this pump's affinity for Na^+ in renal tissue (Beguin *et al.* 2001). Nevertheless, its physiological action remains unclear. Ki-RasA expression and protein levels can increase shortly after aldosterone administration, but not in all aldosterone's target epithelia. Its role is therefore controversial. There is experimental evidence showing Ki-RasA increases the probability of ENaC open channels but reduces ENaC number. It can also activate proliferation of rat cardiac fibroblasts (Spindler & Verrey 1999; Stockand

2002; Stockand & Meszaros 2003; Mastroberardino *et al.* 1998). Its expression in the colon is reportedly stimulated by aldosterone (Brennan & Fuller 2006).

Several other AIPs such as N-Myc downstream regulated gene 2 (NDRG2) (Boulkroun *et al.* 2002; Wielputz *et al.* 2007), endothelin-1 (ET-1) (Wong *et al.* 2007), plasminogen activator inhibitor-1 (PAI-1) (Yuan *et al.* 2007), and connector enhancer of kinase suppressor of ras 3 (CNKSR3) (Ziera *et al.* 2009) have been detected in recent years . The classical mechanism of aldosterone action on epithelial cells via the MR and the most studied AIPs are shown in Figure 1-11.

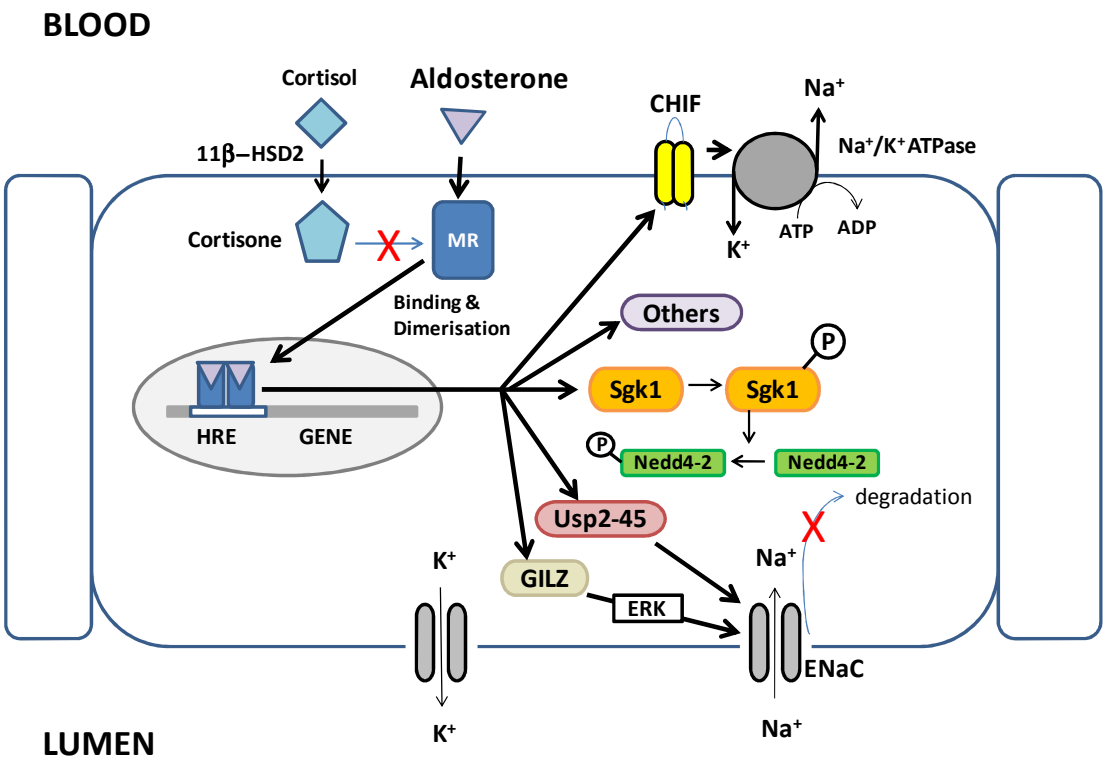


Figure 1-11. Classical mechanism of aldosterone action in epithelial cells. Aldosterone binds to the mineralocorticoid receptor (MR). The complex dimerizes and binds to the hormone-responsive elements (HRE) of target genes to influence their transcription. ENaC: epithelial sodium channel; GILZ: glucocorticoid-induced leucine zipper protein; Usp2-45: ubiquitin-specific protease 2-45; Nedd4-2: neuronal precursor cell-expressed, developmentally down-regulated protein; Sgk1: serum and glucocorticoid-regulated kinase 1; CHIF: channel-inducing factor.

Non-genomic effects

The genomic effects described above require a latent period to produce a physiological effect as it takes some time for the receptor-steroid complex to exert its action as a transcription factor. However, some effects attributed to aldosterone seem to preclude the classical MR mechanism. These non-genomic actions are characterised by being independent of gene transcription, generally insensitive to the classical MR inhibitors, having a rapid time-scale and sometimes occurring in cells without functional nuclei. The mechanisms apparently involve an increase in second messengers, activation of protein kinases, and phosphorylation of effector proteins such as ENaC and the Na^+/H^+ antiporter (Boldyreff & Wehling 2003). There is evidence of cellular actions which can rapidly modify calcium flux and intracellular levels of sodium and potassium (Losel *et al.* 2004). Clinical studies have also shown aldosterone's rapid effects occurring within minutes of an infusion. The earliest clinical observations demonstrated changes in systemic vascular resistance, further confirmed by other studies (Klein & Henk 1963; Wehling *et al.* 1998). Other effects shown include an impairment in baroreflex response (Yee & Struthers 1998), decrease in forearm blood flow (Romagni *et al.* 2003) and increase in intracardiac monophasic action potential (Tillmann *et al.* 2002). Some effects can be blocked by using the MR antagonist spironolactone, and therefore might be mediated via the MR in a different way (Moura & Worcel 1984). However, some others might be mediated by a still unknown receptor (Boldyreff & Wehling 2003).

Non-epithelial actions of aldosterone

Aldosterone action has not only been demonstrated in epithelial cells, but also in vascular (endothelial and smooth muscle), cardiac (vascular and myocyte) and nervous (vascular and neurological) tissue, as well as circulating monocytes (Gomez-Sanchez 1986; Miura *et al.* 2006; Brilla & Weber 1992; Young *et al.* 1994b). This is because the MR is located in all of these sites. However, only in the vascular tissue is the MR protected by coexpression of 11β-HSD2 (Alzamora *et al.* 2000), thus implying that the MR in other tissues would be constitutively bound to glucocorticoids. Moreover, the passage of circulating aldosterone through the blood brain barrier is limited in comparison with circulating glucocorticoids (Funder & Myles 1996). The only exception occurs at the neurons around the nucleus solitary tract, which seem to have a role in controlling sodium appetite (Geerling *et al.* 2006). In tissues with MR but no 11β-HSD2 it remains possible that low levels of aldosterone might be able to activate MR producing a physiological effect, despite the higher concentrations of glucocorticoids able to bind to the same receptor.

It has been suggested that aldosterone activation of MR in the cardiovascular system increases pressor response, collagen deposition and inflammation and, in the central nervous system, it might be involved in blood pressure regulation, thirst and salt appetite, and sympathetic tone (Connell & Davies 2005; Viengchareun *et al.* 2007). In an experimental model, the administration of aldosterone and a high-salt diet stimulated collagen synthesis, leading to left ventricular hypertrophy with tissue fibrosis and myocardial stiffness (Brilla & Weber 1992). Years later it was shown that these effects were humorally mediated and are dependent on salt status but independent of blood pressure (Young *et al.* 1995). More recent studies indicate cardiac fibrosis is mediated by oxidative stress and vascular inflammation (Young *et al.* 2003; Rocha *et al.* 2002; Sun *et al.* 2002). Moreover, experiments with rats and mice highlight the need for a balance between glucocorticoids and mineralocorticoids binding to MR in blood pressure homeostasis and cardiac structure. In rats, aldosterone requires co-administration of corticosterone (the major rat glucocorticoid) to decrease blood pressure and cardiac fibrosis produced by aldosterone alone (Young *et al.* 1994a; Young & Funder 1996). In transgenic mice overexpressing 11β-HSD2 and with low levels of glucocorticoids, development of cardiac hypertrophy, fibrosis and heart failure occurred and could be reversed by blocking the MR with eplerenone (Qin *et al.* 2003). Similar effects have been observed in the CNS, where aldosterone-induced hypertension is attenuated by infusing it together with corticosterone or the MR antagonist RU-28318 (Gomez-Sanchez *et al.* 1990).

Aldosterone action in nonclassic target tissues can regulate other genes which might contribute to its deleterious effects as shown in Table 1-5. Furthermore, aldosterone can also increase the expression of transcriptional factors like activator protein-1 (AP-1) and nuclear factor-κβ which is related to the rise in reactive oxygen species (Fiebeler *et al.* 2001; Sanz-Rosa *et al.* 2005; Johar *et al.* 2006).

Table 1-5. MR target genes and their biological functions in non-epithelial tissues. Adapted from *(Viengchareun et al. 2007).*

Target gene	Tissue/Cell type	Functions
Up-regulated		
Osteopontin (Rocha *et al.* 2002; Sugiyama *et al.* 2005a)	Aortic endothelium	Initiation of inflammation and fibrosis?
ACE (Sugiyama *et al.* 2005b)	Aortic endothelium	Endothelial dysfunction, vascular injury
MDM2 (Nakamura *et al.* 2006)	Smooth muscle	Cell proliferation
EGF-R (Grossmann *et al.* 2007)	Smooth muscle	Increase fibronectin abundance
Collagen I, III, IV (Brilla *et al.* 1994; Nagai *et al.* 2005)	Cardiac fibroblasts Renal fibroblasts	Progression of myocardial fibrosis? Progression of tubunointerstitial fibrosis?
TNX (Fejes-Toth & Naray-Fejes-Toth 2007)	Heart	Cardiac remodeling And Regulation of blood pressure
ADMTS1 (Fejes-Toth & Naray-Fejes-Toth 2007)		
PAI-1 (Fejes-Toth & Naray-Fejes-Toth 2007)		
Orm-1 (Fejes-Toth & Naray-Fejes-Toth 2007)		
RGS2 (Fejes-Toth & Naray-Fejes-Toth 2007)		
Adrenodullin (Fejes-Toth & Naray-Fejes-Toth 2007)		
COX-2 (Rocha *et al.* 2002)	Coronary vasculature	Inflammatory lesions
MCP-1 (Miura *et al.* 2006; Rocha *et al.* 2002)	Coronary vasculature Human peripheral blood mononuclear cells	Pro-inflammatory
bFGF (Fiebeler *et al.* 2001)	Left ventricle	Cardiac hypertrophy, Vasculopathy, fibrosis
IL-1beta (Sanz-Rosa *et al.* 2005)	Aorta	Vascular inflammation
IL-6 (Sanz-Rosa *et al.* 2005)	Aorta and left ventricle	Vascular inflammation
TNF alpha (Miura *et al.* 2006; Sanz-Rosa *et al.* 2005)	Aorta Human peripheral blood mononuclear cells	Vascular inflammation
COL1α1 (Johar *et al.* 2006)	Left ventricle	Pro-fibrosis
Fibronectin (Johar *et al.* 2006)	Left ventricle	Pro-fibrosis
Down-regulated		
G6PD	Coronary artery endothelium	Impairment of vascular reactivity
UPAR	Heart	Cardiac remodeling and Regulation of blood pressure
HAS2	Heart	
UCP1,UCP3	Brown adipocytes	Thermogenesis

ACE:angiotensin converting enzyme; MDM2: murine double minute gene 2; EGF-R:epidermal growth factor receptor; TNX:Tenascin-X; ADMTS1:a desintegrin and metalloproteinase with thrombospondin-like motifs 1;PAI-1: plasminogen activator inhibitor-1; Orm-1:orosomucoid-1; RGS2: regulator of G-protein signaling 2; COX-2:cyclooxygenase-2; MCP-1:macrophage chemoattractant protein 1; bFGF:basic fibroblast growth factor; IL-1:interleukin 1; IL-6:interleukin 6; TNF alpha:tumor necrosis factor alpha; COL1α1:procollagen 1α1; G6PD: glucose-6-phosphate dehydrogenase; UPAR: urokinase-type plasminogen activator receptor; HAS2: hyaluronic acid synthase 2; UCP1:uncoupling protein 1 and UCP3:uncoupling protein 3

1.2.5 Regulation of aldosterone production

Physiological factors

Aldosterone production is principally regulated by the concentration of extracellular K^+ and angiotensin II, the major effector of the renin-angiotensin system (RAAS) (See Figure 1-12).

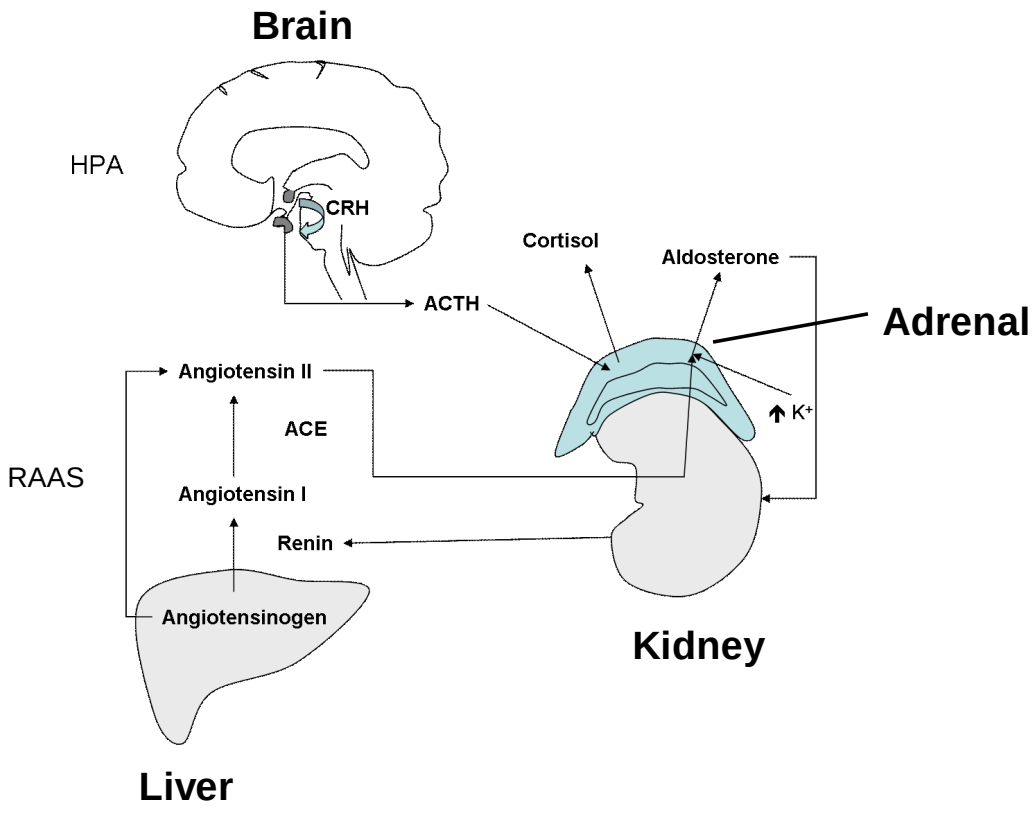


Figure 1-12 Regulation of cortisol and aldosterone synthesis. HPA:hypothalamic-pituitary-adrenal axis; CRH:corticotrophin-releasing hormone; ACTH:adreno-corticotropic hormone; RAAS; renin-angiotensin-aldosterone system; ACE:angiotensin converting enzyme.

The Renin-Angiotensin-Aldosterone-System (RAAS)

Origin and regulation of renin

Renin, a proteolytic enzyme, initiates the series of reactions of the RAAS. Therefore, it is important to understand its origin and regulation.

The juxtaglomerular cells in the kidney synthesize the renin precursor, preprorenin. Preprorenin is converted to prorenin in the endoplasmatic reticulum. A quarter of this is targeted to secretory granules where the prosegment is cleaved to yield renin. The remaining three quarters are secreted into the circulation. This explains why the pro-hormone can be present at concentrations ten to a hundred times that of the active hormone, renin (Atlas *et al.* 1985; Bouhnik *et al.* 1985; Sealey *et al.* 1980). This emphasises the need for specific methods of measuring renin levels (see 1.3.3).

For many years, prorenin was considered to be renin's inactive precursor but the elevated levels of prorenin in diabetic subjects with microvascular complications (Danser *et al.* 2008; Luetscher *et al.* 1989), in pregnant women (Derkx *et al.* 1986; Derkx *et al.* 1987a) and in extrarenal tissues (Krop & Danser 2008) suggests a physiological role. Recently, it has been shown that prorenin can become catalytically active by binding to the prorenin receptor, contributing to tissue angiotensin II generation (Batenburg *et al.* 2007; Nguyen & Contrepas 2008) (Figure 1-13). There is also evidence of *in vitro* activation of intracellular signalling pathways in an angiotensin-independent manner (Nguyen & Contrepas 2008; Saris *et al.* 2006) and elevated blood pressure, plasma aldosterone and/or glomerulosclerosis when the human prorenin receptor is overexpressed in rats (Burckle *et al.* 2006; Kaneshiro *et al.* 2006).

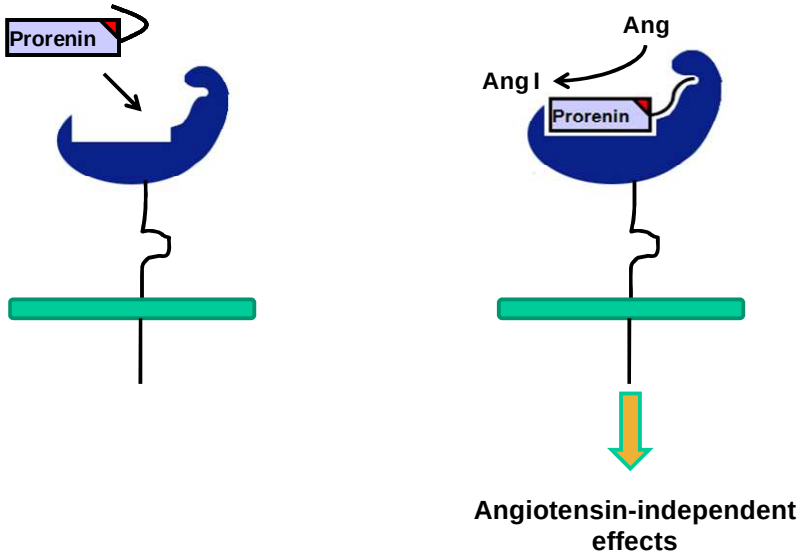


Figure 1-13. Prorenin activation by binding to the prorenin receptor. Adapted from Danser AH *et al*. 2008. Ang: Angiotensinogen; AngI: Angiotensin I

In contrast with prorenin, renin has an established central role in the regulation of blood pressure. Renin is secreted from the kidney mainly in response to a decrease in renal perfusion pressure, due to hypotension or volume depletion, reduced salt intake or stimulation from the sympathetic nervous system. Renin release is largely mediated by the renal perfusion pressure. When there are changes in pressure in the glomerular afferent arteriole, signals are transmitted to the juxtaglomerular cells to alter renin release (Bader & Ganten 2000). The sympathetic nervous system can have opposite effects because alpha stimulation inhibits renin secretion while beta stimulates its release. Prostaglandins like PGI_2 as well as ACTH can also act as renin secretagogues. In addition, ACE inhibitors can increase renin release acutely, decrease prorenin and, chronically, increase both. (Goldstone *et al.* 1983).

Renin can also be found in the placenta, vascular tissue, brain, heart and adrenal cortex (Deschepper *et al.* 1986; Dzau *et al.* 1987; Paul *et al.* 1993). Although the way it is regulated is still unknown, it has been proposed that the same factors that regulate renal secretion are involved. However, the factors may act differently. For example, in the adrenal cortex, angiotensin II and K^+ stimulate renin release but, unlike the kidney, ACTH does not (Inagami *et al.* 1989).

Angiotensin II acts in a negative feedback loop, inhibiting renin release. Increase in plasma potassium concentration (Dluhy *et al.* 1970; Himathongkam *et al.* 1975), increased chloride delivery at the macula densa (Komlosi *et al.* 2004), dopamine (Zhang *et al.* 2009), vasopressin (Aoyagi *et al.* 2008) and atrial natriuretic peptide also inhibit renin release (Cuneo *et al.* 1987).

From renin to angiotensin II and aldosterone

Renin initiates a sequence of reactions (Figure 1-12) which begin with the hydrolytic release from angiotensinogen (Ang), an α-glycoprotein secreted by the liver, of the apparently physiologically inactive decapeptide angiotensin I (AngI). Angiotensin I is rapidly converted to its active form, the octapeptide angiotensin II (AngII), by the angiotensin converting enzyme (ACE), a membrane-bound metalloproteinase. The highest concentration of ACE is on the surface of endothelial cells in the lung, and thus it was believed for some time that most of AngII was produced in pulmonary circulation.

Over the decades, our understanding of the complexity of the RAAS has increased. The components of the system can act on different substrates (i.e. ACE) and the generation of many different peptides has been identified. ACE has an additional substrate with

vasoactive properties called bradykinin (Wahl *et al.* 1996; Zusman 1987). ACE plays an important role in the control of vascular activity by activating AngII, a vasoconstrictor, and inactivating bradykinin, a vasodilator. Another peptide with vasodilator properties, angiotensin 1-7, can also be produced in this series of reactions. Angiotensin converting enzyme 2 (ACE2) acts on AngI producing angiotensin 1-9. In turn, angiotensin 1-9 can be cleaved to angiotensin 1-7 (Donoghue *et al.* 2000). From the series of peptides in the angiotensin family, angiotensin III also releases aldosterone but its pressor effect is less strong (Braley *et al.* 1983). Angiotensin III has low circulatory levels and its effects at tissue level are unclear (Semple *et al.* 1978). The importance of bradykinin, ACE2, angiotensin 1-9 and angiotensin 1-7 is currently under investigation by other groups and will not be addressed in this thesis (Burrell *et al.* 2004; Fyhrquist & Saijonmaa 2008; Madeddu *et al.* 2007).

The concept of local or tissue RAAS has also been introduced. The evidence suggests AngII can also be synthesised in sites such as the kidney, vascular endothelium, adrenal gland and brain (Dzau 1993; Kifor *et al.* 1991; Naftilan *et al.* 1991; Wagner *et al.* 1998). A clinical consequence is that the measurement of renin or AngII in plasma may not be an accurate estimate of these local tissue systems. This is important because some evidence suggests that in patients with essential hypertension with persistent renal vasoconstriction and sodium retention (Redgrave *et al.* 1985) and in patients with stable congestive heart failure, local renin systems may be activated (Schunkert *et al.* 1992).

AngII raises blood pressure directly by acting as a systemic vasoconstrictor, increasing sympathetic nerve activity, myocardial contractility and also enhancing sodium and water reabsorption both directly and indirectly by stimulating aldosterone release. (Carpenter *et al.* 1961; Gavras *et al.* 1976). The stimulation of aldosterone secretion occurs in response to sodium depletion or a decrease in extracellular fluid. AngII also plays a role in the regulation of glomerular filtration rate (GFR) and renal blood flow. (Donoghue *et al.* 2000).

AngII actions occur predominantly via two receptors: AT_1 (subtypes 1a and 1b) and AT_2. Most of the known actions occur through AT_{1a}, but aldosterone secretion is affected through AT_{1b} (Gigante *et al.* 1997). AT_2 seems to counteract several of the actions of AT_1 (Siragy 2004).

Adrenal responses to AngII may be acute or chronic. An acute response occurs within minutes, affecting the early steps of aldosterone biosynthesis. There is rapid conversion of

aldosterone precursors or increased *de novo* synthesis, probably by activating StAR and increasing cholesterol availability in the inner mitochondria. Chronic stimulation with AngII leads to zona glomerulosa hypertrophy and hyperplasia, and an increase in *CYP11B2* expression and aldosterone secretion. This chronic stimulation may be affected by dietary sodium restriction, thereby increasing the adrenal response to AngII, and also by positively stimulating renin release and angiotensin production to increase the ability to retain sodium (Spat & Hunyady 2004; Williams 2005). In the adrenal gland, the best-characterised signalling pathway for AngII is the binding of G-protein-coupled receptors (AT_1) that activates phospholipase C (PLC) which, in turn, stimulates the production of 1,4,5 inositol triphosphate (IP_3) and 1,2 diacylglycerol (DAG). IP_3 frees intracellular calcium and activates calmodulin binding and calcium influx which, in turn, activate transcription factors such as ATF-1, CREB, NGF1B and NURR1 that bind to unique *cis-acting* elements in the 5' region of *CYP11B2.* DAG can activate the PKC pathway or the 12-lipoxygenase pathway. The PKC pathway does not increase transcription of *CYP11B1* or *CYP11B2* and can inhibit *CYP17A1* expression (Bird *et al.* 1996; McAllister & Hornsby 1988). The 12-lipoxygenase pathway leads to the formation of 12-hydroxyeicosatetraenoic acid (12-HETE), which increases the phosphorylation of ATF-1 and CREB. The adrenal AT_1 receptors are also coupled to the src family of tyrosine kinases which inhibit *CYP17A1* expression and increase aldosterone production (Sirianni *et al.* 2001). Although this signalling pathway's activation of several transcription factors helps to explain the expression of *CYP11B2,* further studies are required to elucidate additional factors that may be involved in zone-specific expression of aldosterone synthase.

Potassium (K^+)

Potassium can stimulate aldosterone secretion independently of the RAAS, even with very small increments, for example 0.1mEq/l (Himathongkam *et al.* 1975). An increase in K^+ plasma levels promotes aldosterone release, which then increases renal K^+ excretion. Once the K^+ plasma levels are corrected, aldosterone secretion falls. AngII and potassium appear to act independently on aldosterone secretion but with the same potency. It has been suggested that aldosterone's role is to control renal loss of potassium according to the sodium intake (Young 1988). Interestingly, there is evidence suggesting even very high plasma K^+ concentration is ineffective if renin is absent (Brown *et al.* 1973).

Potassium activates voltage-operated T- and L- type Ca^{2+} channels by depolarizing the membrane of glomerulosa cells, thus, generating a calcium influx and activating calcium calmodulin and calcium calmodulin kinases. These in turn phosphorylate transcription

factors (e.g. NURR1/NGF1B, AT1-F and CREB) that bind to the *CYP11B2* promoter region (Bassett *et al.* 2004; Spat & Hunyady 2004).

ACTH

Adrenocorticotrophin (ACTH) is the principal regulator of cortisol secretion but it is also an acute potent aldosterone secretagogue. Different effects of aldosterone stimulation have been observed according to the dose of ACTH and the route of administration. During continued administration of pharmacological doses, a transient stimulation has been reported, with aldosterone concentration returning to basal levels after a few days (Oelkers 1985; Seely *et al.* 1989). However, there is evidence to suggest this pattern is not followed at low and high doses of ACTH or when there is a pulsatile administration (Oelkers *et al.* 1988; Seely *et al.* 1989). Moreover, findings suggest the ACTH stimulation of aldosterone is influenced by dietary sodium (Kigoshi *et al.* 1980; Weinberger *et al.* 1975), and is independent of renin and AngII (Oelkers 1985). The role of ACTH in aldosterone secretion is further supported by a study including subjects presenting hypopituitarism in which aldosterone's response to sodium restriction and ACTH stimulation is impaired (Williams *et al.* 1971). Despite this, ACTH might be important but not essential in aldosterone regulation. Findings show aldosterone release is not affected in humans treated to suppress ACTH release, after hypophysectomy (Muller 1995).

Other regulators

Weaker secretagogues of aldosterone are endothelin, vasopressin and serotonin (Ganguly & Hampton 1985; Guillon *et al.* 1995; Zeng *et al.* 1992). In addition, aldosterone can be inhibited by somatostatin, atrial natriuretic peptide, beta endorphin, dopamine and digoxin (Aguilera 1993).

The regulation of aldosterone production is complex as several physiological elements are involved. Although AngII and potassium have a predominant role in the regulation of aldosterone, other factors such as ACTH might become important in pathological conditions. (Figure 1-14).

Although ACTH is the main regulator of cortisol synthesis in the zona fasciculata via the hypothalamic/pituitary axis (HPA), a hypothesis generated in our group proposes that, in the long term, ACTH can also affect the zona glomerulosa and aldosterone synthesis, See section 1.4.5. (Connell & Davies 2005).

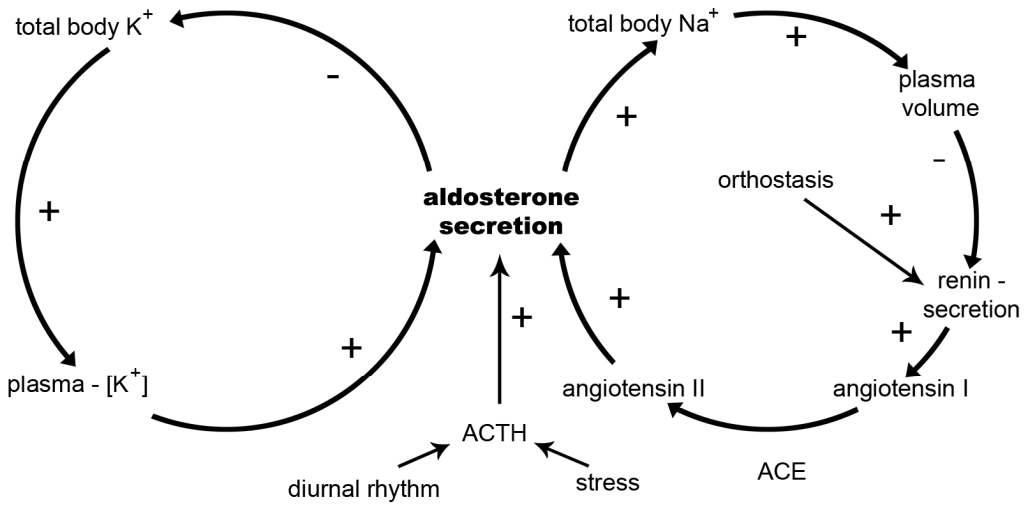


Figure 1-14. Regulation of aldosterone secretion by the main physiological factors taken from Muller *et al* 1995.

Environmental factors

The role of aldosterone in regulating fluid and electrolyte balance makes it susceptible to several environmental factors.

Interestingly, two steps in aldosterone's synthesis may be rate-limiting. For many years, the first step was considered to be the conversion from cholesterol to pregnenolone, dependent on AngII, potassium and ACTH. Nowadays, it is recognized that the first rate-limiting step is in fact the incorporation of cholesterol to the inner mitochondria by StAR. Dluhy *et al.* proposed that aldosterone synthase is a second rate-limiting step, regulated by dietary potassium and sodium (Williams & Dluhy 2005). A low sodium diet is known to increase the sensitivity of aldosterone synthesis to AngII. Experimental studies in humans and rats show that the enzyme's activity becomes more efficient with low sodium or high potassium intake (Menachery *et al.* 1991; Oelkers *et al.* 1974). Sodium-dependant modulation may be tissue-dependent. For example, the pressor response to AngII in vascular tissue is lessened with sodium restriction (Hollenberg *et al.* 1974). The Framingham offspring study confirms a strong correlation between urinary sodium, a measure of intake, and serum aldosterone (R^2 10%) (Vasan *et al.* 2004). A French study supports this observation; it reported that, in the lowest tertile of dietary sodium and

potassium intake, the plasma aldosterone is positively associated with a rise in systolic pressure and with risk of hypertension (Meneton *et al.* 2008).

Aldosterone levels have been reported to vary with age, gender, ethnicity, BMI, time of day, sodium intake, posture, visceral obesity, medications, metabolic clearance and the menstrual cycle, as will be further explained in section 1.3.3 (Fisher *et al.* 1994; Goodfriend *et al.* 1998; James *et al.* 1976; Kang *et al.* 2001; Katz *et al.* 1975; Kidambi *et al.* 2007; Schunkert *et al.* 1997; Tsunoda *et al.* 1986; Weidmann *et al.* 1978). As several anti-hypertensive agents target the RAAS, aldosterone levels are consequently affected, as well as the corresponding aldosterone renin ratio (ARR) (See section 1.3.3). This is supported by the Framingham offspring cohort in which aldosterone levels were higher in individuals treated with diuretics (Kathiresan *et al.* 2005). Also, in the same cohort, the ARR was positively associated with beta-blockers and hormone replacement therapy, and negatively associated with the use of diuretics and ACE inhibitors (Newton-Cheh *et al.* 2007). These influences must be considered in the analysis of population studies, as will be further explored in Chapter 6 of this thesis.

In utero programming may also be relevant in aldosterone production. Recently, Reynolds' study showed that people born with a low birthweight tend to have higher aldosterone levels as adults (Reynolds *et al.* 2009).

Genetics

In addition to environmental factors, genetic factors can also play an important role in the variation of aldosterone secretion. The heritability for serum aldosterone concentration (see section 1.1.3 for definition) of 3326 nonhypertensives in the Framingham Offspring Study was $h^2=0.11$ ($p=0.01$) and the heritability for ARR was 0.36 ($p<10^{-4}$) (Newton-Cheh *et al.* 2007). This is in agreement with the findings of Kotchen *et al.* in 43 hypertensive sibling pairs where heritability of plasma aldosterone concentration was 0.19 (Kotchen *et al.* 2000a). In a study of monozygotic and dizygotic twins, although there was no evidence of a significant genetic influence on plasma aldosterone levels, urinary excretion rate of aldosterone did show genetic influence (Inglis *et al.* 1999). This was later confirmed in a study of nuclear families where the heritability of urinary aldosterone excretion rate was high ($h^2=0.52$, $p<10^{-6}$). These studies provide evidence of an important genetic contribution to the levels of aldosterone in plasma and urine. The conversion of DOC to aldosterone is an important limiting step in aldosterone production and there is evidence to suggest its contribution to the genetic factors (see sections 1.3.1 and 1.4). As aldosterone

levels can be altered in patients with essential hypertension, the aldosterone synthase gene (*CYP11B2)* has been a key candidate gene for hypertension. Furthermore, the proximity and high homology of the *CYP11B2* and *CYP11B1* genes resulted in this locus being identified as an important candidate region affecting blood pressure and aldosterone secretion. This is highlighted by a number of monogenic diseases associated with this locus (see section 1.3.1).

Other potential candidates involved in aldosterone secretion may be the genes encoding for the TWIK-related acid-sensitive K channels (TASK-1 and TASK-3). These channels help to maintain a negative membrane potential with a K^+ current avoiding Ca^{2+} entry that drives aldosterone synthesis and secretion. Both channels are expressed in the zona glomerulosa of the adrenal cortex (Bayliss & Barrett 2008). It has been reported that these channels can affect the membrane potential of the human adrenocortical cell line H295R *in vitro* (Brenner & O'Shaughnessy 2008). Moreover, when TASK channels are deleted in mice (TASK-/-), an overproduction of aldosterone, inappropriate for the level of renin and unresponsive to salt loading and angiotensin type 1 receptor blocker, candesartan, has been shown (Davies *et al.* 2008).

There is also evidence that the cGMP-dependent protein kinase II gene (*PRKG2*) is involved in the control of aldosterone secretion. The cGMP-dependent protein kinase II (cGK-II) is expressed in renal and adrenal cells. It can inhibit renin secretion in juxtaglomerular cells, probably as a downstream molecule of the natriuretic peptides and nitric oxide (possible association with the storage of renin granules). This inhibition can be eliminated when the gene is absent, as demonstrated by cGK II -/- mice or when the cAMP-dependent pathway is working. Moreover, with a low sodium diet, cGK-II is upregulated in the zona glomerulosa and can increase aldosterone secretion by apparently phosphorylating StAR (Vaandrager *et al.* 2005).

Findings support an important role of genetic variation in the secretion of aldosterone. From the genes previously explained, there is strong evidence to suggest that *CYP11B1* and *CYP11B2* can have a considerable influence on the synthesis of aldosterone. Thus, they are the candidate genes chosen for the studies explained in this thesis. A more detailed description of their relevance is contained in section 1.4.

1.3 Adrenal activity and hypertension

More than fifty years ago, functional alterations in the adrenal cortex were already being considered as potential causes of essential hypertension (Genest *et al.* 1956). Since then, reported abnormalities in the urinary excretion and plasma levels of adrenal steroids in essentially hypertensive patients have supported this idea although no convincing mechanism has as yet been devised (Brown *et al.* 1972). As previously mentioned, those forms of secondary hypertension that can be explained in terms of aberrant steroid metabolism may provide clues. They comprise gene defects which increase or decrease mineralocorticoid levels as well as a complex group of anatomical changes in adrenal structure such as neoplastic disease.

1.3.1 Monogenic disorders

Several rare monogenic syndromes involving corticosteroids have provided an insight into the mechanisms involved in essential hypertension. These disorders will be explained in more detail in the following sections.

Congenital adrenal hyperplasia (CAH)

CAH describes a group of disorders characterised by defects in cortisol biosynthesis and autosomal recessive inheritance. Patients with severe loss of 21-hydroxylase activity (*CYP21A2 also called CYP21B*) are born with salt wasting and are hypotensive. Subjects may present with elevated levels of androgens and with salt-loss when there is lack of aldosterone (White & Speiser 2000).

11β-Hydroxylase and 17α-hydroxylase deficiencies are rare causes of CAH. 11β-Hydroxylase deficiency comprises 5-8% of the cases with CAH, while 17α-hydroxylase deficiency comprises approximately 1% (Kater & Biglieri 1994; White *et al.* 1994). Both result in the effects of hypermineralocorticoidism but the compound responsible is not aldosterone but DOC. Although 11β-hydroxylase deficiency is rare in the population, a milder form presents with a variably reduced efficiency; a less distinctive phenotype is likely to have a higher prevalence, similar to 21-hydroxylase deficiency which, in its non-classic form, is more frequent (Merke & Bornstein 2005).

Patients with 11β-hydroxylase or 17α-hydroxylase deficiency have impaired cortisol biosynthesis leading to stimulation of ACTH release. This results in increased levels of 11-deoxycorticosterone (DOC), causing hypermineralocorticoidism and leading to hypertension. With sodium retention, renin suppression and urinary potassium wasting predispose to hypokalemia. The decrease in potassium and renin levels lead to a fall in aldosterone levels. In 11β-hydroxylase deficiency, there is a failure to convert 11-deoxycortisol to cortisol. The onset of hypertension can occur at an early age. The conditions are caused in both homozygous and heterozygous individuals by loss of function mutations either in the *CYP11B1* or *CYP17A1* gene. The mutations responsible for 11β-hydroxylase deficiency are explained in more detail in section 1.4.2. Unlike Familial hyperaldosteronism type I, aldosterone levels are low due to renin suppression (Kater & Biglieri 1994; White & Speiser 1994).

In 11β-hydroxylase deficiency, ACTH stimulation leads to an overproduction of androgens while, in 17α-hydroxylase deficiency, sex steroid biosynthesis is low (Figure 1-15). As a consequence, the main phenotypic differences between 11β-hydroxylase or 17α-hydroxylase deficiencies are that, in the former condition, there is virilization in girls and precocious puberty in boys while, in the latter condition, there is amenhorrea and a failure to go through normal puberty in girls and ambiguous genital development in boys. Diagnosis can be confirmed by screening the *CYP11B1* and *CYP17A1* genes. Glucocorticoids are recommended for treatment to normalize ACTH secretion and buildup of cortisol precursors.

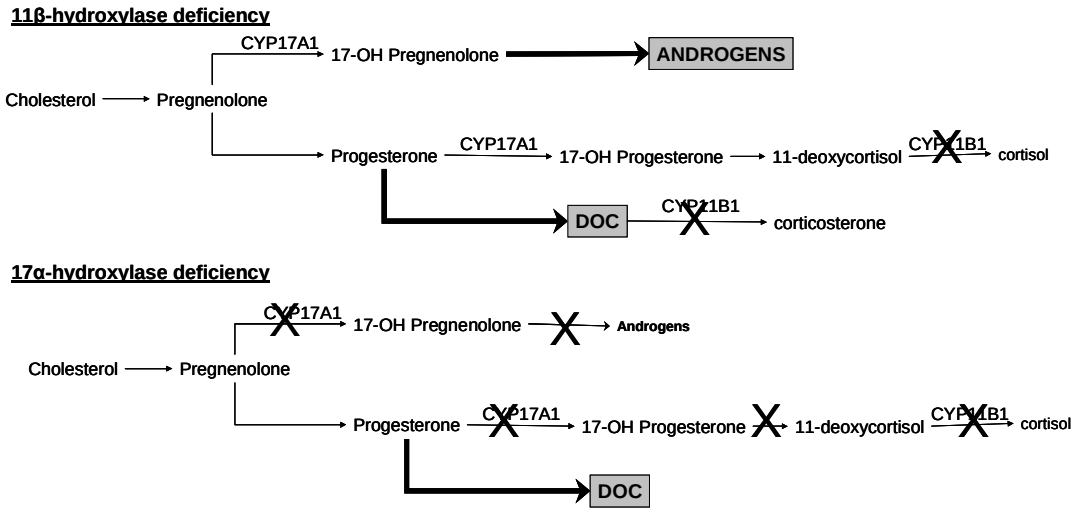


Figure 1-15. Steroid biosynthesis affected in 11β-hydroxylase and 17α-hydroxylase deficiency.

Syndrome of Apparent Mineralocorticoid Excess (SAME)

The syndrome of apparent mineralocorticoid excess is caused by mutations in the *HSD11B2* gene, which encodes the 11β-dehydrogenase 2 (11β-HSD2) enzyme. As a consequence, 11β-HSD2 does not convert cortisol to cortisone, the inactive metabolite, and therefore cortisol, now at higher concentration than aldosterone, can activate the MR, inducing a mineralocorticoid form of hypertension (Stewart *et al.* 1996). Again, mineralocorticoid excess results, not from aldosterone, which is suppressed, but from normal levels of cortisol. SAME usually follows an autosomal recessive pattern of inheritance and has an onset during childhood, although recently a late-onset case has also been reported (Lavery *et al.* 2003). SAME hypertension is associated with suppressed levels of renin, hypokalaemia, and an increased ratio of cortisol:cortisone metabolites in urine. Treatment mainly consists of administration of spironolactone or dexamethasone (Stewart *et al.* 1988; White *et al.* 1997; Williams & Dluhy 2005). It is important to mention that black licorice consumption (Stewart *et al.* 1987) or ectopic ACTH release (i.e. small cell lung cancer) can also lead to the inhibition of 11β-HSD2 (Walker *et al.* 1992; Ulick *et al.* 1992a).

Types of hyperaldosteronism

Familial hyperaldosteronism type I (FH-I)

FH-I is also known as glucocorticoid-remediable aldosteronism (GRA). Its molecular basis was described by (Lifton *et al.* 1992a). Normally, *CYP11B2* encodes aldosterone synthase which catalyses the production of aldosterone by stimulation of AngII. In turn, *CYP11B1* encodes 11β-hydroxylase which is regulated by ACTH and catalyses the final steps in cortisol production. FH-I is caused by a chimeric gene where the 5' region of the *CYP11B1* is fused with the coding region of the *CYP11B2*. This hybrid gene produces aldosterone synthase under the regulation of ACTH. The gene product is expressed in the zona fasciculata where cortisol can also be a substrate for aldosterone synthase, leading to high levels of 18-oxocortisol, 18-hydroxycortisol and aldosterone in these patients irrespective of Na^+ status or AngII. These abnormalities could result from ectopic expression of aldosterone synthase in the zona fasciculata. With the aldosterone excess, plasma renin activity is generally suppressed, although half of the patients tend to be normokalaemic. It is inherited in an autosomal dominant manner with complete penetrance. Usually there is a family history of severe hypertension with early onset; subjects with FH-I are at high risk of a hemorrhagic stroke. The suggested treatment is low dose glucocorticoids to suppress ACTH, an amiloride or spironolactone (Lifton *et al.* 1992a).

The diagnosis can be confirmed with the measurement of urinary aldosterone and genetic testing of the chimeric *CYP11B1/ CYP11B2* gene. A short term suppression of aldosterone with dexamethasone has also been used to screen for FH-I but sometimes can give misleading results (Mulatero *et al.* 1998). Dexamethasone, spironolactone and eplerenone have been used to treat FH-I (Stowasser & Gordon 2001; Stowasser & Gordon 2006).

Familial hyperaldosteronism type II (FH-II)

FH-II is used to describe a familial form of an aldosterone-producing adenoma. Like FH-I, it has an autosomal dominant pattern of inheritance. Patients have a family history of hypertension and hypokalaemia and treatment with spironolactone can be effective. In contrast to FH-I, FH-II aldosteronism cannot be suppressed with dexamethasone. The genetic search for the causative abnormality is still ongoing as the *CYP11B1/CYP11B2* chimeric gene is absent. There is evidence for linkage in the 7p22 locus (Lafferty *et al.* 2000). So far, the only evidence of possible association in this region has been the promoter SNPs in the genes *RBaK* (retinoblastoma-associated Kruppel-associated box gene) and *PMS2* (postmeiotic segregation increased 2) which alter the binding sites for several transcription factors and may have a functional role in FH-II (Jeske *et al.* 2008).

Familial hyperaldosteronism type III (FH-III)

Recently, a novel non-dexamethasone-suppressible form of familial hyperaldosteronism has been described. The reported kindred with this new form of familial hyperaldosteronism is characterized by childhood onset of severe hypertension with an excess of aldosterone, 18-oxocortisol and 18-hydroxycortisol, autonomous cortisol synthesis, decreased androgen production, hyporeninemia and hypokalaemia. The hypertension is resistant to antihypertensive treatment, and neither dexamethasone nor spironolactone changes the steroid profile. Hypertension is only controlled with adrenalectomy. Adrenal glands revealed hyperplasia and cellular atrophy. Patients also presented with left ventricular hypertrophy (Mulatero 2008). Similar to the FH-II, the genetic cause of this recently reported monogenic disorder has not been identified. Potential disease causing mutations in the following genes have not been confirmed: *CYP11B1, CYP11B2,* AngII and ACTH receptors, *DAX-1* (previously implicated in CAH), Ad4BP (transcription factor vital for P450 genes), Nurr77 and Nurr1 (related to adrenal zonation and possibly aldosterone synthesis).

Aldosterone synthase deficiency

This rare deficiency of aldosterone results from the disruption of the final steps of aldosterone synthesis. This enzymatic defect results in hyponatremia, hyperkalemia, increased plasma renin activity, deoxycorticosterone and urinary corticosterone, but normal cortisol and sex steroids. In contrast to the other monogenic disorders, a characteristic clinical feature of this syndrome is hypotension. (White 2004). In addition, the clinical presentation of the deficiency varies with age (Rosler 1984; Ulick *et al.* 1992b).

Two forms of aldosterone synthase deficiency have been described: corticosteroid methyloxidase (CMO) deficiency types I and II (OMIM #203400 and #610600, respectively) (Ulick 1976). Although both disorders have similar clinical features, the profiles of steroids secreted is different (Table 1-6). The increased 18-hydroxycorticosterone to aldosterone ratio either in urine or serum is seen as the best marker for Type II.

The disruption of the final steps of aldosterone synthesis has been mainly attributed to mutations in the aldosterone synthase gene (*CYP11B2).* A more detailed description of these mutations is in section 1.4.2.

Thus, it is now recognized that the classification based on steroid profiles does not always correlate with the genotype or clinical phenotype (Dunlop *et al.* 2003; White 2004). It has been speculated that additional polymorphisms in the contiguous gene, *CYP11B1*, might increase its 18-hydroxylase activity (White 2004), or that aldosterone synthesis might occur via an ACTH-dependent pathway or via another alternative pathway (Lovas *et al.* 2009).

Table 1-6. Characteristic steroid profiles of corticosteroid methyloxidase (CMO) deficiency type I and II

CMO Type I	CMO Type II
Normal or mildly ⬆ 18-hydroxycorticosterone excretion	⬆⬆ 18-hydroxycorticosterone in serum and urine
Aldosterone and its metabolites undetectable	Aldosterone in serum low or normal Mildly ⬆ aldosterone in urine
	⬆⬆ ratio 18-hydroxycorticosterone to aldosterone in plasma

1.3.2 Aldosterone excess: a changing concept

In addition to genetic disorders, corticosteroid excess -mainly mineralocorticosteroid excess associated with hyperplastic and neoplastic changes- can also result in hypertension, providing an insight into the pathophysiological alterations.

Primary aldosteronism (PA)

More than fifty years ago, Dr. Jerome W. Conn described the first case of primary aldosteronism, also known as Conn's syndrome, caused by an aldosterone-producing adenoma (APA). The definition of Dr. Conn has evolved from a specific to a diverse condition in terms of its clinical, pathological and perhaps aetiological features. It is not relevant to give here a detailed classification and differential diagnosis of primary aldosteronism as it is now understood. However, a brief classification for the subtypes of primary aldosteronism is shown in Table 1-7. For the purpose of this thesis, it is important to understand the evolution of the term primary aldosteronism and the role of the screening test, the ARR, in this evolution (Funder *et al.* 2008).

Table 1-7. Subtypes of primary aldosteronism (taken from Young W.F. 2007)

Aldosterone-producing adenoma (APA)
Bilateral idiopathic hyperplasia (IHA)
Primary (unilateral) adrenal hyperplasia
Pure aldosterone-producing adrenocortical carcinoma
Familial hyperaldosteronism
Glucocorticoid-remediable aldosteronism or FH-I
FH type II (APA or IHA)
Ectopic aldosterone-producing adenoma or carcinoma

Evolution of the term, primary aldosteronism

As Dr. Conn described the condition in 1955, aldosterone-producing adrenal tumours caused hypertension and hypokalaemia and their removal cured or significantly ameliorated the clinical abnormalities (Conn 1955). Ten years later, when an assay was developed to measure plasma renin he added as another feature, low plasma renin activity, where renin is suppressed by autonomous aldosterone production (Conn *et al.* 1964). Thus, the simple diagnostic criteria for this rare curable form of hypertension were established. However, variants which did not comply with these were soon described. For example, it was shown that patients with an adrenal adenoma and decreased renin could have normal aldosterone levels (Gunnells, Jr. *et al.* 1970). There followed in the mid 1960s an expansion in the variety of disorders classified as different subtypes of primary aldosteronism.

For example, in 1966, Sutherland and colleagues described a primary aldosteronism variant in two members of a family whose clinical features could be reversed with long-term treatment with a glucocorticoid. This clinical entity was called "glucocorticoid remediable" or "glucocorticoid-responsive" hyperaldosteronism (Sutherland *et al.* 1966). Nowadays this entity is also know as familial hyperaldosteronism type I and has been described previously in section 1.3.1.

Over the years, as a result of systematic screening for Conn's tumours, it became evident that many patients who had excessive aldosterone production with a concomitant suppression of plasma renin activity, did not harbour the typical yellow adenoma but had micro- or macronodular changes in both adrenals (Baer *et al.* 1970; Biglieri *et al.* 1970; Ferriss *et al.* 1975; George *et al.* 1970; Weinberger *et al.* 1979) or even normal glands (Neville 1978). Since 1967, this variety has been variously called bilateral adrenal hyperplasia (Davis *et al.* 1967), idiopathic hyperplasia, idiopathic aldosteronism (Biglieri *et al.* 1970), pseudo-primary aldosteronism (Baer *et al.* 1970) or idiopathic hyperaldosteronism (IHA). The failure to find an adenoma was not the only unusual feature. Subtotal or total adrenalectomy did not cure hypertension despite lowering aldosterone levels (Biglieri *et al.* 1970; Ferriss *et al.* 1978; Weinberger *et al.* 1979). *In vitro* studies suggested the nodules could not secrete aldosterone (Kaplan 1967b). The significance of nodular hyperplasia remains uncertain as the nodules have also been described in patients with essential hypertension and no aldosterone excess, and in normotensive subjects (Gunnells, Jr. *et al.* 1970; Kaplan 1967b; Neville 1978).

The electrolyte abnormalities of sodium retention and potassium depletion characteristic of primary aldosteronism are absent or less pronounced in patients with IHA than in patients with APA (Davies *et al.* 1979). Moreover, in contrast to APAs which are unresponsive to AngII (Davies *et al.* 1979; Fraser *et al.* 1981; Spark *et al.* 1969), there is evidence to suggest that aldosterone secretion in IHA patients is more sensitive than normal to AngII (Davies *et al.* 1979; Fraser *et al.* 1981; Wisgerhof *et al.* 1978). This contrasts with tumorous PA but resembles the enhanced responsiveness to AngII observed in patients with essential and low renin hypertension (described in section 1.3.4)(Davies *et al.* 1979; Fraser *et al.* 1981; Kisch *et al.* 1976; Wisgerhof & Brown 1978). Furthermore, there is a difference in circadian patterns between IHA and Conn's disorder. In the presence of an adenoma, there is a fall in plasma aldosterone levels from 8a.m. to noon (Ganguly *et al.* 1973), suggesting predominant ACTH control and consistent with an important ACTH influence on aldosterone regulation (Espiner & Donald 1980; Schambelan *et al.* 1976). In contrast, in IHA patients ambulant over this morning period, a rise in aldosterone levels is observed; this reflects the overriding dominance of AngII, as in normal subjects (Ganguly *et al.* 1973; Schambelan *et al.* 1976; Weinberger *et al.* 1979).

The differences with clinical Conn's syndrome and the similarities to essential hypertensive patients led some groups to propose that this disorder was a subtype of hypertension rather than primary aldosteronism (Baer *et al.* 1970; Padfield *et al.* 1981). More specifically, several groups reported similarities between low renin hypertension (LRH) patients and IHA (Ferriss *et al.* 1970; Ganguly & Weinberger 1979; Grim 1975; McAreavey *et al.* 1983). For instance in both groups, in addition to hypertension, there is suppressed renin activity, normokalaemia, a sensitive response to AngII and the presence of adrenal nodules. However, the main difference between LRH and IHA is that LRH patients have normal plasma and urinary aldosterone levels but inappropriate PRA levels (Shade & Grim 1975). Aldosterone levels in IHA subjects tend to be lower than in classical PA. Nevertheless, it has been suggested there is a degree of autonomous secretion of aldosterone in IHA and perhaps a progression to autonomous aldosterone secretion in LRH (Connell *et al.* 2003). Although, in recent guidelines, IHA was classified as a subtype of primary aldosteronism (Funder *et al.* 2008), an alternative hypothesis suggesting that IHA is part of a progression from essential hypertension (high-to-normal renin) to low renin and then finally to IHA, is still popular (Lim *et al.* 2002c; Padfield *et al.* 1975).

Cases of primary (unilateral) hyperplasia, type II of familial hyperaldosteronism and ectopic aldosterone-producing adenoma or carcinoma form other subtypes of primary aldosteronism, having clinical characteristics in common with the classical Conn's

syndrome, better known nowadays as aldosterone-producing adenoma (APA) (Young 2007).

A consensus in the terminology might be helpful. However, the main issue is that aldosterone is too high for the level of renin, but is relatively autonomous in aldosterone-related hypertension.

The aldosterone renin ratio (ARR) and primary aldosteronism

In this section the relationship between the use of the ARR and the detection of PA will be described. The details of the test itself are in section 1.3.3.

As the hallmark of PA is the combination of increased aldosterone levels and suppressed renin in plasma, the combination of both parameters in a ratio should emphasize minor abnormalities in both. The first use of this ratio was in 1976 (Dunn & Espiner 1976); later, additional evidence of its efficacy (Hiramatsu *et al.* 1981) increased its use. In the past, only patients presenting with hypokalaemia were considered for diagnosis (Andersen *et al.* 1988; Fishman *et al.* 1968; Kaplan 1967a; Sinclair *et al.* 1987). Based on this, the reported prevalence of PA was <1% in hypertensive patients. The availability of the ARR test and its relative simplicity, together with the recognition that the key biochemical feature of hypokalaemia (and even abnormal aldosterone levels) is present only in a minority of patients (Gordon *et al.* 2005; Mulatero *et al.* 2004), has resulted in the use of ARR as the principal criterion in widespread screening of hypertensive subjects. This has increased the detection and reported prevalence of primary aldosteronism. Recent evidence suggests prevalence estimates of primary aldosteronism, as defined by this new criterion, in hypertensive subjects varies from 5-13% (Fardella *et al.* 2000; Gordon *et al.* 1994; Lim *et al.* 2000; Loh *et al.* 2000; Mosso *et al.* 2003; Schwartz & Turner 2005). This prevalence can vary according to test conditions.

A careful study in newly-diagnosed hypertensive patients made in Italy (Primary Aldosteronism Prevalence in Hypertensives-PAPY) reported that 20.4% of them had an increased ARR. The screening test was not affected by antihypertensive treatment (calcium channel blockers, doxazosin or both). The diagnosis of PA was confirmed in 11.2%, with detailed non-suppressible levels for aldosterone (prevalence of APA of 4.8% and of IHA of 6.4%). The robustness of the study's design gave an accurate estimate of the prevalence of PA in Europeans. The hypertensives in the PA group were older, had higher blood pressure and different levels of PRA, aldosterone and serum potassium compared with the hypertensive individuals without PA (Rossi *et al.* 2006). The selection of antihypertensive

treatment in this study was probably based on previous reports by (Mulatero *et al.* 2002a). They recommended the use of doxasozin and fosinopril when the ARR screening takes place, and also mentioned the drawbacks of beta blockers and ARBs, which will be addressed in more detail in section 1.3.3.

In a selected population of resistant hypertensives, the reported prevalence of PA was higher (20%) when using ARR as a screening test. Although previous studies have reported that black subjects tend to have lower renin activity, in this particular prospective study the prevalence of PA in black subjects was similar to white subjects. This suggests that ancestry might play a role in the levels of plasma aldosterone and renin activity but not necessarily influence PA prevalence (Calhoun *et al.* 2002). This again highlights the relevance of genetic influence that is an important part of this thesis. This new estimate of prevalence has not yet been uniformly accepted as it has been suggested that cases of low renin hypertension can be misclassified as PA (Padfield 2002).

Another consequence of new approaches to diagnosis is that the relative proportions of the main PA subtypes, APA and IHA, have changed. APA has been replaced by IHA as the most common subtype identified (Fardella *et al.* 2000; Lim *et al.* 2000; Stowasser & Gordon 2004). This is not surprising because IHA has milder clinical characteristics and is more likely to be identified by the use of the ARR.

The drawbacks of the ARR as a principal diagnostic criterion, detailed in section 1.3.3, have resulted in varied levels of acceptance among clinicians. Nevertheless, a review of its use from 1966 to 2001, incorporating studies for the screening of PA in more than 3000 patients with essential hypertension, fully supported its use as a case-finding test and highlighted several ways to improve its standardization (the population selection, the conditions of testing, the cut-off value of the ARR, the chosen reference standard and the reporting of the test) (Montori & Young, Jr. 2002). Its use has also been supported in the recent guidelines for detection, diagnosis and treatment of PA. However, there is still no consensus concerning the types of assays, mainly for renin measurement, and units for the component of the ratio. Thus, suggested ARR cut-off levels remain only guidelines (Funder *et al.* 2008).

In summary, all the subtypes of primary aldosteronism have in common an elevated blood pressure and inappropriately low PRA for the concurrent aldosterone level. While the cause of the less common subtypes (i.e. adrenal adenoma or FH type I) is clear, this is not so for the more prevalent subtype, IHA whose aetiology remains unclear.

The use of the ARR as a screening test has increased over the years. It has modified both the definition and the reported prevalence of PA and its main subtypes. The recommendation of widespread ARR screening of the hypertensive population is still controversial (Calhoun 2007; Kaplan 2001; Kaplan 2007; Padfield 2003; Stowasser *et al.* 2001). More robust information about the influence of environmental and genetic factors on the ARR not only in patients with essential hypertension but also in normotensives is lacking. What is clear from this brief survey is that PA now describes a quite different phenomenon from that envisaged by Conn. Aldosterone-related blood pressure is far more prevalent in hypertensive subjects than was first envisaged and it may have an identifiable genetic origin. It is also more intriguing. Thus, understanding the effects of genetic regulation of aldosterone and blood pressure are the principal aims of the work in this thesis.

Two central points arise from the discussion so far. The first is that a plausible source of genetic variation may originate from the genes related to corticosteroid biosynthesis. The second is that the factors determining the related intermediate phenotype, aldosterone status, are by no means clear. In the next section, these are examined.

1.3.3 Assessment of aldosterone status

As described above, the secretion rate of aldosterone in PA may vary from very high to normal but is always excessive in relation to the level of renin. What parameters of the system can be used to assess its physiological status so as to define a category of non-normal? Neither aldosterone itself nor the level of plasma concentration of K^+ is informative in the majority of cases now classified as PA. Currently, the physiological status is based on the relationship of aldosterone to its principal agonist, renin. The rationale is that there is a range of normality of renin level for a given aldosterone level; where the renin level is below this, aldosterone secretion must at least be more sensitive to renin and possibly partially autonomous. Thus the ratio of the two variables will be abnormally high. The test, it is presumed, should identify those patients in whom aldosterone secretion is partly autonomous or is abnormally sensitive to AngII (Schwartz *et al.* 2002). The following sections deal with the assessment of the two independent variables and then the ARR.

Renin

Renin can be measured in terms of its enzymatic activity (PRA) or the concentration of its protein in plasma. The ability to measure renin precisely and accurately at extremely low concentrations is the most critical factor in evaluating the diagnostic reliability of the ARR. The earliest and still most widely used methods measure renin activity. They are based on the incubation of plasma at 37°C in order to convert the endogenous angiotensinogen (Ang) in AngI. AngI is then measured by radioimmunoassay (RIA) and the PRA is calculated from the rate of AngI production (Haber *et al.* 1969; Workman *et al.* 1979). To avoid AngI degradation or AngI to AngII conversion during incubation, inhibitors of degrading and converting enzymes or AngI antibody are added (Millar *et al.* 1980; Sealey 1991). The main advantage of this method is its sensitivity to very low values of renin activity. This can be increased further by extending the incubation time from 90min to 18 hours (Sealey & Laragh 1975).

The rate of AngI production is determined not only by the concentration of renin but also by the concentration of the enzyme's substrate, Ang whose concentration in plasma is approximately equal to the K_m (24×10^{-6} mol/l) of the reaction (Burton & Quinn 1988). This means that the reaction between renin and Ang follows the kinetics of a first-order reaction. Plasma angiotensinogen concentrations may vary in different situations affecting the generation rate of AngI.

The lack of standardisation of the PRA assay made the quantitative comparison of the results between research groups difficult and led to the establishment of the international reference preparation of human renin (Bangham *et al.* 1975; Sealey 1991). However, this has not been widely adopted and the interlaboratory reproducibility remains poor. In addition, the method is time consuming. Thus, the drawbacks have challenged its validity and usefulness in clinical settings (Schwartz *et al.* 2002; Tanabe *et al.* 2003; Montori *et al.* 2001).

The plasma renin concentration (PRC) method was developed as an alternative to overcome the limitations of the PRA assay. The earlier methods were also based on enzyme kinetics. Exogenous Ang was added to ensure its excess so that the reaction velocity depended only on renin concentration. The reaction follows a zero-order kinetics and PRC was measured as the pressor response of a test animal to the injection of incubated plasma (Skinner 1967). Although the addition of exogenous Ang simplifies the kinetics, other complexities are incorporated into the assay in terms of the requirement of

this additional substrate and the procedure to integrate into the assay. As an alternative, the first direct renin RIA was described by Galen and colleagues in 1979 (Galen *et al.* 1979) and further modified by others (Higaki *et al.* 1984). In this initial assay, endogenous angiotensinogen was removed from plasma by acidification (pH 3.5). Then the pH was restored to neutral and exogenous Ang was added at a higher concentration than K_m. The sample was incubated at 37°C and the velocity of AngI generation measured. Finally, the PRC was calculated from a curve relating PRC to velocity. Unfortunately, this assay had several disadvantages. It lacked sensitivity and it could cause nonproteolytic and proteolytic activation of prorenin. In addition, prorenin was detected by the antibodies hampering the quantification of true renin (Derkx *et al.* 1987b; Lumbers 1971; Leckie & McGhee 1980).

A few years later, Menard and colleagues developed an immunoradiometric assay using monoclonal antibodies (Menard *et al.* 1984). The main advantage was that these antibodies could distinguish active and inactive renin in plasma, kidney, amniotic fluid and chorionic cells based on the detection of the active renin site. A multicentre study compared this immunoradiometric assay with the popular PRA assay (Morganti *et al.* 1995). Although it showed the inter- and intra-laboratory variability was higher in the PRA assay than the PRC assay, it was criticized for its high variability at low concentrations, cross-reactivity with prorenin and failure to detect the decrease in renin with age or with supine posture (Sealey *et al.* 1995). Nevertheless, a correlation between PRA and immunoradiometric PRC assays was reported, correcting for the cross-reactivity of the antibodies (Deinum *et al.* 1999; Morganti *et al.* 1995; Ulmer & Meikle 2000). More recently, an automated immunochemiluminometric assay to measure PRC has been made available. Although speed, convenience, significant correlation with PRA results and potential application in several clinical conditions are appealing aspects of this method, so far it is not sensitive enough to replace the traditional method (de Bruin *et al.* 2004; Hartman *et al.* 2004; Olivieri *et al.* 2004; Perschel *et al.* 2004). A comparison of this new PRC assay with the classical PRA assay will be discussed in Chapter 3, and its application in family-based studies in Chapter 4.

Aldosterone

Aldosterone status can be assessed by measuring its concentration in the circulation or its excretion rate in urine. To calculate ARR, the plasma concentration concurrent with PRA/PRC is necessary. Therefore, for the purpose of this thesis, only the assessment of circulating aldosterone will be considered. The concentration of circulating aldosterone is < 1% that of cortisol. For this reason, very sensitive and specific methods are necessary with high-affinity, highly-specific antibodies. Aldosterone in plasma or serum is most frequently measured by RIA (Mayes *et al.* 1970). Early methods required the extraction and purification of aldosterone from cross-reacting steroids prior to its quantification (Fredlund *et al.* 1975). However, direct, sensitive and highly-specific RIAs were later developed. The earliest of these, using tritiated aldosterone, remained cumbersome as an incubation of 18h at 4°C, glass vials for decantation and the use of scintillation liquid were required (Campbell 1982; Poulsen *et al.* 1974; Jowett & Slater 1977). The replacement of tritiated aldosterone by iodinated aldosterone simplified the methodology eliminating the last two steps but the long incubation remained (Nussberger *et al.* 1984; Al Dujaili & Edwards 1978). To improve this, a faster method of measuring plasma aldosterone concentration using an immunometric technique and automated machinery was developed (Perschel *et al.* 2004), validated (Perschel *et al.* 2004), and successfully used in the Framingham Study (Newton-Cheh *et al.* 2007). However, recently, the major manufacturer (Nichols Institute Diagnostics) ceased providing reagents and the current methods rely once again on RIA. This might be inconvenient if the RIA is used together with the recently-automated PRC assays for reporting the ARR, as each component will be measured at a different pace. As a consequence, other methods of high-throughput measurement of aldosterone concentration are being evaluated such as the high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (Taylor *et al.* 2009).

Demographic and environmental factors that affect renin and aldosterone

A previous section (1.2.5) dealt with the control of renin and aldosterone production. Control factors obviously affect circulating levels and it is important in evaluating laboratory values to be aware of these effects. The most relevant factors are show in Table 1-8.

Table 1-8. Demographic and environmental factors affecting plasma renin activity and aldosterone concentration.

Factor	Change in renin activity	Change in aldosterone concentration
Age	⬇ gradually with age (James *et al.* 1986; Tsunoda *et al.* 1986; Weidmann *et al.* 1978)	⬇ gradually with age (James *et al.* 1986; Ogihara *et al.* 1979; Weidmann *et al.* 1978)
Gender	⬆ PRA in males (James *et al.* 1986)	⬆ in female than in male (Sequeira *et al.* 1986; Giacche *et al.* 2000)
Time of day	⬆ early morning, falls during day (Gordon *et al.* 1966; James *et al.* 1986; Katz *et al.* 1975)	⬆ awakening, falls during day (James *et al.* 1976; Katz *et al.* 1975)
Body mass index	Unchanged (Rossi *et al.* 2008)	⬆ in overweight (Bentley-Lewis *et al.* 2007; Goodfriend *et al.* 1998; Rossi *et al.* 2008)
Sodium intake	⬆ as sodium intake falls and vice versa (Sealey 1991)	⬆ as sodium intake falls and vice versa (Luft *et al.* 1979)
Posture	⬆ upright posture (less if seated or supine) (Tuck *et al.* 1975)	⬆ upright posture (less if seated or supine) (Tuck *et al.* 1975)
Menstrual cycle	⬆ during luteal phase (Michelakis *et al.* 1975; Sealey 1991)	Slightly ⬆ during luteal phase (Michelakis *et al.* 1975)
Pregnancy	⬆ (Weinberger *et al.* 1977)	⬆ (Langer *et al.* 1998; Brown *et al.* 1995; Elsheikh *et al.* 2001)
Race	⬇ in black than in white subjects (Sealey 1991; Sever *et al.* 1979)	⬇ aldosterone production in blacks (Pratt *et al.* 1999) ⬇ in black than in white hypertensive subjects with AngII stimulation (Fisher *et al.* 1994) ⬇ secretion in black than in white children (Pratt *et al.* 1989)
Medication		
Antihypertensive drugs	(Sealey 1991)	(Seifarth *et al.* 2002)
ACE inhibitors, ARBs	⬆	⬇
Beta blockers	⬇	Unchanged
Dihydropiridine calcium antagonists	⬇	Unchanged
Diuretics	⬆	⬆
Clonidine, alpha-methyldopa or non-steroidal anti-inflammatory agents	⬇	
Hormonal treatment		
Oral contraceptives	⬆ (Kang *et al.* 2001)	⬆ (Kang *et al.* 2001)
Oral hormone replacement therapy	⬆ (Ichikawa *et al.* 2006)	Unchanged or slightly ⬇ (Harvey *et al.* 1999; Schunkert *et al.* 1997)

Other factors

Prorenin can be converted to renin by cryoactivation during plasma separation, freezing for storage or thawing before its use in the assay. In order to avoid this, the plasma (or serum) should be separated at room temperature. The sample must be stored in a completely frozen state until assayed. For subsequent assays, thawed samples must be kept at room temperature and not on ice or in the refrigerator. Acidification of the sample can also activate prorenin and is therefore avoided in current assays (Sealey 1991).

Plasma Ang is the substrate for the renin-catalysed reaction and its concentration will therefore affect the kinetics of the AngI formation. PRA can be affected in patients with increased plasma Ang concentration. This occurs in situations of increased plasma oestrogen (pregnancy, ovarian-stimulated women, women taking oral contraceptives or hormonal replacement therapy) or treatment with glucocorticoids (Ichikawa *et al.* 2006; Kang *et al.* 2001; Oelkers *et al.* 1992; Weinberger *et al.* 1977). However, PRC is lower in women taking oestrogen replacement therapy than in men, suggesting a down-regulation of renin by oestrogen (Schunkert *et al.* 1997). Ang concentration is likely to be lower in patients with liver disease (i.e. cirrhosis) or reduced hepatic blood flow (i.e congestive heart failure) due to an impaired rate of production.

PRA values can vary between commercial assay kits. Sealey and colleagues proposed a list of conversion factors to improve the inter-laboratory comparability (Sealey 1991), and manufacturers of quality controls for this assay, like BIO-RAD also propose a range according to the commercial kit being used. However, the reproducibility of the assay remains questionable.

Disease states with effect on renin and aldosterone levels

Of particular interest, the ways in which the circulating renin and aldosterone levels are affected in disease contributing to alterations in blood pressure are described in Table 1-9.

Table 1-9. Diseases affecting renin activity and aldosterone concentration and having an effect in blood pressure. HTN: hypertension and HO: hypotension.

Renin	Aldo	Disease	Effect in BP	Adrenal origin	Other features
⬇	⬆	Primary aldosteronism	HTN	✓	Aldosterone excess independent of renin
⬇	⬇	11β-hydroxylase deficiency	HTN	✓	
⬇	⬇	17α-hydroxylase deficiency	HTN	✓	
⬇	⬆	Glucocorticoid remediable aldosteronism	HTN	✓	
⬇	Normal /⬇	DOC adenoma	HTN	✓	
⬆	⬇	Aldosterone synthase deficiency	HO	✓	Salt wasting, Hyperkalaemia
⬇	⬇	Ectopic ACTH		✓	ACTH production from source other than pituitary gland
⬇	⬇	Hyporeninemic hypoaldosteronism	HTN	✓	
⬆	⬇	Primary adrenal insufficiency, including Addison's disease	HO	✓	Inability of adrenal glands to produce steroid hormones
Normal	Normal	Secondary adrenal insufficiency	HO	✓	Inadequate pituitary or hypothalamic stimulation of adrenal
Normal/ ⬇*	Normal/ ⬇*	Cushing's syndrome	HTN	✓	Glucocorticoid excess. *Low levels of renin and aldo with marked hypercortisolism
⬆	⬇	CAH: deficiencies in StAR, CYP11A1, HSD3B2, CYP21A2	HTN or (HO with StAR)	✓	
⬇	⬇	Liddle Syndrome	HTN	×	ENaC dysfunction-salt retention
⬇	⬇	Congenital or acquired 11βHSD2	HTN	×	
⬇	⬇	Mutations MR	HTN	×	
⬇	⬇	Gordon's syndrome	HTN	×	
⬇	⬆	Chronic renal impairment	HTN	×	Fluid overload
⬆	⬆	Renovascular hypertension	HTN	×	Narrowing of kidney arteries
⬆	⬆	Pseudohypoaldosteronism type I (OMIM #264350**)**	HO	×	Mutations in ENaC or mineralocorticoid receptor gene cause salt wasting.
⬆	Normal/⬆	Bartter's syndrome	HO	×	Salt wasting Hypokalaemia limits rise in aldosterone
⬆	Normal/⬆	Gittelman's syndrome	HO	×	
⬆	⬆	Renal artery stenosis or reninoma	HTN	×	Narrowing of renal artery or juxtaglomerular tumor
⬆	⬆	Heart failure (HF), hepatic cirrhosis (HC) and nephrotic syndrome	HF-HTN HC-HO	×	Hypovolemia & sometimes impaired aldo metabolism

Factors affecting the aldosterone-to-renin ratio (ARR) test

The ARR was introduced in 1981 by Japanese investigators as a marker for inappropriate aldosterone activity (Hiramatsu *et al.* 1981). In the 1990s, it rapidly gained widespread acceptance because it is relatively simple, inexpensive and less affected by dietary salt intake, posture or diurnal variation than plasma aldosterone concentration (Ferrari *et al.* 2004; Kaplan 2004). However, several of the factors described as previously affecting one or both components of the ratio can interfere with the ARR. There is divergence in results when the samples used to determine the ARR have been taken in a supine or upright position (Giacchetti *et al.* 2006; Montori *et al.* 2001). It has been suggested that an upright position can increase the specificity and sensitivity of the method (Giacchetti *et al.* 2006). Other possible interferences which must be taken into account are the antihypertensive medication, age, race and hypertensive status. The rate of false-positive results can be increased by the use of beta-blockers -which suppress renin- or by monitoring elderly, black or hypertensive subjects with low PRA. The rate of false-negatives can be increased by the use of diuretics, calcium channel blockers, ACE inhibitors and angiotensin receptor blockers -which inhibit aldosterone biosynthesis- or excessive dietary sodium intake (Brown & Hopper 1999; Fiad *et al.* 1997; Giacchetti *et al.* 2006; Mulatero *et al.* 2002a).

Montori and Young's review revealed a lack of consensus between studies using ARR regarding such variables as the posture of the subject and the sampling time of day, whether or not to interrupt antihypertensive medication, the clinical setting (hypertension clinic versus community-based clinic) and, as mentioned previously, the types of assay, the corresponding units and the cut-off values. In an attempt to standardize the conditions, new guidelines have been published. There is also evidence of a genetic influence on the ratio (Newton-Cheh *et al.* 2007) and observations that a high ratio reflects low PRA and is barely affected by plasma aldosterone concentration (Montori *et al.* 2001). The explanation for this observation is, according to Montori and colleagues, that dividing plasma aldosterone concentration by PRA does not provide a renin measurement independent of aldosterone, but ratios dependant on the inter- and intravariability of PRA. This was supported by their results which showed that, when interindividual variation of PRA was considered, the ARR was strongly and inversely dependent on PRA. All these limitations of the ARR have challenged its use in the clinical practice as the main index to screen for primary aldosteronism and has caused debate over its widespread use in hypertensives (Calhoun 2007; Kaplan 2007). Although there is some evidence in the literature that ARR

and its components can be used as markers of an increased risk of blood pressure elevation, further studies are required. In order to improve the understanding of the ARR variability, its use and its interpretation, as part of the work of this thesis, a new PRC method has been evaluated and has been used in a family-based study with hypertensive and normotensive subjects (See Chapters 3 and 4).

Guidelines for measuring the ARR

To avoid variability due to the above-mentioned factors, new guidelines recommend taking samples in the morning, at least two hours after the patient has been out of bed, and after being seated for 5-15min. There are no restrictions on sodium intake prior to testing but knowledge of the patient's medications is necessary. If possible, for example in mildly hypertensive subjects, antihypertensive medication can be suspended for a washout period or changed to an antihypertensive compound with minimal effect on the ARR (Funder *et al.* 2008).

It has been suggested that an ARR test might be appropriate in the following conditions in order to screen for PA: hypertension and hypokalaemia, resistant hypertension, adrenal incidentaloma and hypertension, hypertension onset at a young age, severe hypertension or whenever secondary hypertension is considered (Funder *et al.* 2008).

1.3.4 Aldosterone, renin, ARR and blood pressure

The dual potential of the RAAS to simultaneously induce vasoconstriction and increase volume is important in essential hypertension as it has been postulated this state is sustained by inappropriate renal sodium retention relative to vascular capacity. The concomitant measurement of renin and aldosterone provides a bipolar analysis of volume and vasoconstriction that helps to evaluate the RAAS disturbances in essential hypertension (Laragh 1973).

Aldosterone levels and blood pressure

A recent study of the Framingham cohort highlights that, even within the normal population, an important relationship exists between aldosterone concentration and blood pressure. Subjects with higher aldosterone levels (top quartile), but within the normal physiological range, had increased risk of elevated blood pressure with age and also of developing hypertension (Vasan *et al.* 2004). Recently, Reynolds showed in an elderly

population that, regardless of the treatment, a difference in blood pressure is observed when the top and bottom tertiles of aldosterone levels are compared (Reynolds *et al.* 2009). In addition, in this study the relationship between aldosterone concentration, age and elevation of blood pressure suggested that regulation of aldosterone may be subject to epigenetic influences, including imprinting.

Renin levels and blood pressure

While aldosterone levels may not be markedly changed in mature adults, an attempt has been made to classify essential hypertensive patients into more homogenous groups according to renin activity and hormonal responses to biological stimuli. Laragh proposed three subgroups while examining the relationships between renin and aldosterone levels, dietary sodium and sodium volume status: low renin activity hypertensives (sustained by volume expansion) high renin (sustained by vasoconstriction) and normal renin (sustained by an imbalance between volume and vasoconstriction). Moreover, according to this classification, it is suggested that the response to antihypertensive treatment can be predicted (Laragh 1973).

Low renin hypertension (LRH)

Before the 1980s, the incidence of LRH was reported to vary 10-50% (Gunnells, Jr. & McGuffin, Jr. 1975). More recently, it was recognized that 20-30% of hypertensive patients have low renin levels (Buhler *et al.* 1984), the finding being more common in black and elderly subjects (Fisher *et al.* 2002; Sagnella 2001). The definition of LRH is arbitrary as the values of renin activity are affected by the reference population, age, salt intake and type of renin assay. With the traditional enzyme kinetic assay, the cut-off for LRH has been set to 0.65-1.0 ng AngI/ml/h; no further agreement has been achieved considering alternative assays (Mulatero *et al.* 2007).

The distinct features of the low renin hypertensive subgroup are salt-sensitivity, low PRA despite sodium restriction, aldosterone levels that are inappropriate to the level of renin, potassium levels within the normal range, and diuretic responsiveness. The low renin activity has been attributed mainly to four mechanisms. The first is excess dietary salt (experimental salt hypertension). The second is impairment in sodium renal excretion due to a renal disturbance which may affect renin secretion. The third is an inability to excrete ingested potassium; as a consequence, a rise in plasma potassium levels would lead to increased aldosterone secretion, volume expansion, increased vascular sensitivity and suppressed plasma renin activity. It has been proposed that, in a compensated state,

potassium balance would be restored and hypertension would be driven by aldosterone induced sodium retention and increased vascular sensitivity to AngII (Ames *et al.* 1965; Brunner *et al.* 1972a). Finally, the fourth proposed mechanism proposed is that adrenal disturbances would increase secretion of aldosterone or another unidentified mineralocorticoid.

Patients with LRH are said to have a better prognosis in related cardiovascular diseases than hypertensives with normal or high levels of renin activity (Buhler *et al.* 1972; Alderman *et al.* 1991; Alderman *et al.* 1997), which might be attributed to less deleterious effects of AngII in the heart (Sim & Qui 2003), better tissue perfusion and a vasodilated vascular system (Laragh 1973). Nevertheless, the subgroup with low PRA is the minority as most of the patients with hypertension have normal or increased PRA values.

High renin hypertension

Brunner and colleagues estimated approximately 16% of essential hypertensive patients present with increased plasma renin activity levels, suggesting potent vasoconstriction (Brunner *et al.* 1972b). In addition, these patients present with increased aldosterone levels and a tendency to hypokalaemia. Although suppression of renin secretion might be expected with the elevation of aldosterone levels, the vasoconstriction induces natriuresis and prevents volume expansion. As one of the main features is the high renin activity, these patients are responsive to beta blockers.

Normal renin hypertension

This is the most common form of hypertension, as it is present in approximately 57% of patients. Not only do they have normal levels of PRA, but also normal aldosterone secretion and a normal renin-aldosterone relationship at all sodium levels. It has been proposed that this type of hypertension begins with a renal disturbance. As a consequence, there is a subtle increase in renin secretion, a rise in AngII, aldosterone secretion and sodium retention, leading to an elevation of blood pressure. With time, renin secretion would be suppressed, AngII levels would fall and the hypertension persist with an increase in volume and vasoconstriction components and subtle sodium retention.

Years after this classification according to renin was suggested, Williams and Hollenberg proposed another system whereby subjects were classified as modulators and non-modulators (Williams *et al.* 1992). Modulators adjust their responsiveness to AngII according to their sodium intake and have appropriate renal vascular responses with AngII infusions. These changes are not observed in non-modulators and are attributed to

abnormal regulation of AngII, which might remain at fixed levels in adrenal and kidneys (Williams *et al.* 1992). This hypothesis is supported by the positive response of these hypertensive patients whose blood pressure is reduced with the administration of ACE inhibitors or angiotensin receptor blockers (ARBs). Non-modulators have normal/high renin levels, are salt-sensitive and tend to be older than modulators. These last two features are similar to LRH. Moreover, there is evidence of a genetic component in nonmodulators. In a study of hypertensive pairs it was shown that nonmudulation aggregates in families and is independent of sodium intake (Dluhy *et al.* 1988). A year later, the same group reported, in a study of hypertensive patients, that 81% behaved as nonmodulators and had a positive family history of hypertension (Lifton *et al.* 1989).

ARR and blood pressure

The classification of hypertensive patients by renin status has been, to some extent, superseded by use of the ratio of aldosterone to renin as a marker of inappropriate sodium retention. Thus, it has been proposed that inappropriate aldosterone production may vary in degree within the hypertensive population in general and that the ARR could be a marker for this (Lim *et al.* 2002a). A range of studies have measured ARR and increases in blood pressure, showing that a high level of ARR is common. However, there has been criticism regarding the arbitrary ARR cut-offs, the use of antihypertensives and variability in assays used. In a recent prospective study of community-based nonhypertensive individuals, ARR was positively associated with untreated hypertension. It was also shown that a higher baseline of the ARR was associated not only with increased risk of blood pressure progression but also with development of hypertension (Newton-Cheh *et al.* 2007). However, Newton-Cheh and colleagues recognised that refinement of the genetic and non-genetic contributors to ARR is crucial for improving our understanding of the RAAS and risk prediction. Thus, as part of this thesis, in Chapter 4, a detailed study of the ARR in families selected via a hypertensive proband is discussed. It is also of interest to evaluate the contribution of the *CYP11B1* and *CYP11B2* genes to ARR and hypertension. The rationale for focusing on these two candidate genes will be described in section 1.4.

1.3.5 Hypotheses to explain inappropriate aldosterone secretion leading to hypertension

Although the above mentioned studies have provided insight into the variations of ARR and its components, few prospective studies have systematically examined changes in volume, renin, aldosterone and blood pressure with age and in hypertensives. Nevertheless,

there is now evidence that small differences in aldosterone production that are genetically determined, possibly originating early in life, can lead, over time, to hypertension with volume expansion reflected by low renin and to enhanced sensitivity to its trophins. This hypothesis, explained in detail in section 1.4.5, may account for recent findings that aldosterone levels within the normal range can predict blood pressure progression over time, as mentioned above (Vasan *et al.* 2004).

There are three other hypotheses devised to explain the mechanisms underlying inappropriate aldosterone secretion, as indicated by raised ARR. The first hypothesis suggests the presence of a stimulator (or stimulators) of aldosterone synthesis, in addition to AngII and potassium. This remains to be identified but various substances (e.g. transcription factors) have been proposed (Higaki *et al.* 1989; Somekawa *et al.* 2009; Lenzini *et al.* 2007; Neri *et al.* 2006; Nogueira *et al.* 2009). The second explanation involves feedback amplification of the RAAS; AngII can upregulate AT_1 receptors in the zona glomerulosa of the adrenal gland and thus the sensitivity to this trophin is enhanced, leading to adrenal hyperplasia (Belloni *et al.* 1998). Finally, the third hypothesis proposes overactivity of the sympathetic nervous system, stimulating aldosterone release; there is *in vitro* evidence to support this hypothesis (Ehrhart-Bornstein *et al.* 1995), and the sympathetic function may play a role in the development of essential hypertension (Grassi *et al.* 2008) but no adrenergic overdrive has been reported in PA (Grassi *et al.* 2009).

It is the genetic hypothesis that is explored in the experimental sections of this thesis. The genetic basis will be explained in the next section.

1.4 *CYP11B1* and *CYP11B2* genes

The previous sections have described the working of the RAAS and reviewed the ways in which it is altered in health and disease. It has been mentioned that the rate of synthesis of aldosterone may contribute to the genetic component of hypertension. To examine this further, an understanding of the genetic variations of the *CYP11B1* and *CYP11B2* genes and their impact on the regulation of blood pressure is crucial. The severe and rare clinical conditions previously mentioned are not the only ones highlighting the importance of the aldosterone synthase and 11β-hydroxylase encoding genes in hypertension. Subtle variants of *CYP11B1* and *CYP11B2* have also been associated with the more common phenotype of essential hypertension. In the following sections variations in *CYP11B1* and *CYP11B2* will

be described in detail, showing the potential for these two candidate genes to explain the variation in ARR and hypertension.

1.4.1 CYP11B1 and CYP11B2 characteristics

CYP11B1 and *CYP11B2* lie in tandem in human chromosome 8, only 40 kb apart (Figure 1-16). In Caucasians, as shown by the HapMap project, they are in relatively tight LD and thus a limited number of haplotypes is expected (http://www.hapmap.org/). In contrast, the LD in Africans, represented by the Yorubans in the HapMap project, appears to be less tight. Each gene has 8 introns and 9 exons. Their coding regions are 95% identical and their intronic regions are 90% identical. The enzymes for which they code are 93% alike, reflecting their shared 11β-hydroxylase and 18-hydroxylation activity. However, they diverge in their 5' untranslated region (73% 240bp of *CYP11B1*), thus accounting for the differences in regulation and expression pattern for each gene (Mornet *et al.* 1989).

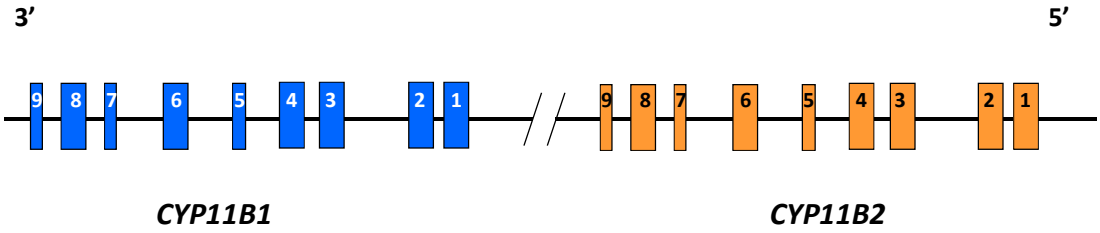


Figure 1-16. Diagram showing the exon/intron structure of the *CYP11B1* and *CYP11B2* genes

To have a better understanding of the different genetic variations within the *CYP11B1* and *CYP11B2* genes, in sections 1.4.2 and 1.4.3 the two groups of rare variations (mutations and structural variations) will be explained alongside their corresponding effects on protein activity levels and phenotype. In section 1.4.4, the polymorphisms within these two genes and their associations with ARR and hypertension will also be explained in detail.

1.4.2 Mutations in CYP11B1 and CYP11B2

Although the terms "mutation" and "polymorphism" are sometimes used interchangeably, the conventional definitions of genetic research should be followed. Being a mutation any genomic variation with a population frequency <1% and a polymorphism any genomic variation with a population frequency >1%. Thus the difference between these two

definitions is attributed to their frequency. Nevertheless, it is important to mention that rare mutations usually have more impact on function than common polymorphisms (Strachan & Read 2004). For the purpose of this thesis, the conventional definition of polymorphism will be followed. However, a mutation will be defined as a change in DNA which alters the genetic message carried by a particular gene.

Mutations in CYP11B1

As shown in Figure 1-17, a variety of mutations are responsible for 11β-hydroxylase deficiency. They are distributed throughout the gene but their frequency is higher in exons 2, 6, and 8. The majority of mutations reported in exons are missense mutations (i.e. Pro42Ser, Leu299Pro). Their effect, reported *in vitro,* varies depending on their position in the enzyme's 3D structure. The activity can be reduced or, if the mutations are situated in a haem-binding or substrate-binding domain, completely lost. In some cases (e.g. Gly267Arg) splicing can be altered. In exons, nonsense mutations (e.g. Trp116STOP and Lys174STOP) and frameshift mutations caused by insertions or deletions (e.g. delC32 or ins2bp) disrupt the reading frame of the coding sequence, leading to a truncated protein. Mutations in the introns can also lead to frameshift and truncation of the protein (for example, G to T transition in intron 3), alter the splice site (318+A-G in intron 5) or produce extremely low activity like the A to G transition in intron 8. Two amino acid changes Ser288Gly and Val320Ala in exon 5 and 6 of *CYP11B1* can confer aldosterone synthetic capacity (Curnow *et al.* 1997).

There is evidence that the mutations which cause a decrease rather than an ablation of activity have been identified in subjects with mild forms of 11β-hydroxylase deficiency, sometimes presenting with mild androgen excess and a slight increase in blood pressure (Joehrer *et al.* 1997).

Mutations in CYP11B2

Mutations in *CYP11B2,* like those in *CYP11B1,* have been reported throughout the gene. Their effects *in vitro* depend on the nature of the mutation and their location in the 3D structure. Mutations that destroy the enzymatic activity of aldosterone synthase *in vitro* are mainly deletions causing a frameshift (e.g. del5bp in exon 1) (Mitsuuchi *et al.* 1993), nonsense mutations (e.g. Glu255STOP) (Peter *et al.* 1997), nonconservative missense mutations (ie. Arg384Pro and Leu461Pro) (Geley *et al.* 1995; Nomoto *et al.* 1997) or are adjacent to the haem-binding domain (e.g. Leu451Phe) (Nguyen *et al.* 2008). Mutations which show a decrease in aldosterone synthase activity are generally missense mutations as

depicted in Figure 1-18. Although several cases have been reported of inactivating mutations in *CYP11B2* corresponding with the clinical features of CMO type I, and of mutations causing enzyme deficiencies being identical in patients with clinical features of CMO type II, there are also cases which do not follow this tidy pattern. Not only are the nature and location of these mutations important, but also their interaction with other variable factors. Zhang and colleagues described a patient with type II deficiency carrying *CYP11B2* mutations that should completely inactivate the enzyme (Thr318Met, essential for catalysis, and a frameshift in exon 6); perhaps the phenotype in this patient was different because the mutations were present in conjunction with mutations related to CMO type II (Arg181Trp and Val386Ala) (Zhang *et al.* 1995). A few years later a case was reported of twins having a type I profile who were homozygous for three variations each having a modest effect on aldosterone synthase activity (Arg173Lys, Glu198Asp and Val386Ala) (Portrat-Doyen *et al.* 1998). Interestingly, in 2004, a compound heterozygote patient for two mutations causing premature stop codons (Tyr265STOP and Gln272STOP) which would be expected to lead to a truncated protein, without essential active site residues or a haem-binding site, displayed low-normal aldosterone levels and the phenotype could not be classified as a classical type I or type II deficiency, leading Williams and colleagues to suggest other genes and regulatory elements might influence the complex genotype-phenotype relationships (Williams *et al.* 2004). A recent study supports this hypothesis; three siblings with a steroid profile of partial aldosterone synthase deficiency type I intriguingly displayed detectable serum aldosterone and urinary THAldo levels but possessed a mutation (Ser308Pro) which lacks activity *in vitro*. In addition, the administration of dexamethasone suppressed aldosterone in serum and urine, suggesting that aldosterone synthesis is mediated *in vivo* via an ACTH-dependent pathway (Lovas *et al.* 2009). It is clear that further studies are required to elucidate the relevance of the different mutations in *CYP11B2*, their influence on aldosterone production and their association with aldosterone-deficient phenotypes.

Animal models, such as the Dahl sensitive rat, have given an insight of the relationship between genotype and phenotype in this locus. In these rats two effects have been observed. The mutations in the *CYP11B1* gene have been associated with altered 11β-hydroxylase activity and blood pressure, while non-synonymous mutations in *CYP11B2* have been associated with increased aldosterone synthase activity (Cicila *et al.* 1993; Cicila *et al.* 2001; Cover *et al.* 1995; Matsukawa *et al.* 1993; Nonaka *et al.* 1998).

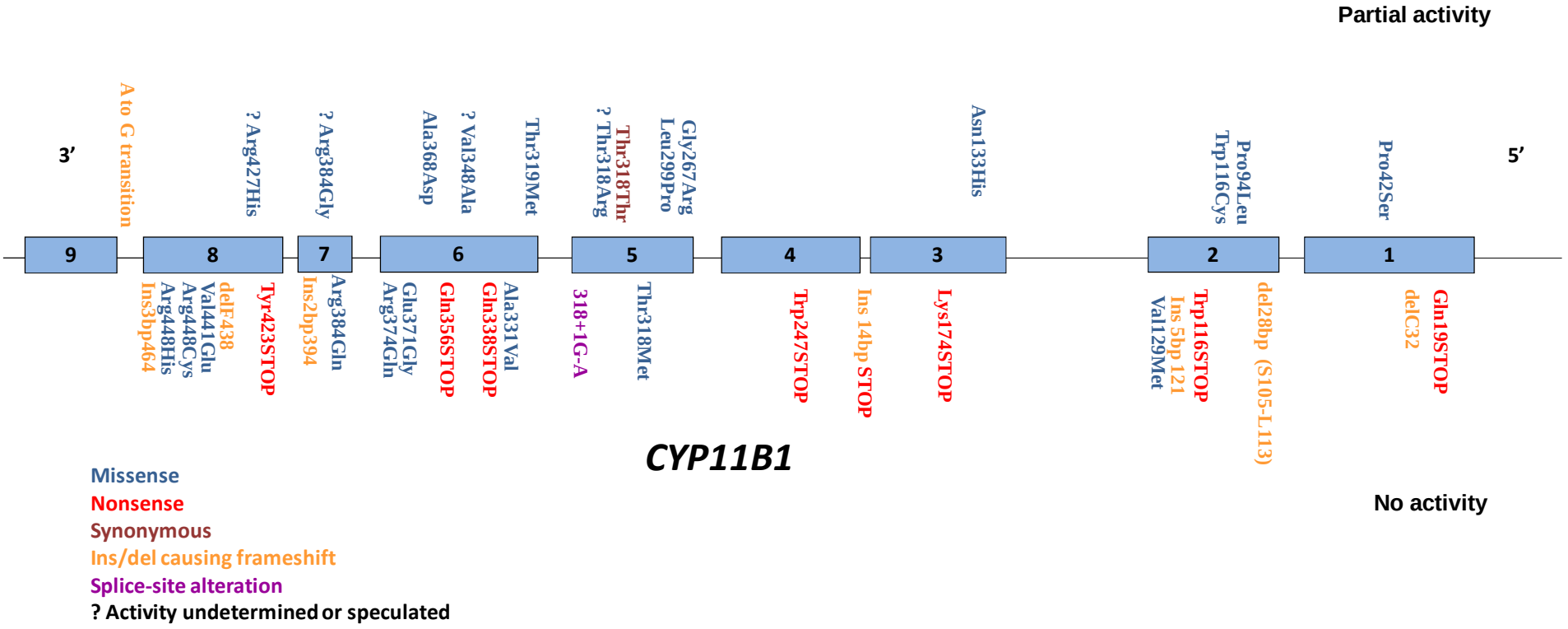


Figure 1-17. Mutations in the *CYP11B1* gene.

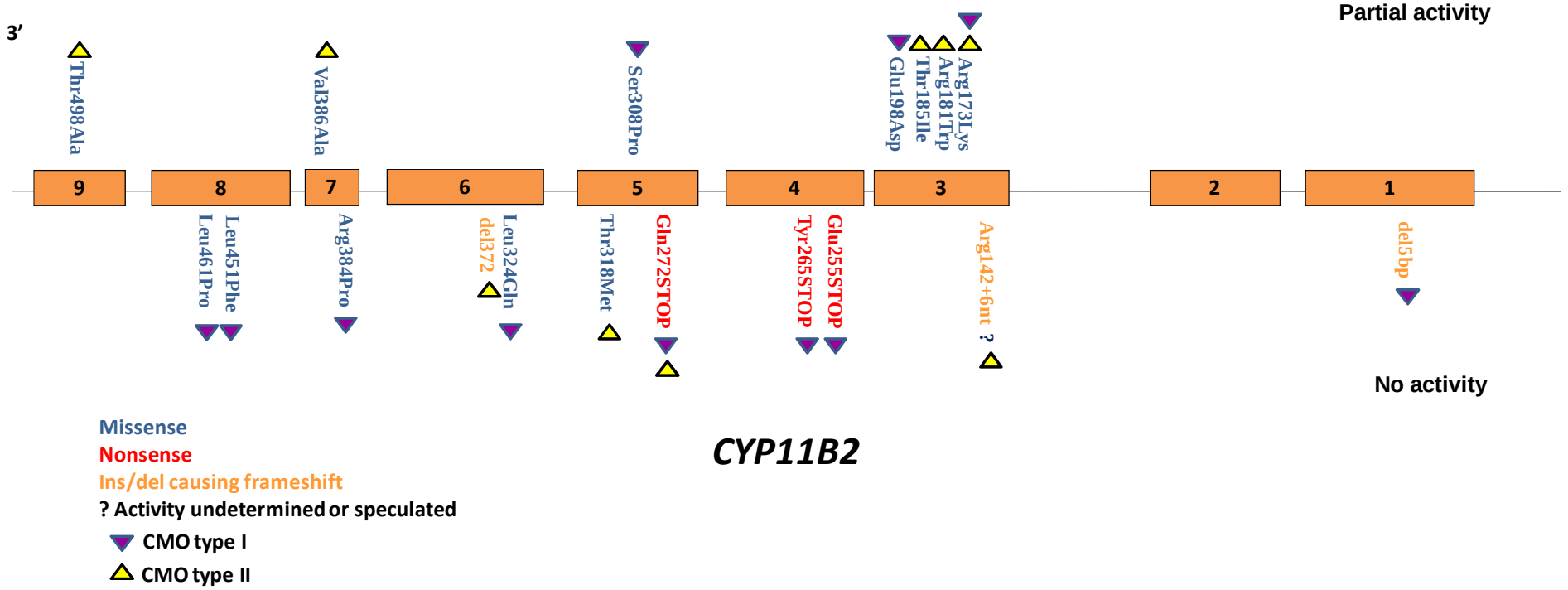


Figure 1-18. Mutations in the *CYP11B2* gene

1.4.3 Structural variations in CYP11B1 and CYP11B2

Structural variations are continually being discovered; they are defined as genomic alterations, typically > 1kb, and include copy number variations (CNV), copy number polymorphisms, segmental duplications, segmental uniparental disomy and balanced changes (inversions and some translocations) (Feuk *et al.* 2006). Structural variations are illustrated by the hybrid genes of *CYP11B1/CYP11B2* reported in FH-I and in 11β-hydroxylase deficiency.

The first evidence of CNVs' influence on disease phenotypes was observed in genomic disorders caused by genomic rearrangements through the mechanism of nonallelic homologous recombination (NAHR) in duplicated genes, gene segments and repeat gene clusters (Lupski 1998). This gene-gene recombination is observed in FH-I. Lifton also proposed that 'uneven crossover' during meiosis of the *CYP11B1* and *CYP11B2* genes can produce a long chromosome or a short chromosome (Lifton *et al.* 1992a). The long chromosome occurs when both wild type genes are present with the hybrid gene in the middle. The short form occurs when both wild type genes are absent and only the reciprocal gene with 5' *CYP11B2* and 3' *CYP11B1* is present. In the reciprocal gene, the promoter and exon 1 to 4 derives from the *CYP11B2* gene and intron 4 to exon 9 from *CYP11B1*. However, very few subjects carry this reciprocal gene, and apparently heterozygous subjects have a normal phenotype, but the presence of the reciprocal gene together with a loss-of-function mutation in the other *CYP11B1* allele lead to 11β-hydroxylase deficiency and congenital adrenal hyperplasia (Hampf *et al.* 2001).

In addition to these uncommon mutations and structural variations, evidence supports the role of the polymorphisms in the *CYP11B1/CYP11B2* locus in altering ARR, its components and blood pressure.

1.4.4 Polymorphisms at the CYP11B1/CYP11B2 locus, ARR and hypertension

Polymorphisms in CYP11B2 and their association with different phenotypes

Two polymorphisms located in the promoter region and intron 2 of *CYP11B2* have been studied in great detail.

The first polymorphism is a SNP in the 5' promoter region at position –344, which sits in a putative binding site for the transcription factor steroidogenic factor-1. This transcriptional protein is involved in the expression of steroid biosynthetic enzymes in the adrenal cortex (White PC & Slutsker L 1995). The two alternative bases at this position are thymine (T) and cytosine (C) (White PC & Slutsker L 1995). Public databases report that the T allele is more frequent than the C allele, and that the T allele is more frequent in subjects of African ancestry (0.8-0.85) than in subjects of European ancestry (0.45-0.57) (http://www.hapmap.org/). These frequencies are comparable to those reported in a recent meta-analysis; the T was more frequent in African Americans and Japanese subjects than in Caucasians (Sookoian *et al.* 2007). These frequencies should be treated with caution because the studies may have been affected by selection bias. Studies have shown that the deletion of this site has no effect on transcription *in vitro.* In addition, the C allele binds more strongly to the steroidogenic factor-1 than the T allele but this has no apparent effect on transcriptional rate, thus suggesting the steroidogenic factor-1 is not a major regulator of *CYP11B2* expression (Bassett *et al.* 2002).

The second polymorphism is a gene conversion (IC) whereby a fragment of the "wild-type (Wt)" intron 2 of *CYP11B2* is replaced with the corresponding intronic fragment of *CYP11B1* (Conv). Due to the nature of this variation and difficulty in genotyping it, its frequency has not been reported in public databases. However, in a small study of essential hypertensive subjects, the conversion allele frequency was much lower in black subjects (0.08) compared with white (0.38) (Zhu *et al.* 2003a). This is consistent with a study in which the conversion allele had a frequency of 0.55 in Caucasian families selected via a hypertensive proband (Keavney *et al.* 2005).

The -344 T/C and intron conversion polymorphisms are in tight LD and three common haplotypes have been reported: T/Conv (38%), C/Wt (45%) and T/Wt (13%), where the first allele corresponds to the -344 polymorphism (T or C) and the second to the intron 2 conversion (Wt or Conv) (Davies *et al.* 1999). Recently, the haplotypes have incorporated

more variations. Moreover, as part of this thesis, a new set of SNPs for haplotypes have been selected and evaluated; these are discussed in Chapters 5 and 7.

Interestingly, the -344 T/C and intron conversion polymorphisms in the *CYP11B2* gene have been associated not only with phenotypes related to this gene, such as hypertension, blood pressure, ARR and its components (PRA and plasma aldosterone concentration) and the urinary metabolite THAldo, but also to phenotypes related to 11β-hydroxylase efficiency. In the following sections the results for these associations will be explained in more detail.

Associations of genetic variations in the *CYP11B2* gene and hypertension have been shown in several studies. In the majority, the -344T allele and the intron conversion (Conv) allele have had a higher frequency in hypertensives than normotensives (Brand *et al.* 1998; Castellano *et al.* 2003; Davies *et al.* 1999; Henderson *et al.* 2004). However, other groups showed no association of the -344 SNP with hypertension or even found that the C allele is more frequent in hypertension (Kupari *et al.* 1998; Mulatero *et al.* 2000; Pojoga *et al.* 1998; Schunkert *et al.* 1999a; Tamaki *et al.* 1999; Tsujita *et al.* 2001). A few studies reported an association with systolic blood pressure but the results are inconsistent (Hautanena *et al.* 1998; Iwai *et al.* 2007; Staessen *et al.* 2001). A similar trend of conflicting results has been observed in hypertensives with raised ARR (also classified as low renin hypertensives); in most, but not all of the studies, the -344T and conversion alleles are more common among hypertensives (Komiya *et al.* 2000; Lim *et al.* 2002b; Nicod *et al.* 2003; Rossi *et al.* 2001; Tamaki *et al.* 1999). Regarding the renin component of the ratio, the associations with the -344T/C have not been consistent (Iwai *et al.* 2007; Staessen *et al.* 2001; Casiglia *et al.* 2005; Tsukada *et al.* 2002; Matsubara *et al.* 2004; Brand *et al.* 1999; Rajput *et al.* 2005; Heller *et al.* 2004; Mulatero *et al.* 2002b) The -344T/C and intron 2 conversion have also been associated with increased aldosterone levels in plasma (Iwai *et al.* 2007; Nicod *et al.* 2003; Paillard *et al.* 1999) and urine (THAldo) (Davies *et al.* 1999; Hautanena *et al.* 1998; Staessen *et al.* 2001) but, again, these findings have not been universal (Pojoga *et al.* 1998; Schunkert *et al.* 1999a). This inconsistency in research findings strongly suggests that other variations at this locus may be involved, as well as demographic differences in the various studies. Using the advantage of scale, a recent meta-analysis that included 42 studies of the -344T/C polymorphism, took into account differences in ancestries, sample size and subject selection criteria which might explain the differences in individual studies. This meta-analysis showed an association between the -344T allele and an increased risk of hypertension. However, not all the discrepancies were explained. There was also no

significant association with systolic or diastolic blood pressure. There was no difference in aldosterone excretion rate or plasma aldosterone levels between genotypes. However, homozygous CC subjects, mainly of Caucasian origin, had lower PRA (Sookoian *et al.* 2007). Alternative explanations suggested for the contrasting findings of associations between these genotypes and phenotypes include increased transcription factor availability in other parts of the gene, transcriptional activation of the steroidogenic acute regulatory gene by a mechanism dependent on steroidogenic factor-1 (Clark & Combs 1999) or the linkage disequilibrium of these polymorphisms with other causative genetic changes or regulatory elements (Nicod *et al.* 2003).

Since the description of the -344T/C and IC *CYP11B2* polymorphisms, other interesting polymorphisms have been reported. For example, the missense mutation in exon 3, where a lysine is replaced by an arginine, K173R (rs4539) associates with hypertension. The 173R allele is more frequent in Chilean subjects with LRH. No *in vitro* differences were found between the variants in their ability to convert deoxycorticosterone to aldosterone and therefore this association has not been explained (Fardella *et al.* 1996). However, the haplotype including -344T and K173 was associated with higher *CYP11B2* expression in adrenal tissue than the -344C and R173 haplotype (Tanahashi *et al.* 2005).

Other SNPs in the *CYP11B2* used in association studies include T4986C (more recently known as T4991C or rs28930074) in exon 7, to investigate the influence with hypertension, blood pressure, plasma aldosterone concentration and urinary excretion rate (Imrie *et al.* 2006; Keavney *et al.* 2005; Kumar *et al.* 2003; Pojoga *et al.* 1998), and A6547G in the 3'UTR region, the minor G allele of which associated with hypertension in a small study (Kumar *et al.* 2003).

Despite the use of several polymorphisms in *CYP11B2*, their causal relationship with hypertension and inappropriate aldosterone production remains unclear.

Polymorphisms in CYP11B2 and CYP11B1 associated with different phenotypes

As mentioned previously, polymorphisms in the *CYP11B2* have also been shown to associate with alterations in 11β-hydroxylase efficiency, which is usually defined by the ratios of the plasma concentrations of 11-deoxycortisol to cortisol (S:F) or DOC to corticosterone (DOC:B), or by the ratios of their respective urinary metabolites, tetrahydrodeoxycortisol (THS) or tetrahydrodeoxycorticosterone (THDOC). An increase in this ratio implies that, to achieve a given level of cortisol synthesis, a higher precursor (11-deoxycortisol, S) concentration is required, and therefore that the enzyme is less efficient. In contrast to the association studies with hypertension or inappropriate aldosterone production, the studies with 11β-hydroxylase efficiency have been more consistent. One of the first studies showing an association between aldosterone synthase (*CYP11B2)* and 11β-hydroxylase related variables was that of (Hautanena *et al.* 1998). Finnish males homozygous for -344T had not only higher urinary aldosterone excretion than CC males but also showed higher 11-deoxycortisol levels in plasma when stimulated with ACTH. Similar findings were reported by (Davies *et al.* 2001) where normal subjects homozygous for the same -344T allele and/or intron conversion had higher levels of precursors for cortisol and corticosterone in plasma compared to those homozygous for -344C and/or normal intron in response to ACTH. A few years later, modest evidence for an association between T4986C in exon 7 of *CYP11B2* and plasma levels of S and S:F ratio was reported (Keavney *et al.* 2005), possible evidence that *CYP11B2* genotypes have associations with 11β-hydroxylase efficiency phenotypes. The associations were reported not only in plasma but also in urine; one small study of normal subjects identified increased THS in individuals with a -344 TT genotype in -344 (Kennon *et al.* 2004). Conversely, SNPs in *CYP11B1* are associated with increased aldosterone levels. These apparently paradoxical associations are not surprising considering the proximity, similarity and high LD between *CYP11B1* and *CYP11B2* and suggest an interaction of phenotypes, plasma and urinary aldosterone levels and 11β-hydroxylase efficiency, in predisposing to hypertension. A large family-based study reported that the strongest association of THaldo excretion was with a polymorphism in *CYP11B1* Intron 3 (rs6387). This SNP accounts for 2.1% of the variability of THaldo (Imrie *et al.* 2006). Because there is a close connection between steroid phenotypes and genotypes and variability in the associations reported for the *CYP11B2*, a recent study of 160 subjects, including normotensive controls, unselected hypertensives and hypertensives with high (≥750) or low (≤200) ARR looked at associations with known polymorphisms in the *CYP11B1*. However, none of the variants in the coding regions of the *CYP11B1* gene could account for increased ARR or hypertension

(Barr *et al.* 2006). Further studies in a Japanese population support these findings as polymorphisms in the *CYP11B1* gene were not associated with plasma aldosterone or blood pressure (Iwai *et al.* 2007). This suggests the variations of interest might be outside the coding regions or in LD with other genetic variants within the *CYP11B1/CYP11B2* locus.

The first two studies looking at polymorphisms in both the coding and non-coding regions of the *CYP11B1* and *CYP11B2* genes reported similar associations with urinary metabolites. (Keavney *et al.* 2005)showed that the strongest association was with a nonsynonymous SNP in exon 1 (rs6410), unlikely to have a functional effect, but explaining 5% of the variability in urinary THS (Keavney *et al.* 2005). (Ganapathipillai *et al.* 2005) simultaneously reported strong LD between polymorphisms in the two genes; adding to their main haplotype rs4538 and rs6391 (*CYP11B2,* Exon 6 and *CYP11B1,* Intron 6 respectively*)* from the core -344, intron conversion (IC) and rs6410 also used by Keavney *et al.* (Ganapathipillai *et al.* 2005). The haplotype combination of these five SNPs (-344, IC, rs4538, rs6410 and rs6391) as TconvGTA showed a significant association with a higher ratio of urinary THS to total cortisol metabolites (tetrahydrocortisone (THE)+tetrahydro 11 deoxycortisol (THF) and 5αTHF) excretion rates, a marker of 11β-hydroxylase deficiency. Moreover, the same haplotype was associated with a higher ARR although this did not achieve conventional statistical significance. This evidence, together with consistent findings in essential hypertensive subjects of a minor impairment in 11β-hydroxylase efficiency (Connell *et al.* 1996; de Simone *et al.* 1985; Honda *et al.* 1977), supports the idea that linked variations in both genes might combine to affect corticosteroid levels, and therefore blood pressure.

A more detailed investigation of the whole locus confirmed the existence of strong genetic linkage across the *CYP11B1/CYP11B2* region (Barr *et al.* 2007). It reported four common haplotypes which together account for 68% of the variability in these two genes. These haplotypes include, in addition to the -344 SNP and the IC, two novel variations in the *CYP11B1* promoter: -1889 G/T and -1859 A/G (Figure 1-19). That these two variations could affect 11β-hydroxylase activity was confirmed by *in vitro* studies that showed a significant effect on *CYP11B1* transcription. When genetic constructs were stimulated *in vitro* with ACTH and forskolin the -1889T and -1859G constructs responded less well than the -1889G and -1859A constructs. This association was confirmed *in vivo*; in hypertensive patients a significantly higher urinary THS/total F was measured in subjects homozygous for the -1889T allele. Those subjects homozyogous for -1859G showed a similar trend although this did not achieve significance (Barr *et al.* 2007). Although this study provides

strong evidence for the alteration of 11β-hydroxylase activity, it gives no further insight into the possible variations in the locus associated with altered aldosterone production and hypertension. From these studies, it is clear that a given *CYP11B1/CYP11B2* genotype may be associated both with higher aldosterone levels, a *CYP11B2*-related phenotype, and reduced 11β-hydroxylase efficiency, a *CYP11B1*-related phenotype, but they do not provide an explanation for the connection between these phenomena and the development of hypertension.

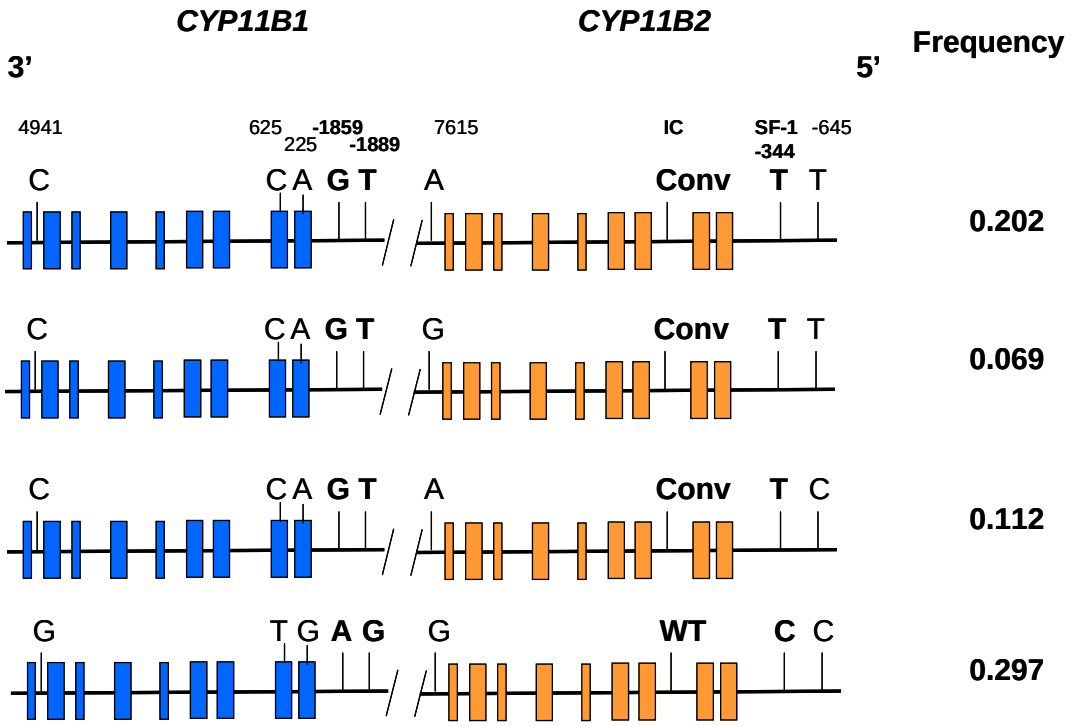


Figure 1-19. Four common haplotypes with a frequency > 5% . (Taken from (Barr *et al.* 2007)).

1.4.5 Hypothesis linking 11β-hydroxylase deficiency, inappropriate production of aldosterone and development of hypertension

Theoretically, two categories of explanations are possible: either that the 11β-hydroxylase deficiency and inappropriate aldosterone production are independent or that they are causally linked. Current evidence favors the second of these, that a mild impairment in 11β-hydroxylase activity is the ultimate cause of higher aldosterone levels. Mild inefficiency would be expected to result in increased ACTH secretion to maintain normal cortisol levels. In consequence, it is suggested that a lifelong exposure to higher ACTH promotes the development of hyperplasia in the zona glomerulosa and zona fasciculata, resulting in increased production of aldosterone, which might lead to hypertension (Connell *et al.* 2003). ACTH can modulate aldosterone levels; is there evidence of altered, possibly ACTH-induced changes in adrenal structure in essential hypertension?

Adrenal hyperplasia is a common finding, not only in hypertensive patients with low renin and normal aldosterone levels or mildly elevated ARR (Komiya *et al.* 1991), but also in patients with primary aldosteronism (Young 2007). There is evidence that ACTH can stimulate the expression of genes required for aldosterone synthesis such as StAR, *CYP11A1* and *CYP21B,* although it is believed that ACTH is not one of the main trophins regulating aldosterone secretion (see section 1.2.5). One of the reasons for not previously considering the possibility that long-term increases in ACTH levels might increase aldosterone levels has been the studies reporting that ACTH, after acute use, suppresses aldosterone synthesis. However, it is important to take into account that the ACTH doses were at supraphysiological concentrations and administered over a short period of time (Oelkers 1985; Whitworth *et al.* 1983; McDougall *et al.* 1980).

High concentrations of ACTH can lead to sodium retention, which will suppress aldosterone secretion and also render it less sensitive to its trophins (Connell *et al.* 1987). However, there is good evidence that ACTH can have a more sustained effect. This is supported by the fact that sodium-restricted subjects receiving an ACTH infusion had a more prolonged stimulation of aldosterone synthesis (Tucci *et al.* 1967). Pratt *et al.* showed that an ACTH infusion over 4 days increased THAldo excretion in males on a high salt diet throughout this period (Pratt *et al.* 1976). These studies suggest ACTH might not have only a transient effect in aldosterone secretion. Also, the hypothesis demands not that ACTH be

transiently very high, but that it is marginally increased for a very long, possibly lifelong, period.

Moreover, the sensitivity of aldosterone plasma levels to ACTH is higher than those of cortisol and DHEA (Daidoh *et al.* 1995). Furthermore, ACTH has the potential to enhance aldosterone synthesis in response to its main stimuli (AngII and potassium) (Miller 1988). A lack of this potential sensitivity is observed in hypopituitary patients in which the response of aldosterone to AngII is impaired (Seifert & Oelkers 1981). Additional evidence of ACTH effects on aldosterone levels derives from patients with Cushing's syndrome. These patients have sustained exposure to ACTH but normal aldosterone levels, suggesting endogenous levels of ACTH can have a different effect to administered ACTH (Biglieri *et al.* 1963; Whitworth *et al.* 2000).

Peptides derived from proopiomelanocortin (POMC), like 16K N-terminal fragment (16K) and its derivative, gamma 3MSH, can stimulate aldosterone production *in vitro* (Pham-Huu-Trung *et al.* 1985). In addition, knockout POMC mice lacking ACTH have low or absent aldosterone levels, suggesting that ACTH is required for aldosterone production (Yaswen *et al.* 1999; Karpac *et al.* 2005; Coll *et al.* 2004). However, it has recently been reported that these mice have secondary hyperaldosteronism and that exogenous administration of ACTH stimulates aldosterone production. The increased basal aldosterone levels are normalized after treatment with dexamethasone, suggesting that aldosterone elevation is a consequence of glucocorticoid deficiency (Linhart & Majzoub 2008).

There is a characteristic synchrony in aldosterone and cortisol secretion (James *et al.* 1976; Katz *et al.* 1975), possibly showing the influence of ACTH on aldosterone regulation during the night (Follenius *et al.* 1992; Richards *et al.* 1986). However, during the daytime, the renin-angiotensin and dopaminergic systems might also play an important role in aldosterone's daily rhythmicity (Takeda *et al.* 1984). Thus it has been suggested that a system comprising the RAAS together with autonomic, dopaminergic and hypothalamic-pituitary adrenal systems might be required for homeostasis (Portaluppi *et al.* 1996). This is supported by studies showing that patients with LRH and APA have abnormal aldosterone rhythmicity (Schambelan *et al.* 1976; Suzuki *et al.* 2008; Cugini *et al.* 1980).

The contribution of ACTH to sustaining the production of adrenal steroids involved in the elevation of blood pressure has been reported in studies of essential hypertensive patients. An early study showed that their blood pressure can be reduced by administration of

dexamethasone (Hamilton *et al.* 1979). The levels of the adrenal androgen, dehydroepiandrosterone sulfate (DHEAS), can be increased in hypertensive individuals (Schunkert *et al.* 1999b). This is consistent with recent findings in a large hypertensive cohort showing a correlation between cortisol, aldosterone and androgen metabolite excretion, but only in patients homozygous for -344T (Freel *et al.* 2007). Moreover, it has also been shown that the variations at -344T/C of *CYP11B2* and -1889 G/T and -1859 A/G of *CYP11B1* can contribute to a susceptibility to impaired regulation of the hypothalamic-pituitary-adrenal axis (Freel *et al.* 2008).

All this evidence favors the hypothesis that genetic changes in 11β-hydroxylase efficiency in the long term, and in combination with other environmental and genetic factors, lead to a chronic increase in ACTH drive and a consequent increase in aldosterone levels. It is of relevance that two older studies have reported increased plasma ratios of DOC:B (de Simone *et al.* 1985) or S:F (Connell *et al.* 1996) in essential hypertension. These findings are consistent with a modest reduction in the efficiency of 11β-hydroxylation . No genetic data were available from these studies, but, they may provide indirect support for this hypothesis. Thus, it has been proposed that ACTH can affect ARR and lead to the development of hypertension in two ways. Firstly, ACTH might act as a growth factor promoting adrenal zonal hyperplasia with an increased sensitivity of aldosterone to its agonists, AngII and potassium. Secondly, ACTH might usurp part of renin's role in the control of aldosterone (Connell *et al.* 2008).

This hypothesis links 11β-hydroxylation and hypertension to an elevated ARR and the *CYP11B* genes. It remains a hypothesis which awaits further evidence (Connell *et al.* 2003; Freel & Connell 2004).

This introduction reveals three important themes in our attempt to understand essential hypertension: the role of adrenal cortex, the crucial contribution of genetic variation and the importance of the correct choice of population in which to study them. In the following experimental section, each of these themes is investigated. In Chapter 3, a new method to measure plasma renin concentration is compared with the classical plasma renin activity assay in order to determine which is more practical to estimate the ARR in a population study. In Chapter 4, ARR is determined in a family-based study. The correlation between the ratio, its components and a range of phenotypes is examined. The heritability of the ratio and its components is determined and their association with variations at the *CYP11B1/CYP11B2* locus tested. Chapter 5 is a thorough study of the linkage disequilibrium, haplotype structure and tagSNPs for the *CYP11B1/CYP11B2* locus in

Caucasians, designed to select the most informative SNPs in the region for an association study. As inconsistent results of association studies in populations with different ancestries have been reported, in Chapter 6, a comparison is made between the allele frequencies and linkage disequilibria at the *CYP11B1/CYP11B2* locus in a population of African ancestry and two populations of Caucasian ancestry. Finally, in Chapter 7, the SNPs selected in Chapter 5 are tested for association with hypertension in Caucasians.

1.5 Hypothesis and aims

Inappropriate aldosterone production is of great relevance to the development and maintenance of high blood pressure. A derangement in adrenal steroid biosynthesis combined with genetic and environmental factors might be responsible for long-term changes that result in a resetting of the level of sensitivity of aldosterone to its main trophins, giving rise to an intermediate phenotype characteristic with an elevated aldosterone to renin ratio (ARR) which may contribute to hypertension and even progress to primary aldosteronism. Over the last decades, several polymorphisms in the genes encoding aldosterone synthase *(CYP11B2)* and 11β-hydroxylase (*CYP11B1)* have been associated with blood pressure, hypertension and a variety of intermediate phenotypes related to aldosterone production such as the ARR. Moreover, functional polymorphisms in the promoter region of the *CYP11B1* gene have recently been characterised. The work presented in this thesis is a detailed study of the ARR and the polymorphisms in the *CYP11B1/CYP11B2* locus in human essential hypertension.

The main hypothesis tested in the experimental section of this thesis was:

Variants in the *CYP11B1/CYP11B2* locus contribute to the biochemical phenotype of increased ARR and this may in turn contribute to the development of hypertension.

The aims to test this hypothesis were:

1. Biochemical studies:
    - Characterisation and evaluation of a plasma renin concentration (PRC) assay for use in population studies.
    - Measurement of PRC and plasma aldosterone concentration to calculate the ARR in hypertensive and normotensive members of a family study
    - The relationship of ARR and its components to other phenotypic characteristics (e.g. age, gender, antihypertensive treatment, left ventricular mass and cortisol) was also analysed.
2. Genetic studies:
    - Determination of the heritability of ARR and its components in a family study.

- Selection of informative single nucleotide polymorphisms for the *CYP11B1/CYP11B2* locus in Caucasians by evaluating frequency, Hardy-Weinberg equilibrium, linkage disequilibrium and functionality.
- Comparison of *CYP11B1/CYP11B2* locus between populations of Caucasian and African ancestry.

3. Association studies of the polymorphisms in the *CYP11B1/CYP11B2* locus with aldosterone and blood pressure phenotypes:
   - With ARR and its components: tested in a family study.
   - With hypertension: tested in a Case-Control study of a population of Caucasian ancestry and replicated in another Case-Control study of the same ethnic origin.

2 Methods

2.1 Renin assays

2.1.1 Measurement of plasma renin activity using a commercial kit (Adaltis MAIA)

The Adaltis MAIA kit is one of the kits commonly used to measure plasma renin activity by radioimmunoassay (RIA) of the generated angiotensin I (AngI) in EDTA samples. Plasma renin activity (PRA) is expressed in ng/ml/h.

Reagents and sample preparation

A 500 µl aliquot of each control (Lyphochek® Hypertension Markers Control Level 1, 2 and 3) was used. The manufacturer's manual reported the mean and range for each concentration and a routine mean and range was also determined for each concentration (Table 2-1). These values were very similar. The average routine coefficient of variation was 13.1% and the average coefficient of variation reported by the manufacturer was 16%.

Table 2-1. Quality controls used to monitor precision in the PRA assay.

	QC1		QC2		QC3	
	Manufacturer	**Routine**	**Manufacturer**	**Routine**	**Manufacturer**	**Routine**
Mean (ng/ml/h)	2.1	2.4	5.3	5.9	18.6	15.1
Range (2SD) (ng/ml/h)	1.4-2.8	1.8-3	3.8-6.8	4.3-7.5	12.3-24.9	11.1-19.1
%CV	16.7	12.5	14.2	13.6	16.9	13.2
Lot. No.	35921		35922		35923	

The three pre-aliquoted quality controls, kit reagents and a maximum of 22 EDTA plasma samples were thawed at room temperature. The calibrators and antibody were reconstituted as stated by commercial kit. A tray with ice was prepared for the ice bath. Once the samples were defrosted they were gently mixed on vortex and uncapped.

Assay procedures

Angiotensin I Generation

For each plasma sample, 500 µl was aliquoted to the corresponding labelled tube and placed in an ice bath along with the defrosted quality controls. Then, 10 µl of the enzymatic inhibitor phenilmethylsulfonyl fluoride (PMSF) and 50µl of pH 6 phosphate buffer were added to each tube and mixed by vortexing. 100 µl was then transferred to each of four tubes.

Two of these new tubes were incubated at 37°C in a water bath and the other two were placed at 4°C on ice for 30 min. After the incubation period, all tubes were kept in the ice bath to then perform the RIA.

Radioimmunoassay (RIA) procedure

The tubes prepared for the calibration included: 2 tubes for total radioactivity (T), 2 tubes for non-specific binding (NSB) each containing 200 µl of "0" standard, 2 tubes for maximum binding (B0) each containing 100 µl of "0" standard; and, for each reconstituted standard (0.2-25ng/ml), 100 µl was aliquoted in duplicate into labelled tubes. All calibration tubes were placed in the ice bath along with the sample tubes.

100 µl Renin Maia Tracer (^{125}I label, maximum radioactivity: 85 kBq) was added to all the tubes. 100 µl antiserum was added to all tubes, except the ones labelled as T and NSB. All tubes were then vortexed and incubated on ice in a refrigerator (4°C) for 18-20 hours.

The tubes were then taken out of the refrigerator and kept on ice. The magnetic separation reagent (1ml) was added to all the tubes except total radioactivity (T). All tubes were mixed, removed from the ice bath and incubated at room temperature for 10 min. After incubation, all tubes were transferred to a magnetic rack and incubated at room temperature for another 10 min, allowing the magnetic suspension to sediment. The tubes were decanted and placed inverted on absorbent paper to remove the drops of liquid adhering to the sides of the tubes.

Radioactivity present in the sediment was counted for 1 min in all the tubes, including the total radioactivity tubes, in an automatic-gamma counter (1261 Multigamma;. LKB Wallac, Turku, Finland). The RiaCalc program (LKB Wallac, Turku, Finland) was used to calculate the standard curve and highlight any value outside the standard range.

Calculations and Results

The PRA is calculated as ng/ml/ hour with the following formula:

PRA= ng/ml at 37°C – ng/ml at 4°C x 2.24 = ng/ml/hour

Where 2.24 is the dilution factor (1.12) of the sample after addition of PMSF and generation buffer divided by the incubation time for angiotensin I generation in hours (0.5h).

PRA intra- and inter-assay had an average coefficient of variation below 15%.

2.1.2 Plasma renin concentration (LIAISON® Direct renin assay)

The LIAISON® Direct Renin assay measures *in vitro* renin concentration in human EDTA-plasma samples by chemiluminescent immunoassay (CLIA) (Diasorin, Saluggia, Italy). As it is an automated method, the test was performed in the LIAISON® Analyser. The renin concentration was measured according to the LIAISON® Direct Renin assay's protocol without modification. For full details of the manufacturer's method, resulting intervals (5th-95th percentile) and performance characteristics see section 9.1.

Briefly, the method involved the following steps:

Reagents and sample preparation

Samples were stored in EDTA tubes and thawed at room temperature. Up to 95 samples could be assayed in a single run (at least two quality controls were also included). Each plasma sample had to be free of any clots or threads and have a unique barcode. The uncapped samples were placed in the corresponding authorized racks. All the samples used had the 500 µl minimum volume required for the reaction.

Assay procedure

Chemiluminescent reaction

After the quality controls and each sample were loaded on the analyser's racks, the renin concentration test required for them was selected in the analyser. The analyser added 20µl of magnetic particles coated with anti-renin/prorenin monoclonal antibody and 100 µl of conjugate (anti-renin monoclonal antibody labelled with isoluminol derivative) to all tubes. The racks were incubated at 36°C±1°C for 31.5min to allow the formation of the sandwich

of renin molecules between the different monoclonal antibodies. The incubation was followed by a wash cycle removing all unbound materials. Then, the starters were added to initiate the flash chemiluminescent reaction. The light signal (λ=420nm) was detected and measured as relative light units (RLU). RLU represents the amount of isoluminol-antibody conjugate bound and is directly proportional to the renin concentration in the calibrators, QCs or samples.The RLU measurement was performed in 3s.

Interpretation of results

The analyser automatically calculated the renin concentrations for the unknown samples based on the recalibrated master curve stored in the analyser's memory.

A detailed description of the performance of this method (analytical and functional sensitivity, precision and stability) is described in Chapter 3.

2.2 Aldosterone assay

2.2.1 Plasma aldosterone measurements using commercial kit (Coat-a-Count)

The Coat-a-Count procedure is a solid-phase RIA. Aldosterone-specific antibodies are adhered to the wall of a coated polypropylene tube. Over 18 hours incubation, the ^{125}I-labeled aldosterone competes for binding sites with the aldosterone present in standards, quality controls (QCs) and samples. Free materials were separated from the bound by aspiration. The bound material was then counted in a gamma counter (1261 Multigamma;. LKB Wallac, Turku, Finland) and the aldosterone concentration determined by RiaCalc program (LKB Wallac, Turku, Finland) based on the calibration curve.

Reagents and samples preparation

The two sets of pre-aliquoted QCs, kit reagents and up to a maximum of 37 plasma samples were thawed at room temperature.

Lithium-heparin samples were used, where available. Where lithium-heparin samples were not available, EDTA samples were used, although the manufacturer suggests EDTA samples result in ~15% overestimate of the true value. Once samples were thawed, they were mixed on a vortex and centrifuged at 2012 x *g* for 5 minutes.

For each set the QCs used were Lyphochek® Immunoassay Plus Control Levels 1, 2 and 3 (500 µl aliquot of each concentration). The manufacturer's manual reported mean and range for each concentration and we determined routine mean and range for each concentration in our laboratory (Table 2-2). A set was included at the beginning and at the end of the assay in order to control for any drift in measurements during the procedure. The average routine coefficient of variation was 9.5% and the average coefficient of variation reported by the manufacturer was 10%.

Table 2-2. Quality controls used to monitor precision in the aldosterone concentration assay.

	QC1		QC2		QC3	
	Manufacturer	**Routine**	**Manufacturer**	**Routine**	**Manufacturer**	**Routine**
Mean (pmol/L)	108	101	728	631	1667	1573
Range (2SD) (pmol/L)	87-130	71-131	582-873	549-713	1334-2000	1349-1797
%CV	10.2	14.9	10.0	6.5	10.0	7.1
Lot. No.	40181		40182		40183	

Two polypropylene tubes were prepared for total count and two for non-specific binding (NSB).100 coated tubes were used per assay: 14 standard tubes and 86 for QCs and samples, all in duplicate. Standards and ^{125}I aldosterone reagent (maximum radioactivity: 259 kBq) were prepared according to the manufacturer's instructions.

Assay procedure

The plasma samples were analysed by RIA (Coat a count, Siemens Medical Solution Diagnostics, Los Angeles, U.S.A.) against a range of standards for aldosterone concentration. The only modification from the standard method was that, after incubating for 18 hours at room temperature, the liquid was carefully aspirated. Aliquots were counted for 1 minute using the gamma counter (1261 Multigamma;. LKB Wallac, Turku, Finland). The amount of aldosterone present in each sample and QC was determined from the calibration curve generated at the beginning of the assay with the standards. After assessing the duplicate readings, the average mean is reported.

Intra- and inter-assay coefficients of variation were below 5.5% and below 13.1%, respectively, as described by the manufacturer.

2.3 Molecular Biology

2.3.1 Genotyping: PCR Amplification and Automated Sequencing

For the purposes of genotyping, five *CY11B1/B2* fragments were amplified by PCR, followed by sequencing. Two fragments were selected in the *CYP11B1* gene (one in the promoter and one in intron 2), and three in the *CYP11B2* gene (the promoter, intron 2 and intron 6) as shown in Figure 2-1. The amplification of each is described in the following sections.

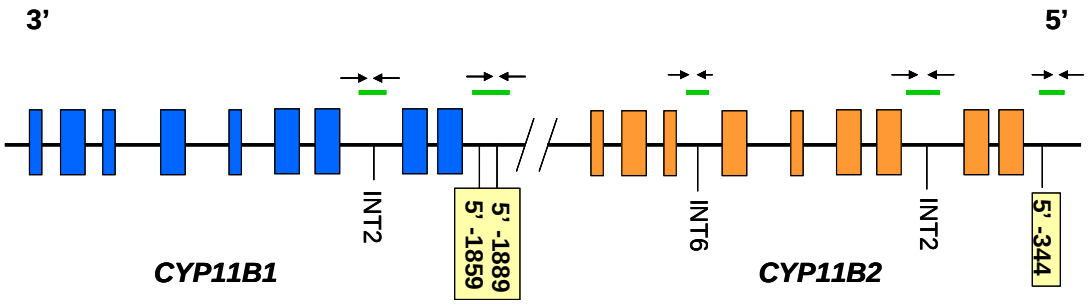


Figure 2-1. Five gene fragments genotyped in the *CYP11B1/CYP11B2* locus. Each fragment is shown in green and the arrows indicate the position of the primers.

Synthetic oligonucleotides were designed for the intron 6 fragment in *CYP11B2* and the intron 2 fragment in *CYP11B1* using the published sequences of these two genes (see section 9.2, Appendix 2) and obtained from a commercial source (Eurofins MGW Operon, Ebersberg, Germany). The primers were designed to have 20-24bp, 50-60% G+C base composition and melting temperatures (Tm) ranging from 53-60 ^{o}C (see Table 9-1 and Table 9-2, Appendix 3).

CYP11B1 Promoter (B1PROM)

This nested PCR method consists of two sequential PCR steps and was previously optimised by (Barr *et al.* 2007). Only the cleanup of the PCR and sequencing products was modified.

B1PROM PCR Amplification 1

	Volume per plate (µl)	Final concentration
Buffer (10x)	250	1x
$MgCl_2$ (25mM)	150	1.5mM
dNTPs	500	200µM each
Enzyme (Thermostart Taq ABGene ABGene)	25	1.25U/reaction
Sense primer, (B1 5'UTR)	100	400nM
Antisense primer, (B1prom-260)	100	400nM
Nuclease free (NF) water	875	----
Total	**2000 µl**	

The above reagents were mixed in a 2ml Eppendorf tube on ice and 20µl of master mix added to 5µl of DNA (5ng/µl), which had been pre-plated on to 96-well PCR plates. The amplification reaction was performed on a 96-well Dyad DiscipleTM sample block powered by the PTC-0221 thermal cycler with heated lid (MJ Research,Waltham, MA, USA) The cycling parameters were as follows:

Program B1PROM

1. 95°C 15 minutes
2. 95°C 30 seconds
3. 60°C 30 seconds
4. 72°C 3 minute
5. Cycle to step 2 for 34 more times
6. 72°C 7 minutes

The PCR products were then diluted 1:10 in nuclease free (NF) water and 1µl used as a template for a second PCR reaction. A second master mix was prepared on ice, this time in a 5ml universal tube:

B1PROM PCR Amplification 2

	Volume per plate (µl)	Final concentration
Buffer (10x)	250	1x
$MgCl_2$ (25mM)	150	1.5mM
dNTPs	500	200µM each
Enzyme (Thermostart Taq ABGene ABGene)	12.5	0.625 U/reaction
Sense primer, (B1 5' 7-32)	100	400nM
Antisense primer, (B1 369-393)	100	400nM
Nuclease free (NF) water	1287.5	----
Total	**2400 µl**	

The reagents were mixed and 24µl of mastermix pipetted into a 96-well PCR plate. 1µl of the 1:10 dilution of the previous PCR product was then added to serve as a template. The amplification conditions were the same as for PCR amplification 1, except that the number of cycles was reduced to 20.

Identification of PCR products

A 1% agarose gel was prepared by adding 1g of agarose to 100ml of TBE buffer, swirling and heating in a 950W microwave oven for 60 seconds. After cooling, 1µl of ethidium bromide (10mg/ml) was added in a fume hood, swirled to mix, poured into a gel mould with Teflon comb and allowed to set for 15min. Subsequently, the comb was removed and gel was submerged in TBE buffer in a standard electrophoresis tank. In each well, 5 µl of PCR product were loaded with 5µl of loading dye. The gel was resolved at 100 volts for 40min. DNA fragments were visualized with Multi-Analyst software v 1.1 (Bio-Rad) by scanning with UV light source at 302nm. The fragment size of the second B1PROM PCR product is 387bp.

PCR product clean-up

The AMPure purification method (Agencourt, USA) was used to remove PCR reagents from PCR product prior to the sequencing reaction. The AMPure was resuspended by vortexing prior to use. The seal of the PCR plate was carefully removed and AMPure added according to the PCR reaction volume so that the resin bound to fragments less than 100bp in size (36µl of AMPure was added to each well). The plate was sealed again, vortexed and centrifuged at 210 x *g* for 30s.The plate was incubated for 3 min at room temperature and then placed on an SPRI (solid phase reversible immobilization) magnet

for 10 min. While still on the magnet, the plate was wrapped in tissue to remove supernatant. Subsequently, 200µl of freshly prepared 70% ethanol was added to each well. The plate was incubated for 30s at room temperature and then the ethanol was discarded by inverting the plate on to tissue. In that position, the plate was centrifuged at 76 x *g* for 30s. The plate was removed from the magnet and air dried at room temperature for at least 20 min before the samples were eluted in 40µl of NF water.

Finally, the plate was placed on the magnet for 5 min. A volume of 10µl of the clear PCR product was transferred to a clean plate for the sequencing reaction.

Sequencing reaction set-up

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

Sequencing reaction mix

	Per well
PCR product	10.0 µl
ABI PRISM BigDye v3.1 Sequencing Buffer (5x)	3.5µl
ABI PRISM BigDye Termination v3.1 ready reaction mix	0.5µl
Sequencing primer (3.2 pmol/µl)	1.0µl
NF water (up to a final volume of 20µl)	5.0 µl
Total	**20.0µl**

The sequencing primer was B1PROM 250-229AS (see Table 9-2).

The programme used for the sequencing reaction was performed on a 96-well Dyad Disciple™ sample block powered by the PTC-0221 thermal cycler with heated lid (MJ Research,Waltham, MA, USA) :

Program: BIGSEQ60

1) Incubate at 96°C for 45s
2) Incubate at 60°C for 4min
3) Cycle to step 1 for 24 more times
4) Incubate at 10°C forever

Sequencing reaction clean-up

The CLEANSEQ Purification method (Agencourt, USA) was used for removing sequencing reaction reagents from the sequencing product prior to automated sequencing. The CLEANSEQ bottle was shaken prior to use. The seal of the plate was carefully removed and 10µl of CLEANSEQ were added along with 62µl of 85% ethanol according to the sequencing reaction volume. The plate was resealed, vortexed, centrifuged at 210 x *g* for 30s, and placed on the SPRI magnet for at least 5min. Once the solution was clear, the seal was carefully removed and the solution discarded. 150µl of 85% ethanol were added to each well and incubated for 30s. Afterwards the plate was inverted on tissue, centrifuged at 210 x *g* for 30s and left to dry for 20min off the magnet. 40µl of NF water were added to each reaction plate. The plate was vortexed, centrifuged at 210 x *g* for 30s and placed on a magnet again in order to transfer 20µl of cleaned sequence product to a barcoded plate.

Automated Cycle Sequencing

Sequencing was performed using Applied Biosystems Big Dye v.3.1 cycle sequencing chemistry (PE Applied Biosystems, Foster City, CA, USA). The technique has been adapted from the dideoxy termination method of sequencing.

The genotypes were assigned in chromatograms using SeqScape v.2.1.1 genotyping software. Samples were excluded when the chromatogram failed or was of poor quality.

CYP11B1 Intron 2 (B1INT2)

B1INT2 PCR Amplification

	Volume per plate (µl)	Final concentration
Buffer (10x)	250	1x
$MgCl_2$ (25mM)	150	1.5mM
dNTPs	500	200µM each
Enzyme (Thermostart Taq ABGene)	25	1.25U/reaction
Sense primer, (B1 3696 S)	100	400nM
Antisense primer, (B1 3937 AS)	100	400nM
Nuclease free (NF) water	1275	
Total	**2400 µl**	

The above reagents were mixed on ice in a 5ml universal vial and 24µl of master mix was added to 1µl of DNA (5ng/µl), which had been pre-plated on to 96-well PCR plates. The amplification reaction was performed on a 96-well Alpha^{TM} sample block powered by the PTC-225 Engine Tetrad(R) Cycler with heated lid (MJ Research, Waltham, MA, USA). The cycling parameters were as follows:

Program PCR57

1. 95°C 15 minutes
2. 95°C 30 seconds
3. 57°C 30 seconds
4. 72°C 1 minute
5. Cycle to step 2 for 34 more times
6. 72°C 7 minutes

The PCR product determination was done by following the steps in section 2.3.1. The length of the B1INT2 product is 261bp. After confirming the expected size of the PCR product, it was cleaned. The sequencing reaction was set up as described in the previous section by using the sequencing primer B1 3696 S for the forward direction or B1 3937 AS for the reverse direction (sequence of both primers is detailed in Table 9-1).

The following programme used for the sequencing reaction was performed on a 96-well Dyad Disciple^{TM} sample block powered by the PTC-0221 thermal cycler with heated lid (MJ Research,Waltham, MA, USA):

Program: BIGSEQ50

1) Incubate at 96°C for 45s
2) Incubate at 50°C for 25s
3) Incubate at 60°C for 4min
4) Cycle to step 1 for 24 more times
5) Incubate at 12°C for 5min

The sequencing product was cleaned and sequenced following the protocol described previously.

CYP11B2 Promoter fragment (B2PROM-344)

The PCR and sequencing for this fragment were previously optimised by (Reynolds *et al.* 2009).

B2PROM-344 PCR Amplification

	Volume per plate (µl)	Final concentration
Buffer (10x)	250	1x
$MgCl_2$ (25Mm)	150	1.5mM
dNTPs	500	200µM each
Enzyme (Thermostart Taq ABGene)	25	1.25U/reaction
Sense primer, (SF1_F)	130	520nM
Antisense primer, (SF1_R)	130	520nM
Nuclease free (NF) water	815	----
Total	**2000 µl**	

The above reagents were mixed on ice and 20µl of master mix added to 5µl of DNA (5ng/µl), which had been pre-plated on to 96-well PCR plates. The amplification reaction was performed on a 96-well Dyad Disciple™ (MJ Research,Waltham, MA, USA) The cycling parameters were as follows:

Program SF1-THERM

1. 94°C 15 minutes
2. 94°C 30 seconds
3. 68°C 30 seconds
4. 72°C 1 minute
5. Cycle to step 2 for 29 more times
6. 72°C 7 minutes
7. Incubate at 10°C forever

PCR products were determined in an agarose gel as described previously (length 232bp). Then the products were cleaned following the steps described earlier. The sequencing reaction set up was the same as described in the previous sections with the corresponding sequencing primer (SF1_F, for more details see Table 9-1) and by using the BIGSEQ50 protocol in the 96-well Dyad Disciple™ (MJ Research,Waltham, MA, USA). The sequencing products were cleaned and sent for automated sequencing as described in the previous sections.

CYP11B2 Intron 2 (B2INCON)

The PCR and sequencing for this fragment were previously optimised by (Reynolds *et al.* 2009).

B2INCON PCR Amplification

	Volume per plate (µl)	Final concentration
Buffer (10x)	250	1x
$MgCl_2$ (25mM)	200	2.0mM
dNTPs	500	200µM each
Enzyme (Thermostart Taq ABGene)	12.5	0.625U/reaction
Sense primer, (ICTAQMAN F)	50	400nM
Antisense primer, (ICTAQMAN R)	50	400nM
Nuclease free (NF) water	437.5	----
Total	**1500 µl**	

The above reagents were mixed on ice and 15µl of mastermix added to 10µl of DNA (5ng/µl), which had been pre-plated on to 96-well PCR plates. The amplification reaction was performed on 96-well Dyad Disciple^{TM} (MJ Research,Waltham, MA, USA) The cycling parameters were as follows:

Program INCON2

1. 95°C 15 minutes
2. 95°C 15 seconds
3. 62°C 30 seconds
4. 72°C 2 minute
5. Cycle to step 2 for 44 more times
6. 72°C 7 minutes

PCR products were determined in an agarose gel (length 600bp). The products were then cleaned-up using AMPure as described previously. 10µl of PCR product was transferred to a new PCR plate. Sequencing reactions were performed using the BigDye Terminator v3.1 (Applied Biosystems, USA) also according to manufacturer's protocol. The corresponding sequencing primer was INTCONR(B1B2) (for more details see Table 9-2) and the protocol for sequencing was BIGSEQ60 as stated in previously. The sequencing products were cleaned and sent for automated sequencing.

CYP11B2 Intron 6 (B2INT6)

B2INT6 PCR Amplification

	Volume per plate (µl)	Final concentration
Buffer (10x)	250	1x
$MgCl_2$ (25mM)	150	1.5mM
dNTPs	500	200µM each
Enzyme (Thermostart Taq ABGene)	25	1.25U/reaction
Sense primer, (B2 6687 S)	100	400nM
Antisense primer, (B2 6995 AS)	100	400nM
Nuclease free (NF) water	1275	
Total	**2400 µl**	

The reaction mix was prepared as described in previous sections. Details of the PCR primers are shown in Table 9-1. The only difference was the cycling conditions, for the B2INT6 fragment the cycling conditions were as follows:

Program B1B2PROM

1. 95°C 15 minutes
2. 95°C 30 seconds
3. 65°C 30 seconds
4. 72°C 1 minute
5. Cycle to step 2 for 34 more times
6. 72°C 7 minutes

PCR products of 329bp were determined by using an agarose gel. Then they were cleaned using AMPure and 10 µl were transferred to a clean 96-well plate to set up the sequencing reaction by using the primer B2 6687 S with a concentration of 3.2 µM. The sequence of B2 6687 S is in Table 9-1. The sequencing programme used was BIGSEQ50. Sequencing products were cleaned using CLEANSEQ prior to automated sequencing.

2.3.2 Genotyping: TaqMan

Ten SNPs were selected for genotyping with the TaqMan method (see Figure 2-2). Five of them are located in *CYP11B1:* rs6410 in exon 1, rs5283 in exon 2, rs6471570 in intron 5, rs5301 and rs4736312 in 3'UTR. Three were located in the intergenic region (rs6471581, rs6471580 and rs4736354) and two in the *CYP11B2*, one in the promoter region (rs1799998) and one in exon 3 (rs4546).

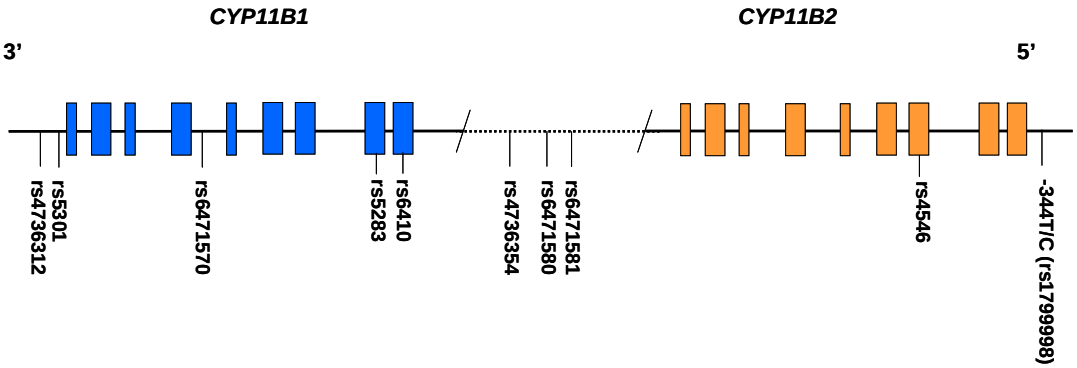


Figure 2-2. Ten SNPs selected for TaqMan assays in the *CYP11B1/CYP11B2* locus.

Details of each assay are shown in Table 2-3. The general method used for all the TaqMan assays, except for rs6471581, rs1799998 and rs4546 used in Chapter 7, was the following:

1µl of each DNA sample (5ng/µl) was plated on to a Micro Amp Optical 96-well reaction plate (Applied Biosystems, U.K.). A volume of 1µl (concentration 5ng/µl) of the positive controls and 1µl of NF water as negative control were added to the designated locations in the plate. The plate was sealed and centrifuged at 210 x *g* for 1 min.

The following reaction mix was prepared in a 0.5 ml amber Eppendorf, while keeping all the reagents on ice. Primers and probes were not exposed to light and were used individually in the reaction mix, based on Table 2-3, according to the SNP being genotyped.

Reagent	Volume per plate (µl)
Taq Man Genotyping Master Mix (Applied Biosystems, CA, USA)	280
Primers & probes	28
NF Water	140
Total volume	**448 µl**

To have a final reaction volume of 5.0 µl, 4.0 µl of master mix were added to each well with pre-plated DNA. The plate was sealed with Therma seal RT film for Real-time PCR (Excel Scientific, Wrightwood CA, USA). Then the plate was placed on the High-Speed Microplate Shaker (Illumina, USA) and pulsed at 480 x *g* for 10s. After that, the plate was centrifuged at 210 x *g* for 1 min.

The amplification reaction was performed on a 96-well Alpha^{TM} sample block powered by the PTC-225 Engine Tetrad(R) Cycler with heated lid (MJ Research, Waltham, MA, USA). The cycling parameters were as follows:

Program ABTAQ2

1. 95°C for 15 min
2. 95°C for 15s
3. 60°C for 1 min
4. Go to step 2, 49 times
5. 10°C forever

After the PCR, the results of the genotyping were read in the ABI Prism 7900 HT. The genotypes were assigned in separate clusters using the allelic discrimination feature of the SDS 2.3 software (Applied Biosystems). Samples that were not within the cluster and with quality values < 95% were excluded. The results were also analysed by the operator in order to remove any incorrect or ambiguous allele assignations made by the software. Finally, the alleles in the genotypes were identified according to the VIC and FAM reference sequences shown in Table 2-3.

For rs6471581 and rs1799998 total reaction volume was 10 µl/well, so the following reaction mix was prepared in a 1.5 ml Eppendorf covered with foil, while keeping all the reagents on ice. 9 µl of master mix was dispensed to each well.

Reagent	Volume per plate (µl)
Taq Man Genotyping Master Mix (Applied Biosystems, CA, USA)	560
Primers & probes	14
NF Water	434
Total volume	**1008 µl**

For rs4546 in Chapter 7, the total reaction volume was also 10 µl/well but with 8µl of reaction mix and 2µl of DNA. Thus, the following modification was done to the reaction mix:

Reagent	Volume per plate (µl)
Taq Man Genotyping Master Mix (Applied Biosystems, CA, USA)	560
Primers & probes	28
NF Water	308
Total volume	**896µl**

The protocol for these three assays was otherwise identical to that for the other SNPs.

Table 2-3. TaqMan assays used for genotyping with their corresponding alleles.

SNP	TaqMan assay type	Applied Biosystems Assay ID	Gene	Location	VIC	VIC ref. seq.	FAM	FAM ref. seq
rs6410	DME	C__11609085_10	*CYP11B1*	Exon 1	C	G	T	A
rs5283	DME	C__11446733_20	*CYP11B1*	Exon 2	A	T	G	C
rs6471570	MtO	C__26236609_10	*CYP11B1*	Intron 5	A	T	C	G
rs5301	DME	C__26236594_10	*CYP11B1*	3' UTR	C	G	T	A
rs4736312	DME	C__27915666_10	*CYP11B1*	3'UTR	A	T	C	G
rs4736354	MtO	C__28011909_10	Intergenic		C	G	G	C
rs6471580	MtO	C__29042875_10	Intergenic		A	T	G	C
rs6471581	MtO	C_____56551_10	Intergenic		G	C	A	T
rs4546	DME	C__30633882_10	*CYP11B2*	Exon 3	A	T	G	C
rs1799998	MtO	C___8896484_10	*CYP11B2*	Promoter	T	A	C	G

DME=Drug metabolising enzyme, inventoried and validated assay; MtO= Made to order, functionally tested; VIC and FAM = names of fluorogenic probes used to discriminate the alleles present in the SNP; ref.seq.= reference sequence in NCBI.

3 Comparison of New Renin Concentration Assay with Renin Activity Assay

3.1 Introduction

The aldosterone to renin ratio is considered to be the most routinely used marker for inappropriate aldosterone production. As renin is an important component of this ratio and is the principal determinant of the level, it is crucial to have an accurate measurement of this enzyme. Measurement of plasma renin has proved to be useful in evaluating the activity of the RAAS in hypertensive states, cardiac failure, renal abnormalities or primary aldosteronism (Catena *et al.* 2007; Lim *et al.* 2002a; Mulatero *et al.* 2005; Sealey *et al.* 2005; Tsutamoto *et al.* 2007). Two different approaches have been used to detect renin. The plasma renin activity (PRA) assay measures the functional activity of renin whereas the plasma renin concentration (PRC) assay measures the quantity of enzyme protein.

In the first approach (Figure 3-1A), the PRA assay detects the generation of AngI by radioimmunoassay (RIA) using an enzyme-kinetic assay. This method is extremely sensitive. The prolonged incubation allows the measurement of low renin activity because the amount of AngI generated eliminates the inaccuracies of non-specific binding (Derkx & Schalekamp 1997). Unfortunately, the method is labour intensive, expensive and has not been standardised, resulting in excessive intra- and interlaboratory variability (Montori & Young, Jr. 2002; Tanabe *et al.* 2003). For this reason, the PRA method has traditionally been established for screening of primary aldosteronism and treatment of congenital adrenal hyperplasia, but is not practical to evaluate more common diseases and the cumbersome nature of the measurement can discourage its use in population studies.

In the second approach (Figure 3-1B), PRC is measured by an immunometric assay using either a radioactive (IRMA) or chemiluminescent label (CLIA). Since the 1990s, a PRC measurement has been possible using IRMA (Derkx *et al.* 1996; Simon *et al.* 1992) which has advantages over the PRA method. It is less laborious and more readily standardised. However, the specificity of the assay has been questioned as the inactive form of renin, prorenin, can cross-react with renin antibodies. This is particularly relevant because, after a prolonged incubation at room temperature, nonproteolytic activation of prorenin occurs (Derkx *et al.* 1996; Sealey & Laragh 1996). The correlation between PRA and IRMA PRC in subjects with low PRA values is unsatisfactory (Derkx & Schalekamp 1997; Ferrari *et al.* 2004). Although the overestimation of renin in plasma samples with high prorenin-to-renin ratios was overcome by increasing the temperature of the assay and decreasing the incubation time (Deinum *et al.* 1999), IRMAs have not achieved the sensitivity and specificity offered by the PRA method, and their use has been unpopular.

Precision and accuracy with CLIA have been improved by calibrating with an international standard and decreasing the amount of manual work by extensive automation. The Nichols Advantage System was the first one commercially available (Nichols Institute Diagnostics, San Clemente, CA, USA) (Sealey *et al.* 2005). It gave results in the expected range in patients with congenital adrenal hyperplasia and mineralocorticoid hypertension and correlated well with PRA in the low renin range indicating potential value in clinical diagnosis (de Bruin *et al.* 2004). However, a later study reports that it correlates poorly at the extremes of the standard curve (Sealey *et al.* 2005) and the company providing the renin kit went into liquidation (Personal communication with Prof. Michael Wallace, Department of Clinical Biochemistry, Glasgow Royal Infirmary, UK). Recently a similar automated CLIA has become available (LIAISON® Direct renin assay), which according to the manufacturer specifications has comparability with the PRA assay. However, it requires independent evaluation.

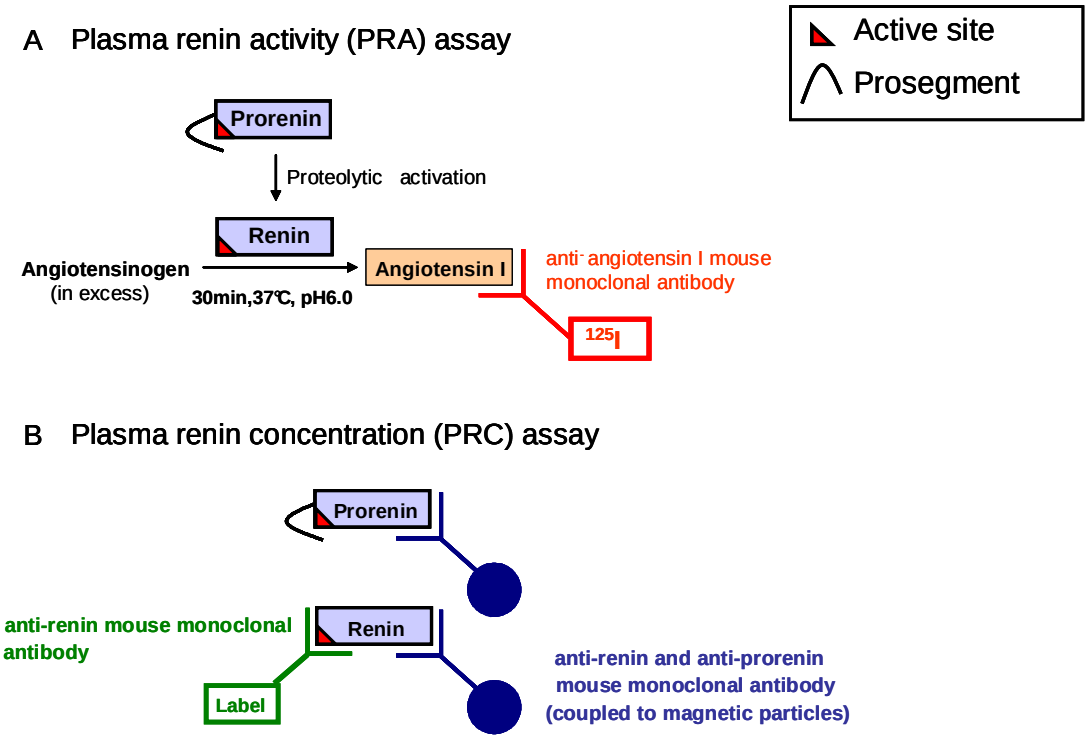


Figure 3-1. Plasma renin activity (PRA) and plasma renin concentration (PRC) assays.

3.2 Aims

The aims of this study were:

1. To evaluate the correlation between the LIAISON® renin concentration (PRC) and the renin activity (PRA) assay in normotensives.

2. To compare the ARR derived from the two different renin assays.

3. To determine the reference range for renin concentration measured by the LIAISON® in individuals with normal blood pressure, without hypertensive medication and without a specified salt intake.

4. To evaluate LIAISON® Direct renin assay for population studies.

3.3 Subjects and Method

3.3.1 Subjects

One hundred and twenty subjects from the BRIGHT study (control normotensive individuals) were selected to compare the two renin assays according to the NCCLS guidelines (Horn & Pesce 2003). The recruitment and eligibility for the study of the subjects were the following:

BRIGHT Controls

The subjects had white British ancestry by grand-parental origin. Their ages were between 18-75. They had no family history of hypertension (or onset <65 years). Their blood pressure recordings were consistently below 130/85 mmHg and BMI < 35 kg/m^2. The consumption of alcohol was less than 21 units per week for men and 14 units for women. Subjects with any clinical evidence of essential hypertension, secondary hypertension, diabetes, kidney disease, anaemia, who had received blood transfusions, who were under treatment for cancer, who had coexisting illnesses which might affect phenotyping or were unable to give informed consent (i.e. with Alzheimer's disease) were excluded from the study.

The majority of the patients' samples (> 95%) were taken when they had been in a seated position for at least 15min. Blood was collected in EDTA-containing tubes and kept at room temperature until it was centrifuged at 2012 x *g* for 10 min. Plasma was then removed, labelled and stored in a –70°C freezer.

3.3.2 Renin measurements

Renin was measured as PRA and PRC in EDTA plasma samples. For PRA, AngI concentration was measured by Ms. Morvern Campbell using a radioimmunoassay (RIA) as described in section 2.1.1. For PRC, renin protein was measured by Mr. Jim Smith using a new semi-automated chemiluminescent immunoassay (CLIA) as described in section 2.1.2.

3.3.3 Aldosterone measurements

Aldosterone was measured by Ms. Morvern Campbell using a solid phase (coated tube) radioimmunoassay technique as detailed in section 2.2.1 in the same plasma samples used for the renin assays.

3.3.4 Statistical analyses

Normally distributed variables are presented as mean ± standard deviation (SD), while data not normally distributed are presented as median and ranges. To compare renin assays' results with different units, a correlation test (Pearson) was used. All analyses were performed in Minitab for Windows version 15.0 (Minitab Ltd, Coventry, U.K.)

3.4 Results

3.4.1 Performance of the LIAISON® Direct renin assay

Analytical Sensitivity: This is defined as the minimum detectable dose that can be distinguished by two standard deviations (SD) above zero. The limit of detection determined as the mean result of the assay plus 2 SD in a series of 20 replicates of the zero standard was 2.1 µIU/ml. After adjusting the results to exclude those outwith 2SD, the sensitivity was of 1.9 µIU/ml.

Functional Sensitivity: This is defined as the lowest renin concentration at which the CV is between 20-22%. In this assay, it was calculated to be < 5 µIU/ml.

Precision: The intra-assay variation of the two controls provided in the assay kit with quoted mean renin concentrations of 25 µIU/ml and 106.9 µIU/ml, was 7.2% and 1.3% respectively. Inter-assay concentration in four samples with renin concentrations from 4.9 to 108 µIU/ml was 3.2-10.4 % (Table 3-1).

Table 3-1. Interassay CVs for LIASION® Direct Renin assay

Renin concentration-mean (SD) µIU/ml	Interassay CV%
4.9 (0.5)	9.2 (n=8)
8.5 (0.9)	10.4 (n=8)
27.6 (2.2)	7.8 (n=19)
108.1 (3.5)	3.2 (n=17)

Stability: To avoid cryoactivation of prorenin, samples were never stored at 4°C but were thawed quickly for use. Repeated freeze/thaw cycles were not permitted.

Other performance criteria such as accuracy, specificity and recovery were provided by the manufacturer. These performance aspects are in the section 9.1, Appendix 1.

3.4.2 Plasma renin activity precision

Three quality control samples were used in each group of PRA assays with low, medium and high concentrations as shown in Table 3-2. Their coefficient of variation based on 10 assays is acceptable for this complex methodology (<15%). All the quality control samples used in the assays for the analysis of the BRIGHT control cohort were within the permitted ranges.

Table 3-2. Quality controls used to monitor precision in the PRA assay.

	QC1	QC2	QC3
Mean (ng/ml/h)	2.4	5.9	15.1
Range (2SD) (ng/ml/h)	1.8-3	4.3-7.5	11.1-19.1
%CV	12.5	13.6	13.2
Lot. No.	35921	35922	35923

3.4.3 Method comparison

The demographic and haemodynamic characteristics of the selected 120 subjects (41 males and 79 females) are summarised in Table 3-3.

Table 3-3. Age, BMI (body mass index), systolic (SBP) and diastolic blood pressures (DBP) from 120 BRIGHT controls

Variables	Mean	Standard Deviation
Age, years	59	7.14
BMI, kg/m^2	24.78	2.93
SBP, mmHg	118.73	8.81
DBP, mmHg	72.74	6.34

A summary of the results for plasma aldosterone concentration, PRA and PRC (CLIA) with the corresponding ARR of the previously mentioned patients is presented in Table 3-4.

Table 3-4. Summary of aldosterone and renin measurements in 120 British normotensives. Subjects with aldosterone <70 pmol/L were considered as 70 pmol/L and PRA <0.3 ng/ml/h considered as 0.3ng/ml/h.

Variable	Median	Ranges (Q_1-Q_3)
Aldosterone, pmol/L	176.0	100.3-260.8
PRA, ng/ml/h	1.3	0.8-2.0
PRC, μIU/ml	17.4	11.7-23.18
ARR, pmol/L:ng/ml/h Aldo/PRA	127.0	78.5-246.8
ARR ,pmol/L: μIU/ml Aldo/PRC	10.0	6.0-15.8

Comparison of PRA and PRC values (Figure 3-2) gave a correlation coefficient of 0.775 ($p < 0.001$). However, the correlation coefficient decreased markedly to 0.411 ($p=0.006$) when only PRA values below 1.0ng/ml/h were compared with their corresponding PRC values (Figure 3-3). When considering the PRA values below the detection limit (<0.3ng/ml/h) the Pearson correlation was 0.355 ($p=0.027$).

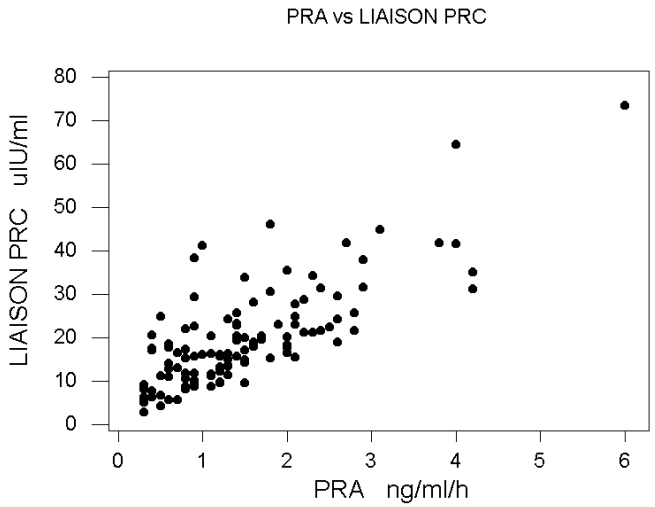


Figure 3-2. Relationship between renin measurements by the PRA and PRC methods (n=120).

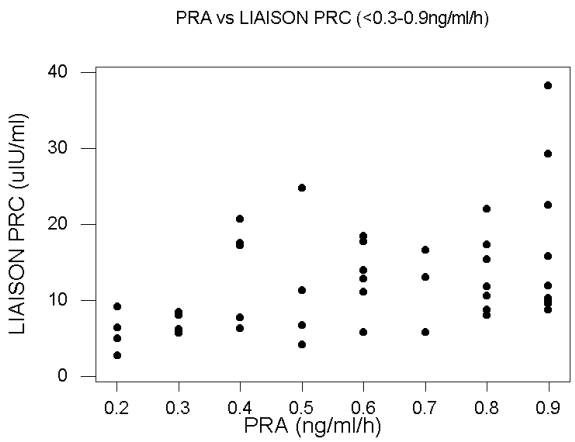


Figure 3-3. Relationship between PRA (<0.3-0.9ng/ml/h) and their corresponding low renin concentration values.

ARR was calculated from either PRA or PRC. The correlation coefficient between ARR values was again determined from 106 samples, giving a value of 0.812 ($p<0.001$). This shows that there is a good relationship between the two ratios (Figure 3-4). Four samples had PRA values below the detectable limit and ten samples had aldosterone values below the detectable limit. When values of undetectable limits were adjusted to equal the respective lowest detectable values (PRA <0.3 to 0.3ng/ml/h and aldosterone <70 for 70 pmol/l), the Pearson correlation increased to 0.854 ($p<0.001$).

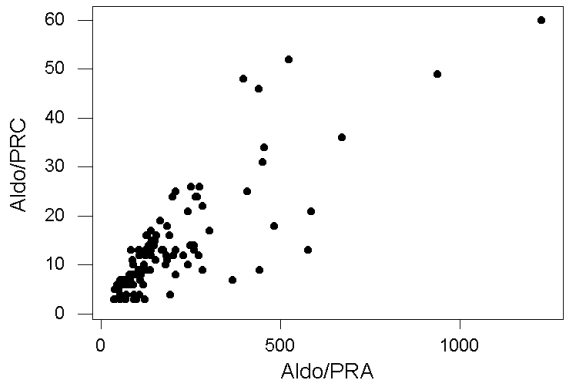


Figure 3-4. Relationship between aldosterone-PRA ratio and aldosterone-PRC method.

The central 95 and 97% intervals that can be used as the reference ranges for each of the measurements are summarized in the Table 3-5.

Table 3-5. Reference range (95 and 97.5 percentiles) for aldosterone, renin and ARR from 120 BRIGHT Study controls, BP < 130/85mmHg, no medication.

Variable	5-95% Reference range	2.5-97.5% Reference range
Aldosterone, pmol/L	<70-404	<70-605
PRA, ng/ml/h	0.3-3.8	<0.3-4.2
PRC, μIU/ml	5.8-41.8	5.0-46.2
ARR, pmol/L:ng/ml/h	50-559	38-759
ARR, pmol/L: μIU/ml	3-48.5	3-64.3

3.5 Discussion

The comparability between the PRA and PRC assays is not direct as their values depend on different variables. The PRC assay is dependent on the amount of renin protein. In contrast, the PRA assay depends on both the concentration of renin and that of its substrate, angiotensinogen. Human angiotensinogen levels are near the Km of renin and any variation in them will therefore affect the reaction rate (Gould & Green 1971). Angiotensinogen levels vary in conditions or pathophysiologies such as pregnancy, adrenal insufficiency, bilateral nephrectomy, variation in thyroid hormones and treatment with ACE inhibitors, glucocorticoids or oestrogens (1993). The advantage of using subjects that were controls for the BRIGHT Study is that those having conditions or pathophysiologies that could affect renin concentration or that of its substrate have been excluded, facilitating the comparison between assays. However, the fact that some of the women might have been taking oestrogen therapy cannot be excluded. This could have led to high renin substrate, low renin concentration, but high renin activity (1993). As data regarding oestrogen treatment are not available, its impact on the results is unknown.

In general, there was a good correlation between the PRA and PRC results, and their derived ARRs (0.775, $p < 0.001$ and 0.812 $p<0.001$, respectively). This is in agreement

with previous studies which had coinciding correlation coefficients using a similar CLIA assay (Nichols Advantage®) (Table 3-6). Studies involving normotensive subjects and patients with different pathophysiologies seem to have slightly higher correlation between types of renin measurements (de Bruin *et al.* 2004; Hartman *et al.* 2004; Olivieri *et al.* 2004; Perschel *et al.* 2004; Unger *et al.* 2004). This may be because the ARR ranges are wider. The cause is still being investigated using the LIAISON® direct renin assay using samples from patients with pathophysiologies affecting renin and aldosterone production.

Table 3-6. Comparison of the correlation between PRA (by RIA), PRC (by CLIA) and ARR in different studies.

Study	Population	Correlation PRA & PRC	Correlation for ARR
BRIGHT Controls	NT (n=120)	r =0.78 (Pearson)	r= 0.81 (Pearson)
Hartman *et al.* 2004 (Hartman *et al.* 2004)	NT & HTN (n=110)	r=0.98	NA
Perschel *et al.* 2004 (Perschel *et al.* 2004)	NT (n=76) PA(n=28)	r=0.72	r=0.73
de Bruin *et al.* 2004 (de Bruin *et al.* 2004)	NT (n=80) PA (n=3) CAH,HF,DM, OCP,PREECL, PREGN,RAS (n=19) Total n=102	r=0.88 (rank-Spearman)	NA
Olivieri *et al.* 2004 (Olivieri *et al.* 2004)	HTN (n=120)	r=0.87	NA
Unger *et al.* 2004 (Unger *et al.* 2004)	NT, HTN and adrenal mass (n=83)	NA	r=0.91

HTN: hypertension; PA: primary aldosteronism; CAH: congenital adrenal hyperplasia; HF:heart failure; DM: diabetes mellitus; insulin dependent; OCP: woman on oral contraceptives; PREECL: preeclamptic women; PREGN: pregnant women; RAS:renal artery stenosis.

The main concern with the CLIA PRC method is the relatively poor correlation with PRA at low concentrations (<5μIU/ml). This could be due to technical performance but could also be because the two approaches measure different aspects of the system and are therefore likely to be affected by different factors. The poor correlation at low concentrations might be a drawback of the PRC method for a clinical setting, as this is the crucial range required to determine the diagnostic reliability of the ARR for PA. Simultaneous to the work of this thesis, a study was undertaken to evaluate the clinical use of the PRC assay for screening PA. The same patient group of hypertensive subjects sent for investigation of possible PA was identified despite concerns of sensitivity and

specificity in this new method. As a result of this comparison with the routinely used PRA assay, the initial screening of renin levels within the clinical practice of North Glasgow NHS trust has changed. The novel PRC assay is used in the screening test, and low renin levels are further confirmed with the PRA assay (Personal communication with Prof. Michael Wallace, Department of Clinical Biochemistry, Glasgow Royal Infirmary, UK).

It was also in the parallel studies to this thesis in which the clinical validation of the PRC assay as part of the ARR in a screening test was done using the receiver operator characteristic (ROC) curves (Dorrian *et al.* 2009). The correlation coefficient used in this chapter is only an indicator of the strength of linear relationship between two variables, in this case PRC and PRA. To further validate a quantitative test, such as the PRC assay, alternative more robust methods can be used. Taking into account the PRA assay is the gold standard test, the methods available are the following. In one method, two hypotheses are tested considering the equation $y = a + bx$ (Barnett 1969). The first hypothesis tested is that b is statistically significantly larger than zero, and the second is that the a-value = 0.000 and b-value = 1.000. If this second hypothesis cannot be confirmed three other criteria for validation can be considered: a squared correlation-coefficient r^2 or intraclass correlation of >95%, or a relative residual variance of <5%. When the new test is validated with one of this criterion the equation $y = a + bx$ is used to calculate the predicted control-test values (Cleophas *et al.* 2009).

The correlation between the two assays might also be improved by considering not only middle-aged and elderly normotensive patients, but also children, young adults and patients with selected diagnoses and treatments known to affect renin values. This would give a better picture of the correlation between methods in the whole population. However, the eventual purpose of this study is to investigate renin dynamics in an essential hypertension cohort where renin levels can be low.

In the present study, the analytical performance of the LIAISON® Direct renin assay is in general acceptable, especially in the higher PRC range (intra-assay CV 1.3% and inter-assay CV 3.2%). Technical performance was also monitored with quality controls (3 for the PRA and 2 for the PRC assay). All of them were in the expected range.

Sensitivity of the methods can be affected by incubation time, affinity of the antibodies used, their respective labelled standards and the way these labels are detected. The immunoassay incubation time varies considerably between the two methods (18h in PRA

assay and ~30min in PRC assay). RIA depends on competitive binding achieving equilibrium before the separation of bound and free fraction. This takes longer in the PRA assay than in the CLIA assay where binding is non-competitive and all the renin in the samples is sandwiched between two antibodies during that incubation time. Hartman *et. al.* reported a decrease in correlation between the PRA and PRC assay (Nichols Advantage System) from 0.98 to 0.77 when comparing the overall correlation in samples with a wide range of PRA levels with samples with low PRAs (<0.65ng/ml/h, n=25), despite leaving these low PRA samples incubating for longer (Hartman *et al.* 2004). This suggests that although a longer incubation increases the sensitivity of the PRA assay, it does not improve the correlation between the two assays. However, when renin levels are low the functional sensitivity of the PRA assay is greater.

The labels used in each assay were also different, a radioisotope in the PRA and isoluminol in PRC. Both the radioactive (^{125}I) and the chemiluminescent (isoluminol) labels can be detected at low levels, but the chemiluminescence offers several advantages (Baeyens *et al.* 1998; Sealey *et al.* 2005). A chemiluminescent label can be detected more sensitively than ^{125}I (Butt 1984). It is more stable and is easier to store and dispose of. Automation is simpler, resulting in more convenience and higher precision. The ^{125}I used in PRA has a half-life of 60.14 days. Therefore, the level of radioactivity available decreases with time, affecting sensitivity and precision unless longer counting times are used. To minimise effects of this decay, the PRA assay kits were used over a short period of time (15 days) and the assay was done in duplicate for each sample. It is also important to mention that, by using % relative binding in all the PRA assays and quality controls, major drifts were not detected. Nevertheless, variations in the precision of the techniques due to using different labels might affect performance at lower renin levels.

Sensitivity is also limited by the ability to monitor the uptake of labelled antibody. Derivatives for isoluminol used as labels for immunoassays match the practical sensitivity of ^{125}I (10^{-18}) in radioimmunoassays and have comparable performances. Radioactive label quantitation is unaffected by environment, being a potential advantage over chemiluminescent labels which can be affected by non-specific luminescence of constituents of biological fluids (Woodhead & Weeks 1985). Nevertheless, this problem has been avoided in the PRC assay by using solid phase antibodies and washing the interfering substances before the light signal is generated and detected.

The specificity of the assays depends on the specificity of the antibodies. In this case, the antibodies used were different as they were targeting different substances, AngI in PRA assay and renin in PRC assay. Substances that could interfere with AngI binding are angiotensinases and peptidases present in the sample (1993). In this study, AngI was protected from these by trapping it with the antibody, incubating and diluting with tracer. Therefore, falsely low values due to the interaction with the substances mentioned above would not be expected in this assay.

Immunoreactive, endogenous AngI and other non-specific substances can also alter the final values of AngI. To detect them, Sealey recommends the use of a blank sample (Sealey 1991) and this was included in the PRA assay. Each sample was divided into four aliquots, two of which were incubated at 37°C to generate AngI, and two were incubated at 4°C as blanks. From the final results obtained from the aliquots incubated at 37°C the corresponding blanks were subtracted to eliminate errors nonspecific interference. No corrections for blanks were made in the PRC assay results.

The specificity of PRC measurements can be affected by prorenin. Prorenin circulates in human plasma in higher concentrations than renin, usually 10 fold in normotensive subjects. Cross-reactivity with prorenin has already been reported by Derkx *et al.* who stated that ~2% of PRC measured in normal plasma by IRMA could be attributed to prorenin (Derkx *et al.* 1996). In contrast, Simon *et al.*, using the same methodology but different antibodies, did not experience problems from prorenin interference (Simon *et al.* 1992). The LIAISON® assay also uses two monoclonal antibodies, one for renin and prorenin and another specific for renin. The affinity parameters are a commercial secret. Although the manufacturer cites cross-reactivity levels, their method of obtaining them has not been made public. The possibility of prorenin binding to the specific renin antibody exists, especially at low renin levels. Further experimental work with the LIAISON® Direct renin assay including prorenin measurements will be helpful in evaluating its performance at detecting PRC.

Another problem encountered in the measurement of renin is the cryoactivation of prorenin at low temperatures. In this study, the samples were handled appropriately to avoid cryoactivation *in vitro,* preventing false high values from this source.

Another way to evaluate the performance in the PRC assay is by comparing the reference ranges obtained in this study with those reported by others.

The PRC reference ranges for the BRIGHT controls cohort are similar to those reported by de Bruin *et al.* (de Bruin *et al.* 2004) and Newton-Cheh *et al.*(Newton-Cheh *et al.* 2007) also using a CLIA assay (Table 3-7). The range reported by de Bruin is broader, perhaps because only males were included and the age range was wider than ours. Comparing the BRIGHT and Newton-Cheh ranges, the former population has a broader range, probably because our samples were taken with the subjects in a seated position at different times of the day whereas the samples of Newton-Cheh study were taken in the morning with the subjects in a supine position.

Table 3-7. Comparison of 95% and 97.5% reference ranges for PRC in different studies.

Study	*Population*	*Central 95% interval*	*Central 97.5%*
BRIGHT Controls	NT male and female (n=120)	5.8-41.8 µIU/ml	5.0-46.2 µIU/ml
de Bruin *et al.* 2004(de Bruin *et al.* 2004)	NT males (n=80)	6-85.5 µIU/ml	
Newton-Cheh *et al.* 2007(Newton-Cheh *et al.* 2007)	NT males (n=760) NT females (n=1013)		9.0-22.5 µIU/ml 7.0-17.0 µIU/ml

The LIAISON® Direct renin assay can be calibrated against an international renin standard. This calibration can also be included to improve precision monitoring.

To choose the best method for population studies, practical issues as well as performance criteria should be considered. LIAISON® offers several advantages over the traditional PRA assay. Its automation allows the efficient processing of more samples at a lower cost and in less time than the PRA assay. Storage and handling processes can improve considerably as the label does not decay. PRC can be more informative of renin itself in patients with fluctuating levels of angiotensinogen (i.e. cardiac failure) (Tsutamoto *et al.* 2007). In large cohorts, the precision of the method can be more important than its sensitivity because a high precision allows distinction of small real differences with greater certainty, making the PRC assay a good choice. The only important restriction LIAISON® presents at the moment is where subjects are expected to have low PRC values.

3.6 Conclusion

The practical advantages of the LIAISON® Direct renin assay make it a better option to measure renin levels in population studies. In general, there is a good correlation between assays. The only limitation of the PRC assay is a decrease in sensitivity at lower concentrations.

Further investigations related with prorenin interference are necessary to see if detection of renin at lower levels can be improved.

Studies including patients with various pathophysiologies will help to decide if the PRC assay is also a better candidate in clinical practice.

4 Phenotypic and genotypic associations of the aldosterone renin ratio (ARR)

4.1 Introduction

The ratio of aldosterone to renin provides a useful marker to screen patients for inappropriate aldosterone production in Primary aldosteronism (PA). Over the past few years, the interest to expand its use from only a screening test for PA to perhaps a test which can help to estimate blood pressure progression, development of hypertension, cardiovascular risk and therapeutic intervention has been increasing (Gradman *et al.* 2005; Himmelmann *et al.* 1996; Jansen *et al.* 2009; Newton-Cheh *et al.* 2007; O'Brien *et al.* 2007; Sever *et al.* 2009).

The reproducibility of the plasma renin activity measurements has hampered the use of ARR in population studies. For this reason a new assay which quantifies renin concentration was validated successfully for this purpose (Chapter 3).

In addition to clinical applications, this intermediate phenotype can also help elucidate functional genetic variants which contribute to inappropriate aldosterone production and the predisposition of hypertension. The investigators of the Framingham Study reported ARR was heritable and had modest linkage to chromosome 11p but had no association with common variants of the gene encoding for renin *(REN)* (Newton-Cheh *et al.* 2007), thus, suggesting the influence of other genetic variants on the ratio and its components. Findings in our group and others have demonstrated association between increased ARR and polymorphisms in the *CYP11B2* gene as described in section 2.3.1 (Lim *et al.* 2002b; Nicod *et al.* 2003), and suggest an association of the ratio with the TConvGTA haplotype in the *CYP11B1/CYP11B2* locus (Ganapathipillai *et al.* 2005). Therefore, it was of interest to test if variations in the *CYP11B1* and *CYP11B2* genes were also associated with measurements of renin, aldosterone and/ or ARR in plasma.

A complex inter-relationship may exist between the components of the ratio. Moreover, physiological, environmental, familial and genetic factors can contribute to their short and long term regulation (Connell & Davies 2005; Connell *et al.* 2008; Montori & Young, Jr. 2002). However, a better understanding of genetic and nongenetic contributors of the ARR and their interactions would improve the interpretation and use of the ratio. Substantial uncertainties remain over the way in which the ratio relates to cardiovascular and steroid phenotypes across the entire physiological range, how familial factors affect the ratio and its components and how they are associated with variations in the *CYP11B1/CYP11B2*

locus. To address these uncertainties a large family study of adults in the United Kingdom was examined.

4.2 Aims

The aims of this study were to examine the correlation between the individual components of the ARR, and the ratio itself with a range of phenotypes, as well as to examine the heritability of renin, aldosterone and their ratio. Finally, markers at the *CYP11B1/CYP11B2* locus were tested for association with renin, aldosterone and ARR in Caucasian families.

4.3 Subjects and Method

4.3.1 Subjects

Oxford Study

The collection strategy of this family study has been described elsewhere (Baker *et al.* 2005). British white families, recruited between 1993-1996, were ascertained through a proband diagnosed with essential hypertension. Proband eligibility required: a mean systolic blood pressure (SBP) >140 mmHg and mean diastolic blood pressure (DBP) >90 mmHg on daytime ambulatory blood pressure monitoring; or more than three office blood pressure readings >160mmHg systolic and >95mmHg diastolic; or current treatment with two or more antihypertensive drugs. Secondary hypertension was excluded using a standard screening protocol applied in the hypertension clinic.

Probands were recruited from the blood pressure clinic at the John Radcliffe Hospital, Oxford, UK. UK resident family members were recruited wherever this was feasible in terms of distance from the research centre; most family members recruited into the study lived within 50 miles of the research centre. All probands and the family members were UK residents of self-reported white ethnicity.

In order to be included in the study, families were required to consist of at least three siblings clinically assessable for blood pressure, with at least one parent available to give blood for DNA analysis; or at least four assessable siblings if no parent was available. The majority of the individuals in the family collection had blood pressures within the

conventionally accepted “normal range”, and the family collection includes some extended families, although most are nuclear. For the purpose of this study, 1425 subjects from 248 families were recruited. The median family size was 5 people, 60% of families comprising between 4 and 6 genotyped and phenotyped members. 71% of families were 2-generation and 29% were 3-generation. 84% of families had an assessable sibship in the generation of the proband, while 16% of families consisted of a proband and their nuclear family (spouse and children over 18 years) only. From the recruited individuals only 1172 subjects had measurements of aldosterone, renin and ARR (464 hypertensives and 708 normotensives).

Participants were visited in their homes by research nurses. Blood pressure was measured for a period of 24 hours in all subjects willing to undergo ambulatory monitoring, using the A&D TM2421 device according to a previously described protocol (Gaukrodger *et al.* 2005). Anthropometric measurements including height, weight and waist and hip circumferences were performed. Body mass index (BMI) was calculated with the formula weight (in kilograms)/height (in square meters). Blood was drawn with the participants sitting comfortably for at least five minutes. The time of day at which blood was drawn was not standardized, although the majority of visits took place in the early evening. DNA was extracted from blood samples using standard methods. Blood samples for analysis of renin and aldosterone levels were taken into EDTA and lithium heparin tubes. To avoid cryo-activation of renin, plasma separation was performed within an hour of the blood draw. Plasma was frozen at -80°C immediately following separation.

4.3.2 Renin measurements

Renin was measured as plasma renin concentration (PRC) in EDTA plasma samples by Mr. Jim Smith. In this assay renin protein was measured using a new semi-automated chemiluminescent immunoassay (CLIA) described in section 2.1.2.

4.3.3 Aldosterone measurements

Aldosterone was measured in lithium-heparin samples by Ms. Morvern Campbell using a solid phase (coated tube) radioimmunoassay technique as detailed in section 2.2.1.

4.3.4 Genotyping

Eight SNPs were genotyped by Keavney and colleagues and had previously been described (Keavney *et al.* 2005). The bi-allelic intron conversion (IC) polymorphism located in the second intron of the *CYP11B2* gene was genotyped using PCR and restriction enzyme digestion. Five other SNPs within the *CYP11B2* were genotyped using PCR and restriction enzyme digestion as described in the same paper: rs1799998 in the promoter region (also known as -344T/C or SF-1), rs4539 in the third exon, rs4538 in the sixth exon, rs28930074 in the seventh exon, and rs3097 in the 3' untranslated region. Three SNPs within the *CYP11B1* were also genotyped using the same method: rs6410 in the first exon, rs6387 in the third intron, and rs5316 in the eighth exon. In addition, two further SNPs within the *CYP11B1* promoter region, rs4313136 and rs4471016 (also known as -1889G/T and -1859A/G respectively), were genotyped by Mrs. Elaine Freel as described in section 2.3.1.

Genotyping was carried out blinded to the phenotypic information, and controls of known genotype were included in each genotyping run. Mendelian inheritance within families was confirmed using the PedCheck program (O'Connell & Weeks 1998). Ten percent of the samples were genotyped in duplicate, with an estimated genotyping error rate of less than 1%.

4.3.5 Statistical Analyses

In Table 1, significance was tested using a two-sample-t-test or ANOVA for normally distributed variables and Kruskal-Wallis for the remainder. Categorical variables were compared with Pearson's chi-square test. Renin, aldosterone and ARR measurements were logarithmically transformed prior to analysis, unless specifically mentioned. In addition, there were cases in which the measurements were further adjusted for covariates such as age, gender and BMI. These residuals are denoted by the word "adjusted" (i.e adjusted ARR = adjusted logarithmically transformed ARR).

The distributions of aldosterone, renin and ARR were evaluated based on hypertensive status and use of the major classes of antihypertensive agents. We used stepwise multivariable regression to evaluate the clinical correlates of ARR, aldosterone and renin. All analyses were performed with SPSS for Windows version 13 (Chicago, IL). The main covariates used, based on stepwise regression, were: age, gender, BMI, daytime systolic

blood pressure adjusted for treatment, potassium, beta-blocker and ACE inhibitor use (for the evaluation of the relation of renin, aldosterone and ARR).

Antihypertensive treatment status was adjusted for in the analysis of quantitative blood pressure data by either including treatment status in the regression model or by adding 10/5 mmHg to the on-treatment blood pressure measurements, using methods adopted in other studies (Newton-Cheh *et al.* 2009a; Tobin *et al.* 2005). Both methods did not substantially alter the final results in the analysis. Quantitative variables were compared across groups using ANOVA, and categorical variables using the Pearson's chi-square test. Differences in proportion of hypertensive and normotensive subjects across deciles of ARR were tested using chi Square test and exact p values determined using 100,000 Montecarlo simulations. Gender comparisons of normotensives and hypertensives using different ARR cut-offs were done using the Pearson's chi-square test. Heritability was calculated using variance component method as implemented in Merlin (Abecasis *et al.* 2002) using residual components after correction for confounding factors as described above. Association analysis was performed using Plink (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell *et al.* 2007).

To perform family based association of quantitative traits, we used the quantitative family based association test (QFAM) procedure in Plink which performs a simple linear regression of phenotype on genotype and uses a permutation procedure to correct for family structure. For this analysis the p-values presented are the empirical p values after 100,000 permutations.

The study received ethical clearance from the appropriate review committees, and corresponded with the principles of the Declaration of Helsinki. All participants gave informed consent to participate in the study.

4.4 Results

Oxford Study

Table 4-1. Demographic, phenotypic and biochemical baseline characteristics

	NT	HTN	*P*
Number of subjects	708	464	
M:F	0.90	0.98	0.51
Age at enrolment	42.0 (13.4)	55.6 (10.5)	<0.0001
BMI, kg/m^2	25.5 (4.0)	26.9 (4.1)	<0.0001
Potassium, mmol/L	3.8 (0.4)	3.8 (0.4)	0.67
Waist, cm	86.0 (12.8)	90.8 (12.1)	<0.0001
Day SBP	126.6 (12.2)	155.6 (13.6)	<0.0001
Day DBP	75.7 (7.3)	97.8 (6.8)	<0.0001
Day MBP	92.5 (8.1)	116.9 (7.7)	<0.0001
Night SBP	110.5 (12.4)	132.2 (15.8)	<0.0001
Night DBP	64.5 (7.5)	82.1 (10.6)	<0.0001
Night MBP	79.6 (8.2)	98.6 (11.5)	<0.0001
Aldosterone, ng/L	75.6[24-662]	82.3[25-621]	0.06
PRC, mIU/L	19.3[1.2-126.4]	18.8[0.5-2015]	0.31
ARR, ng/L per mIU/L	4.1[0.4-94.3]	4.6[0.04-253.2]	0.42
On any antihypertensive	11	330	
On diuretics	5	153	
On beta blockers	5	145	
On ACE inhibitors	0	134	
On calcium antagonists	1	84	
Premenopausal on OCP, %	9.4	0.7	0.02
Premenopausal no OCP, %	22.0	5.5	
Postmenopausal on HRT, %	4.5	7.1	0.16
Postmenopausal no HRT, %	15.9	17.4	
Adjusted ARR Quartiles			
Q1: ≤ -0.63	164(57.7%)	120(42.3%)	
Q2: -0.62 to -0.0046	196(67.1%)	96(32.9%)	
Q3: -0.0045 to 0.58	181(60.5%)	118(39.5%)	
Q4: 0.57+	167(56.2%)	130(43.8%)	

Nonhypertensive participants: NT; hypertensive participants: HTN. Values are reported as mean (SD) for normally distributed traits, and median with range for non-normally distributed values (aldosterone, PRC and ARR). M:F, male to female ratio; BMI, body mass index; % of women in the study taking or not OCP, oral contraceptives or HRT, hormone replacement therapy. SBP: systolic blood pressure; DBP: diastolic blood pressure and MBP: mean blood pressure; PRC: plasma renin concentration. Adjusted ARR quartiles refer to the residuals of ARR adjusted for age, gender, BMI, ACE inhibitors and beta blockers.

From the 1425 subjects recruited, 708 normotensives and 464 hypertensives were evaluated, with an equal proportion of sexes in both groups. Details of the population studied are in Table 4-1. The subjects were middle-aged to elderly and of Caucasian ancestry. Hypertensive subjects had slightly higher BMI and waist circumference and were older than normotensives (56 years and 42 years respectively). This difference may be attributed to the ascertainment of the families as more hypertensives were in the parental than in the offspring generation. The ascertainment also influenced the differences in blood

pressure measurements found between the two groups. No other significant differences were found in the remaining variables. The proportion of women, in a known pre- or post-menopausal state, who were on hormonal therapy is also shown on this table. As the numbers were small, this variable was not considered for further analysis.

Biochemical measurements

Aldosterone measurements in normotensives ranged from 24-662 ng/L, median 76 ng/L (67-1835 pmol/L; median 209 pmol/L) and 25 – 621 ng/L, median 82 ng/L (70 to 1719 pmol/L; median 229 pmol/L) in hypertensives. Measurements were not normally distributed and were logarithmically transformed for further analysis. Renin concentration measurements ranged from 1.2 to 126 mIU/L in normotensives and 0.5 to 2015 mIU/L in hypertensives with respective medians of 19.3 and 18.8 mIU/L. The median ARR in normotensive subjects was 4.1 ng/L per mIU/L (range 0.4 - 94.3) and in hypertensive subjects was 4.6 ng/L per mIU/L (0.04 – 253.2). Like the aldosterone measurements, renin concentration and ARR were not normally distributed and were also logarithmically transformed for further analysis.

Phenotypic, biochemical and therapeutic interactions

A multivariate regression approach was undertaken to determine the amount of variation in logarithmic ARR and its components using significant covariates. The results of the regression analysis for each dependent variable are shown in Table 4-2 and Table 4-3. Regression coefficients were considered to be significantly different from zero at $p<0.05$. In Table 4-2 the 1172 hypertensive and normotensive subjects were considered. The logarithmic ARR was positively related to female gender, age, daytime systolic blood pressure adjusted for treatment, potassium and beta blocker use, and inversely related to BMI and ACE inhibitor use. For logarithmic aldosterone, female gender, potassium, diuretic use and calcium channel blocker use were positively associated, while age and ACE inhibitor use showed negative correlations. The logarithmic renin was positively related to BMI, diuretic use and ACE inhibitor use, and there was an inverse association with female gender, age, daytime systolic blood pressure and beta blocker use. In Table 4-3 only the untreated subjects were considered for the analysis. A positive association was found between logarithmic ARR and female gender, age, daytime systolic blood pressure and potassium, and a negative association with BMI. Logarithmic aldosterone showed a negative association with age and a positive association with female gender and potassium.

Finally, the logarithmic renin showed an inverse association with most of the covariates (female gender, age, daytime systolic blood pressure and potassium) and only a positive association with BMI.

We next examined the proportion of the normotensive and hypertensive subjects above given levels of the unadjusted ARR; these data are shown in Table 4-4. The figures are cumulative; thus, the proportion of subjects shown at each cut-off level represents all subjects above this level. More than 90% of normotensive males had an ARR value of ≤ 10 ng/L per mIU/L, while for females, 80% of normotensive subjects fell into this category ($p< 0.001$ for gender difference). Above this value of ARR, at each cut-off studied, proportionally more of the hypertensive than normotensive subjects were represented. In absolute terms, 11.7% of the male hypertensive subjects and 17% of the female hypertensive subjects had an ARR value >15ng/L per mIU/L compared with 3.8% and 10.1% of normotensive male and female subjects respectively (< 0.01 for difference between genders and comparing normotensive and hypertensive groups); at a cut off of >25ng/L per mIU/L the figures for hypertensive subjects were 4.8% (male) and 8.9% (female) and for normotensive subjects 1.2% (male) and 3.7% (female) (gender and pressure status comparisons all significant at $p<0.05$).

Cardiovascular and steroid interactions

Only a small proportion of the subjects with aldosterone, renin and ARR measurements also had measurements for cardiovascular and steroid phenotypes such as left ventricular mass and plasma cortisol. However, a multivariate approach was also undertaken as shown in Table 4-5. The same criteria of significance to regression coefficients were applied. The logarithmic ARR was only positively related to cortisol (F) in plasma and there was no significant relation with left ventricular mass, deoxycortisol (S) or deoxycortisol to cortisol ratio (S:F). Similarly, the logarithmic aldosterone was only positively related to cortisol (F) but not to any of the other covariates. There was no significant associations with logarithmic renin.

Distribution of ARR

The distributions in normotensive and hypertensive subjects of the residuals of the logarithmically transformed ARR (adjusted for age, gender, BMI, ACE inhibitors and beta blockers use), as well as the proportion of subjects by deciles of logarithmically transformed and adjusted ARR, stratified according to hypertension status, are shown in Figure 4-1. The adjusted ARR measurements in normotensive and hypertensive subjects did not show any significant difference by t-test for equality of means. As expected, the distribution of hypertensive and normotensive subjects by deciles of ARR was Gaussian. The proportion of hypertensives compared with normotensives in deciles of ARR was significantly different, with an increasing proportion of hypertensive subjects from the 8th decile of ARR upwards, (exact $p<0.001$ after 100,000 Montecarlo simulations). This is illustrated in Figure 4-1C, which shows that in the lower 4 deciles of the ARR the proportion of hypertensive and normotensive subjects was similar, there were a greater proportion of normotensive subjects than hypertensives in deciles 5-7, but an increasing proportion of the subjects in deciles 8-10 were hypertensive. However, it should be noted from Figure 4-1 B that the absolute numbers in these top deciles were low.

Table 4-2. Multivariate regression of logarithmic ARR, aldosterone and PRC using only significant covariates at p<0.05 in all subjects with known hypertension status (n=1172)

	ARR (Model R^2=0.181)				Aldosterone (Model R^2=0.068)				PRC (R^2=0.187)			
Covariate	**Partial R^2**	**β(SE)**	**95% CI**	***p***	**Partial R^2**	**β(SE)**	**95% CI**	***p***	**Partial R^2**	**β(SE)**	**95% CI**	***p***
Sex	0.235	0.43(0.06)	1.37-1.74	<0.0001	0.127	0.17(0.04)	1.1-1.28	<0.0001	-0.160	-0.26(0.05)	0.7-0.86	<0.0001
Age at enrolment,y	0.087	0.01(0.002)	1-1.01	0.0126	-0.058	-0.003(0.001)	0.99-1	0.0381	-0.163	-0.01(0.002)	0.99-0.99	<0.0001
Daytime SBP adj treatment	0.107	0.01(0.002)	1-1.01	0.0017				n.s.	-0.108	-0.01(0.002)	0.99-1	0.0013
BMI	-0.124	-0.03(0.01)	0.96-0.99	0.0003				n.s.	0.138	0.03(0.01)	1.01-1.04	<0.0001
Potassium	0.125	1.2(0.32)	1.77-6.29	0.0002	0.109	0.76(0.2)	1.45-3.16	0.0001	-0.063	-0.55(0.28)	0.33-1.01	0.0528
Diuretic use				n.s.	0.216	0.46(0.06)	1.42-1.78	<0.0001	0.087	0.37(0.15)	1.09-1.94	0.0110
Beta-blocker use	0.090	0.43(0.16)	1.11-2.11	0.0092				n.s.	-0.129	-0.55(0.14)	0.44-0.76	0.0001
ACE inhibitor use	-0.344	-1.52(0.14)	0.17-0.29	<0.0001	-0.091	-0.2(0.06)	0.73-0.92	0.0011	0.323	1.31(0.13)	2.88-4.79	<0.0001
Calcium channel blocker use				n.s.	0.060	0.15(0.07)	1.01-1.34	0.0325				n.s.

Table 4-3. Multivariate regression of logarithmic ARR, aldosterone and renin in individuals without antihypertensive treatment (n=831)

	ARR (Model R^2=0.115)			Aldosterone (Model R^2=0.032)			PRC (Model R^2=0.094)		
Covariate	**β(SE)**	**95% CI**	***p***	**β(SE)**	**95% CI**	***p***	**β(SE)**	**95% CI**	***p***
Sex	0.48(0.06)	1.44-1.83	<0.0001	0.2(0.04)	1.12-1.34	<0.0001	-0.28(0.05)	0.68-0.84	<0.0001
Age at enrolment,y	0.01(0.002)	1-1.01	0.0184	-0.003(0.002)	0.99-1	0.0497	-0.01(0.002)	0.99-0.99	<0.0001
Daytime SBP adj treatment	0.01(0.002)	1-1.01	0.0010			n.s.	-0.01(0.002)	0.99-1	0.0022
BMI	-0.03(0.01)	0.96-0.99	0.0002			n.s.	0.03(0.01)	1.01-1.04	<0.0001
Potassium	1.52(0.32)	2.41-8.6	<0.0001	0.86(0.24)	1.46-3.82	0.0005	-0.84(0.28)	0.25-0.75	0.0028

In both models, SE: standard error; 95% CI:95% confidence interval of beta coefficient

Table 4-4. Gender comparisons of normotensives and hypertensives at different cut-offs of unadjusted ARR. Note that the data shown at each cut-off level are cumulative (in other words, 3.8% of normotensive males had an ARR> 15ng/L per mIU/L)

		Male		Female	
		NT	HTN	NT	HTN
ARR	≤10	309(91.7%)	184(80%)	292(78.7%)	176(75.2%)
	>10	28(8.3%)‡	46(20%)	79(21.3%)‡	58(24.8%)
ARR>15		13(3.8%)†	27(11.7%)	38(10.1%)†	40(17%)
ARR>20		5(1.5%)*	17(7.4%)	18(4.8%)*	25(10.6%)
ARR>25		4(1.2%)*	11(4.8%)	14(3.7%)*	21(8.9%)
ARR>30		2(0.6%)	8(3.5%)	8(2.1%)	18(7.7%)
ARR>35		2(0.6%)	8(3.5%)	6(1.6%)	15(6.4%)

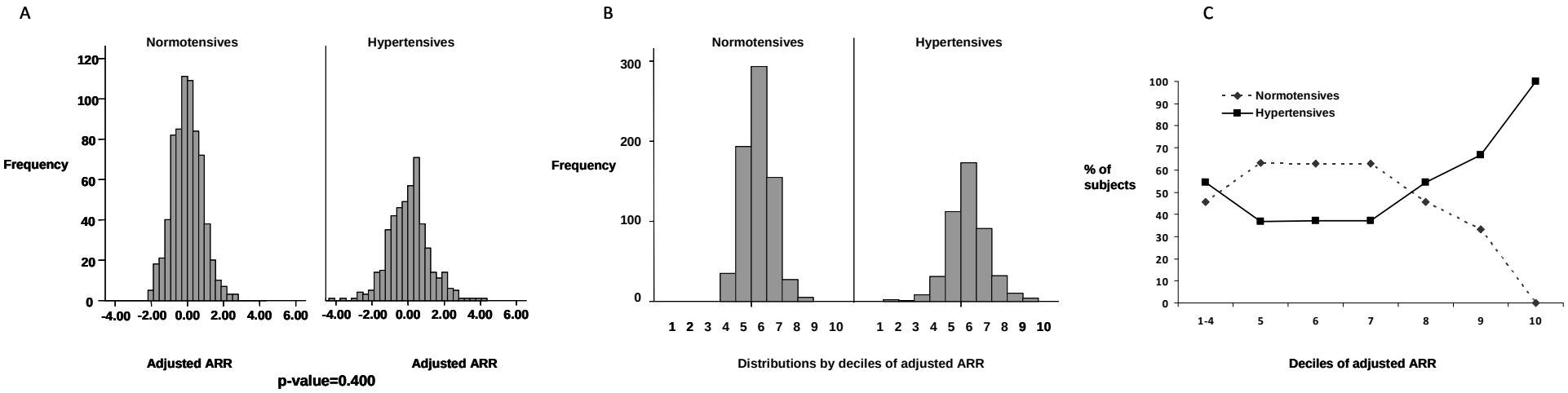
ARR in ng/L per mIU/L. Nonhypertensive participants: NT; hypertensive participants: HTN.
Each group of either hypertensives or normotensives were compared against the remaining. *p<0.05 †<0.01 ‡p<0.001

Table 4-5. Multivariate regression of logarithmic ARR, aldosterone and PRC using cardiovascular and steroid covariates in all subjects.

	Adjusted ARR						Adjusted Aldosterone					Adjusted PRC				
	n	β(SE)	Fold Change	95% CI	*p*	R^2	Beta(SE)	Fold Change	95% CI	*p*	R^2	Beta(SE)	Fold Change	95% CI	*p*	R^2
LVMass	663	0.22(0.12)	1.24	0.98-1.58	0.0788	0.005	-0.1(0.09)	0.90	0.76-1.08	0.2756	0.002	-0.17(0.11)	0.84	0.67-1.05	0.1228	0.0230
F	478	0.23(0.07)	1.26	1.09-1.44	0.0015	0.021	0.26(0.05)	1.29	1.17-1.43	<0.0001	0.054	0.08(0.06)	1.09	0.96-1.24	0.1948	0.0080
S	426	0.05(0.05)	1.05	0.96-1.15	0.2473	0.003	0.04(0.03)	1.04	0.97-1.1	0.2496	0.003	0.01(0.04)	1.04	1.1-1	0.7271	0.0070
S:F	426	-0.02(0.05)	0.98	0.9-1.07	0.6855	0.0004	-0.04(0.03)	0.96	0.9-1.02	0.2150	0.004	-0.01(0.04)	0.99	0.91-1.07	0.8138	0.0070

LVMass: Left ventricular mass in grams adjusted for BMI and blood pressure; F: cortisol in μg/dl, S: deoxycortisol in ng/dl; S:F: deoxycortisol to cortisol ratio. All dependent variables were log transformed for the analysis.

Figure 4-1. Distribution of logarithmically transformed ARR (adjusted for age, gender, BMI, ACE inhibitors and beta blockers use) by hypertensive status.



Panel A shows the distribution of logarithmically transformed ARR (adjusted ARR) measurements in normotensive and hypertensive subjects (No significant difference by t-test for equality of means).

Panels B and C show the distribution of hypertensive and normotensive subjects by deciles of logarithmically transformed ARR (adjusted ARR). There is an increasing proportion of hypertensive subjects from the 8th decile of ARR upwards, (difference exact $p<0.001$ after Montecarlo simulations).

Blood pressure

The relationships between adjusted measurements of aldosterone, PRC or the ARR (adjusted for BMI, gender, age and potassium and treatment) and ambulatory blood pressure measurements corrected for treatment are summarised in Table 4-6. There were no significant differences in blood pressure measurements across the quartiles of aldosterone, PRC or the ARR in this analysis. The relationships among the corrected biochemical variables and blood pressure were then examined using data from subjects not on antihypertensive therapy. These data are shown in Table 4-7; blood pressure measurements did not vary across the aldosterone quartiles. However, there were significant linear relationships for systolic, diastolic and mean blood pressure measurements (both day and night) with PRC and the ARR such that high PRC levels were associated with the lowest blood pressure measurements and the high ARR measurements with the highest blood pressures. Thus, the difference in daytime systolic blood pressure between the highest and lowest quartile of adjusted PRC was approximately 7mmHg; for the ARR, the difference between the lowest and highest quartile was 3.7mmHg.

Table 4-6. Daytime and night-time adjusted blood pressure measurements in all subjects according to adjusted aldosterone (A), PRC (B) and ARR quartiles (C).

A	**Adjusted Aldosterone quartiles**				
	Q1	**Q2**	**Q3**	**Q4**	***p***
SBP day	132.0(17.9)	135.5(19.2)	134.3(16.4)	132.1(16.9)	0.551
DBP day	80.4(11.6)	81.7(12.1)	81.1(11.7)	80.3(11.5)	0.707
MBP day	97.4(13.1)	99.5(13.8)	98.7(12.7)	97.4(12.7)	0.621
SBP night	113.5(15.9)	113.3(14.3)	115.6(16.1)	111.9(13.3)	0.728
DBP night	66.9(10.5)	66.3(10.0)	68.0(10.6)	66.5(8.9)	0.304
MBP night	82.3(11.6)	81.8(10.8)	83.7(11.7)	81.5(9.5)	0.428

B	**Adjusted PRC quartiles**				
	Q1	**Q2**	**Q3**	**Q4**	***p***
SBP day	139.5(20.9)	133.1(15.7)	131.7(17.2)	134.3(20.6)	0.092
DBP day	84.9(12.1)	80.5(10.9)	79.4(11.5)	83.3(14.3)	0.372
MBP day	102.9(14.4)	97.8(11.8)	96.7(12.8)	100.1(15.9)	0.197
SBP night	116.6(17.4)	114.2(14.4)	112.3(14.3)	111.6(16.7)	0.056
DBP night	68.4(9.9)	67.4(9.7)	66(9.4)	67.3(13.4)	0.483
MBP night	84.3(12.0)	82.8(10.5)	81.2(10.3)	81.9(14.1)	0.192

C	Adjusted ARR quartiles				
	Q1	Q2	Q3	Q4	*p*
SBP day	132.7(19.3)	132.9(16.6)	133.6(16.6)	135.0(18.4)	0.150
DBP day	80.8(12.8)	80.0(11.5)	81.3(11.7)	81.6(11.1)	0.317
MBP day	98.0(14.4)	97.4(12.5)	98.6(12.7)	99.2(13.0)	0.211
SBP night	113.2(16.6)	112.9(14.3)	113.3(13.6)	115.1(15.7)	0.248
DBP night	66.7(11.5)	66.7(9.4)	66.8(10.1)	67.8(9.2)	0.347
MBP night	82.0(12.5)	81.9(10.3)	82.1(10.5)	83.4(10.7)	0.268

All blood pressure measurements, reported as mean (SD), were adjusted for age, gender, BMI and treatment. Treatment adjustment was performed by including treatment as a regression variable and by adding 10/5 mmHg for treatment. Neither method altered the final result. SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure. Q1-Q4 indicate increasing quartiles of aldosterone adjusted for gender, treatment and plasma potassium; renin and ARR quartiles were adjusted for age, gender, BMI, ACE inhibitor and beta blocker use.

Table 4-7. Daytime and night-time adjusted blood pressure measurements in untreated subjects according to adjusted aldosterone (A), PRC (B) and ARR quartiles (C).

A	Adjusted Aldosterone quartiles					
	Q1	Q2	Q3	Q4	β	*p*
SBP day	128.5(15.2)	131.4(15.1)	131.4(14.1)	129.7(14.9)	-	0.109
DBP day	78.0(9.7)	79.1(9.9)	79.0(10.2)	78.5(10.1)	-	0.272
MBP day	94.7(10.8)	96.3(10.8)	96.3(10.9)	95.4(11.1)	-	0.161
SBP night	112.1(14.3)	112.2(13.1)	114.8(15.1)	111.7(13.2)	-	0.297
DBP night	65.8(9.2)	65.3(8.5)	67.4(9.9)	66.3(8.6)	-	0.046
MBP night	81.1(10.1)	80.8(9.3)	83(10.9)	81.3(9.2)	-	0.084

B	Adjusted PRC quartiles					
	Q1	Q2	Q3	Q4	β	*p*
SBP day	134.8(17.8)	131.1(14.1)	128.6(14.4)	127.7(15.9)	-0.007	0.002
DBP day	82.4(11.1)	79.0(9.7)	77.2(9.5)	78.4(11.4)	-0.009	0.002
MBP day	99.7(12.8)	96.2(10.4)	94.2(10.5)	94.7(12.3)	-0.009	0.001
SBP night	115.9(16.2)	113.7(14.0)	111.3(13.2)	108.7(13.4)	-0.007	0.002
DBP night	68.0(9.2)	67.0(9.2)	65.2(8.5)	64.9(10.8)	-0.008	0.027
MBP night	83.8(11)	82.4(10.0)	80.4(9.2)	79.4(11.0)	-0.009	0.005

C	Adjusted ARR quartiles					
	Q1	Q2	Q3	Q4	β	*p*
SBP day	127.9(15.4)	130.0(14.3)	131.3(14.8)	131.6(15.3)	0.008	0.006
DBP day	77.3(9.9)	77.8(9.6)	79.6(10.3)	79.7(9.9)	0.012	0.003
MBP day	94.0(11.0)	95.0(10.4)	96.7(11.1)	96.8(11.2)	0.012	0.002
SBP night	110.8(14.0)	112.2(13.7)	113.0(13.4)	114.6(15)	0.008	0.013
DBP night	64.8(9.1)	65.9(8.6)	66.5(9.8)	67.5(8.7)	0.013	0.007
MBP night	80.0(9.9)	81.2(9.5)	81.8(10.3)	83(10.1)	0.013	0.005

All blood pressure measurements, reported as mean (SD), were adjusted for age, gender, BMI. SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure. Q1-Q4 indicate increasing quartiles of aldosterone adjusted for gender and plasma potassium; renin and ARR quartiles were adjusted for age, gender, and BMI.

Heritability

Heritability calculations were made after adjustment of aldosterone, renin and the ARR for significant confounders as described above. Heritability of aldosterone was 28.7% ($P < 10^{-8}$) and heritability of renin was 27.4% ($P < 10^{-8}$). For the ARR, there was a significantly greater degree of heritability with an h^2 value of 38.1% ($P < 10^{-8}$).

Associations with the *CYP11B1/CYP11B2* locus

Based on the linkage disequilibrium structure of this region, 11 polymorphisms were genotyped (see Table 4-8). The average genotyping success rate for assays using amplification of large fragments of genomic DNA is consistent with previous studies using these methods. The allele frequencies of SNPs reported in HapMap (rs6410, rs3097 and rs1799998; http://www.hapmap.org/) were similar to published data (Keavney *et al.* 2005). All polymorphisms except rs5316 and rs28930074, had heterozygosities between 41-53%. There was no significant deviation from Hardy-Weinberg equilibrium in any genotype at any polymorphism in the pedigree founders. There was no association between any SNPs in *CYP11B2* or *CYP11B1* and the ARR; however, one polymorphism in *CYP11B2* (in Intron 2) was associated with variation in plasma aldosterone levels with the "conversion" allele (Conv, beta -0.07, p = 0.04) (see Table 4-9).

Table 4-8. Characteristics of polymorphisms genotyped in the *CYP11B1* and *CYP11B2* genes in the Oxford Study

Chr	Gene	Location	SNP	Position bp	Minor allele	% Genotyping success	Genotyping method	Het	HWE p	MAF
8	*CYP11B1*	Exon 8	rs5316	143953420	T	91.44	RED	0.06	1	0.04
8	*CYP11B1*	Intron3	rs6387	143955429	G	86.95	RED	0.47	1	0.43
8	*CYP11B1*	Exon1	rs6410	143958007	A	89.05	RED	0.49	1	0.47
8	*CYP11B1*	Promoter	rs4471016	143960089	C	75.72	PCR-SEQ	0.43	0.5	0.42
8	*CYP11B1*	Promoter	rs4313136	143960119	A	75.72	PCR-SEQ	0.41	0.36	0.4
8	*CYP11B2*	3'UTR	rs3097	143990317	A	97.68	RED	0.44	0.23	0.29
8	*CYP11B2*	Exon 7	rs28930074	143991268	C	96.28	RED	0.28	1	0.15
8	*CYP11B2*	Exon 6	rs4538	143991704	C	96.07	RED	0.47	1	0.44
8	*CYP11B2*	Exon 3	rs4539	143993541	G	87.30	RED	0.53	0.68	0.45
8	*CYP11B2*	Intron 2	IC	143993985	Conv	92.63	RED	0.52	0.84	0.47
8	*CYP11B2*	Promoter	rs1799998	143996602	C	95.09	RED	0.51	0.85	0.45

The allele frequencies were generated using the founders in each family. Chr, chromosome; SNP, single nucleotide polymorphism; Het, heterozygosity; HWE p, p value for Hardy Weinberg equilibrium; MAF, minor allele frequency; IC, intron conversion. RED, restriction enzyme digestion and PCR-SEQ, PCR followed by automated sequencing.

Table 4-9. Association tests for adjusted ARR, aldosterone and PRC plasma levels.

				ARR		Aldosterone		PRC	
Gene	**SNP**	**Location**	**Minor allele**	**Beta**	***p***	**Beta**	***p***	**Beta**	***p***
CYP11B1	rs5316	Exon 8	T	0.05	0.69	0.12	0.17	0.08	0.48
CYP11B1	rs6387	Intron 3	G	0.05	0.31	-0.01	0.81	-0.06	0.18
CYP11B1	rs6410	Exon 1	A	0.04	0.40	-0.03	0.35	-0.07	0.09
CYP11B1	rs4471016	Promoter	C	0.03	0.54	-0.02	0.50	-0.06	0.18
CYP11B1	rs4313136	Promoter	A	0.03	0.53	-0.03	0.37	-0.07	0.12
CYP11B2	rs3097	3' UTR	A	0.04	0.44	0.02	0.58	-0.03	0.58
CYP11B2	rs28930074	Exon 7	C	0.05	0.48	-0.01	0.81	-0.06	0.31
CYP11B2	rs4538	Exon 6	C	0.01	0.84	-0.04	0.27	-0.05	0.20
CYP11B2	rs4539	Exon 3	G	-0.01	0.90	0.06	0.05	0.07	0.07
CYP11B2	IC	Intron 2	Conv	-0.01	0.81	**-0.07**	**0.04**	-0.07	0.09
CYP11B2	rs1799998	Promoter	C	-0.02	0.73	0.05	0.10	0.07	0.08

4.5 Discussion

The ARR is a clinical marker for inappropriate aldosterone production. Its main use has been promoted as a screening tool for detection of primary aldosteronism (Gordon *et al.* 1994; Mulatero *et al.* 2004) and this has been ratified by recent guidelines published by the Endocrine Society (Funder *et al.* 2008). However, the ratio also reflects an interaction between aldosterone and renin in the regulation of cardiovascular physiology. Few studies have examined the factors that influence the ARR, its distribution in a normal population and compared this in a related hypertensive group.

It has previously been explained (section 1.3.2) that there might be a thin line between the low renin hypertensives and a subtype of primary aldosteronism subjects, those with idiopathic hyperaldosteronism whose aldosterone production might not be adequately regulated by the main trophins. Although it is debatable whether primary aldosteronism is part of a hypertensive subgroup and this was not the purpose of this investigation, it will be discussed that an assessment of the ARR and its appropriate interpretation in a normotensive and hypertensive group may provide a better understanding of the pathophysiology involved.

Several physiologic and environmental factors can affect the components of the ARR and the ratio itself. This was reflected in the multivariate regression analysis as significant phenotypic associations were found with ARR and its individual components (Table 4-2, Table 4-3 and Table 4-5).

The values of R^2 suggest that only a small part of the variation of ARR and its components is accounted for by the number of variables studied. The model in Table 4-2, which included drug treatment, was able to account for more variance in ARR and its components than the model in Table 4-3, and each model was able to account for more variance in ARR and renin than aldosterone.

Although the variation of the ARR and its components accounted in each model is not large, age and gender have substantial effect which may reflect the dominant effect of renin. There was a positive effect of female gender in the ratio and aldosterone, and negative effect with renin, agreeing with the findings reported by Newton-Cheh and colleagues in the Framingham study (Newton-Cheh *et al.* 2007). Significant effects of

gender and sex hormones on renin have been reported previously (James *et al.* 1976; Sealey *et al.* 1994).

Moreover, unadjusted ARR values were higher in females than males, and at each arbitrary cut-off of the ARR >10 ng/L per mIU/L studied it was noted that there were proportionally more females than males (Table 3), irrespective of blood pressure status.

This gender disparity has important implications for selecting values to screen patients using the ratio.

Oral oestrogen administration can increase angiotensinogen leading to a suppression of renin concentration and increase in plasma renin activity (Ichikawa *et al.* 2006; Schunkert *et al.* 1997), possibly being another cofounder in the model. However, as the number of women in this cohort taking oral contraceptives or HRT was small, their influence on ARR or its components was not assessed in this study.

Blood pressure as a quantitative trait and body mass index had minor and contrasting effects in the ARR and renin and no significant effects in aldosterone. In the Framingham study it has previously been reported that blood pressure as a qualitative trait has a positive effect on the ratio which was not observed in this study (Newton-Cheh *et al.* 2007).

Interestingly, there was a positive association of plasma potassium with aldosterone and a negative association with renin, consistent with the known influence of potassium on aldosterone secretion and renin inhibition; a positive association with the ARR was also seen. This finding again emphasises the importance of considering other variables when using the ARR as a clinical screening tool.

Antihypertensive treatment with diuretics, ACE inhibitors and beta blockers had significant effects on the levels of renin, aldosterone and on the ARR. These influences are expected from the known effects of antihypertensive agents on the RAAS system, reflecting the dominant influence of renin on the derived ratio, and are consistent with findings published by other groups (Mulatero *et al.* 2002a). Because of this, in the subsequent analyses individual components of the ratio and the ratio itself were corrected for major confounders, including drug effects. While dietary salt intake might provide another source of confounding, this was not measured in the population studied, and no comments can made regarding its influence. However, it is suggested that in the cohort

studied the interindividual variability in average sodium intake would be relatively low and unlikely to account for a substantial influence in the ARR or its components.

From the cardiovascular and steroid phenotypes, the only one with a significant positive effect on ARR and aldosterone was plasma cortisol levels. This is not surprising considering that our group has recently reported an association between aldosterone and cortisol after suppression with dexamethasone and stimulation by ACTH, suggesting a common component in their regulation (Reynolds *et al.* 2009).

Distribution of ARR in normotensives and hypertensives: Before comparing the relationships between the ARR and ambulatory blood pressure in normotensive and hypertensive subjects, the population distribution of ARR measurements was examined. The findings in this study show that the adjusted ARR is a continuously distributed variable within the normal and hypertensive cohorts. This is in agreement with Kaplan's results of a continuum of ARR value in essential hypertensive patients (Kaplan 1994). The mean adjusted ARR measurements were not different between the two groups; importantly, there was no evidence for a different pattern of distribution in hypertensive subjects. In other words, there was no evidence of a distinct subgroup of subjects with a raised ARR that would identify a separate population of hypertensive patients with PA. Indeed, the continuous distribution suggests that the so-called epidemic of PA, based on identification of a raised ARR in around 10% of subjects with hypertension, may reflect the arbitrary selection of the upper end of a continuous distribution.

Then the proportion of hypertensive and normotensive subjects across the distribution of unadjusted ARR was examined (Table 4-4). The majority of subjects, regardless of blood pressure status, had an ARR ≤ 10ng/L per mIU/L. At each cut-off of ARR >10ng/L per mIU/L studied, a higher proportion of the hypertensive subjects than the normotensive subjects was represented; this was more marked for female subjects, as discussed above. At the higher cut-off levels of the unadjusted ARR the numbers in each group are small, and are likely to be substantially influenced by treatment (e.g. beta blocker) and other effects; it is possible, that some of the potential over- representation of hypertensive subjects at higher levels of the ARR is accounted for by confounding variables that include age, gender and drug use.

The recently published Guidelines on the detection of PA suggest that a cut-off of between 24-50 ng/L per mIU/L could be used in screening for the condition, depending on the degree of stringency required (Funder *et al.* 2008). Although, these recommendations were not based on studies using the assay employed in the present study, an exploratory analysis can be done. A cut-off of > 25 ng/L per mIU/L in our cohort would have identified 4.8% of hypertensive males and 8.9% of hypertensive females; however, 1.2% of normotensive men and 3.7% of normotensive women would have been detected by this arbitrary value. It is of interest that the Framingham study of the ARR, using a different renin concentration method, reported that 8% and 23% of untreated hypertensive men and women respectively, and 3% and 9% of normotensive men and women had an ARR of 26ng/L per mIU/L or higher. These data are, therefore, not dissimilar to the findings in this study. Interestingly, in both studies, normotensive subjects were found even at the highest ARR level selected, and it seems unlikely that they have PA. It should be noted, however, that no dynamic tests were performed to examine the suppressibility of aldosterone and therefore we cannot eliminate the possibility that, within the hypertensive and normotensive subjects with a very high ARR, there may be a smaller cohort who have genuine autonomy of aldosterone production.

ARR and Blood Pressure: After examining ARR distribution, and correcting the biochemical data for treatment and other significant co-variant effects (but not for blood pressure), residual associations between the ARR and systolic and diastolic blood pressure, both day and night, measured by ambulatory recording were demonstrated (Table 4-6 and Table 4-7). These effects were only seen in subjects not taking antihypertensive therapy, suggesting that drug treatment distorts the relationship between renin and aldosterone and blood pressure, and that we were unable to adjust fully for this confounding influence in subjects taking antihypertensive therapy. Clear trends were noted in the untreated subjects, with high renin levels being associated with the lowest blood pressures and the converse being seen for ARR values. There was no relationship between aldosterone quartiles and blood pressure, supporting the notion that renin is the key determinant of the ARR and its haemodynamic associations. Thus, the linear association of renin with blood pressure in the untreated cohort suggests that subjects with low renin levels (and a high ARR) are those with relative volume expansion, which may, in some subjects, reflect excessive and inappropriate (for the level of renin) aldosterone secretion. While the data from this study do not allow ascribing causality to these relationships, it is tempting to speculate that relative aldosterone excess maintains volume expansion that is reflected by a low level of renin and higher levels of blood pressure. In our group it has been proposed elsewhere on

the potential mechanisms which might lead to relative aldosterone excess developing over many years, reflecting an interaction of genetic and environmental factors, so that aldosterone becomes unduly sensitive to its usual trophins, such as angiotensin II and potassium (Connell *et al.* 2003; Connell *et al.* 2008). The current data, which show that the ARR is positively associated with age and is continuously distributed, would be consistent with these suggestions which predict eventual development of high blood pressure associated with an ARR at the upper end of the distribution. This proposal is not inconsistent with the presence of a distinct subgroup of subjects within the cohort with high ARR values who have truly autonomous aldosterone production.

Heritability and genetic influences: Apart from evidence of the physiological effects on ARR and its components, the structure of the cohort was utilised to look for evidence of a familial (and implied genetic) influence on the ARR and its components. First, we demonstrated using the adjusted measurements that the ARR is a strongly heritable phenomenon. This confirms an earlier report from the Framingham study (Newton-Cheh *et al.* 2007) with broadly similar figures and suggests that underlying genetic factors contribute to the level of variations of the ARR within the population. Interestingly, heritability for the ARR was higher than that noted, individually, for levels of aldosterone and renin; this phenomenon was also noted in the Framingham cohort, and may imply that familial (genetic) factors determine the interaction of renin and aldosterone independently of their effect on the individual components.

In the present study, variation in the genes encoding aldosterone synthase (*CYP11B2*) or 11β-hydroxylase (*CYP11B1)* did not account for the heritability of the ARR. Given that the ratio is, predominantly, a reflection of the level of renin, it was not surprising that the well-described polymorphic variants around *CYP11B1/CYP11B2* had no effect on the ARR ratio. Nevertheless, it was confirmed that the polymorphism in intron-2 of *CYP11B2* was associated with variability in aldosterone levels. The conversion allele that we, and others, have previously shown to be associated with high urinary aldosterone excretion (Davies *et al.* 1999; Paillard *et al.* 1999), and with hypertension associated with a high ARR (intron conversion), was also associated with raised plasma aldosterone in this study, although the magnitude of this effect was small (beta -0.7, p =0.04). Interestingly, the Framinghan study did not show any association with 17 common SNPs in the gene encoding for renin (*REN*) (Newton-Cheh *et al.* 2007). Thus, the genetic factors that determine population variability of the ARR remain largely undetermined, although the strong heritability of the ratio indicates that these make an important contribution.

Possible candidate genes might be localised in the regions showing modest linkage in chromosome 11p (LOD 1.89 at 2cM) and chromosome 5p (LOD 1.60 at 30.8cM) reported in the genome-wide linkage analysis performed in the Framingham cohort, using residuals adjusted for treatment. When the residuals were not adjusted for ACE inhibitor and beta blocker use, suggestive evidence of linkage was found on chromosome 7p21-22 (LOD 2.94 and 2.78 at 24.6 cM). According to Newton-Cheh and colleagues, this suggestive linkage might be due to a confounding effect of ACE inhibitor use. However, it is important to mention that also a suggestive linkage has been found in chromosome 7p22 and members of families with Familial hyperaldosteronism type II. Suggesting that there is a gene which is involved in aldosterone regulation in this region (Lafferty *et al.* 2000; Newton-Cheh *et al.* 2007).

Strengths and limitations: This study has a number of strengths in relation to its size and quality of the detailed phenotyping that was carried out, including ambulatory blood pressure recordings, that allow to define with some accuracy the relationship between components of the ARR and blood pressure in both normotensive and hypertensive subjects. Care was taken to adjust initial measurements of aldosterone, renin and the ARR for confounding variables, thus making the subsequent analyses robust. A high throughput assay for measurement of renin concentration, validated in Chapter 3, was used thus avoiding some of the potential pitfalls encountered in more traditional assays of the activity of the enzyme, including variability in endogenous concentrations of angiotensinogen.

There are, also, some limitations: the population included both hypertensives and normotensive subjects and a substantial proportion of the former were taking drugs known to influence renin and aldosterone levels. Although data were corrected for drug influences there is no certainty that this replicates findings in a treatment-naive cohort. Sampling was performed in the evening rather than in the morning. However, it is believed that potential diurnal variation of ARR is unlikely to influence the statistical and association analyses as the time of sampling was consistent across the study. There has been no follow-up of the subjects participating in the study and therefore no information is available regarding individuals with high aldosterone levels who might have developed hypertension. Finally, the population was recruited via a hypertensive proband and it is possible that genetic and familial factors associated with hypertension influenced some of the findings in this study.

In summary, ARR levels were higher in females, and associated with age, BMI, potassium and cortisol. Antihypertensive agents had significant predictable effects on the ARR and the ratio was adjusted accordingly. The adjusted ARR was continuously distributed in both normotensives and hypertensives. Thus, no subgroup of hypertensive subjects with raised ARR that would potentially identify individuals with PA was found. Nevertheless, more hypertensive and female subjects had an increased ratio.

Only in subjects not taking antihypertensives, renin was negatively and ARR positively associated with ambulatory blood pressure readings.

Furthermore, we have shown that there is a significant heritability of ARR and its components confirming the influence of genetic factors in this intermediate phenotype. However, the only genotype-phenotype association was found between plasma aldosterone and the conversion allele in the intron 2 of *CYP11B2*.

5 Characterisation of genetic variation at the *CYP11B1/CYP11B2* locus

5.1 Introduction

Rare variations such as the recombination of the *CYP11B1* and *CYP11B2* in glucocorticoid remediable aldosteronism (GRA) and mutations in the *CYP11B1* can lead to hypertension (see section 1.3.1). More subtle variations in the *CYP11B2* gene (-344T/C and intron conversion) and *CYP11B1* gene (-1889G/T and -1859A/G) have also been variably associated with hypertension as well as other cardiovascular phenotypes, including plasma renin activity and levels of plasma aldosterone, its principle urinary metabolite tetrahydroaldosterone (THAldo), cortisol precursors and their metabolites (see section 1.4.4). This is consistent with the finding of the previous chapter in which plasma aldosterone was associated with the intron conversion.

A recent meta-analysis in 5343 essential hypertensive and 5882 control subjects showed that individuals homozygous for the -344C allele are at 17% lower risk of hypertension compared to TT homozygous individuals. In addition, homozygous CC individuals had lower plasma renin activity, but no significant differences in plasma aldosterone concentration or systolic and diastolic blood pressure (Sookoian *et al.* 2007). It is worth noting that all the studies included in the meta-analysis have small numbers and thus there is a possibility of a bias in the proportion of cases and controls included in the evaluated studies as well as a clear difference in the frequency of alleles according to the ethnic group. Moreover, the only large community-based study showing a significant association of the homozygous TT individuals of the -344 polymorphism with elevated plasma aldosterone levels was in Japanese subjects (Iwai *et al.* 2007).

A more consistent association has been found between the *CYP11B2* polymorphisms and increased ACTH-stimulated levels of the 11-deoxysteroids (DOC and 11-deoxycortisol) indicating a reduced 11β-hydroxylase efficiency (Davies *et al.* 2001; de Simone *et al.* 1985; Hautanena *et al.* 1998). While this apparently shows a paradoxical influence of aldosterone synthase on the cortisol pathway mediated by *CYP11B2*, this perhaps is not entirely surprising considering that the aldosterone synthase gene, *CYP11B2*, and the 11β-hydroxylase gene, *CYP11B1*, lie in tandem in human chromosome 8q22 and are highly homologous in their introns, coding and amino acid sequences (Mornet *et al.* 1989). Moreover, the polymorphisms in one gene might reflect involvement of the other as a result of the linkage disequilibrium between variants in the locus. However, the causal relationship between the polymorphisms and the cardiovascular phenotypes remains unclear and the results of the investigations have been controversial (Brand *et al.* 1998;

Davies *et al.* 1999; Davies *et al.* 2001; Komiya *et al.* 2000; Lim *et al.* 2002b; Paillard *et al.* 1999; Pojoga *et al.* 1998; Tsujita *et al.* 2001).

So far, most studies have focused on -344T/C or intron conversion polymorphisms to look for genotype-phenotype associations. The results are not consistent and it is necessary to do a more comprehensive study of variations across the *CYP11B* region to identify causative associations with different cardiovascular, mineralocorticoid and glucocorticoid phenotypes.

5.2 Aims

The aim of this study was to estimate linkage disequilibrium (LD), haplotype structure and haplotype tagSNPs (htSNPs) for the *CYP11B* locus in Caucasians.

5.3 Subjects and Method

5.3.1 Subjects

Pilot BRIGHT TDT

The MRC BRItish Genetics of HyperTension (BRIGHT) study is a collection of family-based and case-control resources which enable genetic studies of hypertension. In this large multicentre study, 1500 white European families based upon affected sibling pairs as well as 1000 parent-offspring trios were phenotyped. These trios have the optimal family structure for family-based association studies using the transmission disequilibrim test, giving name to this cohort (BRIGHT TDT).

The TDT repository comprises 712 severely hypertensive families, 371 of which are standard trios and 341 single parent families with proband and multiple siblings. Family tree, cardiovascular history, socio-economic and brief medical history and date of birth were recorded. Height, weight, sex, medications, anthropometric measurements (waist-hip ratio, skin fold thickness and body mass index), blood chemistry and urinary analysis were also documented. Detailed blood pressure recordings were taken at the time of the study.

Probands

All hypertensive probands had white British ancestry by grand-parental origin and an onset of hypertension before 60 years of age. Their blood pressure recordings were 150/100mmHg or higher (1 reading), or 145/95mmHg or higher (mean of 3 readings whilst seated). Subjects with diabetes, secondary hypertension, renal disease, BMI > 30 kg/m^2 or consumption of more than 21 units of alcohol per week were excluded. Full details of recruitment strategy have been published elsewhere (http://www.brightstudy.ac.uk/info/sop_TDT.html) (Newhouse *et al.* 2005). From this BRIGHT TDT investigation, seventy nine Caucasian unrelated offspring were selected as the pilot BRIGHT TDT to analyse the *CYP11B1/CYP11B2* locus polymorphisms. From the 79, 50 subjects were hypertensive probands and 29 were selected from the normotensive siblings in single parent families. Ethical approval for the study was granted by the local ethics committees in each of the participating centres and fully informed written consent from all the participants was obtained.

DNA was available and extracted by the BRIGHT consortium. Full details of extraction have been described elsewhere:

http://www.brightstudy.ac.uk/info/sop_9000666.html#processing).

5.3.2 Polymorphisms genotyped with PCR and direct sequencing

In the *CYP11B2,* PCR and direct sequencing were performed in the promoter, intron 2 and intron 6. In the *CYP11B1,* PCR and direct sequencing were performed in the promoter and intron 2 regions. The methodology is described in section 2.3.1.

PCR

Two new protocols of PCR followed by sequencing (B2INT6 and B1INT2) were developed to evaluate two new regions of these genes in this pilot study.

The region of the intron 6 in the *CYP11B2* and intronic 2 region of the *CYP11B1* produced fragments of 329bp and 261bp of length, respectively (Figure 5-1 and Figure 5-2).

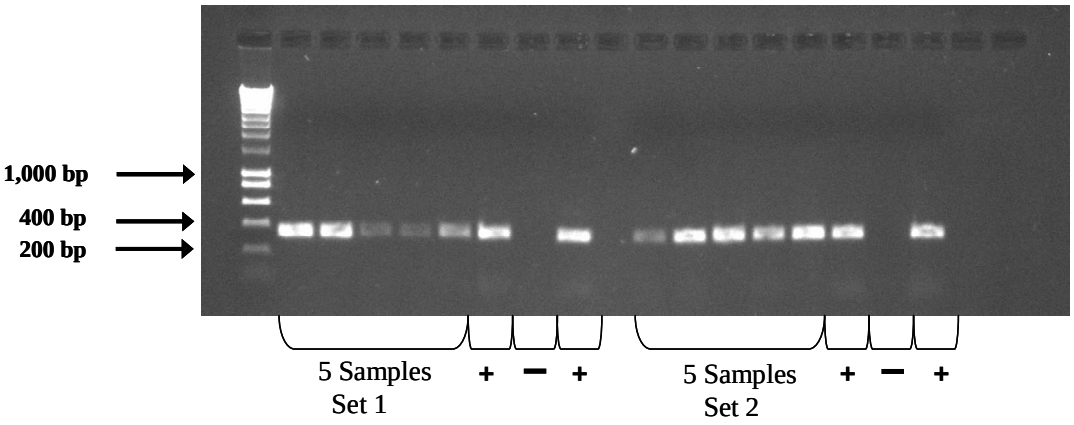


Figure 5-1. PCR fragments of B2INT6 (~330bp) from two sets of BRIGHT TDT (n=10) samples with their corresponding positive and negative controls, resolved on a 1% agarose gel.

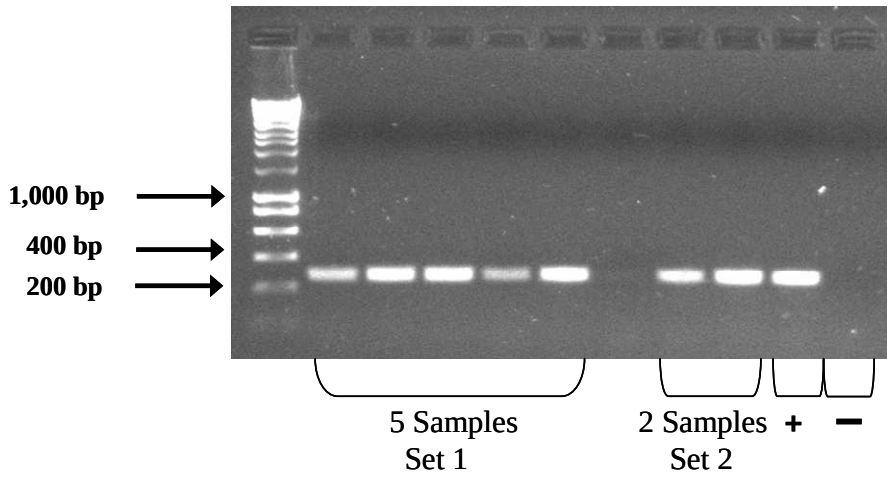


Figure 5-2. PCR fragments of B1INT2 (~260bp) from two sets of BRIGHT TDT (n=7) samples with their corresponding positive and negative controls, resolved on a 1% agarose gel.

Polymorphism identification

Identification by direct sequencing

Polymorphisms were identified by direct sequencing of the fragments of interest, either in the *CYP11B1* or the *CYP11B2* gene. An example of electropherograms of the sequencing in the intron 6 of the *CYP11B2* and intron 2 of the *CYP11B1* are shown in Figure 5-3 and Figure 5-4, respectively. In each figure, examples of homozygous and heterozygous individuals are presented. Eight polymorphisms were characterized in the *CYP11B2* intron 6 fragment – one insertion and seven single nucleotide substitutions, while in the *CYP11B1* intron 2 only one insertion and a single nucleotide substitution were characterized.

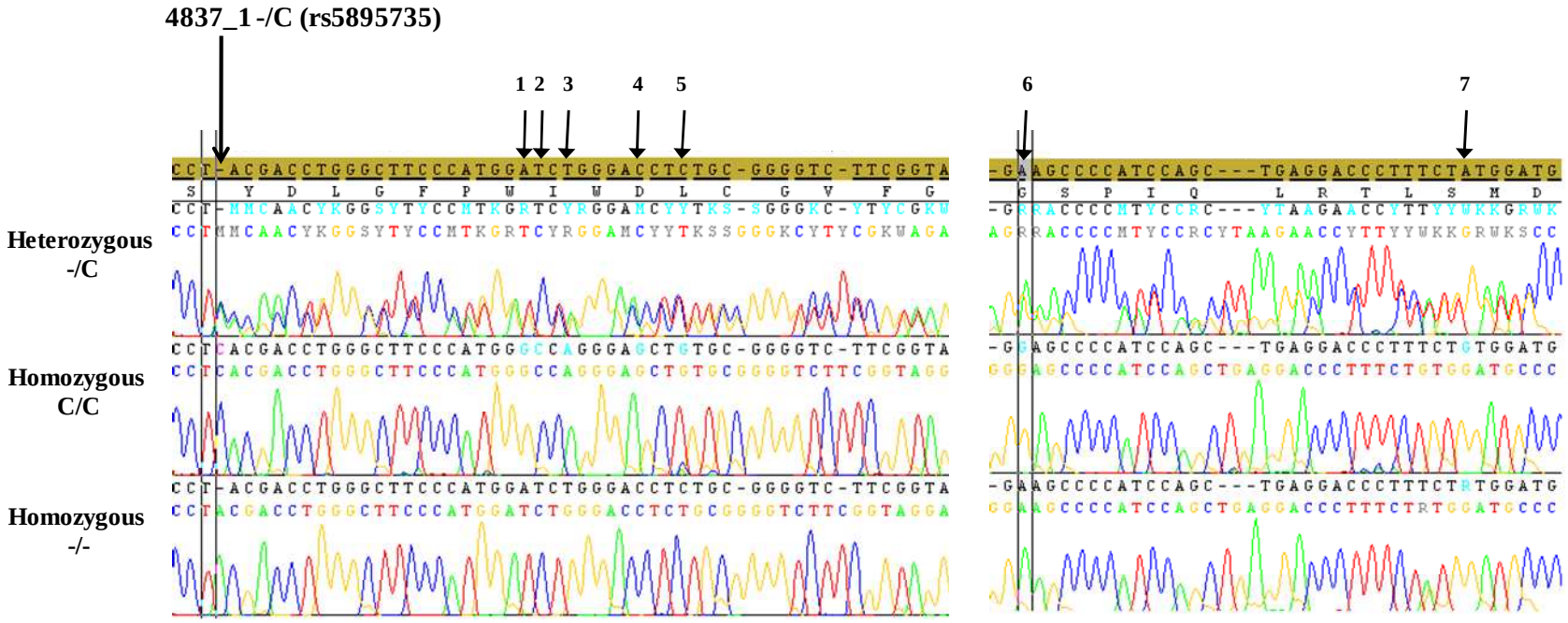


Figure 5-3. Sequence analysis showing one insertion and seven single nucleotide variants within the *CYP11B2* intron 6. Results are shown from one subject from each of the three groups. The first variation at position 4837_1,relative to the first base of *CYP11B2* coding region, corresponds to an insertion of a C. For the rest of the SNPs the alternate forms were only characterised in homozygous individuals were: 1) A/G at position 4858, 2) T/C at position 4859, 3) T/A at position 4861, 4) C/G at position 4866, 5) C/G at position 4969, 6) A/G at position 4908 and 7) A/G at position 4936.

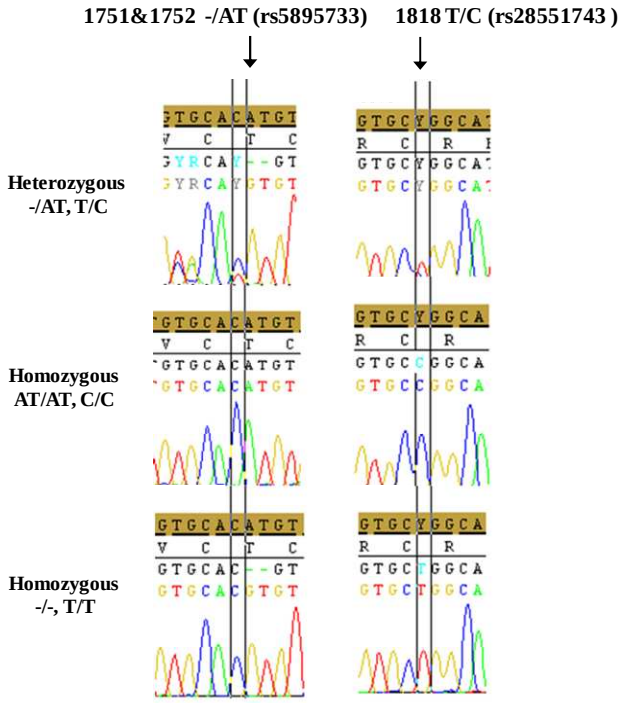


Figure 5-4. Sequence analysis showing one insertion and one single nucleotide variant within the *CYP11B1* intron 2. Results are shown from one subject from each of the three groups. The first variation at positions 1751 and 1752, relative to the first base of *CYP11B1* coding region, corresponds to an insertion/deletion of AT. The other is a substitution which variants were C/T at position 1818.

5.3.3 Polymorphisms genotyped with TaqMan

Polymorphisms located in exon 3 *CYP11B2* (rs4546); intergenic region (rs6471580 and rs4736354), exon 1 (rs6410), exon 2 (rs5283), intron 5(rs6471570) and 3'UTR (rs5301 and rs4736312) in *CYP11B1* were genotyped using the corresponding TaqMan assays (see section 2.3.2)

Genotyping quality control was done by including positive and negative controls in each genotyping run and by genotyping 10% of the samples in duplicate.

Polymorphism identification

Identification by allelic discrimination

The eight SNPs genotyped by the TaqMan method were identified using the allelic discrimination feature of the SDS v.2.3 software (Applied Biosystems, CA, U.S.A.). The homozygous, heterozygous and undetermined or failed samples were automatically categorised by the software, as shown in Figure 5-5 for SNPs rs5301 and rs6410 in the 79 TDT BRIGHT samples. The failed or undetermined samples in between clusters were manually checked. Those not achieving a quality value >90% or giving a low signal located outside the clusters were manually classified as undetermined, as shown in 5 samples in Figure 5-5 B.

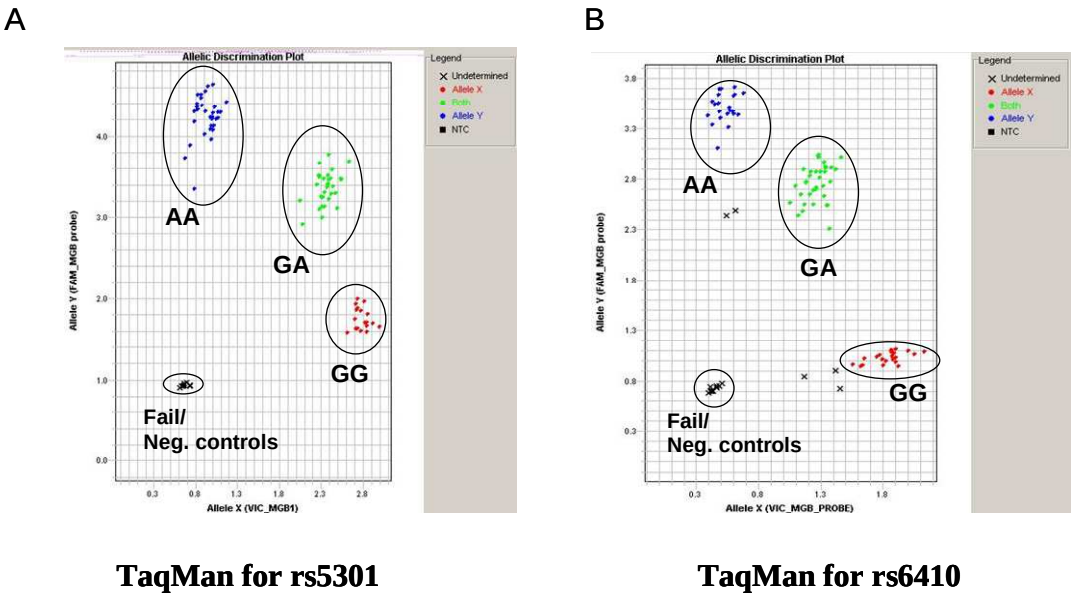


Figure 5-5. Allelic discrimination showing the three possible genotypes in two polymorphisms genotyped by TaqMan, rs5301 and rs6410. Results are shown from the 79 TDT BRIGHT subjects classified as homozygous, heterozygous or fail/negative controls clusters. The classification of the samples was done automatically by the SDS v.2.3 software. Failed or undetermined samples, like the five crosses outwith the clusters shown in panel B on the right, were manually checked to determine the cause of failure.

5.3.4 Statistical Analyses

Haploview v. 3.32 software was used to assess genotyping quality (Hardy Weinberg equilibrium and percentage of genotyping), as well as to analyse, interpret and visualize patterns of linkage disequilibrium, haplotype structure and to help in the manual selection of haplotype tagSNPs (Barrett *et al.* 2005).

JLIN v.1.6.0 was used for calculating, interpreting and visualizing patterns of LD as D' and r^2 (http://www.genepi.org.au/jlin.html) (Carter *et al.* 2006).

5.4 Results

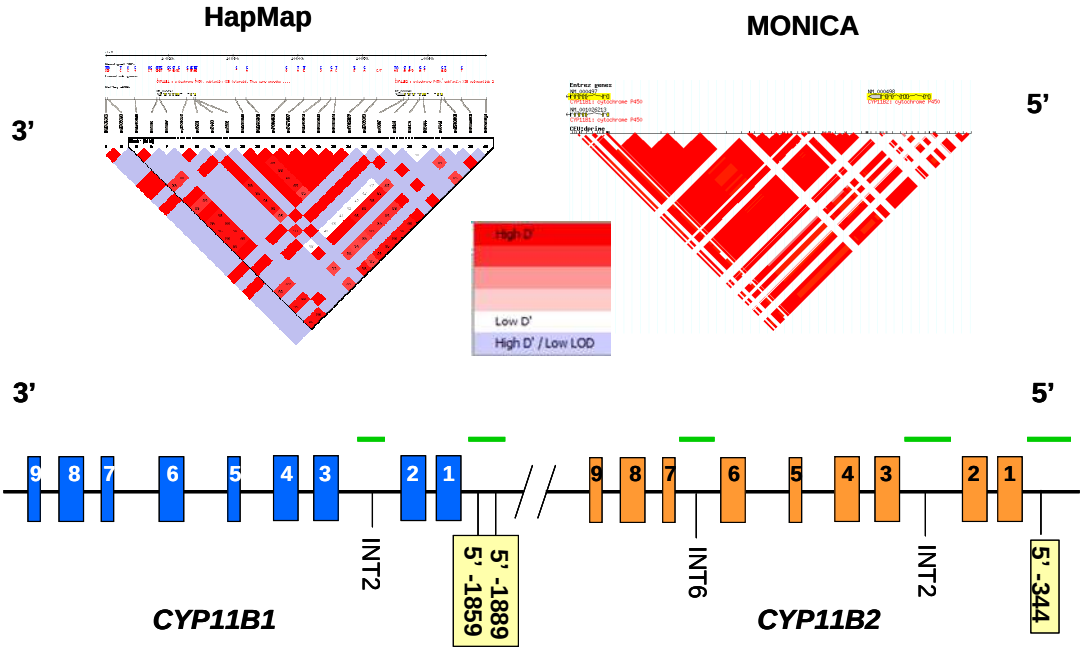


Figure 5-6. Linkage disequilibria plots using HapMap and MONICA data and informative regions in *CYP11B1* and *CYP11B2* genes (highlighted in green). The SNPs highlighted in yellow have already been reported.

The most informative regions across the *CYP11B1/CYP11B2* locus were identified by reviewing linkage disequilibria (LD) plots using the HapMap CEU cohort of the thirty U.S. trios with northern and western European ancestry (HapMap Public Release #20) and 26 normotensives from the the multinational MONItoring of trends and determinants in CArdiovascular disease (MONICA) IV study in Glasgow. We selected three SNPs from previous findings which suggested that variations in the promoter fragments of each gene, -1889 and -1859 in *CYP11B1* and -344 in *CYP11B2*- might play a role in hypertension. (See section 1.4.4). In addition, three regions were identified that were not represented by these three SNPs and these were in the intron 2 and intron 6 of *CYP11B2* gene and the intron 2 in the *CYP11B1* gene (Figure 5-6). These regions were sequenced for identification of polymorphisms. Additionally, when the HapMap database was updated (2007-04-20: HapMap Rel #22), we were able to add eight new polymorphisms (one in exon 3 of *CYP11B2*, two in the intergenic region, and one in exon 1, exon 2, intron 5 and two in 3'UTR of *CYP11B1*) in order to have 90% coverage of this locus.

5.4.1 CYP11B1/CYP11B2 Genotyping

A total of 22 polymorphisms were genotyped in 79 unrelated individuals of the TDT resource (Table 5-1). Four individuals were excluded because their genotype call rate was <50%. Of the polymorphisms, seven SNPs in *CYP11B2* intron 6 departed significantly from Hardy-Weinberg equilibrium ($p<0.001$) and had less than 75% genotyping in the 75 individuals. Only one of the SNPs in intron 6 (rs5895735) was informative as it was in Hardy-Weinberg equilibrium and had a percentage of genotyping >85%. Therefore, it was agreed that in intron 6 only this last SNP would be considered for further analysis.

Table 5-1. Characteristics of polymorphisms genotyped in the *CYP11B1* and *CYP11B2* genes in the Pilot BRIGHT TDT

Variation no.	Chr	Gene	Location	SNP	Position bp	Alleles	MAF	Percentage of Genotyping %	Obs.Het	HWE p
1	8	*CYP11B1*	3'UTR	rs4736312	143950939	T/G	0.49	94.7	0.45	0.51
2	8	*CYP11B1*	3'UTR	rs5301	143952275	A/G	0.43	98.7	0.40	0.13
3	8	*CYP11B1*	Intron 5	rs6471570	143954407	G/T	0.49	97.3	0.44	0.38
4	8	*CYP11B1*	Intron 2	rs28551743	143956414	C/T	0.46	90.7	0.40	0.14
5	8	*CYP11B1*	Intron 2	rs5895733	143956480	-/AT	0.46	85.3	0.48	1.00
6	8	*CYP11B1*	Exon 2	rs5283	143957599	C/T	0.44	100.0	0.32	0.004
7	8	*CYP11B1*	Exon 1	rs6410	143958007	A/G	0.49	98.7	0.45	0.45
8	8	*CYP11B1*	Promoter	rs4471016 (-1859 A/G)	143960096	G/A	0.47	86.7	0.45	0.51
9	8	*CYP11B1*	Promoter	rs4313136 (-1889 G/T)	143960126	T/G	0.49	86.7	0.43	0.35
10	8		Intergenic	rs4736354	143977468	G/C	0.41	93.3	0.40	0.20
11	8		Intergenic	rs6471580	143983703	T/C	0.42	96.0	0.38	0.07
12	8	*CYP11B2*	Intron 6	rs6435	143991323	A/G	0.46	65.3	0.27	<0.001
13	8	*CYP11B2*	Intron 6	rs6397	143991351	A/G	0.42	65.3	0.18	<0.001
14	8	*CYP11B2*	Intron 6	rs6429	143991390	C/G	0.41	68.0	0.12	<0.001
15	8	*CYP11B2*	Intron 6	B2INT6_4866	143991393	C/G	0.42	72.0	0.17	<0.001
16	8	*CYP11B2*	Intron 6	B2INT6_4861	143991398	T/A	0.42	72.0	0.17	<0.001
17	8	*CYP11B2*	Intron 6	B2INT6_4859	143991400	T/C	0.42	72.0	0.17	<0.001
18	8	*CYP11B2*	Intron 6	B2INT6_4858	143991401	A/G	0.42	72.0	0.17	<0.001
19	8	*CYP11B2*	Intron 6	rs5895735	143991422	-/C	0.44	96.0	0.38	0.06
20	8	*CYP11B2*	Exon 3	rs4546	143993555	C/T	0.43	98.7	0.38	0.07
21	8	*CYP11B2*	Intron 2	IC	143993985	Wt/Conv	0.50	96.0	0.47	0.77
22	8	*CYP11B2*	Promoter	rs1799998 (-344 T/C)	143996602	T/C	0.45	97.3	0.51	1.00

Shown here are the 22 *CYP11B1/CYP11B2* locus SNPs genotyped in 79 independent offspring of the TDT BRIGHT to determine LD, haplotype structure and tagging SNPs. SNPs are presented in chromosomal order and their location within the gene indicated. Position in terms of base pair (bp) derived from ENSEMBL release 49 July 2008 Chromosome 8 assembly (ENSEMBL website http://www.ensembl.org). Alleles: major/minor allele; MAF: minor allele frequency; Obs.Het: observed heterozygosity; HWE p: Hardy-Weinberg equilibrium p-value; -/: no insertion; Wt: wild-type; Conv: conversion.

5.4.2 CYP11B1/CYP11B2 Linkage disequilibrium

A graphical representation of the LD is shown in Figure 5-7 following a standard colour scheme: regions with high D' are shown in red or zero D' in white in the upper triangle; regions with high r^2 in blue or zero in white in the lower triangle. In both cases, most of the region is in red or blue indicating high LD. The lowest regions with LD were between the SNPs at the 5' end of the *CYP11B2* gene with the SNPs close to the 3' end in the *CYP11B1* gene.

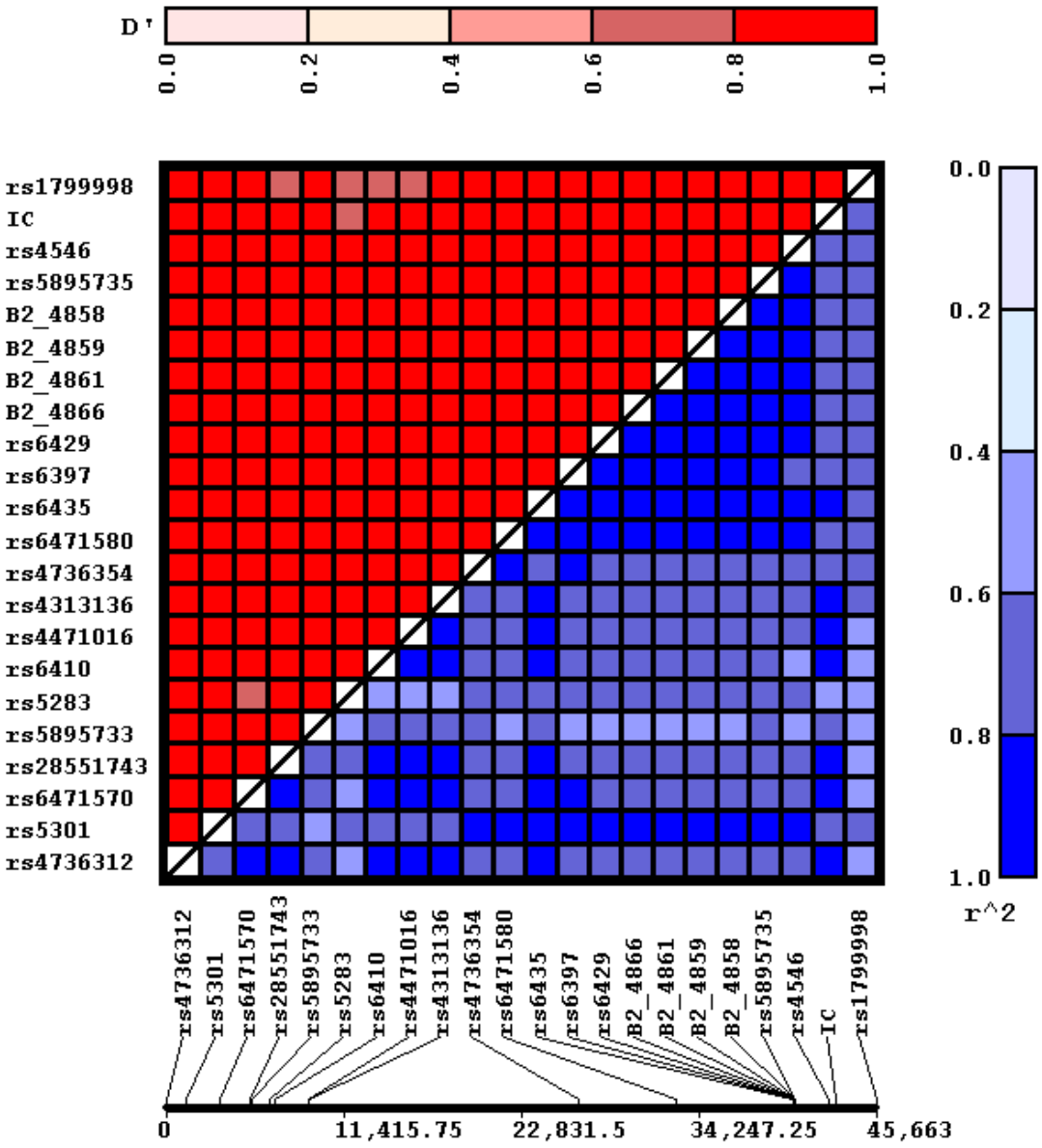


Figure 5-7. Linkage disequilibrium among the SNPs in the *CYP11B1/CYP11B2* locus. Two measures for LD are shown: D' value in the upper left triangle and r^2 in the lower right triangle. Color-coded scales for the D' or r^2 values (measures of LD strength) are above in red for the D' and on the right in blue for r^2.

In Figure 5-8, the 15 polymorphisms chosen to analyse the corresponding haplotypes are shown.

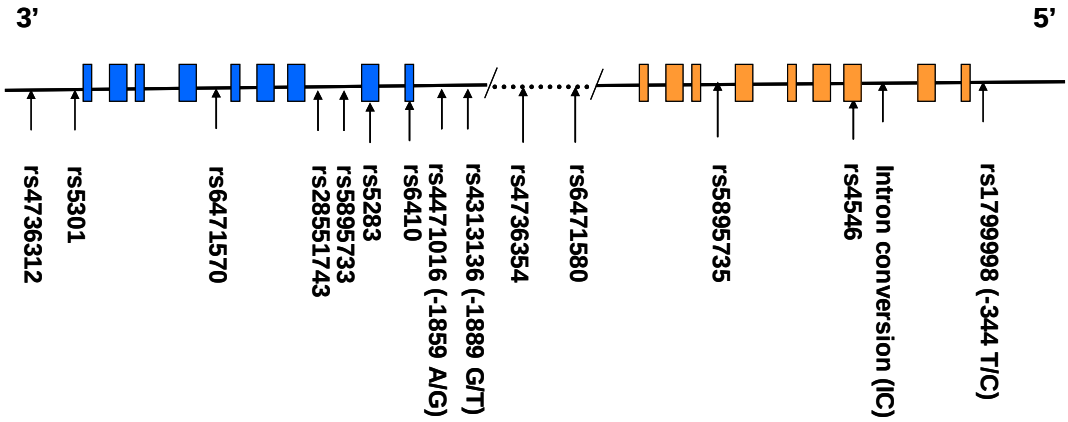


Figure 5-8. Selected SNPs for haplotype analysis in *CYP11B1/CYP11B2* locus.

5.4.3 CYP11B1/CYP11B2 locus haplotypes and haplotype tagSNPs

As there is significant LD in the locus and there is currently no consensus for selecting haplotype blocks or their boundaries, two blocks were chosen to ensure the best selection of haplotype tagSNPs (htSNPs): Block 1 comprised SNPs in the *CYP11B1* plus the intergenic region and Block 2 comprised SNPs in the *CYP11B2* plus the intergenic region.

Considering the block of *CYP11B1* plus the intergenic region, 7 haplotypes were generated with a frequency > 1%; from these, 4 common haplotypes with frequencies > 5% were observed and account for 88% of the possible haplotypes, with the most common haplotypes being in 77% of subjects. Tagging with an r^2 cutoff: 0.8; captured 11 of 11 alleles with mean r^2 of 0.95. By using 4 SNPs highlighted in Figure 5-9, 100% of alleles were captured with r^2 >0.8. The SNPs highlighted with a blue square were the selected htSNPs for this region (rs5301 in the 3'UTR region, rs5895733 in intron 2, rs6410 in exon 1 and rs4313136 in the *CYP11B1* promoter).

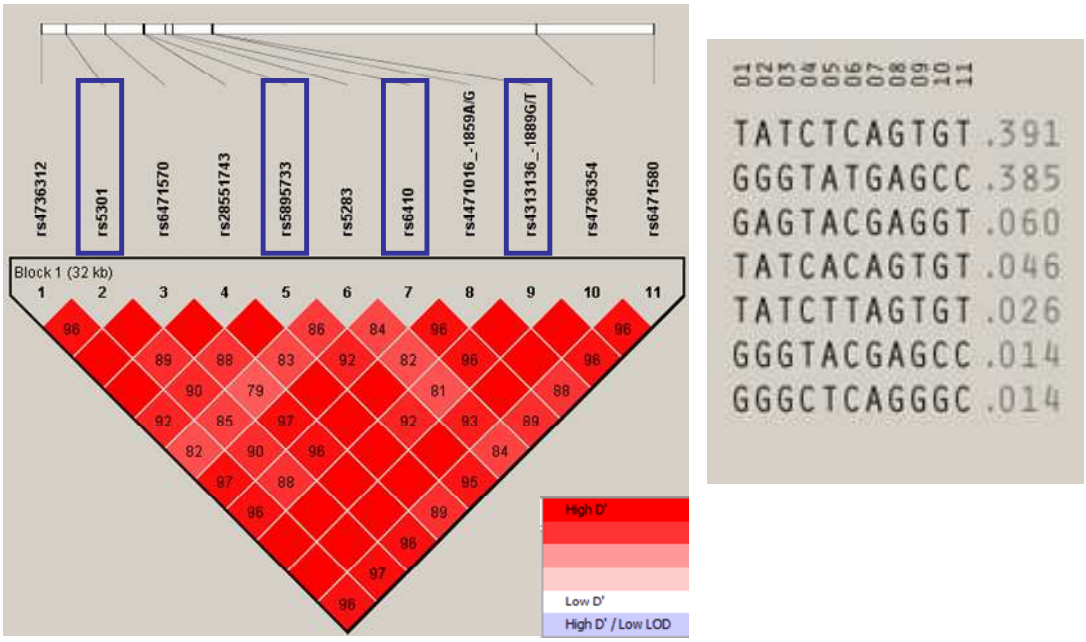


Figure 5-9. LD plot of the block 1 (*CYP11B1* and intergenic region) with their corresponding haplotypes. The SNPs highlighted with a blue square were the selected haplotype tagSNPs for this region.

The second block selected comprised *CYP11B2* plus the intergenic region. Considering this fragment, 7 haplotypes were generated with a frequency > 1%; from these, 3 common haplotypes with frequencies > 5% were observed and account for 88% of the possible haplotypes, with the most common haplotypes being in 83% of subjects. Tagging with r^2 cutoff: 0.8, captured 6 of 6 alleles with mean r^2 of 0.938. By using a subset of 4 SNPs highlighted in Figure 5-10, 100% of alleles were captured with r^2 >0.8. The SNPs highlighted with a blue square were the selected haplotype tagSNPs for this region (rs4736354 in the intergenic region, rs4546 in exon 3, the intron conversion (IC) in intron 2 and rs1799998 in the promoter of the *CYP11B2* gene).

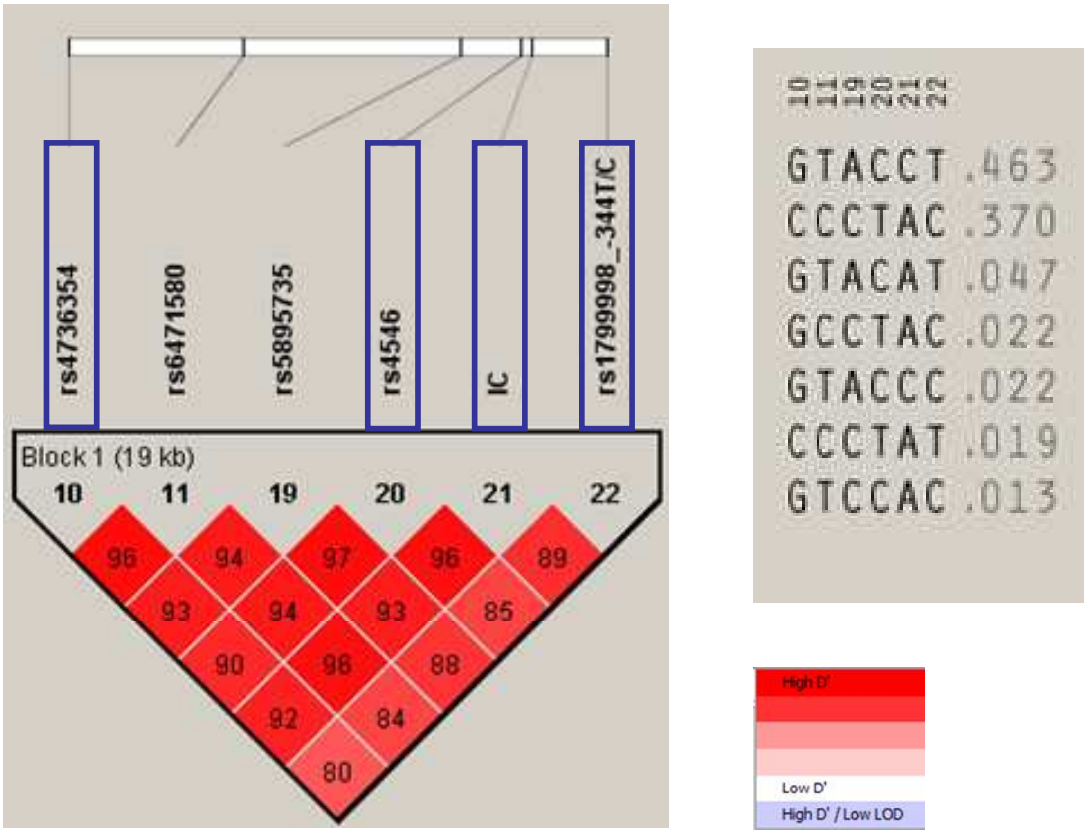


Figure 5-10 LD plot of the block 2 (*CYP11B2* and intergenic region) with their corresponding haplotypes. The SNPs highlighted with a blue square were the selected haplotype tagSNPs for this region.

Therefore, these two blocks, each with a subset of four htSNPs, predicted 90% coverage of the most common haplotypes and the remaining SNPs.

5.5 Discussion

The analysis of the variations in the *CYP11B1* and *CYP11B2* genes and their associations with blood pressure and aldosterone levels started in the 1990s. The first two polymorphisms reported in LD were the rs1799998 (also known as SF-1 or -344T/C) and the conversion in intron 2 of *CYP11B2*. With them, three haplotypes were defined (-344C/Wt, -344T/Wt and -344T/Conv) (White PC & Slutsker L 1995). A few years later, another polymorphism in exon 3 of *CYP11B2*, the Lys173Arg (also known as K173R or 2718A/G, or rs4539) also showed LD with the -344T/C. However, this polymorphism has

only been included in few studies (Fardella *et al.* 1996; Keavney *et al.* 2005; Komiya *et al.* 2000; Matsubara *et al.* 2001).

The polymorphism, V386A (also known as Val386Ala, 4986T/C or 4991T/C depending on the reference position, or rs28930074), has a functional impact by reducing the production of 18-hydroxycorticosterone (Pascoe *et al.* 1992b). Similarly to the polymorphism in exon 3, it has only been used as a marker in a few studies (Keavney *et al.* 2005; Matsubara *et al.* 2001; Pojoga *et al.* 1998), with only one study in Caucasians reporting haplotypes (Keavney *et al.* 2005).

From the polymorphisms in the *CYP11B2*, the only one widely used in association studies has been the -344T/C. As mentioned before, the findings regarding its relationship with a hypertensive and/or mineralocorticoid phenotype are conflicting. The most consistent findings have been those associated between the -344T allele and lower 11β-hydroxylase activity (Davies *et al.* 2001; Hautanena *et al.* 1998). Two studies independently made the first attempt to characterise the *CYP11B1/CYP11B2* locus. Both generated haplotypes and agreed that haplotypes carrying the -344T allele had higher 11-deoxycortisol excretion (Ganapathipillai *et al.* 2005; Keavney *et al.* 2005). Nevertheless, none suggested a plausible functional mechanism to explain this biochemical phenotype. Barr and colleagues reported two polymorphisms in the promoter region of the *CYP11B1* (-1889G/T and -1859A/G) associated with impaired 11β-hydroxylase efficiency (Barr *et al.* 2007). However, functional variants in this locus which could account for increased ARR or blood pressure have not been reported. It has been hypothesized that an impaired activity of 11β-hydroxylase can stimulate a slightly higher ACTH drive which in turn may lead to adrenal hypertrophy. This hypertrophy may cause a small increase in aldosterone levels leading to an increased ARR and consequently high blood pressure (Freel & Connell 2004). A molecular mechanism supporting this hypothesis has not been elucidated.

In addition, despite the acknowledgement of the LD in the *CYP11B1/CYP11B2* locus, the SNPs selected for the previous studies were mainly chosen according to their functionality and what had previously been reported in the literature possibly missing other informative variations. The first study to take advantage of the LD to select the SNPs in this region involved Japanese subjects (Iwai *et al.* 2007) which cannot be applied to Caucasian subjects because the polymorphic variations are different between populations (see Chapter 6).

As SNPs are abundant in this loci and high throughput genotyping is available, it was considered that, to allow fine mapping of a causal variant, a combination of a selection of potential causal variants (-344, -1889 and -1859) and set of several markers (htSNPs) capturing most of the information in the region would be appropriate. Moreover, it has been shown that the power of an association study can be increased by considering two adjacent blocks in LD as one region (Fan & Knapp 2003).

The first step in selecting a set of SNPs that would address all the variation in this region was to use tag SNP strategy. This is a marker selection method to reduce redundancy and increase coverage in the region, based on the linkage disequilibrium statistics derived from genotyping common SNPs within this locus (2005). The markers will not only represent the *CYP11B1* and *CYP11B2* genes but also the common haplotypes of the genes, providing a more robust approach to the detection of phenotypic associations (Akey *et al.* 2001; Stephens *et al.* 2001). However, this requires the availability of comprehensive information on variation in the region. This can be derived from sequencing a small group of individuals in the region, identifying common SNPs and establishing LD structure.

The HapMap project provides extensive allele frequency and linkage disequilibrium information obtained by sequencing a sample of four populations - Han Chinese, Japanese, U.S. residents with European ancestry and Yorubans (http://www.hapmap.org/). However, sequencing of this region is difficult because of the similarity between the genes and therefore the HapMap coverage of this region is incomplete. Barr *et al.* previously sequenced the *CYP11B1* and *CYP11B2* genes extensively in 26 normotensives subjects from the MONICA Study, stratified by the -344 and intron conversion variants (Barr *et al.* 2007). The HapMap data for Caucasians combined with the 26 normotensive subjects from the MONICA study were used to select a set of SNPs that would capture the variability in the region. The subjects from the MONICA study were used to analyze the regions not covered by the HapMap data. By analyzing the LD and the markers in both of them, it was decided that the areas that provided most coverage in the locus were the promoter and the intron 2 in the *CYP11B1* gene and the promoter, intron 2 and the intron 6 in the *CYP11B2* gene. To achieve a more thorough coverage of the locus, 8 SNPs (rs4546, rs6471580, rs4736354, rs6410, rs5283, rs6471570, rs5301 and rs4736312) were added to the analysis when the HapMap data was updated.

In the first part of this study, employing the two new genotyping methods of PCR followed by automated sequencing (B2INT6 and B1INT2), two types of polymorphisms were identified in the 79 individuals: substitutions and insertions.

In intron 6 of *CYP11B2*, an insertion of a C in position 4837_1 (rs5895735) was found. In heterozygous subjects, this insertion altered the alignment of the alleles for more than 100bp and the other SNPs present in the fragment were not identifiable by sequencing in a forward or reverse manner. As a consequence the percentage of genotyping was lower in this intron and only the insertion attained Hardy-Weinberg equilibrium. For homozygous individuals, the other 7 SNPs identified in this region were in positions 4858(A/G), 4859(T/C), 4861(T/A), 4866(C/G), 4869(C/G), 4908(A/G) and 4936(A/G) (Figure 5-3).

In the intron 2 of the *CYP11B1*, an insertion of two bases, AT, was found in position 1751 and 1752, corresponding to the rs5895733. In contrast with the insertion in intron 6, this did not affect the rest of the sequence and another SNP (rs28551743) was found in position 1818(T/C) (Figure 5-4). The hypothesis of effects of small insertion/deletions on genomic size might be an explanation for the two base insertion in this intronic region which apparently has no effect on the reading frame, but it has been speculated that might contribute to evolutionary reductions in genome size (Gregory 2004; Pettersson *et al.* 2009).

The percentage of genotyping of the SNPs in *CYP11B1* intron 2 and the rest of SNPs characterised by PCR and automated sequencing (-344, intron conversion, -1859 and -1889) was as expected and there were no significant deviations from Hardy-Weinberg equilibrium. Similarly, in the second part of the study, in which the 8 TaqMan SNPs were incorporated, the percentage of successful genotyping was high (93-100 %) and the SNPs were in Hardy-Weinberg equilibrium (Table 5-1).

LD in Caucasians is well preserved across the *CYP11B* locus (Figure 5-7). With the entire block, an automatic selection of htSNPs in Haploview Tagger would have reduced considerably the marker density required to represent it. Thus, it was decided to split the block in two in order to have a better coverage of the region. The Tagger software was set to select the SNPs with a minimum r^2 of 0.8 in order to choose the most predictive htSNPs. In addition, other criteria such as the performance of the genotyping assays and the interruption of LD over short distances with the presence of a conversion were also considered for the final selection (Doris 2002; Ke & Cardon 2003; Stephens *et al.* 2001).

From the eight SNPs selected, it was decided one SNP, rs5895733 in *CYP11B1*, would not be genotyped for practical reasons as the PCR and automated sequencing increased considerably the time and cost of genotyping on a large scale. The chosen set of 7 htSNPs allows us to test for associations between a trait and a common variant(s) with small effects and/or a *cis* interaction between two common variants within this locus (Chapman *et al.* 2003).

5.6 Conclusion

This preliminary study, comprising 79 unrelated individuals (50 hypertensives and 20 normotensives), confirms that the *CYP11B1* and *CYP11B2* genes are in high linkage disequilibrium. Using the 15 SNPs in Hardy-Weinberg equilibrium and the highest percentage of genotyping, 3 common haplotypes were generated in block 1 and block 2 which were found in more than 75% of the subjects.

For practical reasons, a set of 7 SNPs: 3 SNPs in *CYP11B1* (rs5301, rs6410, rs4313136 (-1889G/T)), 3 SNPs in *CYP11B2* (rs4546, the intron conversion (IC) and rs1799998 (-344T/C) and one in the intergenic region (rs4736354) were selected as htSNPs. With these SNPs, the haplotype diversity is maximized, the genotyping redundancy minimized and the potential to detect associations in a large case-control study increased (see Chapter 7).

6 Comparison of the SNPs in *CYP11B1/CYP11B2* locus in populations of Caucasian and African ancestry

6.1 Introduction

The allele frequency and linkage disequilibrium (LD) structure of the genetic variations in the *CYP11B* locus can help in the genetic dissection of hypertension and in explaining other intermediate phenotypes such as plasma aldosterone concentration, urinary aldosterone excretion rate and aldosterone renin ratio (ARR) (Barbato *et al.* 2004). The two main polymorphisms most frequently studied are the -344T/C and the intron 2 conversion in the *CYP11B2* gene. Contrasting associations with essential hypertension have been reported in different populations. In some studies, -344T allele showed significant associations in white, Japanese and African American people (Brand *et al.* 1998; Davies *et al.* 1999; Henderson *et al.* 2004; Iwai *et al.* 2007), but other investigations have either failed to find such associations (Matsubara *et al.* 2001; Patel *et al.* 2000; Schunkert *et al.* 1999a; Tsujita *et al.* 2001; Zhu *et al.* 2003a) or have found associations with the -344C allele (Tamaki *et al.* 1999). Although fewer studies have used the intron 2 conversion, there is some evidence of the conversion allele being associated with hypertension in British (Davies *et al.* 1999; Zhu *et al.* 2003a), but not in French or subjects with African ancestry (Brand *et al.* 1998; Zhu *et al.* 2003a).

Only one study has tested the K173R polymorphism in exon 3 of the *CYP11B2* gene for association with hypertension in both whites and blacks, and this showed no association (Zhu *et al.* 2003a).

There are several explanations for the inconsistencies in the association results for SNPs in the *CYP11B1/CYP11B2* locus. Many studies used small sample sizes and thus were underpowered to determine any association. Also, the investigators used diverse study designs (cross-sectional, cases and controls or family-based) and different criteria for the selection of individuals. Moreover, a difference in genetic background exists which can have an impact in allele frequencies or LD blocks.

As genetic variants of the *CYP11B2* gene are in strong LD with variants in the contiguous *CYP11B1* gene, our group and others have started to consider the entire locus comprising both genes for association studies (Barr *et al.* 2007; Ganapathipillai *et al.* 2005; Iwai *et al.* 2007; Keavney *et al.* 2005; Zhu *et al.* 2003a). However, there are practical difficulties in terms of identifying the causative variant in the presence of large regions of strong LD. In this case, one option is to study populations where the LD pattern is not so strong and these have to be characterised. Additionally, there is evidence to suggest variations in genes such

as *ACE, SLC24A5, MCM6, G6PD* and *HBB* can provide insight of ancestry and/or selection of phenotypes such as circulating ACE levels, skin pigmentation, lactose persistence, malarial resistance and susceptibility to β-thalassemias (Cavalli-Sforza *et al.* 1993; Giardina *et al.* 2008; Ingram *et al.* 2009; MacKenzie *et al.* 2008; Verrelli *et al.* 2002). Thus, it is also of interest to analyse if variations in the *CYP11B1/CYP11B2* locus have a pattern of selection related with sodium homeostasis.

6.2 Aims

The aim of this study were:

1. To examine the allele frequency and LD at the *CYP11B1/CYP11B2* locus in a population with African ancestry by genotyping 12 SNPs in the *CYP11B1/CYP11B2* locus in 35 founders of Afro-Caribbean families and to compare them with the allele frequencies and LD of founders of two independent British studies (pilot BRIGHT TDT and Oxford Study).

2. To generate phylogenetic trees of the *CYP11B1* and *CYP11B2* genes.

6.3 Subjects and Methods

6.3.1 Subjects

Jamaican Study

Forty five extended families (334 subjects) were recruited in Jamaica as part of a project to determine the genetic determinants of blood pressure variability. Family tree, cardiovascular history and date of birth were recorded. Height, weight, sex, and anthropometric measurements (waist-hip ratio, skin fold thickness and body mass index) were also documented. Detailed blood pressure recordings were taken at the time of the study.

Probands

The probands were selected from clinical records after attendance at either the hypertension clinic or medical outpatients' clinic. The diagnosis had been established on the basis of three diastolic readings >90 mmHg when the patients were ages 15-49 years.

The probands were subjects with a long-standing clinical diagnosis of hypertension and were taking antihypertensive medication or subjects with a clinical history of hypertension but who were not currently taking medication and who had diastolic blood pressures >95 mmHg when examined for this study. Diabetic subjects were excluded. Full details of recruitment strategy have been published elsewhere (McKenzie *et al.* 1995). The University of West Indies/University Hospital of West Indies Ethics Committee approved the protocol. Families were recruited via a hypertensive proband. DNA was available and extracted by McKenzie and colleagues. Full details of extraction have been described elsewhere (McKenzie *et al.* 1995).

Caucasian Studies

Subjects of the TDT BRIGHT and Oxford Study have been described in Chapter 5 and 4, respectively. From the Oxford Study families, only the 149 founders were selected for the study in this chapter.

HapMap Populations

The genotypes available for the eleven population in the HapMap project release #27 (**ASW**: African ancestry in Southwest USA, **CEU**: Utah residents with Northern and Western European ancestry from the CEPH collection, **CHB**: Han Chinese in Beijing, China, **CHD**: Chinese in Metropolitan Denver, Colorado, **GIH**: Gujarati Indians in Houston, Texas, **JPT**: Japanese in Tokyo, Japan, **LWK**: Luhya in Webuye, Kenya, **MEX**: Mexican ancestry in Los Angeles, California, **MKK**: Maasai in Kinyawa, Kenya, **TSI**: Tuscans in Italy and **YRI**: Yoruban in Ibadan, Nigeria) were downloaded for the following seven genes:

1) *CYP11B1:*11β-hydroxylase (chr8: 143950939.. 143958203, excluding SNPs rs1134096, rs1134095, rs7003319, rs5017238, rs5303, rs1137486)

2) *CYP11B2:* aldosterone synthase (chr8: 143986179.. 143999186, plus SNPs rs4736354 and rs6471580 located in intergenic region)

3) *ACE*: Angiotensin I converting enzyme, including 3 isoforms (chr17:58915909..58952935)

4) *SLC24A5*:solute carrier family 24, member 5 (chr15:46200461..46221880)

5) *MCM6:* minichromosome maintenance deficient 6 (chr2:136313674..136350481)

6) *G6PD*:glucose-6-phosphate dehydrogenase, including 2 isoforms (chrX:153412800..153428427)

7) *HBB*:Beta globin (chr11:5203272..5204877)

6.3.2 CYP11B1/CYP11B2 locus polymorphisms genotyped

Polymorphisms with PCR and direct sequencing

In the *CYP11B2,* PCR and direct sequencing were performed in the promoter (B2PROM-344) and intron 2 (B2INCON). In the *CYP11B1,* PCR and direct sequencing were performed in the promoter (B1PROM). The methodology is detailed in section 2.3.1. Genotyping quality control was carried out by including positive and negative controls in each genotyping run and by genotyping on average 10% of the samples in duplicate. All positive controls in each SNP had the correct genotype, thus excluding mis-genotyping.

Polymorphisms with TaqMan

Polymorphisms located in exon 3 *CYP11B2* (rs4546); intergenic region (rs6471580 and rs4736354), exon 1 (rs6410), exon 2 (rs5283), intron 5(rs6471570) and 3'UTR (rs5301 and rs4736312) in *CYP11B1* were genotyped using the corresponding TaqMan assays (see sections 2.3.2) Genotyping quality control was done by including positive and negative controls in each genotyping run and by genotyping 10% of the samples in duplicate.

6.3.3 Statistical Analyses

Haploview v. 3.32 software was used to analyse, interpret and visualize patterns of LD (Barrett *et al.* 2005). Pearson's chi square test was used to compare allele frequencies. Genetic data analysis v.1.0 software was used to generate gene trees. The software was created by Lewis, P. O., and Zaykin, D. 2001. Genetic Data Analysis: Computer program for the analysis of allelic data, Version 1.0 (d16c) (http://lewis.eeb.uconn.edu/lewishome/software.html).

TreeView v.1.6.6 software was used to visualize the gene trees generated (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (Page 1996).

6.4 Results

6.4.1 Jamaican Study

Table 6-1. Demographic and phenotypic characteristics of only subjects with genotyping data in the Jamaican Study (N=249)

	NT	HTN
N	193	56
M:F	0.69	0.65
Age at enrolment, y	34.5 (13.4)	52.3 (13.3)
BMI, kg/m^2	25.1 (5.2)	27.7 (5.3)
Waist, cm	82.4 (11.8)	91.0 (11.5)
Waist hip ratio	0.84 (0.07)	0.91 (0.08)
SBP, mmHg	123.1 (36.6)	158.2 (27.2)
DBP, mmHg	70.6 (25.7)	92.8 (16.3)

Normotensives participants: NT; hypertensives: HTN. Values are reported as mean (SD) for normally distributed traits. N, number of subjects; M:F, male to female ratio; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

CYP11B1/CYP11B2 Genotyping

The 12 polymorphisms were genotyped in 249 individuals of the Jamaican Study (Table 6-2). All of them were in Hardy-Weinberg equilibrium and had minor allele frequencies (MAF) > 1%.

Table 6-2. Characteristics of polymorphisms genotyped in the *CYP11B1* and *CYP11B2* genes in the Jamaican Study

Variation no.	Chr	Gene	Location	SNP	Position bp	Status	Minor allele	MAF	% Genotyping	Obs.Het	HWE p
1	8	*CYP11B1*	3'UTR	rs4736312	143950939	ALL	T	0.43	98.7	0.63	0.17
						HTN				0.56	0.60
						NT				0.71	0.16
2	8	*CYP11B1*	3'UTR	rs5301	143952275	ALL	G	0.31	97.5	0.44	1.00
						HTN				0.47	1.00
						NT				0.40	1.00
3	8	*CYP11B1*	Intron 5	rs6471570	143954407	ALL	T	0.47	98.7	0.60	0.32
						HTN				0.56	0.65
						NT				0.65	0.34
4	8	*CYP11B1*	Exon 2	rs5283	143957599	ALL	T	0.06	99.6	0.06	0.09
						HTN				0.06	0.09
						NT				0.06	1.00
5	8	*CYP11B1*	Exon 1	rs6410	143958007	ALL	G	0.39	98.3	0.43	0.72
						HTN				0.44	1.00
						NT				0.41	0.62
6	8	*CYP11B1*	Promoter	rs4471016 (-1859 A/G)	143960096	ALL	A	0.34	93.3	0.46	1.00
						HTN				0.44	1.00
						NT				0.47	1.00
7	8	*CYP11B1*	Promoter	rs4313136 (-1889 G/T)	143960126	ALL	T	0.26	94.5	0.34	0.66
						HTN				0.33	1.00
						NT				0.35	0.57
8	8		Intergenic	rs4736354	143977468	ALL	C	0.14	98.3	0.23	0.53
						HTN				0.17	0.27
						NT				0.29	1.00
9	8		Intergenic	rs6471580	143983703	ALL	T	0.37	99.2	0.57	0.29
						HTN				0.56	0.65
						NT				0.59	0.59
10	8	*CYP11B2*	Exon 3	rs4546	143993555	ALL	T	0.14	98.3	0.29	1.00
						HTN				0.17	1.00
						NT				0.41	1.00
11	8	*CYP11B2*	Intron 2	IC	143993985	ALL	Conv	0.10	97.5	0.21	1.00
						HTN				0.29	1.00
						NT				0.12	1.00
12	8	*CYP11B2*	Promoter	rs1799998 (-344 T/C)	143996602	ALL	C	0.16	94.5	0.31	1.00
						HTN				0.19	1.00
						NT				0.44	0.54

Shown are the characteristics for the 12 *CYP11B1/CYP11B2* locus SNPs of the 35 founders in the Jamaican Study and the percentage of genotyping for the entire cohort (249 individuals). SNPs are presented in chromosomal order and their location within the gene indicated. Position in base pair (bp) derived from ENSEMBL release 49 July 2008 Chromosome 8 assembly (ENSEMBL website http://www.ensembl.org). Status: ALL, includes the 35 founder subjects; HTN, includes only the hypertensives; NT, includes only the normotensive members. MAF, minor allele frequency; Obs.Het, observed heterozygosity;HWE p, Hardy-Weinberg equilibrium p-value.

Allelic variation

Genotype analysis of twelve SNPs in common between two cohorts across the *CYP11B1/CYP11B2* locus revealed differences in allele frequencies in relation to ancestry (Figure 6-1). For example, the C allele of the -344T/C (rs1799998) was more frequent in British with Caucasian ancestry (represented by the 79 BRIGHT subjects (0.45)) but was much less frequent in Jamaicans (0.16). A similar trend was noted in the IC, rs4546, rs4736354 and rs5283 SNPs. A less pronounced difference in frequency was also observed for rs4736312, rs4313136 (-1889 G/T) and rs6471580; in the Caucasian cohort these are classified as major alleles but were found as minor alleles in the population of African ancestry. All the SNPs are in Hardy-Weinberg equilibrium for both populations as shown in Table 6-2 of this chapter and in Chapter 5.

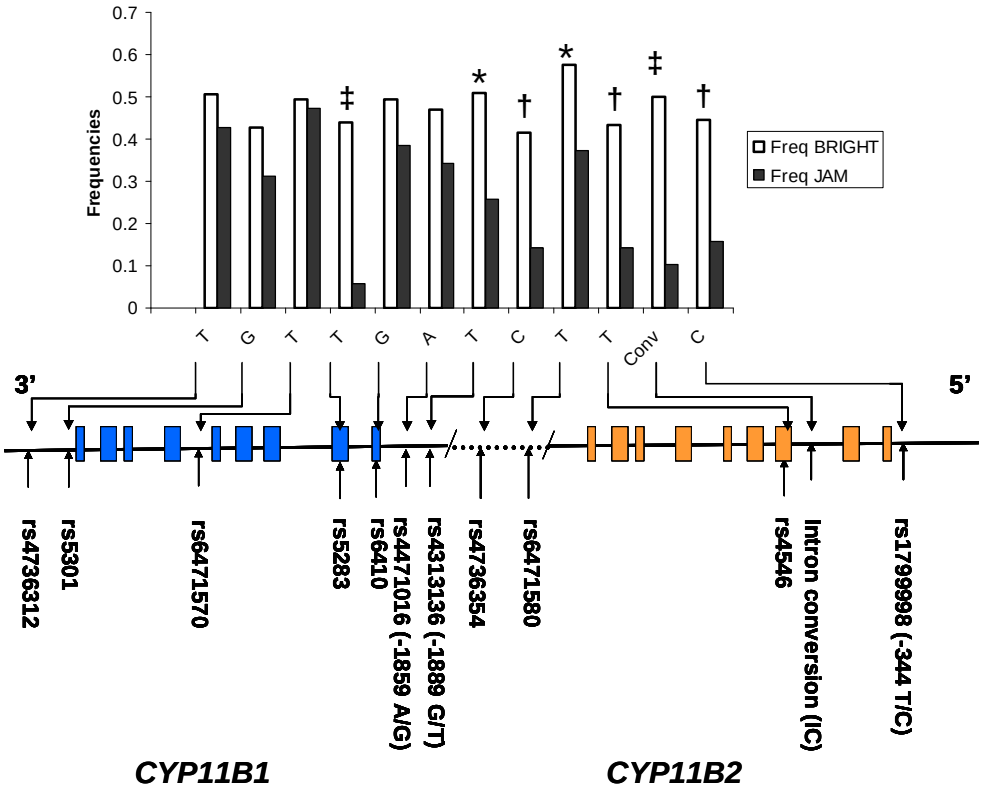


Figure 6-1. Differences in allele frequencies between Jamaican founders and 79 Caucasians of the pilot BRIGHT study. *p<0.05 †<0.01 ‡p<0.0001

A similar pattern in allele frequency differences was observed in the 5 common SNPs between the Jamaican founders (35 individuals) and the Oxford study founders (149 individuals) (Keavney *et al.* 2005) as shown in Table 6-3.

Table 6-3. Comparison of allele frequencies of common SNPs in an Afro-Caribbean and a Caucasian population from Oxford, UK

			Frequency		
Marker	**rs number**	**Allele**	**Afro-Caribbeans**	**Caucasians (Oxford Study)**	***p***
CYP11B1 Exon 1 A/G	rs6410	G	0.386	0.534	0.15
CYP11B1 -1859 C/T	rs4471016	A	0.343	0.441	0.28
CYP11B1 -1889 A/C	rs4313136	T	0.257	0.424	0.07
CYP11B2 Intron conversion (IC)		Conv	**0.103**	**0.551**	**<0.0001**
CYP11B2 -344 C/T	rs1799998	C	**0.156**	**0.467**	**<0.0001**

LD and Haplotype Structure

Differences in LD were also found between the populations of Caucasian ancestry (TDT BRIGHT or Oxford Study) and that of African ancestry (Figure 6-2 and Figure 6-3). The pattern of any LD block in the British samples showed a large contiguous region of high LD in the *CYP11B* locus, highlighted by large areas in red (high D'). The BRIGHT TDT pilot study has regions in higher LD compared with the Oxford Study because the SNPs selected for the BRIGHT TDT had a better coverage of the locus. In contrast, the pattern of LD in the Jamaican samples showed the *CYP11B* locus was broken into smaller regions with high LD (high D' in red) interspersed by regions of low D', in white, or high D' with low log of the likelihood odds ratio (LOD) in gray. LOD is a measure of confidence in the value of D'.

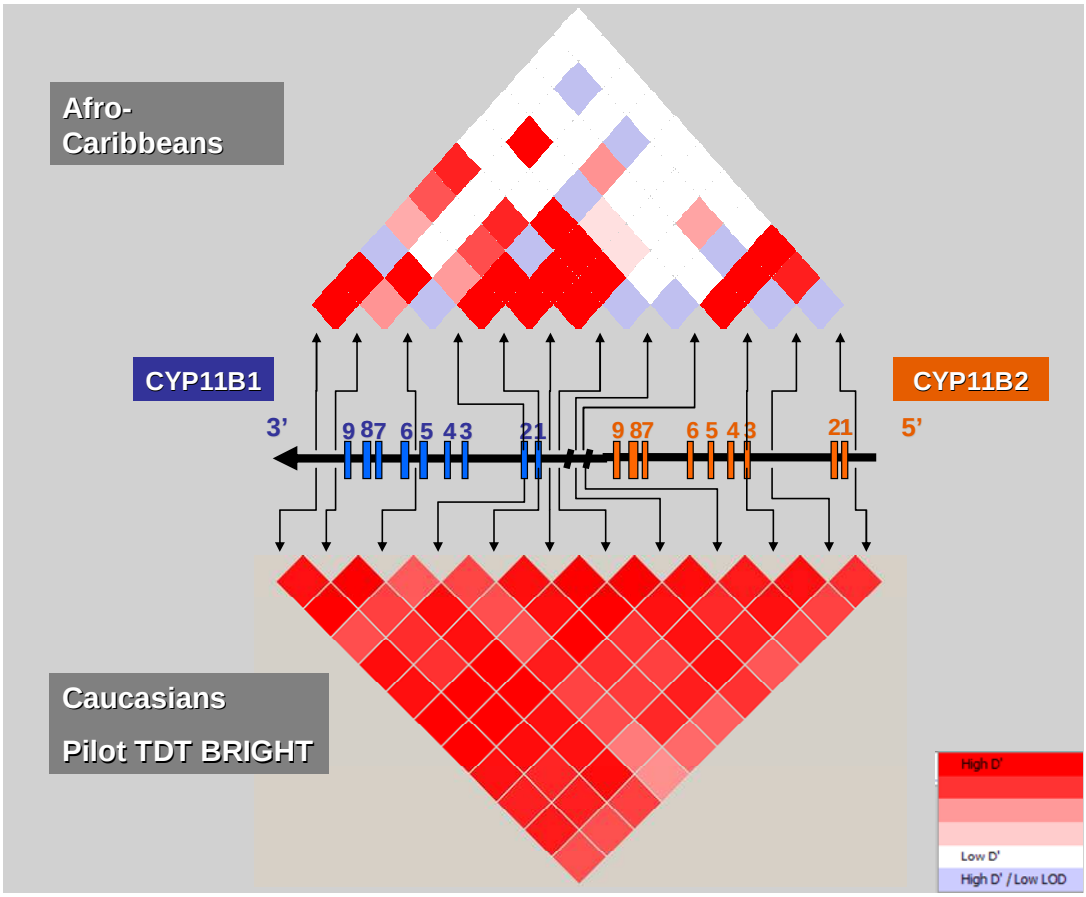


Figure 6-2. LD block comparison between the 12 SNPs genotyped in the Jamaicans and the 79 pilot BRIGHT TDT.

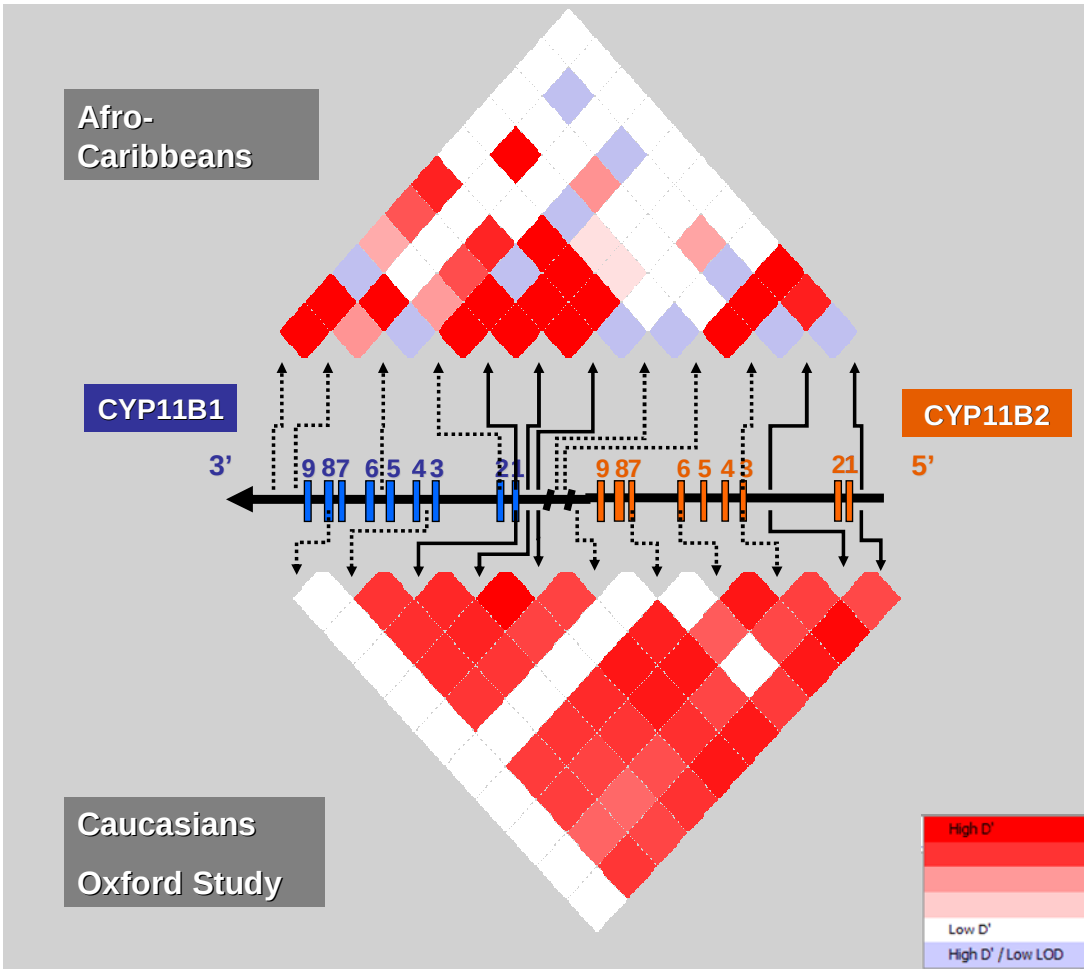


Figure 6-3. LD block comparison between the 12 SNPs genotyped in the Jamaicans and the 11 SNPs genotypes in the Oxford Study. The solid lines indicate the 5 SNPs genotyped in common in both studies and the dotted lines indicate the different SNPs genotyped in each study.

Phylogenetic trees

Phylogenetic distributions of the genes *CYP11B1, CYP11B2, ACE, SLC24A5, MCM6, G6PD* and *HBB* in different populations are presented in Figure 6-4, Figure 6-5 and Figure 6-6.

In the *CYP11B1* and *CYP11B2* phylogenetic trees (Figure 6-4), three main branches can be distinguished: a Caucasian branch, an African branch and an Asian branch. This shows higher relatedness between populations of similar ancestry. However, these branches are distributed differently in each gene. There is a shorter distance between the Caucasian and African branches compared to the Asian branch in the *CYP11B1* gene. This distribution changes in the *CYP11B2* gene, as the distance is shorter between the Caucasian and Asian branches and is longer with the African branch. Thus, in the *CYP11B2* gene, there is a clearer difference between Africans and non-Africans.

The distribution of the branches in the generated *ACE* tree (Figure 6-5A) resembles the *CYP11B2* tree. There is branch of African ancestry, a branch of Caucasian ancestry and a branch of Asian ancestry, the last two being closer, with a similar split between African and non-African populations. Despite the similarities with the *CYP11B2* phylogenetic tree, in the *ACE* tree the Tuscans are clustered with the African branch and not the Caucasian branch.

The clustering of populations in the phylogenetic tress of the remaining four genes (*SLC24A5, MCM6, G6PD* and *HBB)* follows a different pattern (Figure 6-5 and Figure 6-6). The only tree demonstrating certain similarities with the *CYP11B* genes is the *G6PD* tree as an African and a non-African branch can be identified. This shows the evolution of variations in these genes is probably affected by different factors.

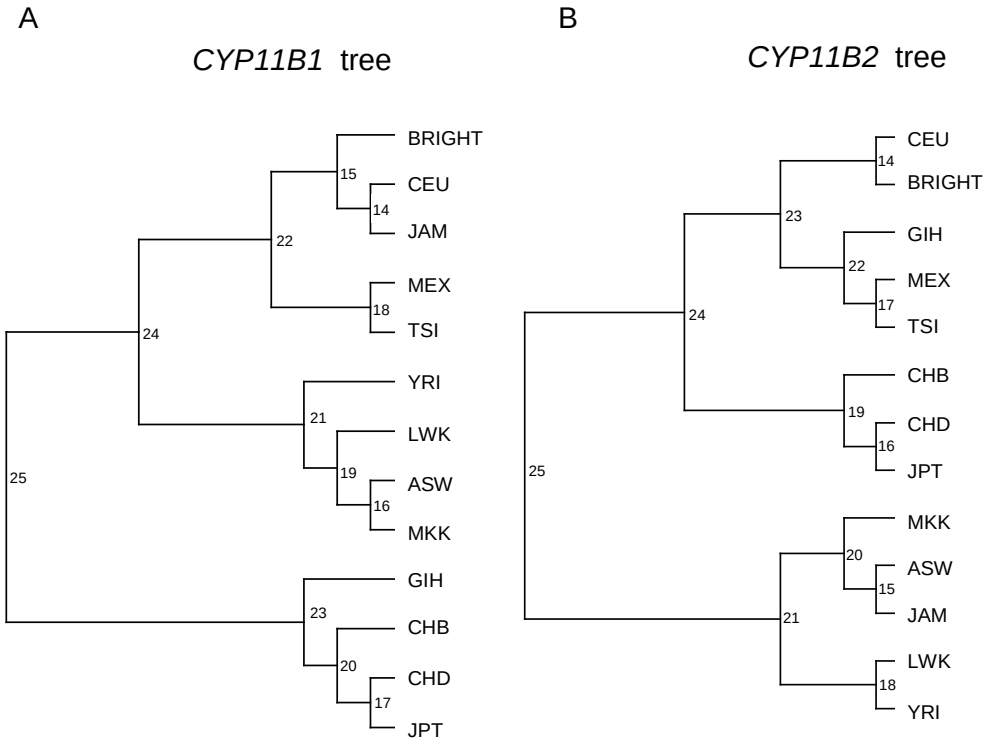


Figure 6-4. Phylogenetic trees of the *CYP11B1* gene (panel A) and *CYP11B2* (panel B) generated with the 11 populations of the HapMap, the pilot BRIGHT TDT and the Jamaican Study. ASW: African ancestry in Southwest USA, BRIGHT: pilot BRIGHT TDT from Britain, CEU: Utah residents with Northern and Western European ancestry from the CEPH collection, CHB: Han Chinese in Beijing, China, CHD: Chinese in Metropolitan Denver, Colorado, GIH: Gujarati Indians in Houston, Texas, JAM: Afro-Caribbeans from Kingston, Jamaica, JPT: Japanese in Tokyo, Japan, LWK: Luhya in Webuye, Kenya, MEX: Mexican ancestry in Los Angeles, California, MKK: Maasai in Kinyawa, Kenya, TSI: Tuscans in Italy and YRI: Yoruban in Ibadan, Nigeria.

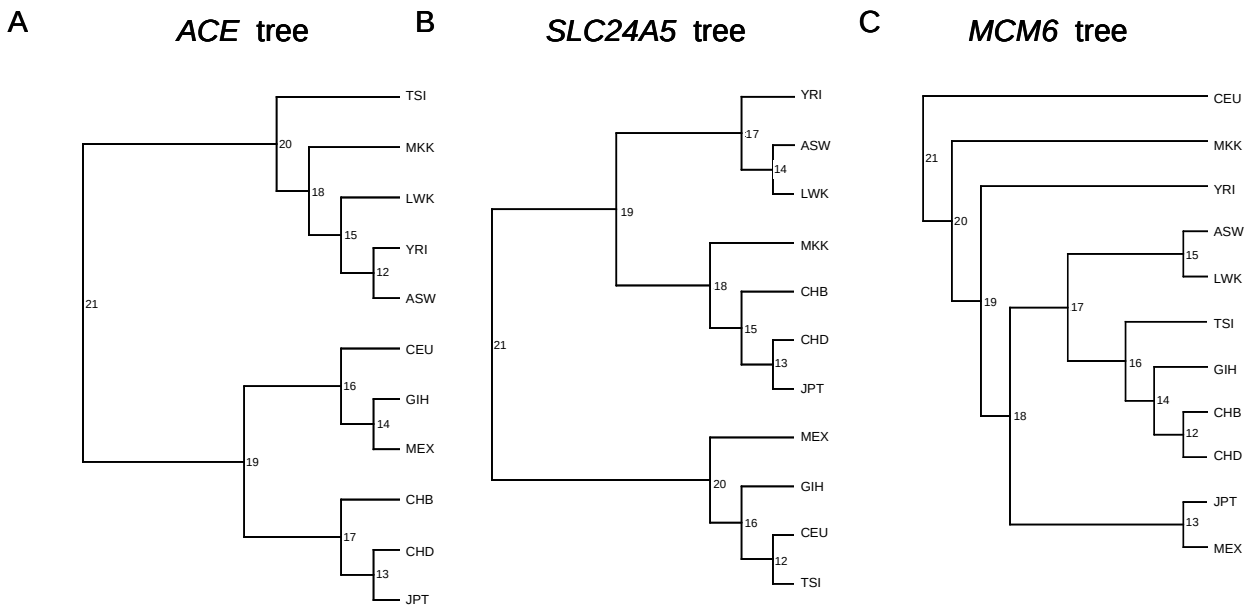


Figure 6-5. Phylogenetic trees of the *ACE* gene (panel A), *SLC24A5* (panel B) and *MCM6* (panel C) generated with the 11 populations of the HapMap. ASW: African ancestry in Southwest USA, CEU: Utah residents with Northern and Western European ancestry from the CEPH collection, CHB: Han Chinese in Beijing, China, CHD: Chinese in Metropolitan Denver, Colorado, GIH: Gujarati Indians in Houston, Texas, JPT: Japanese in Tokyo, Japan, LWK: Luhya in Webuye, Kenya, MEX: Mexican ancestry in Los Angeles, California, MKK: Maasai in Kinyawa, Kenya, TSI: Tuscans in Italy and YRI: Yoruban in Ibadan, Nigeria.

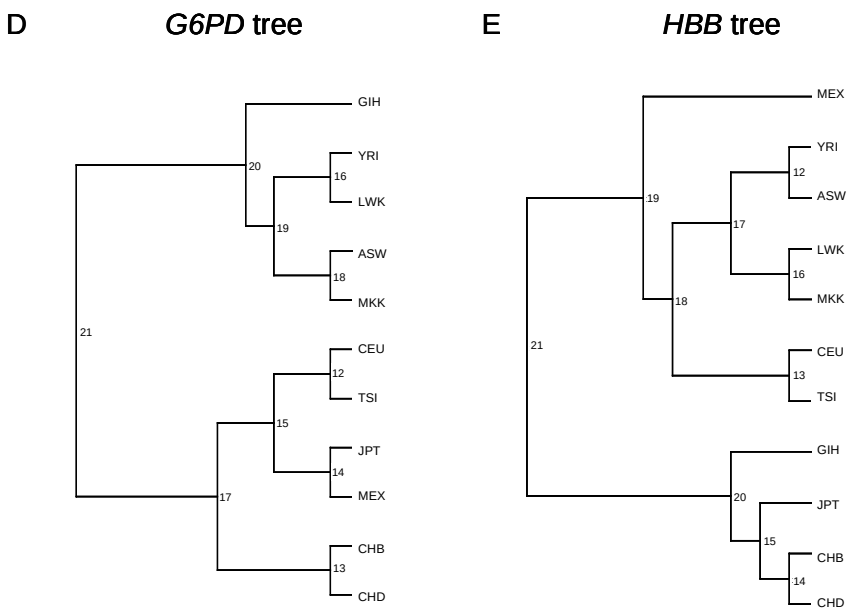


Figure 6-6. Phylogenetic trees of the *G6PD* (panel D) and *HBB (*panel E) generated with the 11 populations of the HapMap. ASW: African ancestry in Southwest USA, CEU: Utah residents with Northern and Western European ancestry from the CEPH collection, CHB: Han Chinese in Beijing, China, CHD: Chinese in Metropolitan Denver, Colorado, GIH: Gujarati Indians in Houston, Texas, JPT: Japanese in Tokyo, Japan, LWK: Luhya in Webuye, Kenya, MEX: Mexican ancestry in Los Angeles, California, MKK: Maasai in Kinyawa, Kenya, TSI: Tuscans in Italy and YRI: Yoruban in Ibadan, Nigeria

6.5 Discussion

Previous studies of some variants in the *CYP11B1* and *CYP11B2* genes have shown correlation with human essential hypertension and intermediate phenotypes without a common consensus. This prompted us to perform an analysis of allele frequencies and LD in a population of African ancestry and compare it with populations of Caucasian ancestry. Although a complete characterization of all haplotypes sequencing the whole *CYP11B* locus in many individuals would be ideal (Carlson *et al.* 2003; Crawford *et al.* 2004), it was shown in Chapter 5 that the selection of certain SNPs can give enough information for haplotype-based association studies in the locus. This initial framework using a Jamaican population can be used for further studies of this locus in individuals of African ancestry and also to document the diversity of the patterns in these two hypertension candidate genes which can help understand their evolution.

To make the genotyping of the Jamaican subjects feasible and economical, 12 SNPs were chosen from the 15 used in the Caucasian study in Chapter 5 and are shown in Table 6-2. The allele frequencies are similar to the ones reported in the public database HapMap (http://hapmap.org) for Africans (YRI). The minor allele frequency ranged from 0.06 to 0.47. The frequencies of the same SNPs in the pilot BRIGHT TDT study described in Chapter 5, were less variable and therefore a comparison between populations of Caucasian and African ancestry was made. The genotyping of each SNP was very successful ranging from 93-99%. These 12 SNPs differ considerably in genetic variability with some having very low heterozygosity (i.e. rs5283, Het=0.06) and others having medium heterozygosity, values ranging from 0.2 to 0.6. There was no significant deviation from Hardy-Weinberg equilibrium in any of the SNPs genotyped in the Jamaican Study.

When a more detailed comparison was made between allele frequencies in the *CYP11B* locus in subjects with Caucasian and African ancestry, considerable variation was observed (Figure 6-1 and Table 6-3). More significant discrepancy in allele frequencies was found towards the 5' region of the *CYP11B1* and *CYP11B2* genes or in the intergenic region. This suggests the variations might be located in regulatory fragments of the genes and not in regions after exon 5 which can be more deleterious to the production of the enzymes, as previously mentioned by (Pascoe *et al.* 1992a). In some SNPs (rs4736312, rs4313136 and rs6471580) the differences in frequencies were small but enough to be classified as minor alleles in one population and major in the other.

Several evolutionary mechanisms have been proposed to contribute to allelic population differentiation (Akey *et al.* 2002; Bamshad & Wooding 2003; Bowcock *et al.* 1991; Lewontin & Krakauer 1973). From them, founder effects, bottlenecks and balancing selection might be involved. Founder effects and bottlenecks can contribute to a loss in genetic variation. The former mechanism occurs when a new population is a started by a few members of a larger population. The latter mechanism occurs when a population size is decreased. Balancing selection is based on the hypothesis of Theodosius Dobzhansky of a selection regime that results in the preservation of certain variants at a locus in a population by favoring heterozygotes (Hedrick 2007).

It is important to also consider the implications of the difference of allele frequencies in the power of association studies as there is a direct relationship between them. In addition, the power is also affected by the magnitude of the effect of the variant on the disease risk and the sample size. Thus to evaluate an effect of the same magnitude of an allele in the *CYP11B* locus in populations with different ancestry the sample size will vary. Care should also be taken in selection criteria of the subjects being studied to avoid population stratification which can cause false positive associations (Ardlie *et al.* 2002; Cardon & Palmer 2003; Hirschhorn & Daly 2005). Characterisation of allele frequencies in other populations with ancestries different from Caucasian and African populations might be useful to arrive at a better understanding of the associations found in this region.

A manifestation of the differences in allele frequencies is the variation in the patterns of LD between the populations. The variation in LD can be mainly observed in differences in length or in haplotypes (Hanchard *et al.* 2007). This agrees with the findings in this chapter as it was observed that Jamaicans, with an African ancestry, have shorter LD patterns compared with any of the two studies with Caucasian ancestry. The upper triangles in Figure 6-2 and Figure 6-3 corresponding to the Jamaican population have smaller regions with high LD, shown in red, compared with the continuous block in high LD either in the pilot BRIGHT TDT or the Oxford study (lower triangles). The differences in pattern of LD in subjects from African and Caucasian ancestry is similar to what has been reported for several regions across the human genome (Conrad *et al.* 2006; Ke *et al.* 2004). Evidence suggests human migration, natural selection, recombination and evolution have shaped these patterns (2003; McVean *et al.* 2004; Plagnol & Wall 2006; Reich *et al.* 2001; Sabeti *et al.* 2002).

The high level of LD in the pilot BRIGHT TDT and the Oxford study has both an important advantage and a disadvantage for association mapping (Altshuler *et al.* 2000). The advantage is the extensive LD in the *CYP11B1/CYP11B2* locus that can help to reduce the number of SNPs required for genotyping in the region to test for association with a specific phenotype, i.e. hypertension or ARR. Nevertheless, the high level of LD will make it more difficult to distinguish which markers are causal variants and those which are association markers (Jorde *et al.* 2001). It is important to emphasise that it is not only coding region variants that can have a biological role. The promoter and regulatory region variants can also influence gene expression (Felder *et al.* 2002). The results in this chapter suggest that these latter regulatory elements might play an important role in the expression of *CYP11B1* and *CYP11B2* genes and the phenotypes of interest.

The differences in LD were also demonstrated by differences in SNP coverage between Europeans (BRIGHT Study=90%, Oxford Study =87%) and Afro-Caribbeans (53%).

The populations with African ancestry, such as the one studied here, show a lower level of LD and this differing LD pattern can be valuable in dissecting the actual functional variants in the *CYP11B* locus which are associated with hypertension and the other intermediate phenotypes of interest. This approach has already been used successfully for the ACE gene and hypertension (Felder *et al.* 2002; Zhu *et al.* 2000).

To explore further the differences between populations in the *CYP11B1/CYP11B2* locus a phylogenetic tree for each gene was constructed using the information from the Jamaican Study, the TDT BRIGHT pilot study (explained in more detailed in Chapter 5) and the eleven populations available from HapMap (Figure 4). To validate this approach, to compare the relationship between populations and to have a possible path to infer the evolutionary pattern, trees were also generated for five other genes (*ACE*, *SLC24A5, MCM6, G6PD* and *HBB)* using only the eleven HapMap populations .

From the methods available to generate phylogenetic trees, the Unweighted Pair-Group Method using artithmetic Averages (UPGMA) method was chosen. The data used are the distances in a gene between two populations. First, the observed number of nucleotide differences between a pair of sequences is calculated. If a proportion of bases is the same, the Jukes-Cantor distance is estimated. Then, the intercluster distance is calculated as the average in the pairwise Jukes-Cantor distances for members in two clusters. The lesser the distance, the more similar the populations. The first cluster chosen in this type of tree was

the population pair with the closest distance. This cluster will have the shortest branches. Then, to continue building the tree the distance between the above mentioned cluster 1 and the other populations is measured. Cluster 2 will be generated by grouping cluster 1 and the closest population to it. The process of comparing distances and generating clusters continues until all the populations are included in the tree (Weir 1996).

This method was selected because it allows a simple way to construct and interpret trees. In addition, the results it provides are similar to the maximum likelihood method which, in a test of methods, gave the least error in reconstructing trees (Astolfi *et al.* 1981). However, it is important to consider that the UPGMA has two important limitations. The first limitation is the assumption that there is the same evolutionary rate in all the branches of the tree producing a rooted tree. The meaning in this case by a rooted tree is that one of its branches is used as a common ancestor for all the population in the tree. The second limitation is the data are reduced to a set of distances, ignoring other possible correlations (Cavalli-Sforza *et al.* 1993).

In the *CYP11B1* and *CYP11B2* phylogenetic trees the divergence in distribution suggests that each gene might have evolved in a different way and that perhaps different factors are involved in this evolution. The BRIGHT population of Caucasian ancestry is located, as expected, in the *CYP11B1* and *CYP11B2* trees within the Caucasian branch close to the CEU population (Northern and Western European ancestry). This indicates that the variations between these two populations are small. Interestingly, a different pattern is observed with the Jamaican Study. For *CYP11B1*, the Jamaican Study is in the branch of Caucasians, showing more similarities with the CEU population. However, for *CYP11B2* the Jamaican Study is located in the African branch, sharing more similarities with the African Americans (ASW). This is not surprising as the Jamaicans are primarily of African descent with an admixture of Caucasian and Native American ancestry (Benn-Torres *et al.* 2008). This suggests that variations in Jamaicans of African ancestry might have more influence on the *CYP11B2* gene whereas the variations derived from the other populations could have more influence in the *CYP11B1*.

The *ACE* gene is also a candidate gene in hypertension and has been extensively studied. Similar to the *CYP11B1* and *CYP11B2* genes, the *ACE* has less LD among blacks (Zhu *et al.* 2003c). In addition, a haplotype analysis suggested that African Americans and European Americans have different inheritance patterns for different haplotypes (Zhu *et al.* 2003b). A study in Africans, Afro-Caribbeans and African Americans showed that,

although the majority of the variations have similar allele frequency, there are a few in which the frequency is influenced by European admixture (Bouzekri *et al.* 2004).

The distribution of the branches in the generated *ACE* tree (Figure 6-5A) follows relatedness according to ancestry similar to the *CYP11B2* tree. However, the Italians from Tuscany, instead of sharing more similarities with the Caucasian branch, share similarities with the African branch. It has been hypothesized that there is an ancient origin and possible specific genetic identity of the Tuscan population, with variations shared with populations from North Africa and Near East (Achilli *et al.* 2007; Francalacci *et al.* 1996). This might be a reasonable explanation for observing more similarity between the Italians and Africans in the *ACE* gene.

The trees generated with the *SLC24A5* and *MCM6* genes follow a different pattern in the distribution of populations compared with the *CYP11B* trees (Figure 6-5B and C). These differences help to validate the approach with UPGMA method. An explanation of possible factors contributing to these differences in the trees of the *SLC24A5* and *MCM6* genes will be given with more detail.

The *SLC24A5* gene encodes an intracellular membrane protein involved in the diversity of skin pigmentation (Sturm 2009). Although several factors have an influence in skin color, such as intensity of solar radiation and availability of vitamin D, either produced by the body or supplied by the diet (Cavalli-Sforza *et al.* 1993), an effort has been made to elucidate the mechanisms by which allele variation in pigmentation genes affect the phenotype. The variations in this gene have recently been proposed as ancestry informative markers (Soejima & Koda 2007). The *SLC24A5* tree does not follow the same relatedness to ancestry as the *ACE* or the *CYP11B* genes but there is still a certain trend. According to a study of Italian, African and Asian subjects, the heterozygosis in the *SLC24A5* varies between populations, being more homogeneous in Caucasians (Giardina *et al.* 2008). This might explain the closeness of the African and Asian branches as they tend to be more heterogeneous. Moreover, there is still a debate as to whether the variations in this gene are affected by adaptative, natural or sexual selection. This selection process might also influence the clusters in the branches of the tree.

The *MCM6* gene encodes a protein involved in the formation of DNA replication forks. However, is of interest as it acts as a marker for gene involvement in lactase activity being in close proximity with the lactase gene. The branches of the tree generated with this gene,

instead of following relatedness according to ancestry, might follow relatedness according to the different combinations of SNPs leading to lactase persistence. The SNP frequent in lactose persistent Europeans is rare in individuals from Africa and the Middle East where lactose persistency is also present (Ingram *et al.* 2009). Three other studies revealed that other sequence variants (Enattah *et al.* 2008; Ingram *et al.* 2007; Tishkoff *et al.* 2007) are present in East Africans and one of the variants is also present in subjects from Saudi Arabia (Imtiaz *et al.* 2007). It has been suggested the variants could have arisen in different geographic locations. This could explain why the distances in this tree are larger.

Two traditional examples of selection in the human genome are the mutations in the *G6PD* and *HBB* genes (Figure 6-6D and E). The *G6PD* encodes the glucose-6-phosphate dehydrogenase. Mutations in it usually result in reduced enzyme activity which can affect resistance to malaria. The *HBB* gene encodes for the beta chain in human adult haemoglobin. Mutations in this chain can lead to anemias or thalassemias but can also confer resistance to malaria. As both genes are subject to a selection in malarial areas, they have a similar pattern of variation according to the geographic distribution of the disease (Cavalli-Sforza *et al.* 1993).

Similar to *CYP11B2*, in *G6PD*, there is division between the African and non-African populations. This has already been reported (Verrelli *et al.* 2002) and is in agreement with the finding of highest malaria transmission in Africa. The β thalassemias are more common in Africa and Europe and less common in East and Southeast Asia (Cavalli-Sforza *et al.* 1993). This geographic distribution of disease prevalence might be an explanation of having the Asian branch further apart in the *HBB* gene tree.

From the structure of the additional phylogenetic trees of genes affected by environmental factors, perhaps the ones with a closer structure to the *CYP11B* genes are the *ACE* and *G6PD* which suggests that variations in the *CYP11B* genes are related according to ancestry and might follow an adaptive selection.

The differences found in the *CYP11B1* and *CYP11B2* phylogenetic trees might imply evolutionary diversity perhaps affected by environmental factors. The diversity of *CYP11B1* might be influenced by stress, while other factors such as the need to adapt to sodium conservation according to climate and salt availability might contribute to *CYP11B2* diversity.

6.6 Conclusion

There is significant disparity in LD structure between ethnic groups. This along with the differences in allele frequencies would result in a degree of population stratification that would increase type 1 error. The high levels of LD across these regions in Caucasians, while advantageous in designing efficient association studies do not allow any identification of causal SNPs. African ancestry is more conducive to the identification of such variants. Studies in different ethnic groups are essential to dissect the role of this locus in hypertension and the evolution of the variants in this locus in different populations.

7 Association of *CYP11B1/CYP11B2* polymorphisms in Case-Control Populations

7.1 Introduction

The *CYP11B1/CYP11B2* polymorphisms which have previously been reported to be associated with hypertension and its related phenotypes are described in Chapter 1. These associations have been inconsistent, reported on small samples and not replicated (Sookoian *et al.* 2007). The only large study which found a significant association in this region, at the -344T/C polymorphism, with plasma aldosterone levels, urinary sodium excretion and at systolic blood pressure used a community-based Japanese cohort (Iwai *et al.* 2007). In Chapter 6, I established that there are differences in LD patterns in this region depending on the ethnicity of the population. Thus the characterisation of this region made by Iwai *et al.* is not applicable for a Caucasian population. For this reason, the study in Chapter 5 demonstrated that there is a strong LD in the *CYP11B1/CYP11B2* locus of Caucasians. A robust approach was used to select a set of 7 htSNPs that can identify common haplotypes, and can be used to impute more SNPs in the locus and to detect association in a large-scale study. It was therefore of interest to determine whether or not this set of polymorphisms associated with hypertension in a large case-control study.

7.2 Aims

The aim of this study was to test for association with hypertension in a Caucasian case-control population and to test for replication in an independent population.

7.3 Subjects and Method

7.3.1 Subjects

All samples used for the discovery stage study and the replication study are from Caucasian individuals.

Discovery stage study: BRIGHT Cases-Controls

As part of the MRC BRIGHT Study, unrelated hypertensive cases (n=1643) and normotensive controls (n=1697) of white European ancestry were recruited for association testing.

BRIGHT Cases

For the 1643 cases, the selection criteria for the subjects have already been described in section 5.3.1, http://www.brightstudy.ac.uk/ and (Caulfield *et al.* 2003). The inclusion criteria, in addition to the characteristics described in section 5.3.1, considered that the hypertensives' ages were between 18-85 years of age.

BRIGHT Controls

The 1697 healthy controls were age and sex-matched to the cases. Like the cases, the controls were of white British ancestry by grand-parental origin. Their characteristics have been described in section 3.3.1.

DNA was available and extracted by the BRIGHT consortium. Full details of extraction have been described elsewhere:

http://www.brightstudy.ac.uk/info/sop_9000666.html#processing).

Replication study: Swedish Cases-Controls

Swedish Cases

The criteria for inclusion in the replication study were as follows: Hypertensive cases had at least two consecutive blood pressure measurements of ≥160mmHg systolic and ≥100mmHg diastolic blood pressure, with the diagnosis made below 60 years of age. Two thousand cases were identified according to these criteria in the Nordic Diltiazem study (NORDIL) study; of these, 1612 individuals were genotyped. These hypertensive subjects represent the top 1.7% blood pressure distribution in the Swedish population.

The design and main results of the NORDIL Study have been previously described in detail (Hansson *et al.* 2000). Briefly, the study included 10881 Swedish and Norwegian hypertensive patients who were randomised to receive either diltiazem-based (n=5410) or diuretic- and/or β-blocker-based (n=5471) antihypertensive treatment in order to compare incident cardiovascular events during a mean follow-up of 4.5 years. Hypertension was defined as a diastolic blood pressure of > 100mmHg on at least two occasions. The combined primary end-point in the NORDIL study was fatal and non-fatal stroke, fatal and non-fatal myocardial infarction, and other cardiovascular death (Hansson *et al.* 2000). All primary end-points were assessed by an independent end-point committee, according to pre-specified criteria. Primary end-points occurred in 403 patients in the diltiazem group

and in 400 patients in the diuretic and β-blocker group. There was no difference in the incidence of primary endpoints between two treatment groups. Of the NORDIL patients, only those from Swedish centres participated in the genetic sub-study and DNA was obtained from 5280 Swedish patients, from which 2000 were selected for the replication study.

Swedish Controls

Control subjects were drawn from the Malmö Diet and Cancer study (MDC) cohort and had blood pressure ≤ 120mmHg systolic and ≤ 80mmHg diastolic. They were at least 50 years of age, free from cardiovascular events (coronary events and stroke during 10 year follow up) and not on any antihypertensive medication. Of the entire MDC population, 9.2% met these criteria and thus 1317 subjects were selected as hyper-controls with low cardiovascular risk.

The Malmö Diet and Cancer study (MDC) is a Swedish population-based sample aged 40-70 years (n=28098) originally collected to examine the relationship between dietary patterns and future developments of cancer (Berglund *et al.* 1993). The age of the MDC is similar to that of the NORDIL patients and recruitment/baseline examination was performed during the same time period as the NORDIL (1991-1996). At the baseline exam, blood pressure was recorded twice in the supine position and the mean of the two blood pressure measurements was recorded. MDC also includes detailed medical history, medication, anthropometry and life style factors (smoking, diet, alcohol intake, physical activity and socioeconomic index). DNA was isolated from whole blood and granulocyte preparations. The MDC has been followed for 10.5 years from baseline for cardiovascular endpoints (fatal and non-fatal coronary events and stroke) by linkage of the Swedish 10-digit personal number to the National Hospital Discharge register and the National Cause of Death register. At baseline, there were 860 prevalent coronary/stroke events and, during the follow-up time, 2100 incident coronary and stroke events occurred.

From a parallel genome wide association analysis in the Swedish Study, there was access to information of population stratification obtained through Eigenstrat v.3 (a method based on principal components analysis to model ancestry differences between cases and controls (Price *et al.* 2006).Thus, the analysis of the *CYP11B1/CYP11B2* locus was adjusted using data extracted from the genome wide association study.

7.3.2 Genotyping

The most informative htSNPs obtained from the BRIGHT TDT pilot study in Chapter 5 were used in the discovery stage with the BRIGHT Case-Controls to test for association with hypertension at the *CYP11B1/CYP11B2* locus.

Discovery stage

Many of the informative SNPs, identified in the pilot BRIGHT TDT study, had already been genotyped in the BRIGHT Cases and Controls or were in tight LD with the htSNPs making further genotyping unnecessary. The SNP rs4313136 in the *CYP11B1* was genotyped by Mrs. Elaine Friel and Mr. Lim Wei Yao. From the *CYP11B2* promoter region rs1799998 (in LD with rs28659182) and IC were genotyped by Mrs. Elaine Friel. The protocols followed are described in section 2.3.1.

The remaining four SNPs (rs6410, rs5301, rs4546 and rs4736354) were genotyped using TaqMan assays (see section2.3.2).

Genotyping quality control was carried out by including positive and negative controls in each genotyping run and by genotyping, on average, 5% of the samples in duplicate. All positive controls in each SNP had the correct genotype, thus excluding mis-genotyping.

Replication stage

For replication purposes, the most significant regions in the BRIGHT Cases-Controls were genotyped in the Swedish Cases-Controls by Mrs. Elaine Friel and Mrs. Christine Holloway. The SNPs in the *CYP11B1* promoter, rs4471016 and rs4313136, were genotyped following the protocol in section 2.3.1. The intron conversion in the *CYP11B2* was genotyped using PCR followed by automated sequencing (section 2.3.1). A SNP in the intergenic region, rs6471581, was selected as a proxy SNP for rs6414 in exon 3 of *CYP11B2.* This intergenic SNP, along with rs4546 and rs1799998 were genotyped using the corresponding TaqMan assay (see section 2.3.2).

The SNP in exon 1 of *CYP11B1,* rs6410, was genotyped using Illumina 610 quad chip and extracted from the results of a previous genome wide association study (Personal communication with Dr.Sandosh Padmanabhan, BHF Glasgow Cardiovascular Research Centre, UK). Genotyping quality control was performed in the TaqMan assays by genotyping on average 5% of the samples in duplicate. All genotype assignments were

performed blind with regard to clinical data. Test of deviations from Hardy-Weinberg equilibrium were performed using PLINK v.1.05 (http://pngu.mgh.harvard.edu/purcell/plink/) (Purcell *et al.* 2007).

7.3.3 Statistical Analyses

For the discovery study, shown in Table 7-1, significant differences between cases and controls were tested using a two-sample t-test for normally-distributed variables and Mann-Whitney for the rest. Categorical variables were compared using Pearson's chi-square test.

The characteristics for each SNP genotyped (minor allele, minor allele frequency, percentage of genotyping, observed heterozygosity and Hardy-Weinberg probability value) were calculated by using PLINK v.1.05 (Purcell *et al.* 2007).

In the discovery stage, absent and non-genotyped SNPs in the *CYP11B1/CYP11B2* locus were imputed using PLINK v.1.05 and data available from Caucasians with European ancestry (CEU) in the HapMap (release 24) (Purcell *et al.* 2007). The confidence threshold for making an imputed call was set at 0.9. Of the 37 SNPs in the region, 29 SNPs were imputed according to this criterion.

The tests of association with each individual SNP and also the three SNPs sliding window haplotype analysis were computed using PLINK v.1.05 (Purcell *et al.* 2007).

The three-marker sliding window haplotype analysis graph and LD plot was generated using SNP.plotter (http://cbdb.nimh.nih.gov/~kristin/snp.plotter.html) (Luna & Nicodemus 2007). Regarding the meta-analysis, combined analysis of association datasets was carried out by pooling of log-transformed odds ratios with the inverse variance method for the fixed-effects model. These calculations were performed using R (2.5.1) statistical software.

7.4 Results

BRIGHT Cases-Controls

Table 7-1. Phenotypic baseline characteristics for BRIGHT Study

	NT	HTN
N	1697	1643
M:F	0.62	0.62
Age at enrolment, y	59.4(9.1)	59.4 (9.1)
BMI, kg/m^2	25.3 [17.1-34.8]*	27.3 [15.0-45.0]
SBP, mmHg	123.6 (10.4)*	155.3 (21.0)
DBP, mmHg	76.8 (7.0)*	94.0 (11.2)

*p<0.001, showing significant differences between the normotensive and hypertensive group

From the BRIGHT Study, selected for the discovery stage, 1697 controls and 1643 cases were evaluated, with more females present in both groups, as described in Table 7-1. The subjects were middle-aged to elderly and of Caucasian ancestry. Hypertensive subjects had slightly higher BMI and higher blood pressure as expected.

Swedish Cases-Controls

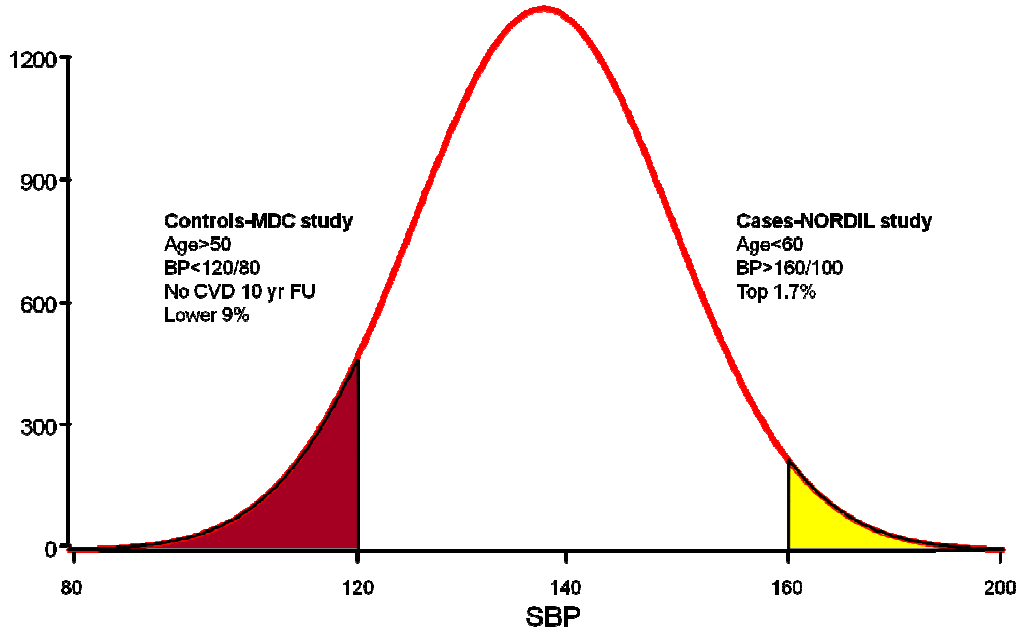


Figure 7-1. Phenotypic baseline characterisitics for Swedish Study

From the Swedish Study, selected for the replication stage, 1317 controls and 1612 cases were evaluated, as described in Figure 7-2. The subjects were middle-aged to elderly and of Caucasian ancestry. Similar to the BRIGHT Study, the ascertainment influences the differences in blood pressure between the groups.

CYP11B1/CYP11B2 Genotyping

The polymorphisms were genotyped in 3340 unrelated individuals of the BRIGHT Case Control cohort and 2929 unrelated individuals of the Swedish Study for the replication stage (Table 7-2). The SNPs for controls were in Hardy-Weinberg equilibrium ($p \geq 0.01$). The percentage of successful genotyping was above 80% for all of the SNPs. The study outlined in Chapter 5 established the 7 htSNPs that would be used for the discovery stage of this association study, considering the high LD in the region. As the PCR and automated sequencing of the promoter region in the *CYP11B1* gene allows the genotyping of rs44710161 (-1859A/G) in addition to the chosen htSNP rs4313136 (-1889 G/T), both have been reported. The results of the eight SNPs for the BRIGHT Study are shown in Table 7-2. The allele frequencies correspond with those reported by public databases (HapMap) in a population with Caucasian ancestry and they are all in Hardy-Weinberg equilibrium. There was good genotyping success ranging from 91-99%. The lowest genotyping success was found in the SNPs located in the *CYP11B1* promoter. In this nested PCR, a longer amplicon is required to ensure specificity, subsequently this influences the success of the genotyping. The quality control in each plate was monitored by using positive and negative controls alongside the samples. The observed heterozygosity ranged from 0.46-0.52, showing considerable genetic variability in each polymorphism. For the replication stage (Swedish Study) only the significant SNPs in the discovery association test were chosen. Thus, the discovery and replication studies had six SNPs genotyped in common. The SNP rs6414 was imputed in the BRIGHT Study and a proxy SNP in the intergenic region, the rs6471581, was chosen in the Swedish Study. In both studies, the minor allele frequencies and observed heterozygosities of the genotyped SNPs were similar. The replication study also had a good genotyping success ranging from 82 to 100% and the SNPs from the unaffected subjects were in Hardy-Weinberg equilibrium.

Table 7-2. Characteristics of the polymorphisms genotyped in the *CYP11B1* and *CYP11B2* genes in the BRIGHT and Swedish Cases and Controls.

								BRIGHT				Swedish			
Variation no.	Chr	Gene	Location	SNP	Position bp	Status	Minor allele	MAF	% Genotyping	Obs.Het	HWE p	MAF	% Genotyping	Obs.Het	HWE p
1	8	*CYP11B1*	3'UTR	rs5301	143952275	ALL	C	0.46	98.6	0.50	1.00	NA	NA	NA	NA
						HTN				0.50	0.58				
						NT				0.49	0.63				
2	8	*CYP11B1*	Exon 1	rs6410	143958007	ALL	T	0.48	96.8	0.49	0.53	0.46	82.4	0.48	0.04
						HTN				0.51	0.58			0.49	0.56
						NT				0.48	0.14			0.47	0.02
3	8	*CYP11B1*	Promoter	rs4471016	143960089	ALL	G	0.48	91.5	0.50	0.80	0.4688	100.0	0.53	$1X10^{-3}$
				(-1859 A/G)		HTN				0.52	0.19			0.56	$3.53X10^{-7}$
						NT				0.48	0.10			0.49	0.44
4	8	*CYP11B1*	Promoter	rs4313136	143960119	ALL	T	0.45	91.5	0.49	0.47	0.4474	100.0	0.52	$2.2X10^{-3}$
				(-1889 G/T)		HTN				0.52	0.10			0.55	$7.92X10^{-6}$
						NT				0.46	0.01			0.49	0.74
5	8		Intergenic	rs4736354	143977468	ALL	G	0.43	98.4	0.49	1.00	NA	NA	NA	NA
						HTN				0.49	0.84				
						NT				0.49	0.84				
6	8		Intergenic	rs6471581	143986919	ALL	C	NA	NA	NA	NA	0.4548	100.0	0.53	$4.0X10^{-4}$
						HTN								0.57	$8.44X10^{-8}$
						NT								0.48	0.54
7	8	*CYP11B2*	Exon 3	rs4546	143993555	ALL	T	0.43	96.6	0.49	0.78	0.4286	100.0	0.53	$2.88X10^{-6}$
						HTN				0.48	0.54			0.56	$1.76X10^{-9}$
						NT				0.50	0.84			0.49	0.78
8	8	*CYP11B2*	Intron 2	IC	143993985	ALL	Conv	0.47	91.1	0.49	0.19	0.4554	100.0	0.53	$1.0X10^{-3}$
						HTN				0.51	0.32			0.56	$1.91X10^{-7}$
						NT				0.46	0.01			0.48	0.43
9	8	*CYP11B2*	Promoter	rs1799998	143996602	ALL	C	0.44	99.3	0.50	0.26	0.4326	100.0	0.53	$3.05X10^{-6}$
				(-344 T/C)		HTN				0.49	0.72			0.56	$2.67X10^{-9}$
						NT				0.51	0.20			0.50	0.78

Shown are the most informative SNPs genotyped in the BRIGHT and Swedish Cases and Controls at the *CYP11B1/CYP11B2* locus and their corresponding characteristics for quality control purposes. SNPs are presented in chromosomal order and their location within the gene indicated. Position base pair (bp) derived from ENSEMBL release 49 July 2008 Chromosome 8 assembly (ENSEMBL website http://www.ensembl.org). Status: ALL, includes the Case Control subjects; HTN, includes only the hypertensive cases (n=1643 BRIGHT, n=1612 Swedish) ; NT, includes only the normotensive members (n=1697 BRIGHT, n=1317 Swedish). MAF, minor allele frequency; Obs.Het, observed heterozygosity;HWE p, Hardy-Weinberg equilibrium p-value.

7.4.1 Discovery stage

CYP11B1/CYP11B2 Imputation and single marker analysis for BRIGHT

In the discovery stage, the single marker association analysis results for the eight SNPs genotyped in the BRIGHT Study and the additional 29 SNPs that were generated through imputation are shown in Table 7-3. The strongest evidence for association with hypertension was found in the intron conversion (IC) polymorphism. Subjects with the conversion allele in intron 2 of *CYP11B2* have a higher risk of hypertension (OR=1.23, p value=1.23×10^{-4}). This result remained after correcting by means of a permutation test. Twenty-two of the genotyped and imputed SNPs showed p-values < 0.05 and were distributed across the entire locus, which is in high LD. However, the results did not remain significant after correction.

Table 7-3. Results of association analysis with single markers, using 37 SNPs.

Method	SNP	Position bp	m	MAF	HWEp	Freq cases	Freq controls	M	CHISQ	OR [95%CI]	p
Imputed	rs3819497	143921054	A	0.46	1	0.44	0.48	G	7.438	0.87 [0.79-0.96]	0.01
Imputed	rs7826809	143922611	T	0.46	1	0.44	0.48	C	7.438	0.87 [0.79-0.96]	0.01
Imputed	rs3753122	143923929	T	0.46	1	0.44	0.48	G	7.438	0.87 [0.79-0.96]	0.01
Imputed	rs2078674	143925235	T	0.46	1	0.44	0.48	G	7.438	0.87 [0.79-0.96]	0.01
Imputed	rs4418320	143941140	A	0.46	1	0.44	0.48	G	7.438	0.87 [0.79-0.96]	0.01
Imputed	rs4736346	143948851	A	0.46	1	0.44	0.48	C	7.438	0.87 [0.79-0.96]	0.01
Genotyped	rs5301	143952275	C	0.46	1	0.44	0.48	T	7.438	0.87 [0.79-0.96]	0.01
Imputed	rs6471570	143954407	A	0.45	0.05	0.47	0.44	C	3.422	1.1 [0.99-1.22]	0.06
Genotyped	rs6410	143958007	T	0.48	0.53	0.50	0.47	C	4.135	1.11 [1-1.23]	0.04
Genotyped	rs4471016	143960089	G	0.48	0.80	0.49	0.47	A	2.286	1.08 [0.98-1.2]	0.13
Genotyped	rs4313136	143960119	T	0.45	0.47	0.47	0.44	G	2.955	1.1 [0.99-1.22]	0.09
Imputed	rs4736349	143964434	T	0.43	0.78	0.42	0.45	C	5.302	0.89 [0.8-0.98]	0.02
Imputed	rs4736350	143966034	G	0.49	0.80	0.50	0.47	A	4.135	1.11 [1-1.23]	0.04
Imputed	rs7004383	143972165	G	0.43	0.78	0.42	0.45	C	5.302	0.89 [0.8-0.98]	0.02
Imputed	rs4641039	143974821	T	0.45	0.05	0.47	0.44	C	3.415	1.1 [0.99-1.22]	0.06
Genotyped	rs4736354	143977468	G	0.43	1	0.42	0.45	C	5.048	0.89 [0.8-0.99]	0.02
Imputed	rs7819943	143979063	A	0.43	1	0.42	0.45	G	5.048	0.89 [0.8-0.99]	0.02
Imputed	rs7011830	143979394	A	0.45	0.05	0.47	0.44	C	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs7824229	143981066	A	0.45	0.05	0.47	0.44	C	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs10096393	143981317	T	0.45	0.05	0.47	0.44	C	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs6651273	143983502	G	0.45	0.05	0.47	0.44	A	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs6471580	143983703	G	0.46	1	0.44	0.48	A	7.438	0.87 [0.79-0.96]	0.01
Imputed	rs6987382	143984193	A	0.46	1	0.44	0.48	G	7.438	0.87 [0.79-0.96]	0.01
Imputed	rs4736357	143984220	T	0.45	0.05	0.47	0.44	A	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs4736359	143984429	T	0.45	0.05	0.47	0.44	G	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs4379428	143985050	T	0.45	0.05	0.47	0.44	A	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs6471583	143988094	G	0.45	0.05	0.47	0.44	A	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs6433	143990642	C	0.45	0.05	0.47	0.44	T	3.415	1.1 [0.99-1.22]	0.06
Imputed	rs6414	143993436	G	0.49	0.80	0.50	0.47	A	4.135	1.11 [1-1.23]	0.04
Genotyped	rs4546	143993555	T	0.43	0.78	0.43	0.45	C	3.786	0.9 [0.82-1]	0.05
Genotyped	IC	143993985	Conv	0.47	0.19	0.50	0.45	A	14.75	1.23 [1.11-1.37]	$1.23x10^{-4}$
Genotyped	rs1799998	143996602	C	0.44	0.26	0.43	0.46	A	5.041	0.89 [0.81-0.99]	0.02
Imputed	rs10087214	143996728	A	0.44	0.26	0.43	0.46	G	5.041	0.89 [0.81-0.99]	0.02
Imputed	rs7831617	143999285	T	0.44	0.26	0.43	0.46	G	5.041	0.89 [0.81-0.99]	0.02
Imputed	rs9643358	144000140	C	0.44	0.26	0.43	0.46	G	5.041	0.89 [0.81-0.99]	0.02
Imputed	rs7011889	144002262	C	0.44	0.26	0.43	0.46	A	5.041	0.89 [0.81-0.99]	0.02
Imputed	rs7016924	144003075	A	0.46	1	0.44	0.48	G	7.438	0.87 [0.79-0.96]	0.01

The 29 SNPs imputed and 8 SNPs genotyped are described in the Method column. m, minor allele; MAF, minor allele frequency; HWEp, overall Hardy-Weinberg p-value; M, major allele; CHISQ, basic allelic test chi square with a single SNP (1 degree of freedom); p, asymptotic p-value for this single SNP association test; OR, estimated odds ratio for minor allele; 95% CI, 95% confidence interval for odds ratio.

Three-marker sliding window haplotype analysis

For the discovery stage, a three-marker sliding window haplotype analysis was performed, in which consecutive combinations of three SNPs were used to assess each haplotype; thus 35 windows of 3 SNPs were generated using the 37 SNPs (See section 9.4, Appendix 4) Only four of the 35 windows showed a strong association with hypertension, achieving significant p values $<10^{-4}$ (Table 7-4, Figure 7-2 and Figure 7-3). These windows were located either in *CYP11B1* from the promoter to exon 1, or in *CYP11B2* from the promoter to exon 3. In the *CYP11B1* region, window 9 comprised rs6410, rs4471016 and rs4313136 ($p=2.08x10^{-5}$). The most significant haplotype in this window was T/A/G ($p=3.5x10^{-5}$), present in approximately 2% of the cases. In the *CYP11B2* region, there were three neighbouring sliding windows where association tapered towards the 5'region. Window 29 comprising the SNPs rs6414, rs4546 and IC, had the most significant haplotype (A/T/Conv $p=2.96x10^{-8}$). Window 30 comprised rs4546, IC and rs1799998 and its most significant haplotype was T/Conv/C ($p=2.37x10^{-6}$). The significant haplotypes in windows 29 and 30 were also present in approximately 2% of the cases. Finally, window 31 comprised IC, rs1799998 and rs10087214 and had two significant haplotypes: Conv/C/A and Wt/C/A (p=0.0003 and p=0.0006, respectively). The first haplotype was only present in 3% of the cases, but the second haplotype is much more common with a frequency of approximately 40% in the BRIGHT hypertensives.

Table 7-4. Results of association analysis with three SNPs sliding window haplotypes with significant p value ($p<10^{-3}$, -logp>3.0) using 37 SNPs

Locus window	Gene	SNP1	SNP2	SNP3	Haplotype	Freq cases	Freq controls	CHISQ	DF	p
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	**Overall**	**NA**	**NA**	**24.380**	**3**	**2.08×10^{-5}**
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	T/G/T	0.46	0.44	1.767	1	0.77
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	T/G/G	0.02	0.03	0.708	1	0.14
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	**T/A/G**	**0.02**	**0.01**	**20.650**	**1**	**3.5×10^{-5}**
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	C/A/G	0.49	0.52	4.896	1	0.04
WIN29	*CYP11B2*	rs6414	rs4546	IC	**Overall**	**NA**	**NA**	**46.820**	**5**	**6.19×10^{-9}**
WIN29	*CYP11B2*	rs6414	rs4546	IC	**A/T/Conv**	**0.02**	**0.01**	**36.320**	**1**	**2.96×10^{-8}**
WIN29	*CYP11B2*	rs6414	rs4546	IC	G/C/Conv	0.46	0.44	3.730	1	0.20
WIN29	*CYP11B2*	rs6414	rs4546	IC	G/T/Wt	0.02	0.02	0.055	1	0.82
WIN29	*CYP11B2*	rs6414	rs4546	IC	A/T/Wt	0.39	0.43	10.520	1	3.06×10^{-4}
WIN29	*CYP11B2*	rs6414	rs4546	IC	G/C/Wt	0.02	0.01	2.043	1	0.15
WIN29	*CYP11B2*	rs6414	rs4546	IC	A/C/Wt	0.09	0.09	0.769	1	0.46
WIN30	*CYP11B2*	rs4546	IC	rs1799998	**Overall**	**NA**	**NA**	**35.860**	**4**	**3.10×10^{-7}**
WIN30	*CYP11B2*	rs4546	IC	rs1799998	**T/Conv/C**	**0.02**	**0.005**	**22.270**	**1**	**2.37×10^{-6}**
WIN30	*CYP11B2*	rs4546	IC	rs1799998	C/Conv/C	0.01	0.01	1.527	1	0.22
WIN30	*CYP11B2*	rs4546	IC	rs1799998	T/Wt/C	0.40	0.45	11.080	1	1.49×10^{-5}
WIN30	*CYP11B2*	rs4546	IC	rs1799998	C/Conv/T	0.47	0.44	7.990	1	0.15
WIN30	*CYP11B2*	rs4546	IC	rs1799998	C /Wt/T	0.09	0.10	1.770	1	0.34
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	**Overall**	**NA**	**NA**	**25.090**	**3**	**1.48×10^{-5}**
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	**Conv/C/A**	**0.03**	**0.02**	**13.290**	**1**	**3.0×10^{-4}**
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	**Wt/C/A**	**0.40**	**0.44**	**11.630**	**1**	**6.0×10^{-4}**
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	Conv/T/G	0.47	0.43	8.186	1	0.004
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	Wt/T/G	0.10	0.11	0.995	1	0.32

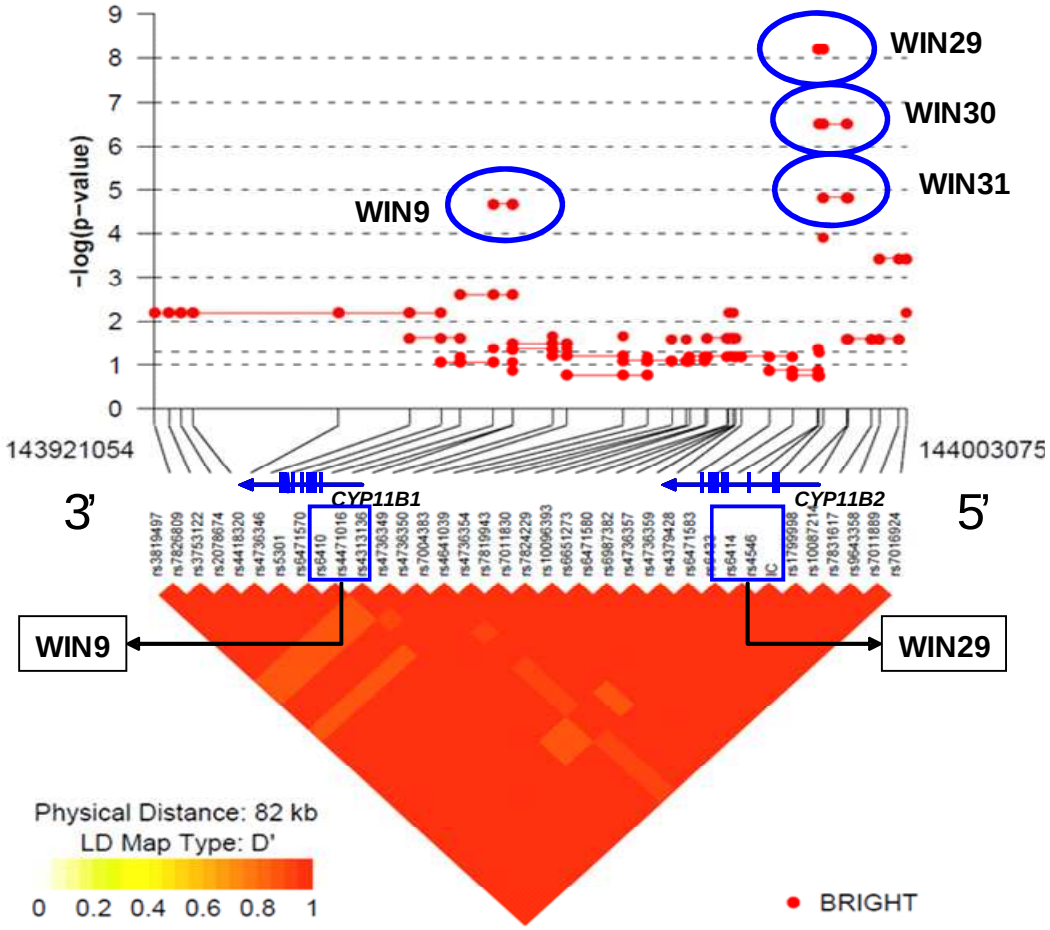


Figure 7-2 Three-marker sliding window haplotype analysis LD plot and negative logarithmic p-values. The SNPs of the most significant windows, WIN9 and WIN29, in each gene are marked by a blue rectangle.

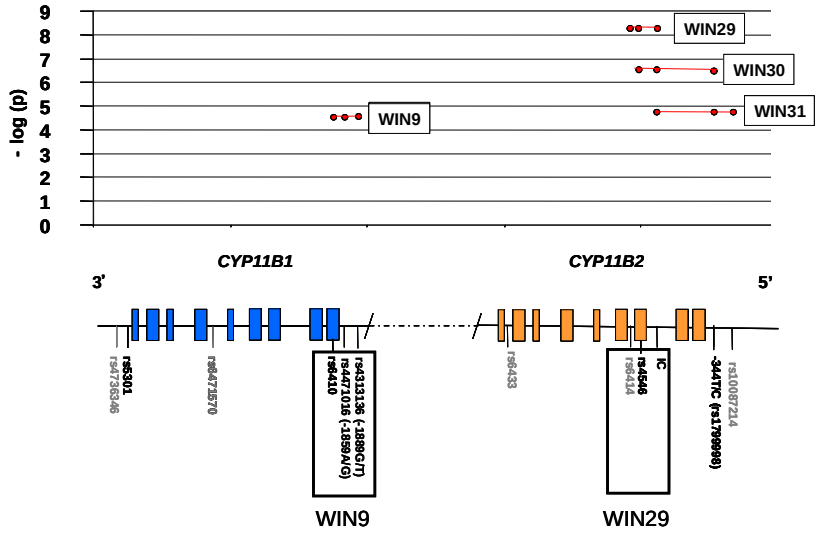


Figure 7-3. SNPs in each window in the *CYP11B1/CYP11B2* locus with most significant association with hypertension in the discovery phase.

7.4.2 Replication stage

CYP11B1/CYP11B2 single marker analysis for BRIGHT, Swedish and Meta-analysis

The 7 SNPs with the most significant results in the discovery stage (rs6410, rs4471016, rs4313136, rs6414, rs4546, IC and rs1799998) were chosen for replication in an independent population with Caucasian ancestry. All of them were genotyped in the Swedish Study, except rs6414 where a proxy SNP was chosen (rs6471581). The genotyping results for the Swedish study have been shown in Table 7-2. A single marker analysis was performed with the Swedish Study and the results were compared with those obtained from the BRIGHT. The results from both studies were also included in a meta-analysis (Table 7-5). The minor allele frequency of the 7 SNPs selected for replication was similar in the BRIGHT and Swedish Studies. There was no significant deviation from Hardy-Weinberg equilibrium at any polymorphism. The odds ratio for the SNPs included in both studies are in the same direction. Two of the SNPs in the Swedish Study achieved borderline significance: rs6471581 (p=0.01) and IC (p=0.02). This is in agreement with the results obtained from the BRIGHT Study where rs6414 had also borderline significance and IC was significant. However, the suggestive significance of two other SNPs in the BRIGHT, rs6410 (p=0.04) and rs1799998 (p=0.02), was not observed in the Swedish cohort, despite having similar minor allele frequencies. In the meta-analysis, strong evidence for association between the intron conversion variant and hypertension was found (OR=1.19, 95%CI 1.10-1.29, $p=1.06 \times 10^{-5}$). From the remaining six SNPs, only the ones in *CYP11B1* and intron 3 in *CYP11B2* (rs6414/rs6471581) showed p-values less than 0.05.

Table 7-5. Results of single marker association analysis and meta-analysis of 7 SNPs at the *CYP11B1/CYP11B2* locus in the discovery study subsequently replicated in an independent cohort

			Discovery Study: BRIGHT				Replication Study: Swedish				Combined Meta-analysis	
Method	**SNP**	**m**	**MAF**	**HWEp**	**OR [95% CI]**	**p**	**MAF**	**HWEp**	**OR [95% CI]**	**p**	**OR [95% CI]**	**p**
Genotyped	rs6410	T	0.48	0.14	1.11 [1.00-1.23]	0.04	0.46	0.02	1.09 [0.96-1.24]	0.17	1.10 [1.02- 1.19]	0.01
Genotyped	rs4471016	G	0.48	0.10	1.08 [0.98-1.20]	0.13	0.47	0.44	1.11 [0.99-1.25]	0.09	1.09 [1.01-1.18]	0.02
Genotyped	rs4313136	T	0.45	0.01	1.10 [0.99-1.22]	0.09	0.45	0.74	1.12 [1.00-1.26]	0.05	1.11 [1.03-1.20]	0.01
Imputed/Genotyped	rs6414/rs6471581	G	0.49	0.32	1.11 [1.00-1.23]	0.04	0.45	0.54	1.16 [1.03-1.31]	0.01	1.13 [1.05-1.22]	$1.6X10^{-3}$
Genotyped	rs4546	T	0.43	0.84	0.90 [0.82-1.00]	0.05	0.43	0.78	1.01 [0.89-1.13]	0.92	0.95 [0.88-1.02]	0.17
Genotyped	**IC**	**Conv**	**0.47**	**0.01**	**1.23 [1.11-1.37]**	**$1x10^{-4}$**	**0.46**	**0.43**	**1.14 [1.02-1.29]**	**0.02**	**1.19 [1.10-1.29]**	**$1.06X10^{-5}$**
Genotyped	rs1799998	C	0.44	0.20	0.89 [0.81-0.99]	0.02	0.43	0.78	1.02 [0.90-1.15]	0.77	0.94 [0.87-1.02]	0.12

The 7 SNPs of the BRIGHT Study replicated in the Swedish Study are described in the Method column. m, minor allele; MAF, minor allele frequency; HWEp, Hardy-Weinberg p-value for unaffected subjects; OR, estimated odds ratio for minor allele; 95% CI, 95% confidence interval for odds ratio p, asymptotic p-value for this single SNP association test.

Haplotype association analysis for BRIGHT, Swedish and Meta-analysis

Haplotype association analysis was also performed in the Swedish population and compared with the results obtained from the BRIGHT, then included in a meta-analysis with both populations (Table 7-6 and Figure 7-4). The most significant haplotype in the *CYP11B1* gene, combining rs6410, rs4471016 and rs4313136, T/A/G, was successfully replicated in the Swedish Study with similar p-values and OR in the same direction. This is reflected in the combined meta-analysis, as the significance of this haplotype increased to $2.99x10^{-12}$ and the combined OR of 3.14 (95%CI: 2.27-4.32) shows a higher risk of hypertension in carriers of this haplotype. In the populations studied, this haplotype is present in ~2% of the whole cohort in these two populations with Caucasian ancestry. With the same combination of SNPs, the haplotype C/A/G tended towards significance in the BRIGHT and meta-analysis, and was significant in the Swedish study. It is present in approximately 50% of the subjects but, in contrast to T/A/G, the OR indicates the C/A/G is a protective haplotype (meta-analysis OR 0.85, 95%CI: 0.79-0.92, $p=5.39x10^{-5}$). The other common haplotype, T/G/T, present in ~40% of these populations with a Caucasian background did not show any significant association but the directionality of the OR suggests a subtle predisposition for hypertension.

Of the other two significant haplotypes in the BRIGHT study, A/G/G and A/T/Conv, the first reached significance in the Swedish population and the second did not, but both showed the same directional change in OR. When the results were combined in the meta-analysis, both haplotypes achieved significance (p values 3.90×10^{-8} and 1.11×10^{-7}, respectively) and the OR showed an increased risk of hypertension (OR 2.64 and 4.64, respectively). Similar to the significant haplotype in *CYP11B1*, the significant haplotypes here, either combining SNPs from both genes (two from the *CYP11B1* promoter and one from the intron 3 in *CYP11B2* in A/G/G), or from *CYP11B2* (one in the intron 3, one in exon 3 and the intronic 2 conversion in A/T/Conv) have a low frequency in the British and Swedish populations studied (~1%). Observing these significant haplotypes in Table 6, when the last allele present is the alternate allele, A/G/A or A/T/Wt, the frequency is higher (40-50% in all the individuals studied) and the analysis shows the carriers have a lower risk of hypertension, despite achieving a result which tended towards significance (OR 0.88, $p=9x10^{-4}$ and OR 0.87, $p=1.6x10^{-3}$, respectively).

The last combination of SNPs from *CYP11B2* used to analyse haplotypes was rs4546, IC and rs1799998 (-344T/C). None of the haplotypes achieved significance in the meta-analysis. However, the T/Wt/C haplotype achieved significance in the BRIGHT and the C/Wt/T haplotype achieved significance in the Swedish Study. These two haplotypes not only share the Wt allele but also the tendency of being protective haplotypes for the carriers. The third haplotype, C/Conv/T, although not significant in any of the studies or in the meta-analysis, is present in more than 40% of the subjects and shows a trend for higher risk of hypertension.

Figure 7-4. SNPs in each haplotype in the *CYP11B1/CYP11B2* locus with most significant association with hypertension in the combined meta-analysis.

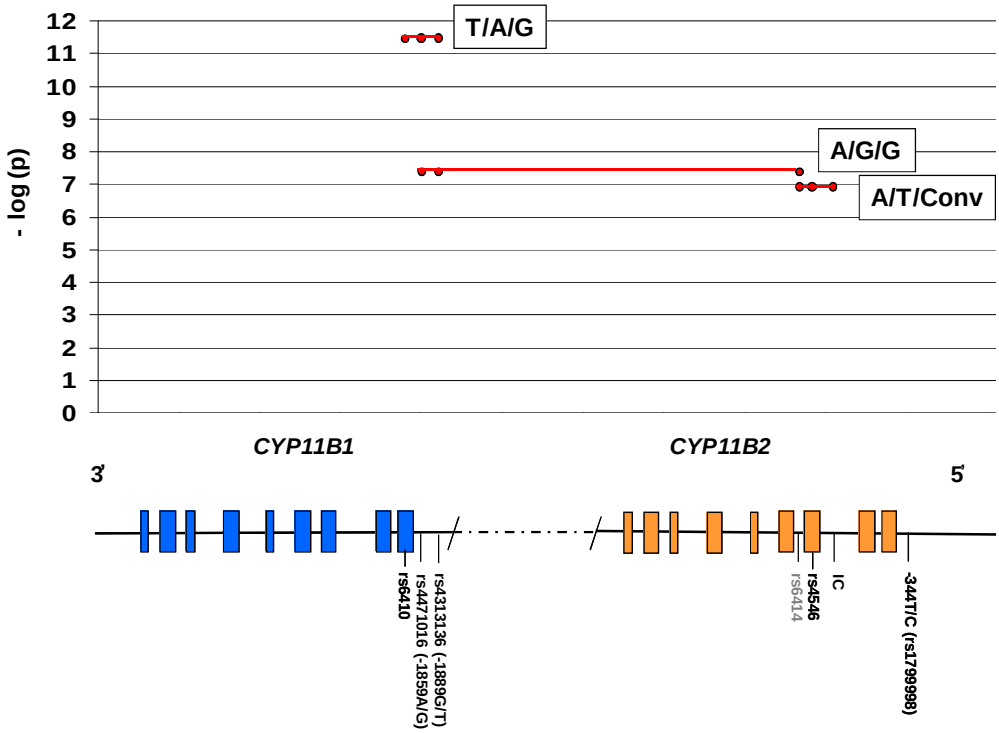


Table 7-6. Results of the haplotype association analysis and meta-analysis from the *CYP11B1/CYP11B2* locus of the discovery study then replicated in an independent cohort

					Discovery Study: BRIGHT			Replication Study: Swedish			Combined Meta-analysis	
Gene	**SNP1**	**SNP2**	**SNP3**	**Haplotype**	**Freq**	**OR**	**p**	**Freq**	**OR**	**p**	**OR [95% CI]**	**p**
CYP11B1	rs6410	rs4471016	rs4313136	T/G/T	0.44	1.01	0.77	0.43	1.01	0.84	1.01 [0.96- 1.07]	0.73
CYP11B1	rs6410	rs4471016	rs4313136	T/G/G	0.02	0.78	0.14	0.02	0.80	0.28	0.79 [0.62-1.02]	0.07
CYP11B1	**rs6410**	**rs4471016**	**rs4313136**	**T/A/G**	**0.02**	**2.38**	**$3.5x10^{-5}$**	**0.02**	**4.83**	**$1.95x10^{-9}$**	**3.14 [2.27-4.32]**	**$2.99x10^{-12}$**
CYP11B1	rs6410	rs4471016	rs4313136	C/A/G	0.51	0.90	0.04	0.51	0.80	$1.18x10^{-4}$	0.85 [0.79-0.92]	$5.39x10^{-5}$
CYP11B1/CYP11B2	rs4471016	rs4313136	rs6414	G/T/G	0.44	1.02	0.67	0.44	1.10	0.12	1.05 [0.98-1.13]	0.20
CYP11B1/CYP11B2	**rs4471016**	**rs4313136**	**rs6414**	**A/G/G**	**0.02**	**2.44**	**$2.46x10^{-5}$**	**0.01**	**3.19**	**$3.32x10^{-4}$**	**2.64 [1.87-3.74]**	**$3.90x10^{-8}$**
CYP11B1/CYP11B2	rs4471016	rs4313136	rs6414	G/G/G	0.02	0.78	0.14	0.002	0.60	0.41	0.77 [0.56-1.05]	0.10
CYP11B1/CYP11B2	rs4471016	rs4313136	rs6414	A/G/A	0.51	0.90	0.04	0.52	0.85	$6.88x10^{-3}$	0.88 [0.81-0.95]	$9.0x10^{-4}$
CYP11B1/CYP11B2	rs4313136	rs6414	rs4546	G/A/T	0.41	0.87	0.01	0.42	0.99	0.93	0.92 [0.85-0.99]	0.03
CYP11B1/CYP11B2	rs4313136	rs6414	rs4546	T/G/C	0.44	1.04	0.44	0.44	1.1	0.10	1.07 [0.99-1.15]	0.10
CYP11B1/CYP11B2	rs4313136	rs6414	rs4546	G/G/C	0.02	1.68	$3.92x10^{-3}$	0.01	2.41	$1.49x10^{-3}$	1.87 [1.39-2.51]	$3.33x10^{-5}$
CYP11B1/CYP11B2	rs4313136	rs6414	rs4546	G/A/C	0.10	1.00	1.00	0.12	0.69	$5.0x10^{-5}$	0.69 [0.58-0.83]	$5.0x10^{-5}$
CYP11B2	rs6414	rs4546	IC	G/C/Conv	0.44	1.07	0.20	0.45	1.15	0.02	1.10 [1.02-1.19]	0.01
CYP11B2	rs6414	rs4546	IC	A/T/Wt	0.40	0.83	$3.06x10^{-4}$	0.42	1.01	0.92	0.87 [0.79-0.95]	$1.6x10^{-3}$
CYP11B2	rs6414	rs4546	IC	A/C/Wt	0.09	0.94	0.46	0.12	0.72	$2.08x10^{-4}$	0.82 [0.73-0.93]	$1.8x10^{-3}$
CYP11B2	**rs6414**	**rs4546**	**IC**	**A/T/Conv**	**0.02**	**5.36**	**$2.96x10^{-8}$**	**0.001**	**1.04**	**0.97**	**4.64 [2.63-8.19]**	**$1.11x10^{-7}$**
CYP11B2	rs4546	IC	rs1799998	T/Wt/C	0.41	0.80	$1.49x10^{-5}$	0.43	1.01	0.82	0.92 [0.86-0.98]	$8.4x10^{-3}$
CYP11B2	rs4546	IC	rs1799998	C/Conv/T	0.44	1.07	0.15	0.45	1.15	0.02	1.10 [1.02-1.18]	0.01
CYP11B2	rs4546	IC	rs1799998	C/Wt/T	0.11	0.92	0.34	0.12	0.71	$2.03x10^{-4}$	0.82 [0.73-0.93]	$1.3x10^{-3}$

The haplotypes of the BRIGHT Study (n=3340) replicated in the Swedish Study (n=2929) are described in the Haplotype column, with the SNPs contributing to each allele described in the three previous columns. Freq, frequency of the haplotype in the population studied; OR, estimated odds ratio for the haplotype; 95% CI, 95% confidence interval for odds ratio; p, probability value of the association test.

7.5 Discussion

Most studies of polymorphisms at the *CYP11B1/CYP11B2* locus have focused on the -344T/C (rs1799998) and their association with hypertension has been tested in groups of less than 500 subjects. Moreover, ethnic background, phenotyping criteria and the study design have not been uniform. Consequently, the findings reported have been inconsistent. For hypertension, Sookoian *et al.* did a valid systematic review of 19 studies (11 225 individuals) and showed that the -344C allele was associated with a decreased risk of hypertension. However, ethnic heterogeneity had an important influence and, when the studies were subclassified by ethnicity, only in Caucasians did the association remain significant; in Japanese subjects it was only suggestive (Sookoian *et al.* 2007). The methods used by Sookoian *et al.* have been criticized, but the informativeness of their results remains valid and the need for more efficient association studies in this locus was highlighted (Staessen *et al.* 2007).

In studies of more than 500 Caucasian subjects, the results are conflicting. The cross-sectional studies agree that the -344T allele is associated with high blood pressure (Castellano *et al.* 2003; Brand *et al.* 1998), but the only prospective study shows that individuals with a -344CC genotype have a higher risk of hypertension (Staessen *et al.* 2001).

The study in Chapter 6 showed there are differences not only in allele frequencies between populations of different ancestries, but also in LD patterns. Thus, population admixture, which can lead to inconsistent results and false-positive associations, should be avoided in any study (Newton-Cheh & Hirschhorn 2005).

Aware of the lack of large case-control studies in Caucasian populations to evaluate effects of the *CYP11B1* and *CYP11B2* genes in hypertension, it was decided to carry out the study described in this chapter. To improve the design of the study, the criteria proposed by the National Cancer Institute-National Human Genome Research Institute (NCI-NHGRI) were followed whereby an initial or discovery study for variations at the *CYP11B1/CYP11B2* locus associated with hypertension was followed by a replication study in an independent population of the same ancestry (Chanock *et al.* 2007).

A case-control study was chosen over a TDT for three reasons. Firstly, a case-control study is economically efficient because the siblings and parents are not genotyped. Secondly, it

has been reported that it can be as powerful as a TDT study (Morton & Collins 1998). Thirdly, evidence for population stratification can be evaluated and corrected. Regarding the BRIGHT Study, the WTCCC genotyped BRIGHT cases and did not find any evidence of population stratification (2007). For the Swedish Study, population stratification was evaluated and corrected using a principal component analysis.

From Chapter 5, it is known that the htSNPs selected effectively capture the genetic variation of the *CYP11B1/CYP11B2* locus in Caucasians. However, there has been recent interest in studying the SNPs not covered by this technology. An imputation method uses the LD structure of the region to infer alleles of the SNPs which have not been directly genotyped (Halperin & Stephan 2009). This imputation can increase the efficiency of the association inference without adding significantly to the bias (Dai *et al.* 2006). For this reason, an imputation was performed in the discovery stage with the BRIGHT Study. The CEU population (Utah residents with Northern and Western European ancestry from the CEPH collection) from the HapMap database (release 24) was selected as the reference population. It was assumed that the LD structure and haplotype distribution is shared by the reference population and by the cases and controls from the BRIGHT. By imputing SNPs, the possibility of finding a plausible causal variant increases because, even if a SNP is not included in the direct genotyping, it might be included in the imputed SNPs (Halperin & Stephan 2009). By using the PLINK v.1.05 software, 29 SNPs were imputed. Adding these to the SNPs previously genotyped, gave a total of 37 SNPs which could be used for the association tests. The 37 SNPs provided maximum haplotype information for each of the genes and ensured coverage of intergenic regions which may harbour regulatory polymorphisms.

Two approaches were chosen for the association analysis in order to identify genetic variant(s) which directly predispose to hypertension or are in LD with such variants. These approaches were the single SNP and the sliding window haplotype analysis. Despite not yet reaching a consensus as to the best strategy (Liu *et al.* 2007), the haplotype-based analysis seems to offer several advantages over the single SNP approach. It can have more power to detect association by incorporating LD information from multiple markers that can originate and predispose independently (Morris & Kaplan 2002); the analysis is more robust (Akey *et al.* 2001) and; as it allows a combined effect of multiple variants, the interaction of *cis-acting* elements can also be detected (Epstein & Satten 2003). Although the use of a large number of haplotypes can decrease the efficiency, this can be corrected (Akey *et al.* 2001).

For the discovery stage using the BRIGHT cases and controls only one significant association was revealed between the intron conversion and hypertensives, as shown in Table 3. The addition of imputed SNPs did not alter the results suggesting the htSNPs selection was appropriate. Moreover, this is consistent with previous studies showing an association with this polymorphism despite being underpowered (Davies *et al.* 1999; Zhu *et al.* 2003a).

For the haplotype analysis, the haplotypes were inferred from the genotype data of the BRIGHT cases and controls, once it had been verified that there was no significant deviation in the Hardy-Weinberg equilibrium for any SNP. Considering that there are regions with less strong LD, specific haplotypes employing all the identified SNPs could not be selected. In addition, no proxies were found among the SNPs, meaning that each of them is independent and does not predict associations due to others. Consequently, a sliding window haplotype-based analysis was chosen.

The only significant window in *CYP11B1* comprised the SNPs rs6410, rs4471016 (-1859A/G) and rs4313136 (-1889G/T). SNP rs6410 is located in exon 1 and is synonymous (Leu75Leu). The other two SNPs are located in the promoter region of this gene and previous functional studies performed by our group showed that they can alter *CYP11B1* transcription (Barr *et al.* 2007). In addition, the three polymorphisms have been associated with impaired 11β-hydroxylase activity, either in hypertensives or in families with hypertensive probands (Barr *et al.* 2007; Keavney *et al.* 2005). However, the most significantly susceptible haplotype in this window was T/A/G ($p=3.50x10^{-5}$, OR=2.38) which does not contain the two alleles (G/T) which have been reported to be associated with decreased 11β-hydroxylase efficiency by (Barr *et al.* 2007). The previously reported alleles are part of a more common haplotype, in contrast with this haplotype which is only present in ~2% of the case subjects. These results suggest the combination of these alleles may be driving the low p-value. These results will be discussed further when the discovery stage results are compared with the replication study.

In *CYP11B2,* three consecutive windows achieved significance. The window showing the most significant association (WIN29) included rs6414, rs4546 and the intron 2 conversion. Two of the SNPs are located in intronic regions (rs6414 in intron 3 and the conversion in intron 2) and their functional relevance remains unknown. The SNP in exon 3, rs4546, is synonymous and although it might not be functionally relevant, it is probably in LD with a causal marker. This SNP is only 4 bp from the Lys173Arg (rs4539) described previously in

Caucasian, Chilean and Japanese populations (Fardella *et al.* 1996; Keavney *et al.* 2005; Matsubara *et al.* 2001). The Lys173Arg is a nonsynonymous SNP. *In vitro* studies recreating this amino acid change showed no significant effect on the conversion of deoxycorticosterone to aldosterone (Fardella *et al.* 1996). Thus, there is no *in vitro* evidence to explain why the most significantly susceptible haplotype in WIN29 was A/T/Conv ($p=2.96x10^{-8}$, OR= 5.36).

The second window in *CYP11B2*, WIN30, also achieved a significant association ($p<10^{-6}$). This window included rs4546, the intron 2 conversion and rs1799998 (-344T/C). So far, for the first two SNPs, no relevant functional information has been published, as discussed with WIN29. For rs1799998, despite the initial interest of its location within a putative *cis* element capable of binding steroidogenic factor 1 (White PC & Slutsker L 1995), the *in vitro* experiments show no difference in transcription between the two alternative forms (Bassett *et al.* 2002). However, a later study assessing mRNA levels in normal adrenal tissue and aldosterone-producing adenomas suggest that the -344T/Lys173 haplotype is associated with higher levels of gene expression compared to the -344C/Arg173 haplotype (Tanahashi *et al.* 2005). In WIN30, the most significant haplotype was T/Conv/C ($p=2.37x10^{-6}$), although, it had very low frequency in the BRIGHT controls and, for this reason, was not selected for replication. The other haplotype with suggestive significance was T/Wt/C, being protective for the risk of hypertension ($p=1.49x10^{-5}$, OR 0.80). It is not known how alleles in rs4546 are associated with Lys173Arg, nor how these interact with the most significant alleles in WIN30 (T from rs4546, the conversion from IC and the C in the -344T/C).

For WIN9, 29 and 30, the most significant haplotypes were present in only ~2% of the cases while a less significant one in WIN30 was present in 45% of cases. This suggests a rare combination of alleles might increase the susceptibility of developing hypertension and perhaps explain some of the discrepancies observed in previous association studies examining variations in this locus and hypertension.

The third, and last window in *CYP11B2* showing a significant association with hypertension was WIN31, including the intron 2 conversion, rs1799998 (-344) and rs10087214. In this case, the third SNP added to the window, rs10087214, is located in the promoter close to -344T/C, with which it is in very tight LD. The possible functional influence of this third SNP is unknown. However, it is important to emphasise that its

incorporation in the window to test for association decreases the significance of the association.

The evidence suggests that further studies in this region are needed to elucidate the causal variant(s) which influences blood pressure and hypertension status. To confirm the associations observed in the two genes, a replication study of the most significant regions was performed in a Swedish case-control population, also with Caucasian ancestry.

In the single marker association test for the replication stage, the strong significance observed for the IC is in agreement with the increased risk of hypertension reported previously by our group and others (Davies *et al.* 1999; Zhu *et al.* 2003a). The G allele from rs4471016, also known as -1859, and the T allele from rs4313136 (-1889) showed a tendency towards susceptibility to hypertension in both the BRIGHT and the Swedish Studies and a borderline significance in the same direction in the meta-analysis. Although the results for these two SNPs did not achieve significant values in the single marker analysis, this susceptibility to hypertension supports previous findings of a possible impairment in 11β-hydroxylase efficiency in carriers of these alleles (Barr *et al.* 2007).

The C allele of the -344T/C (rs1799998) showing protection against hypertension had borderline significant in the BRIGHT Study but was not significant in the Swedish Study nor in the meta-analysis. This is consistent with the suggestions of previous association studies that the -344T/C might be in LD with another causal variant (Sookoian *et al.* 2007).

The haplotype association analysis showed, from the SNPs being studied, that the intron conversion variant is the only one achieving significant association with hypertension as a single marker and in combination with other alleles. It was observed that, when the conversion allele of this polymorphism is present in any haplotype, there is a pattern of susceptibility to hypertension that can achieve a significant association or not depending on the accompanying alleles. It is possible that a causal variant might be located in this fragment of the gene or is in LD with this fragment. A thorough study of this fragment in *CYP11B2* is suggested in order to elucidate plausible molecular mechanisms which might explain this association with hypertension. Perhaps this fragment encodes or interacts with elements affecting gene expression like microRNAs (miRNAs) (Lau *et al.* 2001). In addition, it was shown that when the SNPs of the *CYP11B1* promoter were incorporated into a haplotype, the alleles of the rs4471016 (-1859) and rs4313136 (-1889) present in the most significant haplotypes were A and G respectively, in contrast to the G and T alleles

which have been shown to have an effect on 11β-hydroxylase efficiency (Barr *et al.* 2007). Although, initially, this seems paradoxical, it has been observed that the interaction with the third allele (in this case from either rs6410 or rs6414) might be crucial to determining whether the haplotype increases or decreases the risk of hypertension. This suggests the interaction between the alleles in the SNPs is driving the significant result rather than the individual alleles.

This study suggests that rare haplotypes in the studies had a tendency to more significant effects than common haplotypes. It may be that, for common haplotypes at this locus to have a significant association with hypertension, their interaction with other elements affecting gene expression like miRNA, methylation and transcription factors should also be considered.

There are several explanations for the lack of replication of some of the results obtained in BRIGHT Study. Although both populations had similar genetic background and genotyping, there were differences in environmental exposure. In addition, only the SNPs showing association in the original study were selected for replication. SNPs in strong LD with the significant SNPs in BRIGHT or other SNPs in the regions of interest demonstrating potential association with hypertension were not explored. Moreover, it has already been suggested that the first study generally shows a stronger association than subsequent studies (Ioannidis & Lau 2001; Ioannidis *et al.* 1999). It is unlikely that associations identified in the BRIGHT Study are spurious because the results of the Swedish study show a similar directionality confirmed by the meta-analysis. However, it has been suggested that differences in the strengths of association can be attributed to adjustments in the appropriate measurement of association or perhaps to a marked gene effect in specific subpopulations (Ioannidis *et al.* 2001).

The major strengths of this study were the use of large scale case-control populations, with detailed phenotyping and controls to avoid population stratification. The individuals participating in the BRIGHT Study had British ancestry up to the level of grandparents and the individuals participating in the Swedish Study were corrected for population stratification. Although the size of both studies was similar and they shared the same ancestry, the selection criteria for cases and controls were stricter in the Swedish study. This may have an impact on the frequency of alleles and haplotypes studied compared in the populations. Another limitation of the study in Caucasians is that the strong LD at this locus hampers the identification of the causal variant(s). As established in Chapter 6, the

LD at this locus is shorter in populations of African ancestry. Thus, using a similar approach in an African population to the one used in Caucasians might be useful in localizing the causal variants as was the case with the angiotensin-1 converting enzyme locus (McKenzie *et al.* 2001).

In summary, using a combination of single marker and haplotype analysis at the *CYP11B1/CYP11B2* locus, we have narrowed and replicated the region most strongly associated with hypertension in Caucasians to a region between intron 2 and intron 3 of the *CYP11B2* gene, and the promoter and exon 1 of the *CYP11B1* gene. In addition, functional studies are indicated to elucidate the mechanism by which these genetic variations lead to alterations in aldosterone and cortisol production and to subsequent hypertension.

7.6 Conclusion

This study reports the replication of an association of the *CYP11B1/CYP11B2* locus with a common complex disorder, hypertension. The same locus has been strongly implicated in the biochemical and haemodynamic abnormalities in the Dahl salt-sensitive hypertensive rat. It is of interest that two distinct regions (in *CYP11B1* and *CYP11B2)* are reported in the rodent model, similar to the findings of this thesis, suggesting that there is a digenic effect that may account for some of the risk of hypertension. Further, both *CYP11B1* and *CYP11B2* are clearly implicated in rare autosomal forms of hypertension. Previous studies have shown that common polymorphic variation at this locus has clear effects on intermediate biochemical phenotype. In the studies that I report there is variability in the strength of association according to allele frequencies, and this suggests that the locus is involved in hypertension in a complex manner and may well contribute to overall population risk.

Thus, for the first time a candidate gene locus that causes rare autosomal forms of a common complex human disorder, which has clear and plausible functional effects, and which also accounts for a similar phenotype in a rodent model has been reproducibly implicated in a common human condition. Further studies are now indicated to identify precisely the causal polymorphisms and establish how they lead to functional effects that result in high blood pressure.

8 General Discussion

Hypertension and cardiovascular diseases are an important cause of morbidity and mortality and have a well-established genetic component. In Chapter 1, the case has been made for genes expressed in the adrenal cortex, and in particular the production of aldosterone and cortisol as significant contributors to this genetic influence over essential hypertension. Aldosterone production and 11β-hydroxylase efficiency are altered in essential hypertension, and there is strong evidence that the genes encoding aldosterone synthase and 11β-hydroxylase are plausible vehicles for this change. Several common polymorphisms within the *CYP11B1/CYP11B2* locus associate with hypertension, blood pressure, ARR, and levels of mineralocorticoids and glucocorticoids in plasma and urine. However, several studies have not detected these associations, particularly those related to hypertension and aldosterone production. Possible reasons for these inconsistencies have been discussed and include differences in ARR measurement, selection criteria for hypertensive subjects and ethnicity. These must be considered in order to generate more reliable and reproducible results. The work presented in this thesis addresses these aspects. It includes a detailed study of ARR and of the *CYP11B1/CYP11B2* locus in essential hypertension. The main objectives were to examine the factors that influence ARR measurement and to examine the relationship between ARR, blood pressure and genetic variation at the *CYP11B1/CYP11B2* locus using both family-based and case-control designs. Briefly, it was found that genetic and non-genetic factors influence the ARR, which is not a pathological marker of PA; that the pattern of genetic variation in the *CYP11B1/CYP11B2* locus varies according to ancestry and that two regions in the locus strongly associate with hypertension in Caucasians.

The case-control design was chosen for its efficient practical and powerful approach, as less recruitment and genotyping is required to achieve an adequate power compared with a family-based design. However, one of the main concerns in this design is population stratification which, in this study, has been avoided by selecting cases and controls from the same ancestry and also by using a family-based design. This latter design is more robust to population stratification because unaffected family members are used as controls. In addition, subjects in the populations studied were ascertained for hypertension by means of common criteria, thus making the results easier to compare with each other.

When the ARR is selected as a phenotype of interest, it is important to consider the assays of each of its components, particularly renin because, as described in section 1.3.3, there is no consensus as to which method to use. In Chapter 3, the plasma renin activity and concentration assays were compared. The convenience of the PRC assay, the performance

of which was not significantly inferior to the PRA alternative, made it preferable for population studies, despite the potential interference of prorenin and a decrease in sensitivity at lower levels. Further studies are currently under way in the laboratory to assess this interference and correct for it. In this respect, addition of recombinant prorenin to plasma samples is being used to evaluate cross-reactivity and trypsin conversion of prorenin to renin is being measured.

On the basis of a careful comparison of the PRA and PRC assay methodology (Chapter 3), PRC and plasma aldosterone concentration, measured by direct RIA, were used to estimate ARR in a family-based study. In Chapter 4, this study confirmed that the ratio is affected by genetic and non-genetic factors. The non-genetic influence has been more thoroughly reported in the literature as there is arguably greater awareness of the effects of such factors as age, sex and the use of hypertensive or hormonal treatment medication on the ARR. However, this study was the first to examine the distribution of the ARR in hypertensive and normotensive family members. There was no evidence of a bimodal distribution, suggesting the ratio is not a marker of a distinct pathological abnormality specific to patients with high blood pressure but forms part of a single normal distribution. However, the findings do suggest that a larger proportion of hypertensive subjects have an increased ARR than the normotensive controls. This supports the hypothesis (section 1.4.5) that the ratio is a continuum in hypertensive patients, with patients presenting with inappropriately high (to the level of renin) aldosterone concentrations at the higher end.

The existence of a genetic component in ARR variation is the subject of relatively recent interest. The study described in Chapter 4 confirmed that ARR is a strongly heritable phenotype. However, neither the ratio nor the PRC were associated with the selected variations in the *CYP11B1/CYP11B2* locus. Of the two components of the ARR, only plasma aldosterone concentration associated with the intron 2 conversion in *CYP11B2*. This is not surprising as the ratio is mainly determined by renin whereas *CYP11B2* is specifically involved in aldosterone production. These results suggest the genetic influence on the ARR is not the effect of a single genetic variant in *CYP11B2*, as this would be evident either by showing very strong association or by a high frequency of such variants in carriers of the relevant phenotype. Possibly, the genetic component might be attributed to other genes or molecules involved in the regulation of renin and/or aldosterone production. Only the genes related to corticosteroid synthesis were studied here.

There is evidence to suggest that the intron conversion or genetic variations in LD with it might play an important role in the development of hypertension. In Chapter 7, this

polymorphism had the most significant association with hypertension in the single marker analysis in the BRIGHT Study, the replication in the Swedish study and the meta-analysis, and also had the most significant association in the sliding windows haplotype analysis in combination with a SNP in intron 3 (rs6414) and another in exon 3 (rs4546). Thus, the different analytical strategies used in this work consistently indicated that the intron conversion variation, or a variation in LD with it, probably plays a significant role in the variation of plasma aldosterone concentration and the development of hypertension.

In the same study, another statistically significant combination of SNPs in *CYP11B1,* including a synonymous SNP in exon 1 (rs6410) and two SNPs in the promoter region, was shown to associate strongly with hypertension. Interestingly, the variations in the promoter had previously been shown to affect 11β-hydroxylase efficiency. Earlier investigations by our group failed to show an association of these polymorphisms with hypertension, possibly due to underpowered study design (Barr *et al.* 2007). Power was improved here by increasing the sample size and using well-phenotyped controls. Moreover, the results were replicated in another case-control study with Caucasian ancestry, thus validating our results. It was not possible to test association of ARR or its components with the *CYP11B1/CYP11B2* locus in this case-control cohort because the data are not yet available. The ARR will be measured in the near future.

The findings of this study are of interest, as they suggest that there may be two independent influences related to *CYP11B1* and *CYP11B2* that affect blood pressure. This may not be surprising, given that the two genes have distinct autosomal conditions that lead, through quite different mechanisms, to conditions characterised by low (*CYP11B2* deficiency) or high (*CYP11B1* deficiency and GRA) blood pressure. Additionally, the concept of both genes being involved in blood pressure is a feature of the Dahl hypersensive salt sensitive rat. Thus, the findings here are of considerable interest, showing, for the first time, a positive association of a locus that is known to be involved in a rodent model of hypertension, rare autosomal human disorders, and functional changes that lead to an altered biochemical intermediate phenotype.

There is a high degree of sequence variation in the *CYP11B1/CYP11B2* locus and also a high variability of LD and allele frequencies for these variations between populations. The study described in Chapter 6 compared a population of African ancestry with two populations of Caucasian ancestry. These variations in LD influence the number of possible haplotypes that can exist in each population. The population with African ancestry

had smaller regions with high LD and therefore many SNPs would have been required to represent the whole locus. In contrast, in Caucasians, there are larger regions in high LD, thus decreasing the number of haplotypes that can be generated and tagSNPs which can be selected. As shown in Chapter 5, only 7 SNPs (rs5301, rs6410, rs4313136, rs4546, IC and rs1799998) were required to describe the total locus haplotype, decreasing the cost and effort of genotyping. These 7 SNPs included untranslated regions at the locus (5', 3' and intergenic region), as well as common variations. After genotyping the 7 SNPs and imputing other SNPs in the locus of interest, 37 SNPs were then analysed with a single marker and a sliding windows approach giving consistent results and maintaining directionality of effect. The case-control study in this thesis had appropriate power, minimal multiple testing and no population stratification, and the results have been reproduced in another study. This is the first time the results of an association study of a candidate gene have been replicated in an independent study of the same ancestry.

The limitations of the studies described in this thesis have been listed elsewhere (see the discussion section in each chapter). To facilitate the dissection of the genetic influence on the ARR and hypertension, future family-based or population studies might consider the following points:

1) The association studies should not only be focused on hypertension and blood pressure as phenotypes; ARR and urinary steroids as intermediate phenotypes may show clearer association with variations in the *CYP11B1/CYP11B2* locus and provide information on the mechanisms of effects on ultimate phenotype.
2) When ARR is used as an intermediate phenotype, careful standardisation of measurements as well as awareness of factors which affect it should improve detection of associated factors. A 24-hour urine collection (for the evaluation of electrolytes and steroid metabolites) is recommended. The follow-up of patients is essential to evaluate the change in genetic contribution with time. The PRC assay using the LIAISON platform is recommended for reproducible results.
3) For investigation of causal variants in the *CYP11B1/CYP11B2* locus, genotyping a population of essential hypertensive patients of African ancestry would be useful due to the smaller regions in LD in this locus.
4) In the studies presented in this thesis, only the common variations (>1%) were studied. In future studies, it would be important also to consider rare variants, CNVs or other molecules that could affect the expression of the *CYP11B1* and *CYP11B2* genes. This might include transcription factors and miRNAs. These studies are currently under way.

5) Thorough genotyping of the *CYP11B1* and *CYP11B2* genes in different populations is required to provide a better understanding of the evolution of variations in these genes. The study in this thesis suggests that different populations have different patterns.
6) In the narrower regions that significantly associate with hypertension, (Chapter 7), causal variants are potentially present. Once these possible causal variants have been identified, further studies *in vitro,* for example using reporter gene constructs to analyse the expression of the *CYP11B1* and/or *CYP11B2,* would be recommended.
7) Ethnicity might not be the only factor causing the discrepancies in association analyses between different studies. Studies looking at epistasis (gene-gene interactions) not only between the *CYP11B* genes but also between the *CYP11B* locus and other genes, gene-environment interactions and epigenetics (heritable changes in gene expression that occur by other mechanisms which do not alter DNA sequence (Wolffe & Matzke 1999)) might give a better overall picture of the genetic control of aldosterone production and 11β-hydroxylase efficiency in hypertension.
8) The experimental and analytical strategies used in the studies described in this thesis reflect recently-published recommendations (Chanock *et al.* 2007) thus increasing the potential to compare the conclusions presented here with those of other similar studies. Further studies also meeting these criteria will help to improve the association analyses and to dissect the functional variants of clinical relevance at the *CYP11B1/CYP11B2* locus.

In summary, the experimental studies in this thesis contribute significantly to an explanation for:

- The influence of genetic and non-genetic factors on the ARR.
- The ARR as a marker of physiological abnormality rather, than a pathological marker.
- The effect of ethnicity on the genetic variations at the *CYP11B1/CYP11B2* locus, and
- The association of a region in *CYP11B2* (between intron 2 and intron 3) and *CYP11B1* (between promoter and exon 1) with hypertension in Caucasians.

In addition, the results in this thesis further add support to the theory explained in section 1.4.5, that a mild impairment of 11β-hydroxylase activity can lead to increased aldosterone

production and the development of hypertension. This is the first time significant associations with variations in both genes have been consistently shown not only with intermediate phenotypes (e.g. plasma aldosterone concentration), but also with an ultimate phenotype (i.e. hypertension). Thus, more evidence has been provided to support a link between the *CYP11B1/CYP11B2* locus, increased ARR and hypertension. Although the causal variants in the locus remain to be determined, the evidence in this thesis shows that differences in genotype at the *CYP11B1/CYP11B2* locus might play an important role in increasing or decreasing the adverse effects of exposure to inappropriate aldosterone levels, which affect blood pressure and contribute to end-organ damage. Early identification of predisposed subjects through the screening of the ARR and, in the future, identification of susceptible genotypes at the *CYP11B1/CYP11B2* locus will enable the provision of early lifestyle intervention (e.g. restricting sodium intake) or a targeted therapeutic agent, such as the currently-available MR antagonists or, in time, specific aldosterone synthase inhibitors, to slow the progression towards hypertension and its attendant cardiovascular diseases.

9 Appendices

9.1 Appendix 1: LIAISON® Direct renin assay, manufacturer's information

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LIAISON® Direct Renin (310470)

1. INTENDED USE
The LIAISON® Direct Renin assay uses chemiluminescent immunoassay (CLIA) technology for the *in vitro* quantitative determination of renin in human EDTA-plasma specimens. The kit is intended for performannce evaluation only.The test has to be performed on the LIAISON® Analyzer.

2. SUMMARY AND EXPLANATION OF THE TEST
The proteolytic enzyme renin (molecular weight: about 42 kDa) is mainly synthesized by the juxtaglomerular cells of the kidneys as prorenin and is stored in granules as prorenin or renin. It is released in response to physiological stimuli like decreased blood volume and blood pressure, and sodium depletion. Renin inactive precursor is prorenin, which is converted into renin by two steps. Firstly, prorenin undergoes a reversible conformational change (which gives rise to activated prorenin); secondly, 46 amino acids of prorenin are proteolytically cleaved to produce active renin, a glycoprotein formed of 340 amino acid residues. Part of prorenin escapes proteolytic cleavage to renin and is released into the circulation. Prorenin can be activated by different ways, like cryoactivation, acidification or partial proteolysis. Prorenin secretion does not appear to be tightly regulated, whereas renin secretion is strictly controlled. Blood concentration of prorenin is approximately ten-fold greater than that of renin. Renin is termed a double-domain enzyme, because the N- and C-terminal ends are quite similar. Each domain contains a single aspartic acid residue, critical for catalytic activity. Renin catalyzes the formation of angiotensin I (a decapeptide) by proteolytic cleavage of renin substrate, called angiotensinogen, a glycoprotein synthesized in the liver. Angiotensin-converting enzyme (ACE), in turn, converts angiotensin I to angiotensin II, an octapeptide, that promotes aldosterone release and inhibits renin secretion by a negative feedback mechanism.
The renin-angiotensin-aldosterone system (RAS) plays a paramount role in water homeostasis and electrolyte balance, and in the regulation of arterial pressure. Measurement of plasma renin and aldosterone is therefore considered a marker of the renin-angiotensin-aldosterone system activity. Measurement of total renin (prorenin plus active renin) or of prorenin are of lesser clinical interest. Theoretically, angiotensin II may work as a better marker, but angiotensin II has a very short half-life, and it is difficult to distinguish from angiotensin I. Angiotensinogen, renin substrate, is the limiting factor in angiotensin II production. In fact, its availability contributes to a certain degree to the stimulation or inhibition of the renin-angiotensin-aldosterone system. Angiotensin is not stored in liver cells where it is synthesized, by contrast with renin, which is stored in kidneys. Increased levels of thyroid hormones (hyperthyroidism), estrogens (oral contraceptives, pregnancy) as well as glucocorticoids (Cushing's syndrome, corticotherapy) all lead to increased renin substrate levels. Angiotensin II is involved in control of glomerular filtration and renal blood flow. Renin is secreted by kidneys in response to reduction in renal arteryperfusion (intrarenal baroceptor), reduction in distal tubular resorption of sodium ions (sodium leakage), hypokalaemia or stimulation of β-adrenergic receptors. In addition, renin secretion is reduced (by negative feedback) in the presence of high plasma concentrations of angiotensin II.

Increased renin levels are found in	Lowered renin levels are found in
Secondary aldosteronism (severe hypertension of renal origin). Direct renin measurement helps in differentiation of primary from secondary hyperaldosteronism In conjunction with aldosterone assay.	Primary aldosteronism. Direct renin measurement helps in differentiation of primary from secondary hyperaldosteronism In conjunction with aldosterone assay.
Addison's disease.	Salt-retaining steroid therapy.
Low-sodium diet, administration of diuretics, haemorrhage.	Renal artery stenosis.
Chronic renal failure.	Vasopressin (ADH) therapy.
Salt-losing status because of gastrointestinal disease.	Congenital adrenal hyperplasia with 17-hydroxylase deficiency.
Renin-producing kidney tumours.	
Essential hypertension.	
Hypokalaemia.	
Bartter's syndrome (high renin levels without hypertension).	

As a general rule, people with systolic blood pressure consistently above 160 mmHg and/or diastolic blood pressure over 95-100 mmHg (hypertension) need anti-hypertensive treatment. The yearly death rate related to hypertension is estimated as five million worldwide.
Hypertension is of two main types:
Essential or primary hypertension (90-95% overall), where the cause is unknown in origin.
Secondary hypertension (5-10%), e.g. due to an underlying cause, which may have the following origins:
– Hypertension related to kidney failure (2-3% of vascular origin; 2-3% of parenchymal origin).
– Hypertension related to hormonal disorders.
– Hypertension related to endocrine tumours (very rare).
– Iatrogenic hypertension (e.g., due to oral contraceptives, 1%).
Renin should be measured in accordance with medical judgement, whenever:

– Diastolic blood pressure exceeds 90 mmHg (guidelines of the European Society of Hypertension and European Society of Cardiology).
– Systolic blood pressure exceeds 140 mmHg (guidelines of the European Society of Hypertension and European Society of Cardiology).
– Hypokalaemia is present (to establish differential diagnosis of secondary hyperaldosteronism or primary hypermineralocorticism).
– Response to current anti-hypertensive treatment is insufficient.
– Functional character of a renal artery stenosis is investigated (by renin measurement in the renal veins during acute inhibition of angiotensin-converting enzyme).
– Cancer is linked to increased blood pressure (to establish differential diagnosis of ectopic production of renin).

3. PRINCIPLE OF THE PROCEDURE

The method for the quantitative determination of renin is a sandwich chemiluminescence immunoassay.
A specific mouse monoclonal antibody is coated on the magnetic particles (solid phase), that recognizes both renin and prorenin; another monoclonal antibody (specific for renin) is linked to an isoluminol derivative (isoluminol-antibody conjugate).
During the incubation, renin present in calibrators or controls as well as renin and prorenin present in samples bind to the solid phase monoclonal antibody, and subsequently the antibody conjugate reacts with renin already bound to the solid phase. A sandwich is formed only in the presence of renin molecules that bridge both antibodies. After incubation, the unbound material is removed with a wash cycle.
Subsequently, the starter reagents are added and a flash chemiluminescence reaction is thus induced. The light signal, and hence the amount of isoluminol-antibody conjugate, is measured by a photomultiplier as relative light units (RLU) and is directly proportional to renin concentration present in calibrators, samples or controls. To prevent renin overestimation, sample handling conditions which may activate prorenin must be avoided (see paragraph Specimen Collection and Preparation).

4. MATERIALS PROVIDED

The order of reagents reflects the layout of containers in the reagent integral.

Reagent Integral for 100 determinations	
2.3 mL	Magnetic particles (suspension) coated with anti-renin/prorenin monoclonal antibody (mouse).
13 mL	Conjugate solution: anti-renin monoclonal antibody (mouse), labelled with isoluminol derivative.

Included with Integral

5 x 2.0 mL	Calibrator A (lyophilized): recombinant human renin (active 340-amino acid protease produced in human embryonic kidney cells transfected with a human renin expression construct), phosphate buffer, bovine serum albumin, preservatives.
5 x 2.0 mL	Calibrator B (lyophilized): recombinant human renin (active 340-amino acid protease produced in human embryonic kidney cells transfected with a human renin expression construct), phosphate buffer, bovine serum albumin, preservatives.
5 x 2	Bar-coded labels for calibrator A and for calibrator B.
Conjugate and magnetic particles are provided ready-to-use. Calibrators are provided lyophilized.	

Materials required but not provided
LIAISON® Module (code 319130).
LIAISON® Starter Kit (code 319102).
LIAISON® Light Check (code 319101).
LIAISON® Wash/System Liquid (code 319100).
LIAISON® Waste Bags (code 450003).

Additionally required materials
LIAISON® Direct Renin controls, levels 1 and 2 (code 310471).
LIAISON® Cleaning Kit (code 310990).

5. WARNINGS AND PRECAUTIONS

For *in vitro* diagnostic use.

6. SAFETY PRECAUTIONS

Do not eat, drink, smoke or apply cosmetics in the assay laboratory.
Do not pipette solutions by mouth.
Avoid direct contact with all potentially infectious materials by using protective clothing such as lab coats, protective glasses and disposable gloves. Wash hands thoroughly at the end of each assay.
Avoid splashing or forming an aerosol. Any reagent spills should be washed with a 5% sodium hypochlorite solution and disposed of as though potentially infectious.
All samples, biological reagents and materials used in the assay must be considered potentially able to transmit infectious agents. They should therefore be disposed of in accordance with the prevailing regulations and guidelines of the agencies holding jurisdiction over the laboratory, and the regulations of each Country.

7. REAGENT PREPARATION
REAGENT INTEGRAL

Before removing the seals from the containers, gently and carefully shake the reagent integral horizontally. Avoid formation of foam. Remove the seal from each container and turn the thumb wheel at the bottom of the magnetic particle container to and fro until the suspension turns brown. This procedure initiates resuspension of magnetic particles. Carefully wipe the surface of each septum to remove residual liquid. Then, place the integral into the reagent area of the Analyzer with the barcode label facing left and let it stand for 30 minutes before using. The Analyzer automatically stirs and completely resuspends the magnetic particles. Follow the Analyzer Operator's Manual to load the specimens and start the run.

CALIBRATORS
LIAISON® Direct Renin calibrators are supplied lyophilized.
– Reconstitute the vial contents with 2.0 mL deionized or distilled water.
– Allow the vials to stand for 10-15 minutes at 18-25°C to achieve complete dissolution.
– Mix vials thoroughly by gentle inversion; avoid foaming.
– The reconstituted solution of each calibrator must be transferred to a 12 x 75mm polystyrene tube. Affix the proper barcoded label to the calibrator tube and load on to the instrument. Each calibrator solution allows three calibrations to be performed.
Once reconstituted refer to paragraph 8 to store the calibrators.
For details on the use of the calibrators on board the instrument, refer to the LIAISON® Operator's Manual.
Renin concentrations in the calibrators are printed on the vial labels, coded in the bar-coded labels provided separately and in the reagent integral bar codes.
CONTROLS
Refer to the LIAISON® Direct Renin Control Set instructions for use section for proper preparation and handling instructions.

8. REAGENT STORAGE AND STABILITY
REAGENT INTEGRAL
– **Sealed**: Stable at 2-8°C until the expiry date.
– **Opened on board or at 2-8°C** : Minimum stability six weeks.
After this period, it is still possible to keep on using the reagent integral provided that the controls are found within the
expected ranges.
– Use always the same LIAISON® Analyzer for a reagent integral already opened.
– Use storage rack provided with the LIAISON® Analyzer for upright storage of reagent integral.
– Do not freeze.
– Keep upright for storage to facilitate later proper resuspension of magnetic particles.
– Keep away from direct light.
CALIBRATORS
– **Lyophilized**: Stable at 2-8°C until the expiry date.
– **Reconstituted**: Stable for two weeks when properly stored at 2-8°C either in their sealed vials or in stoppered transfer tubes.
Do not leave the reconstituted calibrators at room temperature longer than the time required to process them on the LIAISON®.
During handling, use appropriate precautions to avoid bacterial contamination of calibrators.

9. SPECIMEN COLLECTION AND PREPARATION
When prorenin, the inactive precursor of renin, is cryoactivated to renin during sample handling, falsely elevated results are obtained. Cryoactivation occurs when patient samples are chilled to temperatures of 4°C or below for extended periods of time, and when samples are chilled but still liquid (i.e., not frozen). Cryoactivation of prorenin to renin occurs more rapidly in serum. Prorenin blood concentration is approximately ten-fold greater than that of renin. The only sample material validated is human EDTA-plasma. Use of serum, heparinized plasma, and citrated plasma samples provides lower renin values, and is therefore not recommended.
Careful standardization of the patient preparation and sampling conditions is strongly recommended. Collect blood at room temperature by venipuncture, in siliconized glass tubes, vacutainers (violet cap) or equivalent, containing EDTA as anticoagulant. The presence of haemolysis may indicate mistreating during sample collection or handling.
Fasting specimens are recommended but not required. Record the time of day and the patient's posture during blood collection (supine, upright or seated). Do not pre-chill EDTA blood collection tubes nor store tubes on ice, but process blood at room temperature. Centrifuge tubes in a non-refrigerated centrifuge, separate EDTA-plasma from cells immediately after centrifugation, then aliquot and deep-freeze at –20°C or below immediately.
Carefully thaw before testing, mix the thawed samples and check for and remove air bubbles before assaying.
Grossly haemolyzed or lipaemic samples as well as samples containing particulate matter or exhibiting obvious microbial contamination should not be tested.
Do not use clotted samples.
Avoid repeated freeze-thaw cycles.
It is recommended to test plasma samples immediately after loading on to the instrument.
The minimum volume required for a single determination is 350 µL specimen (200 µL specimen + 150 µL dead volume).

10. CALIBRATION
Assay of calibrators contained in the reagent integral box allows the Analyzer to recalibrate the stored master curve, as indicated via the bar codes on the reagent integral label.
Calibrators must be used only with the reagent integral lot they are matched with. Do not use calibrators matched with a different
reagent integral lot in the same assay. For correct lot matching, calibrator lot number is printed also on the reagent integral label.
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The Analyzer should be calibrated in triplicate whenever one of the following conditions occurs:
– A new lot of Starter Kit is used.
– The previous calibration was performed more than one week before.
– Each time a new lot of integral is used.
– The Analyzer has been serviced.
– Control values lie outside the expected ranges.

11. ASSAY PROCEDURE

Strict adherence to the Analyzer Operator's Manual ensures proper assay performance. Each test parameter is identified via the bar codes printed on both the reagent integral label and the bar-coded calibrator labels. In case of malfunction of the bar code reader, the data can be entered manually. For details, refer to the Analyzer Operator's Manual.

The Analyzer operations are as follows:

200 µL	Calibrators, controls or specimens.
+ 20 µL	Coated magnetic particles.
+ 100 µL	Conjugate.
31.5 minutes	Incubation followed by a wash cycle.
3 seconds	Measurement.

12. QUALITY CONTROL

Quality control must be performed by running LIAISON® Direct Renin controls (a) at least once per day of use, (b) whenever a new reagent integral is used, (c) whenever the kit is calibrated, (d) whenever a new lot of Starter Reagents is used, (e) to assess adequacy of performance of the open integral beyond six weeks, or in agreement with guidelines or requirements of local regulations or accredited organizations.

Warning: LIAISON® controls should be run in singlicate to monitor the assay performance. Control values must lie within the expected ranges: whenever one or both controls lie outside the expected ranges, calibration should be repeated and controls retested. If control values obtained after successful calibration lie repeatedly outside the predefined ranges, the test should be repeated using a freshly reconstituted control vial. If control values lie outside the expected ranges, patient results must not be reported.

The performance of other controls should be evaluated for compatibility with this assay before they are used. Appropriate value ranges should then be established for quality control materials used.

13. INTERPRETATION OF RESULTS

The Analyzer automatically calculates renin concentrations for the unknown samples. For details, refer to the Analyzer Operator's Manual.

Measuring range: The Analyzer directly calculates renin concentration up to 500 µIU/mL.

Reference standard: The assay is referenced to the World Health Organization International Reference Preparation, NIBSC
code 68/356. The results are expressed as µIU/mL.

Expected values: Each laboratory should establish its own range of expected values for the population taken into consideration.

To assess the expected reference range, a study was performed in 178 EDTA-plasma samples (89 subjects).

Samples were collected from a fasting population formed of male (n = 49) and female (n = 40) apparently healthy blood donors of Caucasian, African-American and Hispanic origin, who meet the following inclusion criteria:
adult subjects, 18-65 years of age, with normal blood pressure and normal fasting glucose levels.

The following criteria prevented inclusion in this study: age below 18 years; need for prescription medications; need for doctor-prescribed restricted diet; pregnancy; breast feeding; administration of oral contraceptives.

Blood was collected between 7:00 a.m. and 10:00 a.m. with the subjects either in an upright or supine position. Upright samples were collected when individuals sat down to have their blood withdrawn, after standing for 30 minutes; supine samples were collected after the individuals lay in supine position for at least 30 minutes. The resulting intervals (5th-95th percentile) are the following: 4.4-46.1 µIU/mL (upright/sitting posture) and 2.8-39.9 µIU/mL (supine posture).

14. LIMITATIONS OF THE PROCEDURE

– The reagents should be used only in the LIAISON® System.
– Calibrators are kit lot specific and must not be interchanged with a reagent integral from a different lot.
– Single components of the reagent integral should not be removed from the integral.
– This kit must not be used after the expiry date printed on the package label.
– A skillful technique and strict adherence to the instructions are necessary to obtain reliable results.
– Bacterial contamination or heat inactivation of the specimens may affect the test results.
– A result within the expected range does not rule out the presence of disease and should be interpreted together with the patient's clinical picture and other diagnostic procedures.
– Test results are reported quantitatively. However, diagnosis of a disease should not be based on the result of a single test, but should be determined in conjunction with clinical findings in association with medical judgement. Any therapeutical decision must also be taken on a case-by-case basis.
– The LIAISON® Direct Renin assay has been developed for the determination of the analyte in its intact and unaltered state.
Degradation of the molecule or prorenin cryoactivation may affect final results.
– Although HAMA-neutralizing agents are added, extremely high HAMA (human anti-mouse antibodies) concentrations may occasionally influence results.
– Renin levels in paediatric age have not been investigated.
– No interference due to drug administration has been investigated.
– Interference may be rarely observed in the presence of extremely high serum concentrations of streptavidin antibodies as well as in plasma of patients administered with high doses of biotin (vitamin H) because of manufacturing optimization.

15. SPECIFIC PERFORMANCE CHARACTERISTICS

15.1. Analytical specificity

Analytical specificity may be defined as the ability of the assay to accurately detect specific analyte in the presence of potentially interfering factors in the sample matrix (e.g., haemolysis, lipaemia, bilirubinaemia).

Interference. Controlled studies of potentially interfering substances or conditions showed that the assay performance was not affected by concentrations of bilirubin up to 20 mg/dL, haemoglobin up to 1000 mg/dL or triglycerides up to 3000 mg/dL.

15.2. Precision

Different samples, containing different concentrations of specific analyte, were assayed to estimate repeatability and reproducibility of the assay (i.e., within- and between-assay variability). The results refer to the groups of samples investigated and are not guaranteed specifications, as differences may exist between laboratories and locations.

Repeatability. Twenty replicates were performed in the same run to evaluate in-house repeatability.

Repeatability	A	B	C	D	Control 1	Control 2
Number of determinations	20	20	20	20	20	20
Mean (µIU/mL)	15.1	33.8	82.2	258.0	27.1	99.0
Standard deviation (µIU/mL)	0.6	0.9	1.7	3.1	1.5	2.3
Coefficient of variation (%)	3.7	2.8	2.0	1.2	5.6	2.4
Min value (µIU/mL)	13.4	32.4	78.5	252.8	24.1	95.3
Max value (µIU/mL)	15.7	35.9	84.6	264.4	29.9	102.5

Reproducibility. Twenty replicates were performed in different days (one or two runs per day) to evaluate reproducibility. The tests were performed in two sites, in house (site 1) and in an independent laboratory (site 2) using the same instruments.

Reproducibility - Site 1	A	B	C	D	Control 1	Control 2
Number of determinations	20	20	20	20	20	20
Mean (µIU/mL)	15.0	40.6	98.5	317.4	31.6	118.4
Standard deviation (µIU/mL)	1.3	2.1	7.1	34.6	1.4	7.8
Coefficient of variation (%)	8.6	5.2	7.2	10.9	4.3	6.6
Min value (µIU/mL)	13.1	36.7	86.3	267.9	29.2	102.4
Max value (µIU/mL)	18.9	45.8	108.5	365.3	34.5	132.5

Reproducibility - Site 2	A	B	C	D	Control 1	Control 2
Number of determinations	20	20	20	20	20	20
Mean (µIU/mL)	15.8	34.8	85.4	260.8	26.8	103.2
Standard deviation (µIU/mL)	2.7	2.1	5.2	26.5	2.8	7.5
Coefficient of variation (%)	17.1	6.1	6.1	10.2	10.6	7.3
Min value (µIU/mL)	12.9	32.3	75.2	214.5	22.1	91.0
Max value (µIU/mL)	22.7	38.4	93.7	306.2	31.7	116.3

15.3. Linearity by dilution test

Plasma samples containing high renin concentrations were tested as such and after serially diluting with a renin-free plasma. Measured versus expected renin concentrations were analyzed by linear regression. The correlation coefficients (r) ranged from 0.999 to 1.000.

Dilution	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery	Dilution	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery
neat	–	333.4	–	neat	–	> 500.0	–
1:2	166.7	190.5	114.3	1:2	–	396.5	–
1:4	83.4	91.7	110.1	1:4	198.3	200.0	100.9
1:8	41.7	47.3	113.5	1:8	99.1	103.1	104.0
1:16	20.8	24.7	118.6	1:16	49.6	51.3	103.6
1:32	10.4	12.1	116.3	1:32	24.8	26.2	105.8

15.4. Trueness by recovery test

Two sets formed of a high- and a low- to normal-renin sample (samples X and Y in set 1, and samples W and Z in set 2) were mixed in 1:5, 1:2, 1:1, 2:1 and 5:1 ratios and assayed. Percent recoveries were determined from results of undiluted samples. Measured versus expected renin concentrations were analyzed by linear regression. The correlation coefficients (r) ranged from 0.996 to 1.000.

Set 1	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery	Set 2	Expected concentration, µIU/mL	Measured concentration, µIU/mL	% Recovery
X neat	–	5.3	–	W neat	–	23.9	–
5:1	25.8	24.5	95.0	5:1	65.4	62.3	95.2
2:1	46.3	49.4	106.6	2:1	106.9	103.4	96.7
1:1	66.9	69.3	103.6	1:1	148.4	148.4	100.0
1:2	87.4	92.1	105.4	1:2	189.9	186.6	98.2
1:5	107.9	108.0	110.1	1:5	231.4	230.0	99.4
Y neat	–	128.4	–	Z neat	–	272.9	–

15.5. Carryover

The carryover effect was investigated by testing one renin-free plasma sample before and after four samples containing increasing renin concentrations. The results obtained demonstrate that no carryover is observed when using the LIAISON® Analyzer.

15.6. High-dose hook effect

The high-dose hook effect (HDH) was determined by addition of recombinant renin to a human plasma pool up to to a maximum of 150,000 µIU/mL.

Whenever samples containing extremely high analyte concentrations are tested, the high-dose hook effect can mimic concentrations lower than real. Analysis of high-dose hook effect was evaluated by testing one high-concentration renin-spiked sample. The sample resulted in a calculated concentration value above the measuring range, indicating no sample misclassification.

15.7. Analytical sensitivity

Analytical sensitivity, defined as the minimum detectable dose that can be distinguished from zero by two standard deviations (that is, two standard deviations above zero), ranges from 0.13 µIU/mL to 0.24 µIU/mL (as assessed by several assay runs, kit lots and instruments).

9.2 Appendix 2: Nucleotide sequences of human *CYP11B1* and *CYP11B2* genes

The nucleotide sequences obtained from Ensembl release 46 August 2007

Gene sequence information for OTTHUMG00000139952,

>chromosome:NCBI36:8:143948707:144006243:-1

THIS STYLE: Location of *CYP11B2* exons

THIS STYLE: Location of *CYP11B1* exons

THIS STYLE: Location of untranslated regions and introns

THIS STYLE: Oligonucleotides for PCR and sequencing listed in Appendix 3. Each of the oligonucleotide is indicated by name above relevant highlighted sequence. Where sequences overlap the second sequence is underlined as is the primer name. Sense and antisense oligonucleotides are indicated by > or <.

CYP11B2

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TTGCTGATGACGCTCTTTGAGCTGGCTCGGAACCCCAACGTGCAGCAGGCC
CTGCGCCAGGAGAGCCTGGCCGCCGCAGCCAGCATCAGTGAACATCCCCAG
AAGGCAACCACCGAGCTGCCCTTGCTGCGTGCGGCCCTCAAGGAGACCTTG
CGGTGGGTGCTGGCTGAGGCCTCCCTGTGGCCCTGGCCCCCTGCTGGAGAG
CAGCCCCCACTGGGTGGTGGCAGACAGAATCTGGGGCTGATAAACAGCGTC
ACCCAGCAGCCCATTCCCCTGCACCTGCTCTTCCTCCCCCTCAAGGACAGG
GAGCTCTTCTTTCTCTGGAATCCCTCTTCAACGCCCTGGGGATTAACGTGG
AGCATGTCCTTCTGCGCTCGGGGCTGCTTAAGTTAGGGGAGGTTTGGCCGG

GCTCAGCAGGTGCAAGGAAGCACTTCCTACGACCTGGGCTTCCCATGGATC
TGGGACCTCTGCGGGTTCTTCGGTAGGAAGGGTGCAGAGAGCACAGGAAGC
CCCATCCAGCTGAGGACCCTTTCTATGGATGCCCCCACCTCCAG**GCTCTAC**
CCTGTGGGTCTGTTTCTGGAGCGAGTGGCGAGCTCAGACTTGGTGCTTCAG
AACTACCACATCCCAGCTGGGGTGAGTGAGCCCCACACCCCTCGAGCTGAG
AACCTCCCTCCCCAGTCATTCCCTGATCCCCGCTCTGCACCGTCCGCAG**AC**
ATTGGTGCGCGTGTTCCTCTACTCTCTGGGTCGCAACCCCGCCTTGTTCCC
GAGGCCTGAGCGCTATAACCCCCAGCGCTGGCTAGACATCAGGGGCTCCGG
CAGGAACTTCTACCACGTGCCCTTTGGCTTTGGCATGCGCCAGTGCCTTGG
GCGGCGCCTGGCAGAGGCAGAGATGCTGCTGCTGCTGCACCATGTGAGCAG
GCCCGGGCTGGGGAGGGGCCTGGGCGGGGTCTGGGCAGCATGGGCGGGGCT
TGAGCAATGTGGGACTGGCCTCGGCAGAGTGGGAGTGGCCTGCATGTTTCC
TGGACTGGGCAGAGCCGGTACTGGGAGAACCTGGGCCAGGTTGAGGCTGTG
TAGGTCCTGGGCAGGAGTTGGTATGGTGAGGAGCGTACCATCTGGGTGAGG
TTGCTGCTAAACCGGGTCAGGTGGGAACTGGGGAAGTCGGGTGGAGCCTGT
ACAGGATAGTGGGGCTTGGGCAATACCTGGGCTGGATGAATTCTGGGCCTG
GGCTGTAAGGTGGGGCTGGTCAGGAATGAAACAGGTTGGAGGCCAGGCTGC
TGTTCCCCCTTCAGCATAATCTCTGCAACTTTGAGGGTCTGAGAAGGCTGC
ACCACGTGCATGGGCTGCGGACCAAGCCAGATGGAAACCCGGCTTCTGTCC
TAG**GTGCTGAAACACCTCCAGGTGGAGACACTAACCCAAGAGGACATAAAG**
ATGGTCTACAGCTTCATATTGAGGCCCAGCATGTTCCCCCTCCTCACCTTC
AGAGCCATCAACTAATCACGTCTCTGCACCCAGGGTCCCAGCCTGGCCACC
AGCCTCCCTTTCTGCCTGACCCCAGGCCACCCCTCTTCTCTCCCACATGCA
CAGCTTCCTGAGTCACCCCTCTGTCTAACCAGCCCCAGCACAAATGGAACT
CCCGAGGGCCTCTAGGACCAGGGTTTGCCAGGCTAAGCAGCAATGCCAGGG
CACAGCTGGGGAAGATCTTGCTGACCTTGTCCCCAGCCCCACCTGGCCCTT
TCTCCAGCAAGCACTGTCCTCTGGGCAGTTTGCCCCCATCCCTCCCAGTGC
TGGCTCCAGGCTCCTCGTGTGGCCATACAAGGGTGCTGTGGTTTTGTCCCT
TGCCTTCCTGCCTAGTCTCACATGTCCCTGTTCCTCTTCCCCTGGCCAGGG
CCCCTGCGCAGACTGTCAGAGTCATTAAGCGGGATCCCAGCATCTCAGAGT
CCAGTCAAGTTCCCTCCTGCAGCCTGACCCCTAGGCAGCTCGAGCATGCCC
TGAGCTCTCTGAAAGTTGTCACCCTGGAATAGGGTCCTGCAGGGTAGAATA
AAAAGGCCCCTGTGGTCACTTGTCCTGACATCCCCATTTTCAAGTGATACA
ACTGAGTCTCGAGGGACGTGTGTTCCCCAGCTGATCGTGTCAGCCTCATGC
CCCAGGCCTCATCTTTCATGGACCAGGCCTTGTTCCAGGAGTGGGTGTTGG
GTCCTCTGCTTCCTGTGCTGTCCCCTGGGGAAGGTCCCGAGGATGCTGTCA
GGAGATGGAAGAGTCATGTGGGGTGGGAACCTGGGGTGTGGTTCCAGAAAT
GTTTTTGGCAACAGGAGAGACAGGATTGGGCCAACAAGGACTCAGACGAGT
TTTATTGACTCATTTCTCTGGTTGATACGGAGCCATGTCATGTGCCACGAC
CTGGGGTGGGCACAGGGAGGCTGCAGTTTCCTGCGTGAACCTGCCTTGGGC
CTCATCTGCTCCTAGCCCAGCAGAGAGAGTTGACCCCTCCTGAACTGGCCA
CTCCCCAGTGCTCCCGTGCAGGGAGAGGAAGCACCCAGTTTGAGAGTGTAC
CCAGCCAGACTGTCTTTATCTCCATGGATTTTCATCAGGGCAAAGATCACA
GCAGCCAGCTCCTGGTGGCTGATGAGGATCAGAGCATTTGTTCCCCCATGA
AAGGGGAAATCCCTATGGCATCATTCCAGTGGTGGTCAGTAGATCCAGGAC
GCCCTGCAGGACTCAGCCTGTACAGGGAGATGAAGTGCCCCAGGTTGGGAG
CACACCTAGCTAGAGTTATTTGTGTTAATCTATTCAGGATGCTCTAAGAAA
ACGCCATAGACTGGGTTGCTGACAAACATCAGAATTCTATTTCTCATGGTT
CTGAAGGCTTAGAAGGCTAAGATCAAGGTGTCAGCAGGTTCTGTGTCTGGT
GAGGACCCACCTCTTGTTTCATAGATAAAACCTTCTTGCTGTGTCCTCACA
TGGTGGAAAAGGGCAAGACAGCTCACAGAGACCTCTTTTATAAGGACGCCA
GTTCCATTCAAGAGGGCACTGCCTGCATTCCCTTCCCCCCTCAAAGGCCCG
GCCCTGCTATTATCGTCACCTTGGTGACTAGATTTCAGCCTATGAATTCTG
GAGGCGGACACAAAAATTCAGATTAAACTACTCTGCTTCTGTTTCTCTAAA
ATCTACATCCTTCTCATATACAAAATACATGCATTTCTCCCCAGTAGCCCC
AAATATCAACTCATTTTAGCATCAACTTTAAAATTGTAGTCCAAAGTGTGA
TTTAAATGTTATCTGAGTCAAACATGGGTAAGACTCAAGGTACAATTTATC
CTGAGTCCAACTGTTCTTGCTGCGAACCTATGAAATCAAACATGTTATGTG
CTTCCAAAATACAGTGGTTGGAGAGGCATAGGATAAACATTCCCATTCCAG
AAAGGAGAAACAGGAAAGAAGAAAGGAGTAACAAGTTTCAAGCAACTCCAA
AATTGAATGTGACAAACAGTATTAAACCTTAAGCCTCAAGAATAATCTCCT

TTGACTGGATTTTCCATCTTCTGAACATAGTGGTGCAGGGATTGTTTCCCT
CAGGGCTTCAGGATGCTCTGCCCCTGCAGCTTGGCTGGGTGCAGCTGCAGT
GCAGCTCTCATGAGTTGGAGTTGCATTCCTGCAGGTCTCCCAGACTGGAAT
GACTCACCGGTAGCTTCACCTACCTGGGATCCTGGGCTGTCCTGCTCTGCT
GCCTCCACTAGGCATGG

9.3 Appendix 3: PCR and sequencing primers for *CYP11B1* and *CYP11B2*

Table 9-1. Sequences of PCR primers

PCR

Protocol	Gene	Location	SNP positions	PCR	Fragment length	Primer direction	Primer name	Sequence	No. bp	Tm (℃)
B1PROM	*CYP11B1*	Promoter	-1889 and -1859*	1st	1738 bp	Sense	B1 5'UTR	5'-TCCTTCGCATCCCTTGTAAGTT-3'	22	58.4
						Antisense	B1prom-260	5'-CTTGGATTATTCATCTCCTTGCAAGG-3'	26	61.6
B1PROM	*CYP11B1*	Promoter	-1889 and -1859*	2nd	387 bp	Sense	B1 5' 7-32	5'-GCATCCCTTGTAAGTTGGATTCCTAA-3'	26	61.6
						Antisense	B1 5' 369-393	5'-AAGCATTCCCTTTGAAAACTGGTAC-3'	25	59.7
B1INT2	*CYP11B1*	Intron 2	1751,1752 & 1818*	1	261 bp	Sense	B1 3696 S	5'-TTTGCACGTGTTGTGTGTG-3'	19	54.5
						Antisense	B1 3937 AS	5'-ATTCCTGCAAAACTCAAAGC-3'	20	53.2
B2PROM-344	*CYP11B2*	Promoter	-344*	1	232bp	Sense	SF1_F	5'-GTGTCAGGGCAGGGGGTA-3'	18	60.5
						Antisense	SF1_R	5'-AGGCGTGGGGTCTGGACT-3'	18	60.5
B2INCON2	*CYP11B2*	Intron 2	Several linked SNPs within intron 2	1	600bp	Sense	ICTAQMAN F	5'-GATGGCATGAAGCACAAAGCT-3'	21	57.9
						Antisense	ICTAQMAN R	5'-CCTTGGGCGACAGCACA-3'	17	57.6
B2INT6	*CYP11B2*	Intron 6	Several SNPs in the region from bases 4837 to 4936*	1	329 bp	Sense	B2 6687 S	5'-ATTCCCCTGCACCTGCTCTT-3'	20	59.4
						Antisense	B2 6995 AS	5'-AGCTCACCACTCGCTCCAAA-3'	20	59.4

* Relative to first base *CYP11B1* or *CYP11B2* coding region

Table 9-2. Sequences of sequencing primers

SEQUENCING

Protocol	Region	Location	SNP positions	Primer direction	Primer name	Sequence	No. bp	Tm (℃)	Program
B1PROM	*CYP11B1*	Promoter	-1889 and -1859*	Sense	NA				
				Antisense	B1 Prom 250-229 AS	5'-AAGTCAAATTGTCTCTGTTTG-3'	21	52	BIGSEQ60
B1INT2	*CYP11B1*	Intron 2	1751,1752 & 1818	Sense	B1 3696 S	5'-TTTGCACGTGTTGTGTGTG-3'	19	54.5	BIGSEQ50
				Antisense	**B1 3937 AS**	5'-ATTCCTGCAAAACTCAAAGC-3'	20	53.2	BIGSEQ50
B2PROM-344	B2PROM	Promoter	-344*	Sense	SF1_F	5'-GTGTCAGGGCAGGGGGTA-3'	18	60.5	BIGSEQ50
				Antisense	NA				
B2INCON2	B2INT2	Intron 2	Several linked SNPs within intron 2	Sense	NA				
				Antisense	INTCONR(B1B2)	5'-GTGTTCGAGCTGCAGCCTTTTC-3'	22	62.1	BIGSEQ60
B2INT6	B2INT2	Intron 6	Several SNPs in the region from bases 4837 to 4936*	Sense	B2 6687 S	5'-ATTCCCCTGCACCTGCTCTT-3'	20	59.4	BIGSEQ50
				Antisense	NA				

* Relative to first base *CYP11B1* or *CYP11B2* coding region. Primer in bold produced a more efficient reaction.

9.4 Appendix 4: Results of association analysis with sliding window approach using 37 SNPs in the discovery phase

GML: Gene encoding glycosylphosphatidylinositol-anchored molecule-like protein; Interg, intergenic region; CYP11B1: gene encoding11β-hydroxylase; CYP11B2: gene encoding aldosterone synthase; Freq: Frequency, CHISQ: chi square, DF: degrees of freedom; p: p-value chi square test

Locus window	Gene	SNP1	SNP2	SNP3	Haplotype	Freq cases	Freq controls	CHISQ	DF	P
WIN1	*GML*	rs3819497	rs7826809	rs3753122	A/T/T	0.4413	0.4761	7.438	1	0.006386
WIN1	*GML*	rs3819497	rs7826809	rs3753122	G/C/G	0.5587	0.5239	7.438	1	0.006386
WIN2	*GML*	rs7826809	rs3753122	rs2078674	T/T/T	0.4413	0.4761	7.438	1	0.006386
WIN2	*GML*	rs7826809	rs3753122	rs2078674	C/G/G	0.5587	0.5239	7.438	1	0.006386
WIN3	*GML/Interg*	rs3753122	rs2078674	rs4418320	T/T/A	0.4413	0.4761	7.438	1	0.006386
WIN3	*GML/Interg*	rs3753122	rs2078674	rs4418320	G/G/G	0.5587	0.5239	7.438	1	0.006386
WIN4	*GML/Interg*	rs2078674	rs4418320	rs4736346	T/A/A	0.4413	0.4761	7.438	1	0.006386
WIN4	*GML/Interg*	rs2078674	rs4418320	rs4736346	G/G/C	0.5587	0.5239	7.438	1	0.006386
WIN5	*Interg/CYP11B1*	rs4418320	rs4736346	rs5301	A/A/C	0.4413	0.4761	7.438	1	0.006386
WIN5	*Interg/CYP11B1*	rs4418320	rs4736346	rs5301	G/C/T	0.5587	0.5239	7.438	1	0.006386
WIN6	*Interg/CYP11B1*	rs4736346	rs5301	rs6471570	Overall	NA	NA	7.511	2	0.02339
WIN6	*Interg/CYP11B1*	rs4736346	rs5301	rs6471570	C/T/A	0.4629	0.4385	3.649	1	0.05612
WIN6	*Interg/CYP11B1*	rs4736346	rs5301	rs6471570	A/C/C	0.4381	0.4724	7.2	1	0.007291
WIN6	*Interg/CYP11B1*	rs4736346	rs5301	rs6471570	C/T/C	0.09902	0.0891	1.751	1	0.1857
WIN7	*CYP11B1*	rs5301	rs6471570	rs6410	Overall	NA	NA	8.133	4	0.08681
WIN7	*CYP11B1*	rs5301	rs6471570	rs6410	T/A/T	0.4628	0.4384	3.649	1	0.05611
WIN7	*CYP11B1*	rs5301	rs6471570	rs6410	C/C/T	0.01593	0.01759	0.251	1	0.6163
WIN7	*CYP11B1*	rs5301	rs6471570	rs6410	T/C/T	0.01665	0.01287	1.497	1	0.2212
WIN7	*CYP11B1*	rs5301	rs6471570	rs6410	C/C/C	0.4221	0.4548	6.571	1	0.01037
WIN7	*CYP11B1*	rs5301	rs6471570	rs6410	T/C/C	0.08249	0.07636	0.7811	1	0.3768

Locus window	Gene	SNP1	SNP2	SNP3	Haplotype	Freq cases	Freq controls	CHISQ	DF	P
WIN8	*CYP11B1*	rs6471570	rs6410	rs4471016	Overall	NA	NA	14.33	3	0.002487
WIN8	*CYP11B1*	rs6471570	rs6410	rs4471016	A/T/G	0.4596	0.4372	3.045	1	0.08099
WIN8	*CYP11B1*	rs6471570	rs6410	rs4471016	C/T/G	0.02609	0.02857	0.3484	1	0.555
WIN8	*CYP11B1*	rs6471570	rs6410	rs4471016	A/T/A	0.0165	0.007649	10.01	1	0.001558
WIN8	*CYP11B1*	rs6471570	rs6410	rs4471016	C/C/A	0.4978	0.5266	4.973	1	0.02575
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	Overall	NA	NA	24.38	3	2.08E-05
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	T/G/T	0.461	0.4432	1.767	1	0.77
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	T/G/G	0.0231	0.02663	0.7077	1	0.14
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	T/A/G	0.02456	0.0091	20.65	1	3.50E-05
WIN9	*CYP11B1*	rs6410	rs4471016	rs4313136	C/A/G	0.4914	0.5211	4.896	1	0.04
WIN10	*CYP11B1/Interg*	rs4471016	rs4313136	rs4736349	Overall	NA	NA	8.138	3	0.04325
WIN10	*CYP11B1/Interg*	rs4471016	rs4313136	rs4736349	A/G/T	0.4163	0.4466	5.21	1	0.02245
WIN10	*CYP11B1/Interg*	rs4471016	rs4313136	rs4736349	G/T/C	0.4634	0.4435	2.245	1	0.134
WIN10	*CYP11B1/Interg*	rs4471016	rs4313136	rs4736349	G/G/C	0.02338	0.02725	0.8388	1	0.3597
WIN10	*CYP11B1/Interg*	rs4471016	rs4313136	rs4736349	A/G/C	0.09687	0.08269	3.442	1	0.06357
WIN11	*CYP11B1/Interg*	rs4313136	rs4736349	rs4736350	Overall	NA	NA	8.777	3	0.03241
WIN11	*CYP11B1/Interg*	rs4313136	rs4736349	rs4736350	T/C/G	0.4597	0.4397	2.439	1	0.1184
WIN11	*CYP11B1/Interg*	rs4313136	rs4736349	rs4736350	G/C/G	0.04368	0.03334	4.377	1	0.03643
WIN11	*CYP11B1/Interg*	rs4313136	rs4736349	rs4736350	G/T/A	0.4185	0.4489	5.7	1	0.01696
WIN11	*CYP11B1/Interg*	rs4313136	rs4736349	rs4736350	G/C/A	0.07811	0.07798	0.0003296	1	0.9855
WIN12	*Interg*	rs4736349	rs4736350	rs7004383	Overall	NA	NA	5.536	2	0.06279
WIN12	*Interg*	rs4736349	rs4736350	rs7004383	T/A/G	0.4213	0.4512	5.479	1	0.01925
WIN12	*Interg*	rs4736349	rs4736350	rs7004383	C/G/C	0.4972	0.47	4.486	1	0.03417
WIN12	*Interg*	rs4736349	rs4736350	rs7004383	C/A/C	0.08145	0.07881	0.1426	1	0.7057

Locus window	Gene	SNP1	SNP2	SNP3	Haplotype	Freq cases	Freq controls	CHISQ	DF	P
WIN13	*Interg*	rs4736350	rs7004383	rs4641039	Overall	NA	NA	5.033	3	0.1694
WIN13	*Interg*	rs4736350	rs7004383	rs4641039	G/C/T	0.4673	0.4433	3.525	1	0.06047
WIN13	*Interg*	rs4736350	rs7004383	rs4641039	A/G/C	0.4213	0.4497	5.009	1	0.02522
WIN13	*Interg*	rs4736350	rs7004383	rs4641039	G/C/C	0.02972	0.02824	0.118	1	0.7313
WIN13	*Interg*	rs4736350	rs7004383	rs4641039	A/C/C	0.08176	0.07875	0.1865	1	0.6658
WIN14	*Interg*	rs7004383	rs4641039	rs4736354	Overall	NA	NA	5.053	2	0.07992
WIN14	*Interg*	rs7004383	rs4641039	rs4736354	G/C/G	0.4225	0.451	5.048	1	0.02465
WIN14	*Interg*	rs7004383	rs4641039	rs4736354	C/T/C	0.4659	0.4424	3.415	1	0.06461
WIN14	*Interg*	rs7004383	rs4641039	rs4736354	C/C/C	0.1116	0.1066	0.3892	1	0.5327
WIN15	*Interg*	rs4641039	rs4736354	rs7819943	Overall	NA	NA	5.053	2	0.07992
WIN15	*Interg*	rs4641039	rs4736354	rs7819943	C/G/A	0.4225	0.451	5.048	1	0.02465
WIN15	*Interg*	rs4641039	rs4736354	rs7819943	T/C/G	0.4659	0.4424	3.415	1	0.06461
WIN15	*Interg*	rs4641039	rs4736354	rs7819943	C/C/G	0.1116	0.1066	0.3892	1	0.5327
WIN16	*Interg*	rs4736354	rs7819943	rs7011830	Overall	NA	NA	5.053	2	0.07992
WIN16	*Interg*	rs4736354	rs7819943	rs7011830	C/G/A	0.4659	0.4424	3.415	1	0.06461
WIN16	*Interg*	rs4736354	rs7819943	rs7011830	G/A/C	0.4225	0.451	5.048	1	0.02465
WIN16	*Interg*	rs4736354	rs7819943	rs7011830	C/G/C	0.1116	0.1066	0.3892	1	0.5327
WIN17	*Interg*	rs7819943	rs7011830	rs7824229	Overall	NA	NA	5.053	2	0.07992
WIN17	*Interg*	rs7819943	rs7011830	rs7824229	G/A/A	0.4659	0.4424	3.415	1	0.06461
WIN17	*Interg*	rs7819943	rs7011830	rs7824229	A/C/C	0.4225	0.451	5.048	1	0.02465
WIN17	*Interg*	rs7819943	rs7011830	rs7824229	G/C/C	0.1116	0.1066	0.3892	1	0.5327
WIN18	*Interg*	rs7011830	rs7824229	rs10096393	A/A/T	0.4659	0.4424	3.415	1	0.06461
WIN18	*Interg*	rs7011830	rs7824229	rs10096393	C/C/C	0.5341	0.5576	3.415	1	0.06461
WIN19	*Interg*	rs7824229	rs10096393	rs6651273	A/T/G	0.4659	0.4424	3.415	1	0.06461
WIN19	*Interg*	rs7824229	rs10096393	rs6651273	C/C/A	0.5341	0.5576	3.415	1	0.06461

Locus window	Gene	SNP1	SNP2	SNP3	Haplotype	Freq cases	Freq controls	CHISQ	DF	P
WIN20	*Interg*	rs10096393	rs6651273	rs6471580	Overall	NA	NA	7.51	2	0.0234
WIN20	*Interg*	rs10096393	rs6651273	rs6471580	C/A/G	0.4381	0.4724	7.2	1	0.007292
WIN20	*Interg*	rs10096393	rs6651273	rs6471580	T/G/A	0.4629	0.4385	3.649	1	0.05612
WIN20	*Interg*	rs10096393	rs6651273	rs6471580	C/A/A	0.09902	0.0891	1.751	1	0.1857
WIN21	*Interg*	rs6651273	rs6471580	rs6987382	Overall	NA	NA	7.51	2	0.0234
WIN21	*Interg*	rs6651273	rs6471580	rs6987382	A/G/A	0.4381	0.4724	7.2	1	0.007292
WIN21	*Interg*	rs6651273	rs6471580	rs6987382	G/A/G	0.4629	0.4385	3.649	1	0.05612
WIN21	*Interg*	rs6651273	rs6471580	rs6987382	A/A/G	0.09902	0.0891	1.751	1	0.1857
WIN22	*Interg*	rs6471580	rs6987382	rs4736357	Overall	NA	NA	7.51	2	0.0234
WIN22	*Interg*	rs6471580	rs6987382	rs4736357	A/G/T	0.4629	0.4385	3.649	1	0.05612
WIN22	*Interg*	rs6471580	rs6987382	rs4736357	G/A/A	0.4381	0.4724	7.2	1	0.007292
WIN22	*Interg*	rs6471580	rs6987382	rs4736357	A/G/A	0.09902	0.0891	1.751	1	0.1857
WIN23	*Interg*	rs6987382	rs4736357	rs4736359	Overall	NA	NA	7.51	2	0.0234
WIN23	*Interg*	rs6987382	rs4736357	rs4736359	G/T/T	0.4629	0.4385	3.649	1	0.05612
WIN23	*Interg*	rs6987382	rs4736357	rs4736359	A/A/G	0.4381	0.4724	7.2	1	0.007292
WIN23	*Interg*	rs6987382	rs4736357	rs4736359	G/A/G	0.09902	0.0891	1.751	1	0.1857
WIN24	*Interg*	rs4736357	rs4736359	rs4379428	T/T/T	0.4659	0.4424	3.415	1	0.06461
WIN24	*Interg*	rs4736357	rs4736359	rs4379428	A/G/A	0.5341	0.5576	3.415	1	0.06461
WIN25	*Interg*	rs4736359	rs4379428	rs6471583	T/T/G	0.4659	0.4424	3.415	1	0.06461
WIN25	*Interg*	rs4736359	rs4379428	rs6471583	G/A/A	0.5341	0.5576	3.415	1	0.06461
WIN26	*Interg/CYP11B2*	rs4379428	rs6471583	rs6433	T/G/C	0.4659	0.4424	3.415	1	0.06461
WIN26	*Interg/CYP11B2*	rs4379428	rs6471583	rs6433	A/A/T	0.5341	0.5576	3.415	1	0.06461

Locus window	Gene	SNP1	SNP2	SNP3	Haplotype	Freq cases	Freq controls	CHISQ	DF	P
WIN27	*Interg/CYP11B2*	rs6471583	rs6433	rs6414	Overall	NA	NA	4.055	2	0.1317
WIN27	*Interg/CYP11B2*	rs6471583	rs6433	rs6414	G/C/G	0.4659	0.4424	3.415	1	0.06461
WIN27	*Interg/CYP11B2*	rs6471583	rs6433	rs6414	A/T/G	0.0324	0.03024	0.2366	1	0.6267
WIN27	*Interg/CYP11B2*	rs6471583	rs6433	rs6414	A/T/A	0.5016	0.5274	4.041	1	0.0444
WIN28	*CYP11B2*	rs6433	rs6414	rs4546	Overall	NA	NA	6.349	4	0.1746
WIN28	*CYP11B2*	rs6433	rs6414	rs4546	T/G/T	0.01764	0.01966	0.3378	1	0.5611
WIN28	*CYP11B2*	rs6433	rs6414	rs4546	T/A/T	0.4084	0.4329	3.773	1	0.05207
WIN28	*CYP11B2*	rs6433	rs6414	rs4546	C/G/C	0.4643	0.4422	3.004	1	0.08307
WIN28	*CYP11B2*	rs6433	rs6414	rs4546	T/G/C	0.01485	0.01059	2.217	1	0.1365
WIN28	*CYP11B2*	rs6433	rs6414	rs4546	T/A/C	0.09481	0.09459	0.0008653	1	0.9765
WIN29	*CYP11B2*	rs6414	rs4546	IC	Overall	NA	NA	46.82	5	6.19E-09
WIN29	*CYP11B2*	rs6414	rs4546	IC	A/T/Conv	0.02487	0.005958	36.32	1	2.96E-08
WIN29	*CYP11B2*	rs6414	rs4546	IC	G/C/Conv	0.4643	0.4396	3.73	1	0.02
WIN29	*CYP11B2*	rs6414	rs4546	IC	G/T/Wt	0.02078	0.02165	0.05453	1	0.8154
WIN29	*CYP11B2*	rs6414	rs4546	IC	A/T/Wt	0.3862	0.4272	10.52	1	3.06E-04
WIN29	*CYP11B2*	rs6414	rs4546	IC	G/C/Wt	0.01861	0.01397	2.043	1	0.1529
WIN29	*CYP11B2*	rs6414	rs4546	IC	A/C/Wt	0.08527	0.09168	0.7688	1	0.46
WIN30	*CYP11B2*	rs4546	IC	rs1799998	Overall	NA	NA	35.86	4	3.10E-07
WIN30	*CYP11B2*	rs4546	IC	rs1799998	T/Conv/C	0.01762	0.004878	22.27	1	2.37E-06
WIN30	*CYP11B2*	rs4546	IC	rs1799998	C/Conv/C	0.01488	0.01125	1.527	1	0.2166
WIN30	*CYP11B2*	rs4546	IC	rs1799998	T/Wt/C	0.403	0.4456	11.08	1	1.49E-05
WIN30	*CYP11B2*	rs4546	IC	rs1799998	C/Conv/T	0.4727	0.4363	7.99	1	0.15
WIN30	*CYP11B2*	rs4546	IC	rs1799998	C/Wt/T	0.09176	0.102	1.77	1	0.34

Locus window	Gene	SNP1	SNP2	SNP3	Haplotype	Freq cases	Freq controls	CHISQ	DF	P
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	Overall	NA	NA	25.09	3	1.48E-05
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	Conv/C/A	0.0324	0.01786	13.29	1	0.000267
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	Wt/C/A	0.4005	0.4436	11.63	1	0.0006485
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	Conv/T/G	0.4659	0.4295	8.186	1	0.004221
WIN31	*CYP11B2*	IC	rs1799998	rs10087214	Wt/T/G	0.1012	0.109	0.9952	1	0.3185
WIN32	*CYP11B2/Interg*	rs1799998	rs10087214	rs7831617	C/A/T	0.4329	0.4615	5.041	1	0.02475
WIN32	*CYP11B2/Interg*	rs1799998	rs10087214	rs7831617	T/G/G	0.5671	0.5385	5.041	1	0.02475
WIN33	*CYP11B2/Interg*	rs10087214	rs7831617	rs9643358	A/T/C	0.4329	0.4615	5.041	1	0.02475
WIN33	*CYP11B2/Interg*	rs10087214	rs7831617	rs9643358	G/G/G	0.5671	0.5385	5.041	1	0.02475
WIN34	*Interg*	rs7831617	rs9643358	rs7011889	T/C/C	0.4329	0.4615	5.041	1	0.02475
WIN34	*Interg*	rs7831617	rs9643358	rs7011889	G/G/A	0.5671	0.5385	5.041	1	0.02475
WIN35	*Interg*	rs9643358	rs7011889	rs7016924	Overall	NA	NA	18.39	3	0.0003648
WIN35	*Interg*	rs9643358	rs7011889	rs7016924	C/C/A	0.4049	0.4453	10.15	1	0.00144
WIN35	*Interg*	rs9643358	rs7011889	rs7016924	G/A/A	0.03641	0.03087	1.444	1	0.2295
WIN35	*Interg*	rs9643358	rs7011889	rs7016924	C/C/G	0.02798	0.01623	9.821	1	0.001726
WIN35	*Interg*	rs9643358	rs7011889	rs7016924	G/A/G	0.5307	0.5076	3.245	1	0.07164

10 References

1988 Intersalt: an international study of electrolyte excretion and blood pressure. Results for 24 hour urinary sodium and potassium excretion. Intersalt Cooperative Research Group. *BMJ* **297** 319-328.

1993 *The Renin-Angiotensin System*. London: Mosby.

2002 Multi-center genetic study of hypertension: The Family Blood Pressure Program (FBPP). *Hypertension* **39** 3-9.

2003 The International HapMap Project. *Nature* **426** 789-796.

2005 A haplotype map of the human genome. *Nature* **437** 1299-1320.

2007 Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls. *Nature* **447** 661-678.

Abecasis GR, Cherny SS, Cookson WO & Cardon LR 2002 Merlin--rapid analysis of dense genetic maps using sparse gene flow trees. *Nat.Genet.* **30** 97-101.

Achilli A, Olivieri A, Pala M, Metspalu E, Fornarino S, Battaglia V, Accetturo M, Kutuev I, Khusnutdinova E, Pennarun E, Cerutti N, Di Gaetano C, Crobu F, Palli D, Matullo G, Santachiara-Benerecetti AS, Cavalli-Sforza LL, Semino O, Villems R, Bandelt HJ, Piazza A & Torroni A 2007 Mitochondrial DNA variation of modern Tuscans supports the near eastern origin of Etruscans. *Am.J.Hum.Genet.* **80** 759-768.

Aguilera G 1993 Factors controlling steroid biosynthesis in the zona glomerulosa of the adrenal. *J.Steroid Biochem.Mol.Biol.* **45** 147-151.

Ahmad N, Romero DG, Gomez-Sanchez EP & Gomez-Sanchez CE 2004 Do human vascular endothelial cells produce aldosterone? *Endocrinology* **145** 3626-3629.

Akey J, Jin L & Xiong M 2001 Haplotypes vs single marker linkage disequilibrium tests: what do we gain? *Eur.J.Hum.Genet.* **9** 291-300.

Akey JM, Zhang G, Zhang K, Jin L & Shriver MD 2002 Interrogating a high-density SNP map for signatures of natural selection. *Genome Res.* **12** 1805-1814.

Al Dujaili EA & Edwards CR 1978 The development and application of a direct radioimmunoassay for plasma aldosterone using 125I-labeled ligand--comparison of three methods. *J.Clin.Endocrinol.Metab* **46** 105-113.

Alderman MH, Madhavan S, Ooi WL, Cohen H, Sealey JE & Laragh JH 1991 Association of the renin-sodium profile with the risk of myocardial infarction in patients with hypertension. *N.Engl.J.Med.* **324** 1098-1104.

Alderman MH, Ooi WL, Cohen H, Madhavan S, Sealey JE & Laragh JH 1997 Plasma renin activity: a risk factor for myocardial infarction in hypertensive patients. *Am.J.Hypertens.* **10** 1-8.

Altshuler D, Daly M & Kruglyak L 2000 Guilt by association. *Nat.Genet.* **26** 135-137.

Alzamora R, Michea L & Marusic ET 2000 Role of 11beta-hydroxysteroid dehydrogenase in nongenomic aldosterone effects in human arteries. *Hypertension* **35** 1099-1104.

Ames RP, Borkowski AJ, Sicinski AM & Laragh JH 1965 Prolonged infusions of angiotensin II and norepinephrine and blood pressure, electrolyte balance, and aldosterone and cortisol secretion in normal man and in cirrhosis with ascites. *J.Clin.Invest* **44** 1171-1186.

Andersen GS, Toftdahl DB, Lund JO, Strandgaard S & Nielsen PE 1988 The incidence rate of phaeochromocytoma and Conn's syndrome in Denmark, 1977-1981. *J.Hum.Hypertens.* **2** 187-189.

Aoyagi T, Izumi Y, Hiroyama M, Matsuzaki T, Yasuoka Y, Sanbe A, Miyazaki H, Fujiwara Y, Nakayama Y, Kohda Y, Yamauchi J, Inoue T, Kawahara K, Saito H, Tomita K, Nonoguchi H & Tanoue A 2008 Vasopressin regulates the renin-angiotensin-aldosterone system via V1a receptors in macula densa cells. *Am.J.Physiol Renal Physiol* **295** F100-F107.

Ardlie KG, Lunetta KL & Seielstad M 2002 Testing for population subdivision and association in four case-control studies. *Am.J.Hum.Genet.* **71** 304-311.

Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE & Evans RM 1987 Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. *Science* **237** 268-275.

Ashraf SS, Guenther R & Agris PF 1999 Orientation of the tRNA anticodon in the ribosomal P-site: quantitative footprinting with U33-modified, anticodon stem and loop domains. *RNA.* **5** 1191-1199.

Astolfi P, Kidd KK & Cavalli-Sforza LL 1981 A comparison of methods for reconstructing evolutionary trees. *Syst.Zool.* **30** 391-400.

Atlas SA, Christofalo P, Hesson T, Sealey JE & Fritz LC 1985 Immunological evidence that inactive renin is prorenin. *Biochem.Biophys.Res.Commun.* **132** 1038-1045.

Bader M & Ganten D 2000 Regulation of renin: new evidence from cultured cells and genetically modified mice. *J.Mol.Med.* **78** 130-139.

Baer L, Sommers SC, Krakoff LR, Newton MA & Laragh JH 1970 Pseudo-primary aldosteronism. An entity distinct from true primary aldosteronism. *Circ.Res.* **27** 203-220.

Baeyens WR, Schulman SG, Calokerinos AC, Zhao Y, Garcia Campana AM, Nakashima K & De Keukeleire D 1998 Chemiluminescence-based detection: principles and analytical applications in flowing streams and in immunoassays. *J.Pharm.Biomed.Anal.* **17** 941-953.

Baker M, Gaukrodger N, Mayosi BM, Imrie H, Farrall M, Watkins H, Connell JMC, Avery PJ & Keavney B 2005 Association between common polymorphisms of the proopiomelanocortin gene and body fat distribution - A family study. *Diabetes* **54** 2492-2496.

Bamshad M & Wooding SP 2003 Signatures of natural selection in the human genome. *Nat.Rev.Genet.* **4** 99-111.

Bangham DR, Robertson I, Robertson JI, Robinson CJ & Tree M 1975 An international collaborative study of renin assay: establishment of the international reference preparation of human renin. *Clin.Sci.Mol.Med.Suppl* **2** 135a-159s.

Barbato A, Russo P, Siani A, Folkerd EJ, Miller MA, Venezia A, Grimaldi C, Strazzullo P & Cappuccio FP 2004 Aldosterone synthase gene (*CYP11B2*) C-344T polymorphism, plasma aldosterone, renin activity and blood pressure in a multi-ethnic population. *J.Hypertens.* **22** 1895-1901.

Barlassina C, Dal Fiume C, Lanzani C, Manunta P, Guffanti G, Ruello A, Bianchi G, Del Vecchio L, Macciardi F & Cusi D 2007 Common genetic variants and haplotypes in renal CLCNKA gene are associated to salt-sensitive hypertension. *Hum.Mol.Genet.* **16** 1630-1638.

Barnett DV 1969 Simultaneous pairwise linear structural relationships. *Biometrics* **28** 129-142.

Barr M, MacKenzie SM, Friel EC, Holloway CD, Wilkinson DM, Brain NJ, Ingram MC, Fraser R, Brown M, Samani NJ, Caulfield M, Munroe PB, Farrall M, Webster J, Clayton D, Dominiczak AF, Connell JM & Davies E 2007 Polymorphic variation in the 11beta-hydroxylase gene associates with reduced 11-hydroxylase efficiency. *Hypertension* **49** 113-119.

Barr M, MacKenzie SM, Wilkinson DM, Holloway CD, Friel EC, Miller S, MacDonald T, Fraser R, Connell JM & Davies E 2006 Functional effects of genetic variants in the 11beta-hydroxylase (*CYP11B1*) gene. *Clin.Endocrinol.(Oxf)* **65** 816-825.

Barrett JC, Fry B, Maller J & Daly MJ 2005 Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* **21** 263-265.

Bassett MH, White PC & Rainey WE 2004 The regulation of aldosterone synthase expression. *Mol.Cell Endocrinol.* **217** 67-74.

Bassett MH, Zhang Y, Clyne C, White PC & Rainey WE 2002 Differential regulation of aldosterone synthase and 11beta-hydroxylase transcription by steroidogenic factor-1. *J.Mol.Endocrinol.* **28** 125-135.

Batenburg WW, Krop M, Garrelds IM, de Vries R, de Bruin RJ, Burckle CA, Muller DN, Bader M, Nguyen G & Danser AH 2007 Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. *J.Hypertens.* **25** 2441-2453.

Bayliss DA & Barrett PQ 2008 Emerging roles for two-pore-domain potassium channels and their potential therapeutic impact. *Trends Pharmacol.Sci.* **29** 566-575.

Beevers DG, Brown JJ, Cuesta V, Davies DL, Fraser R, Lebel M, Lever AF, Morton JJ, Oelkers W, Robertson JI, Schalekamp MA & Tree M 1975 Inter-relationships between plasma angiotensin II, arterial pressure, aldosterone and exchangeable sodium in normotensive and hypertensive man. *J.Steroid Biochem.* **6** 779-784.

Beguin P, Crambert G, Guennoun S, Garty H, Horisberger JD & Geering K 2001 CHIF, a member of the FXYD protein family, is a regulator of Na,K-ATPase distinct from the gamma-subunit. *EMBO J.* **20** 3993-4002.

Belloni AS, Andreis PG, Macchi V, Gottardo G, Malendowicz LK & Nussdorfer GG 1998 Distribution and functional significance of angiotensin-II AT1- and AT2-receptor subtypes in the rat adrenal gland. *Endocr.Res.* **24** 1-15.

Benn-Torres J, Bonilla C, Robbins CM, Waterman L, Moses TY, Hernandez W, Santos ER, Bennett F, Aiken W, Tullock T, Coard K, Hennis A, Wu S, Nemesure B, Leske MC, Freeman V, Carpten J & Kittles RA 2008 Admixture and population stratification in African Caribbean populations. *Ann.Hum.Genet.* **72** 90-98.

Bentley-Lewis R, Adler GK, Perlstein T, Seely EW, Hopkins PN, Williams GH & Garg R 2007 Body mass index predicts aldosterone production in normotensive adults on a high-salt diet. *J.Clin.Endocrinol.Metab* **92** 4472-4475.

Berglund G, Elmstahl S, Janzon L & Larsson SA 1993 The Malmo Diet and Cancer Study. Design and feasibility. *J.Intern.Med.* **233** 45-51.

Bernhardt R, Muller A, Uhlmann H, Grinberg A, Muller JJ & Heinemann U 1998 Structure of adrenodoxin and function in mitochondrial steroid hydroxylation. *Endocr.Res.* **24** 531-539.

Berube D, Luu T, V, Lachance Y, Gagne R & Labrie F 1989 Assignment of the human 3 beta-hydroxysteroid dehydrogenase gene (HSDB3) to the p13 band of chromosome 1. *Cytogenet.Cell Genet.* **52** 199-200.

Bhalla V, Soundararajan R, Pao AC, Li H & Pearce D 2006 Disinhibitory pathways for control of sodium transport: regulation of ENaC by SGK1 and GILZ. *Am.J.Physiol Renal Physiol* **291** F714-F721.

Bianchi G, Ferrari P & Staessen JA 2005 Adducin polymorphism: detection and impact on hypertension and related disorders. *Hypertension* **45** 331-340.

Biglieri EG, Hane S, Slaton PE, Jr. & Forsham PH 1963 *In vivo* and *in vitro* studies of adrenal secretions in Cushing's syndrome and primary aldosteronism. *J.Clin.Invest* **42** 516-524.

Biglieri EG, Schambelan M, Slaton PE & Stockigt JR 1970 The intercurrent hypertension of primary aldosteronism. *Circ.Res.* **27** 195-202.

Bird IM, Pasquarette MM, Rainey WE & Mason JI 1996 Differential control of 17 alpha-hydroxylase and 3 beta-hydroxysteroid dehydrogenase expression in human adrenocortical H295R cells. *J.Clin.Endocrinol.Metab* **81** 2171-2178.

Biron P, Mongeau JG & Bertrand D 1976 Familial aggregation of blood pressure in 558 adopted children. *Can.Med.Assoc.J.* **115** 773-774.

Bochud M, Bovet P, Elston RC, Paccaud F, Falconnet C, Maillard M, Shamlaye C & Burnier M 2005 High heritability of ambulatory blood pressure in families of East African descent. *Hypertension* **45** 445-450.

Boldyreff B & Wehling M 2003 Rapid aldosterone actions: from the membrane to signaling cascades to gene transcription and physiological effects. *J.Steroid Biochem.Mol.Biol.* **85** 375-381.

Bolte E, Coudert S & Lefebvre Y 1974 Steroid production from plasma cholesterol. II. *In vivo* conversion of plasma cholesterol to ovarian progesterone and adrenal C19 and C21 steroids in humans. *J.Clin.Endocrinol.Metab* **38** 394-400.

Borkowski AJ, Levin S, Delcroix C, Mahler A & Verhas V 1967 Blood cholesterol and hydrocortisone production in man: quantitative aspects of the utilization of circulating cholesterol by the adrenals at rest and under adrenocorticotropin stimulation. *J.Clin.Invest* **46** 797-811.

Bouhnik J, Fehrentz JA, Galen FX, Seyer R, Evin G, Castro B, Menard J & Corvol P 1985 Immunologic identification of both plasma and human renal inactive renin as prorenin. *J.Clin.Endocrinol.Metab* **60** 399-401.

Boulkroun S, Fay M, Zennaro MC, Escoubet B, Jaisser F, Blot-Chabaud M, Farman N & Courtois-Coutry N 2002 Characterization of rat NDRG2 (N-Myc downstream regulated gene 2), a novel early mineralocorticoid-specific induced gene. *J.Biol.Chem.* **277** 31506-31515.

Bouzekri N, Zhu X, Jiang Y, McKenzie CA, Luke A, Forrester T, Adeyemo A, Kan D, Farrall M, Anderson S, Cooper RS & Ward R 2004 Angiotensin I-converting enzyme polymorphisms, ACE level and blood pressure among Nigerians, Jamaicans and African-Americans. *Eur.J.Hum.Genet.* **12** 460-468.

Bowcock AM, Kidd JR, Mountain JL, Hebert JM, Carotenuto L, Kidd KK & Cavalli-Sforza LL 1991 Drift, admixture, and selection in human evolution: a study with DNA polymorphisms. *Proc.Natl.Acad.Sci.U.S.A* **88** 839-843.

Boyd C & Naray-Fejes-Toth A 2005 Gene regulation of ENaC subunits by serum- and glucocorticoid-inducible kinase-1. *Am.J.Physiol Renal Physiol* **288** F505-F512.

Braley LM, Menachery AI, Underwood RH & Williams GH 1983 Is the adrenal angiotensin receptor angiotensin II--or angiotensin III like? *Acta Endocrinol.(Copenh)* **102** 116-121.

Brand E, Chatelain N, Mulatero P, Fery I, Curnow K, Jeunemaitre X, Corvol P, Pascoe L & Soubrier F 1998 Structural analysis and evaluation of the aldosterone synthase gene in hypertension. *Hypertension* **32** 198-204.

Brand E, Schorr U, Ringel J, Beige J, Distler A & Sharma AM 1999 Aldosterone synthase gene (*CYP11B2*) C-344T polymorphism in Caucasians from the Berlin Salt-Sensitivity Trial (BeSST). *J.Hypertens.* **17** 1563-1567.

Brennan FE & Fuller PJ 1999 Transcriptional control by corticosteroids of CHIF gene expression in the rat distal colon. *Clin.Exp.Pharmacol.Physiol* **26** 489-491.

Brennan FE & Fuller PJ 2006 Mammalian K-ras2 is a corticosteroid-induced gene in vivo. *Endocrinology* **147** 2809-2816.

Brenner T & O'Shaughnessy KM 2008 Both TASK-3 and TREK-1 two-pore loop K channels are expressed in H295R cells and modulate their membrane potential and aldosterone secretion. *Am.J.Physiol Endocrinol.Metab* **295** E1480-E1486.

Brilla CG & Weber KT 1992 Mineralocorticoid excess, dietary sodium, and myocardial fibrosis. *J.Lab Clin.Med.* **120** 893-901.

Brilla CG, Zhou G, Matsubara L & Weber KT 1994 Collagen metabolism in cultured adult rat cardiac fibroblasts: response to angiotensin II and aldosterone. *J.Mol.Cell Cardiol.* **26** 809-820.

Bromley C, Chaudhury M, Craig R, Deverill C, Erens B, Fuller E, Gray L, Herrick K, Hirani V, Kelly Y, Leyland A, MacGregor A, Moody A, Prescott A, Pickering K, Primatesta P, Scholes S, Shelton N, Speight S, Stamatakis E, Wardle H & Zaninotto P 2005 Blood pressure. In *Scottish Health Survey*, pp 321-351. Eds C Bromley, K Sproston & N Shelton. Edinburgh: Blackwell.

Brookes AJ 1999 The essence of SNPs. *Gene* **234** 177-186.

Brown JJ, Chinn RH, Fraser R, Lever AF, Morton JJ, Robertson JI, Tree M, Waite MA & Park DM 1973 Recurrent hyperkalaemia due to selective aldosterone deficiency: correction by angiotensin infusion. *Br.Med.J.* **1** 650-654.

Brown JJ, Fraser R, Love DR, Ferriss JB, Lever AF, Robertson JI & Wilson A 1972 Apparently isolated excess deoxycorticosterone in hypertension. A variant of the mineralocorticoid-excess syndrome. *Lancet* **2** 243-247.

Brown MA, Thou ST & Whitworth JA 1995 Stimulation of aldosterone by ACTH in normal and hypertensive pregnancy. *Am.J.Hypertens.* **8** 260-267.

Brown MJ & Hopper RV 1999 Calcium-channel blockade can mask the diagnosis of Conn's syndrome. *Postgrad.Med.J.* **75** 235-236.

Brunner HR, Chang P, Wallach R, Sealey JE & Laragh JH 1972a Angiotensin II vascular receptors: their avidity in relationship to sodium balance, the autonomic nervous system, and hypertension. *J.Clin.Invest* **51** 58-67.

Brunner HR, Laragh JH, Baer L, Newton MA, Goodwin FT, Krakoff LR, Bard RH & Buhler FR 1972b Essential hypertension: renin and aldosterone, heart attack and stroke. *N.Engl.J.Med.* **286** 441-449.

Buhler FR, Bolli P, Kiowski W, Erne P, Hulthen UL & Block LH 1984 Renin profiling to select antihypertensive baseline drugs. Renin inhibitors for high-renin and calcium entry blockers for low-renin patients. *Am.J.Med.* **77** 36-42.

Buhler FR, Laragh JH, Baer L, Vaughan ED, Jr. & Brunner HR 1972 Propranolol inhibition of renin secretion. A specific approach to diagnosis and treatment of renin-dependent hypertensive diseases. *N.Engl.J.Med.* **287** 1209-1214.

Bulow HE & Bernhardt R 2002 Analyses of the CYP11B gene family in the guinea pig suggest the existence of a primordial CYP11B gene with aldosterone synthase activity. *Eur.J.Biochem.* **269** 3838-3846.

Burckle CA, Jan Danser AH, Muller DN, Garrelds IM, Gasc JM, Popova E, Plehm R, Peters J, Bader M & Nguyen G 2006 Elevated blood pressure and heart rate in human renin receptor transgenic rats. *Hypertension* **47** 552-556.

Burrell LM, Johnston CI, Tikellis C & Cooper ME 2004 ACE2, a new regulator of the renin-angiotensin system. *Trends Endocrinol.Metab* **15** 166-169.

Burton J & Quinn T 1988 The amino-acid residues on the C-terminal side of the cleavage site of angiotensinogen influence the species specificity of reaction with renin. *Biochim.Biophys.Acta* **952** 8-12.

Butt WR 1984 *Practical Immunoassay*. New York: CRC Press.

Butterworth MB, Edinger RS, Frizzell RA & Johnson JP 2009 Regulation of the epithelial sodium channel by membrane trafficking. *Am.J.Physiol Renal Physiol* **296** F10-F24.

Calhoun DA 2007 Is there an unrecognized epidemic of primary aldosteronism? Pro. *Hypertension* **50** 447-453.

Calhoun DA, Nishizaka MK, Zaman MA, Thakkar RB & Weissmann P 2002 Hyperaldosteronism among black and white subjects with resistant hypertension. *Hypertension* **40** 892-896.

Campbell DJ 1982 Effect of composition of incubation medium on aldosterone and corticosterone production by isolated rat zona glomerulosa cells. *J.Endocrinol.* **94** 211-224.

Campese VM 1994 Salt sensitivity in hypertension. Renal and cardiovascular implications. *Hypertension* **23** 531-550.

Capurro C, Coutry N, Bonvalet JP, Escoubet B, Garty H & Farman N 1996 Cellular localization and regulation of CHIF in kidney and colon. *Am.J.Physiol* **271** C753-C762.

Cardon LR & Palmer LJ 2003 Population stratification and spurious allelic association. *Lancet* **361** 598-604.

Carey AH, Waterworth D, Patel K, White D, Little J, Novelli P, Franks S & Williamson R 1994 Polycystic ovaries and premature male pattern baldness are associated with one allele of the steroid metabolism gene CYP17. *Hum.Mol.Genet.* **3** 1873-1876.

Carlson CS, Eberle MA, Rieder MJ, Smith JD, Kruglyak L & Nickerson DA 2003 Additional SNPs and linkage-disequilibrium analyses are necessary for whole-genome association studies in humans. *Nat.Genet.* **33** 518-521.

Carpenter CC, Davis JO & Ayers CR 1961 Relation of renin, angiotensin II, and experimental renal hypertension to aldosterone secretion. *J.Clin.Invest* **40** 2026-2042.

Carretero OA & Oparil S 2000 Essential hypertension. Part I: definition and etiology. *Circulation* **101** 329-335.

Carter KW, McCaskie PA & Palmer LJ 2006 JLIN: a java based linkage disequilibrium plotter. *BMC.Bioinformatics.* **7** 60.

Casiglia E, Tikhonoff V, Mazza A, Rynkiewicz A, Limon J, Caffi S, Guglielmi F, Martini B, Basso G, Winnicki M, Pessina AC & Somers VK 2005 C-344T polymorphism of the aldosterone synthase gene and blood pressure in the elderly: a population-based study. *J.Hypertens.* **23** 1991-1996.

Castellano M, Glorioso N, Cusi D, Sarzani R, Fabris B, Opocher G, Zoccali C, Golin R, Veglio F, Volpe M, Mantero F, Fallo F, Rossi GP, Barlassina C, Tizzoni L, Filigheddu F, Giacche M & Rossi F 2003 Genetic polymorphism of the renin-angiotensin-aldosterone system and arterial hypertension in the Italian population: the GENIPER Project. *J.Hypertens.* **21** 1853-1860.

Catena C, Colussi G, Nadalini E, Chiuch A, Baroselli S, Lapenna R & Sechi LA 2007 Relationships of plasma renin levels with renal function in patients with primary aldosteronism. *Clin.J.Am.Soc.Nephrol.* **2** 722-731.

Caulfield M, Munroe P, Pembroke J, Samani N, Dominiczak A, Brown M, Benjamin N, Webster J, Ratcliffe P, O'Shea S, Papp J, Taylor E, Dobson R, Knight J, Newhouse S, Hooper J, Lee W, Brain N, Clayton D, Lathrop GM, Farrall M & Connell J 2003 Genome-wide mapping of human loci for essential hypertension. *Lancet* **361** 2118-2123.

Cavalli-Sforza LL, Menozzi P & Piazza A 1993 *The history and geography of human genes*. Princeton, N.J.: Princeton University Press.

Chang YP, Liu X, Kim JD, Ikeda MA, Layton MR, Weder AB, Cooper RS, Kardia SL, Rao DC, Hunt SC, Luke A, Boerwinkle E & Chakravarti A 2007 Multiple genes for essential-hypertension susceptibility on chromosome 1q. *Am.J.Hum.Genet.* **80** 253-264.

Chanock SJ, Manolio T, Boehnke M, Boerwinkle E, Hunter DJ, Thomas G, Hirschhorn JN, Abecasis G, Altshuler D, Bailey-Wilson JE, Brooks LD, Cardon LR, Daly M, Donnelly P, Fraumeni JF, Jr., Freimer NB, Gerhard DS, Gunter C, Guttmacher AE, Guyer MS, Harris EL, Hoh J, Hoover R, Kong CA, Merikangas KR, Morton CC, Palmer LJ, Phimister EG, Rice JP, Roberts J, Rotimi C, Tucker MA, Vogan KJ, Wacholder S, Wijsman EM, Winn DM & Collins FS 2007 Replicating genotype-phenotype associations. *Nature* **447** 655-660.

Chapman JM, Cooper JD, Todd JA & Clayton DG 2003 Detecting disease associations due to linkage disequilibrium using haplotype tags: a class of tests and the determinants of statistical power. *Hum.Hered.* **56** 18-31.

Charmandari E, Kino T, Ichijo T & Chrousos GP 2008 Generalized glucocorticoid resistance: clinical aspects, molecular mechanisms, and implications of a rare genetic disorder. *J.Clin.Endocrinol.Metab* **93** 1563-1572.

Chaudhury M 2008 Hypertension. In *Health Survey for England 2006*, pp 43-61. Eds R Craig & J Mindell. Leeds: The Information Centre.

Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr. & Roccella EJ 2003 Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension* **42** 1206-1252.

Chua SC, Szabo P, Vitek A, Grzeschik KH, John M & White PC 1987 Cloning of cDNA encoding steroid 11 beta-hydroxylase (P450c11). *Proc.Natl.Acad.Sci.U.S.A* **84** 7193-7197.

Cicila GT, Garrett MR, Lee SJ, Liu J, Dene H & Rapp JP 2001 High-resolution mapping of the blood pressure QTL on chromosome 7 using Dahl rat congenic strains. *Genomics* **72** 51-60.

Cicila GT, Rapp JP, Wang JM, St Lezin E, Ng SC & Kurtz TW 1993 Linkage of 11 beta-hydroxylase mutations with altered steroid biosynthesis and blood pressure in the Dahl rat. *Nat.Genet.* **3** 346-353.

Clark BJ & Combs R 1999 Angiotensin II and cyclic adenosine 3',5'-monophosphate induce human steroidogenic acute regulatory protein transcription through a common steroidogenic factor-1 element. *Endocrinology* **140** 4390-4398.

Clark BJ, Wells J, King SR & Stocco DM 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *J.Biol.Chem.* **269** 28314-28322.

Cleophas TJ, Zwinderman AH, Cleophas TF & Cleophas EP 2009 Validating Quantitative Diagnostic Tests. In *Statistics Applied to Clinical Trials*, edn 4th, pp 423-431. Netherlands: Springer.

Coll AP, Challis BG, Yeo GS, Snell K, Piper SJ, Halsall D, Thresher RR & O'Rahilly S 2004 The effects of proopiomelanocortin deficiency on murine adrenal development and responsiveness to adrenocorticotropin. *Endocrinology* **145** 4721-4727.

Colombo L, Dalla VL, Fiore C, Armanini D & Belvedere P 2006 Aldosterone and the conquest of land. *J.Endocrinol.Invest* **29** 373-379.

Conn JW 1955 Presidential address. I. Painting background. II. Primary aldosteronism, a new clinical syndrome. *J.Lab Clin.Med.* **45** 3-17.

Conn JW, Cohen EL & Rovner DR 1964 Suppression of plasma renin activity in primary aldosteronism. *JAMA* **190** 213-221.

Connell JM & Davies E 2005 The new biology of aldosterone. *J.Endocrinol.* **186** 1-20.

Connell JM, Fraser R, MacKenzie S & Davies E 2003 Is altered adrenal steroid biosynthesis a key intermediate phenotype in hypertension? *Hypertension* **41** 993-999.

Connell JM, Jamieson AJ, Davies E, Ingram M, Soro A & Fraser R 1996 11 beta-Hydroxylase activity in glucocorticoid suppressible hyperaldosteronism: lessons for essential hypertension? *Endocr.Res.* **22** 691-700.

Connell JM, MacKenzie SM, Freel EM, Fraser R & Davies E 2008 A lifetime of aldosterone excess: long-term consequences of altered regulation of aldosterone production for cardiovascular function. *Endocr.Rev.* **29** 133-154.

Connell JM, Whitworth JA, Davies DL, Lever AF, Richards AM & Fraser R 1987 Effects of ACTH and cortisol administration on blood pressure, electrolyte metabolism, atrial natriuretic peptide and renal function in normal man. *J.Hypertens.* **5** 425-433.

Conrad DF, Jakobsson M, Coop G, Wen X, Wall JD, Rosenberg NA & Pritchard JK 2006 A worldwide survey of haplotype variation and linkage disequilibrium in the human genome. *Nat.Genet.* **38** 1251-1260.

Cooper RS, Kaufman JS & Ward R 2003 Race and genomics. *N.Engl.J.Med.* **348** 1166-1170.

Cooper RS, Wolf-Maier K, Luke A, Adeyemo A, Banegas JR, Forrester T, Giampaoli S, Joffres M, Kastarinen M, Primatesta P, Stegmayr B & Thamm M 2005 An international comparative study of blood pressure in populations of European vs. African descent. *BMC.Med.* **3** 2.

Cover CM, Wang JM, St Lezin E, Kurtz TW & Mellon SH 1995 Molecular variants in the P450c11AS gene as determinants of aldosterone synthase activity in the Dahl rat model of hypertension. *J.Biol.Chem.* **270** 16555-16560.

Cowley AW, Jr. 1992 Long-term control of arterial blood pressure. *Physiol Rev.* **72** 231-300.

Cowley AW, Jr. 2006 The genetic dissection of essential hypertension. *Nat.Rev.Genet.* **7** 829-840.

Crawford DC, Carlson CS, Rieder MJ, Carrington DP, Yi Q, Smith JD, Eberle MA, Kruglyak L & Nickerson DA 2004 Haplotype diversity across 100 candidate genes for inflammation, lipid metabolism, and blood pressure regulation in two populations. *Am.J.Hum.Genet.* **74** 610-622.

Cugini P, Manconi R, Serdoz R, Mancini A, Meucci T & Scavo D 1980 Rhythm characteristics of plasma renin, aldosterone and cortisol in five subtypes of mesor-hypertension. *J.Endocrinol.Invest* **3** 143-147.

Cuneo RC, Espiner EA, Nicholls MG, Yandle TG & Livesey JH 1987 Effect of physiological levels of atrial natriuretic peptide on hormone secretion: inhibition of angiotensin-induced aldosterone secretion and renin release in normal man. *J.Clin.Endocrinol.Metab* **65** 765-772.

Curnow KM, Mulatero P, Emeric-Blanchouin N, Aupetit-Faisant B, Corvol P & Pascoe L 1997 The amino acid substitutions Ser288Gly and Val320Ala convert the cortisol producing enzyme, CYP11B1, into an aldosterone producing enzyme. *Nat.Struct.Biol.* **4** 32-35.

Dai JY, Ruczinski I, LeBlanc M & Kooperberg C 2006 Imputation methods to improve inference in SNP association studies. *Genet.Epidemiol.* **30** 690-702.

Daidoh H, Morita H, Mune T, Murayama M, Hanafusa J, Ni H, Shibata H & Yasuda K 1995 Responses of plasma adrenocortical steroids to low dose ACTH in normal subjects. *Clin.Endocrinol.(Oxf)* **43** 311-315.

Danser AH, Batenburg WW, van Esch JH & Krop M 2008 Prorenin anno 2008. *J.Mol.Med.* **86** 655-658.

Davies DL, Beevers DG, Brown JJ, Cumming AM, Fraser R, Lever AF, Mason PA, Morton JJ, Robertson JI, Titterington M & Tree M 1979 Aldosterone and its stimuli in normal and hypertensive man: are essential hypertension and primary hyperaldosteronism without tumour the same condition? *J.Endocrinol.* **81** 79P-91P.

Davies E, Holloway CD, Ingram MC, Friel EC, Inglis GC, Swan L, Hillis WS, Fraser R & Connell JM 2001 An influence of variation in the aldosterone synthase gene (*CYP11B2*) on corticosteroid responses to ACTH in normal human subjects. *Clin.Endocrinol.(Oxf)* **54** 813-817.

Davies E, Holloway CD, Ingram MC, Inglis GC, Friel EC, Morrison C, Anderson NH, Fraser R & Connell JM 1999 Aldosterone excretion rate and blood pressure in essential hypertension are related to polymorphic differences in the aldosterone synthase gene *CYP11B2*. *Hypertension* **33** 703-707.

Davies E & MacKenzie SM 2003 Extra-adrenal production of corticosteroids. *Clin.Exp.Pharmacol.Physiol* **30** 437-445.

Davies LA, Hu C, Guagliardo NA, Sen N, Chen X, Talley EM, Carey RM, Bayliss DA & Barrett PQ 2008 TASK channel deletion in mice causes primary hyperaldosteronism. *Proc.Natl.Acad.Sci.U.S.A* **105** 2203-2208.

Davis WW, Newsome HH, Jr., Wright LD, Jr., Hammond WG, Easton J & Bartter FC 1967 Bilateral adrenal hyperplasia as a cause of primary aldosteronism with hypertension, hypokalemia and suppressed renin activity. *Am.J.Med.* **42** 642-647.

de Bruin RA, Bouhuizen A, Diederich S, Perschel FH, Boomsma F & Deinum J 2004 Validation of a new automated renin assay. *Clin.Chem.* **50** 2111-2116.

de Simone G, Tommaselli AP, Rossi R, Valentino R, Lauria R, Scopacasa F & Lombardi G 1985 Partial deficiency of adrenal 11-hydroxylase. A possible cause of primary hypertension. *Hypertension* **7** 204-210.

de Wardener HE, He FJ & Macgregor GA 2004 Plasma sodium and hypertension. *Kidney Int.* **66** 2454-2466.

Deinum J, Derkx FH & Schalekamp MA 1999 Improved immunoradiometric assay for plasma renin. *Clin.Chem.* **45** 847-854.

Denner K, Doehmer J & Bernhardt R 1995 Cloning of *CYP11B1* and *CYP11B2* from normal human adrenal and their functional expression in COS-7 and V79 Chinese hamster cells. *Endocr.Res.* **21** 443-448.

Derkx FH, Alberda AT, de Jong FH, Zeilmaker FH, Makovitz JW & Schalekamp MA 1987a Source of plasma prorenin in early and late pregnancy: observations in a patient with primary ovarian failure. *J.Clin.Endocrinol.Metab* **65** 349-354.

Derkx FH, de Bruin RJ, van Gool JM, van den Hoek MJ, Beerendonk CC, Rosmalen F, Haima P & Schalekamp MA 1996 Clinical validation of renin monoclonal antibody-based sandwich assays of renin and prorenin, and use of renin inhibitor to enhance prorenin immunoreactivity. *Clin.Chem.* **42** 1051-1063.

Derkx FH & Schalekamp MA 1997 More on renin. *Clin.Chem.* **43** 694-697.

Derkx FH, Schalekamp MP & Schalekamp MA 1987b Two-step prorenin-renin conversion. Isolation of an intermediary form of activated prorenin. *J.Biol.Chem.* **262** 2472-2477.

Derkx FH, Steunkel C, Schalekamp MP, Visser W, Huisveld IH & Schalekamp MA 1986 Immunoreactive renin, prorenin, and enzymatically active renin in plasma during pregnancy and in women taking oral contraceptives. *J.Clin.Endocrinol.Metab* **63** 1008-1015.

Deschepper CF, Mellon SH, Cumin F, Baxter JD & Ganong WF 1986 Analysis by immunocytochemistry and in situ hybridization of renin and its mRNA in kidney, testis, adrenal, and pituitary of the rat. *Proc.Natl.Acad.Sci.U.S.A* **83** 7552-7556.

Diakov A & Korbmacher C 2004 A novel pathway of epithelial sodium channel activation involves a serum- and glucocorticoid-inducible kinase consensus motif in the C terminus of the channel's alpha-subunit. *J.Biol.Chem.* **279** 38134-38142.

Dickson ME & Sigmund CD 2006 Genetic basis of hypertension: revisiting angiotensinogen. *Hypertension* **48** 14-20.

Dluhy RG, Hopkins P, Hollenberg NK, Williams GH & Williams RR 1988 Heritable abnormalities of the renin-angiotensin-aldosterone system in essential hypertension. *J.Cardiovasc.Pharmacol.* **12 Suppl 3** S149-S154.

Dluhy RG, Underwood RH & Williams GH 1970 Influence of dietary potassium on plasma renin activity in normal man. *J.Appl.Physiol* **28** 299-302.

Donoghue M, Hsieh F, Baronas E, Godbout K, Gosselin M, Stagliano N, Donovan M, Woolf B, Robison K, Jeyaseelan R, Breitbart RE & Acton S 2000 A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ.Res.* **87** E1-E9.

Doris PA 2002 Hypertension genetics, single nucleotide polymorphisms, and the common disease:common variant hypothesis. *Hypertension* **39** 323-331.

Dorrian CA, Toole BJ, Alvarez-Madrazo S, Kelly A, Connell JMC & Wallace A.M. 2009 A screening procedure for primary aldosteronism based on the Diasorin Liaison automated chemiluminescent immunoassay for direct renin. *Ann.Clin.Biochem.*

Drumm K, Kress TR, Gassner B, Krug AW & Gekle M 2006 Aldosterone stimulates activity and surface expression of NHE3 in human primary proximal tubule epithelial cells (RPTEC). *Cell Physiol Biochem.* **17** 21-28.

Dunlop FM, Crock PA, Montalto J, Funder JW & Curnow KM 2003 A compound heterozygote case of type II aldosterone synthase deficiency. *J.Clin.Endocrinol.Metab* **88** 2518-2526.

Dunn PJ & Espiner EA 1976 Outpatient screening tests for primary aldosteronism. *Aust.N.Z.J.Med.* **6** 131-135.

Dzau VJ 1993 Tissue renin-angiotensin system in myocardial hypertrophy and failure. *Arch.Intern.Med.* **153** 937-942.

Dzau VJ, Ellison KE, Brody T, Ingelfinger J & Pratt RE 1987 A comparative study of the distributions of renin and angiotensinogen messenger ribonucleic acids in rat and mouse tissues. *Endocrinology* **120** 2334-2338.

Edwards CR, Stewart PM, Burt D, Brett L, McIntyre MA, Sutanto WS, de Kloet ER & Monder C 1988 Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. *Lancet* **2** 986-989.

Ehrhart-Bornstein M, Bornstein SR, Gonzalez-Hernandez J, Holst JJ, Waterman MR & Scherbaum WA 1995 Sympathoadrenal regulation of adrenocortical steroidogenesis. *Endocr.Res.* **21** 13-24.

Elliott P, Stamler J, Nichols R, Dyer AR, Stamler R, Kesteloot H & Marmot M 1996 Intersalt revisited: further analyses of 24 hour sodium excretion and blood pressure within and across populations. Intersalt Cooperative Research Group. *BMJ* **312** 1249-1253.

Elsheikh A, Creatsas G, Mastorakos G, Milingos S, Loutradis D & Michalas S 2001 The renin-aldosterone system during normal and hypertensive pregnancy. *Arch.Gynecol.Obstet.* **264** 182-185.

Enattah NS, Jensen TG, Nielsen M, Lewinski R, Kuokkanen M, Rasinpera H, El Shanti H, Seo JK, Alifrangis M, Khalil IF, Natah A, Ali A, Natah S, Comas D, Mehdi SQ, Groop L, Vestergaard EM, Imtiaz F, Rashed MS, Meyer B, Troelsen J & Peltonen L 2008 Independent introduction of two lactase-persistence alleles into human populations reflects different history of adaptation to milk culture. *Am.J.Hum.Genet.* **82** 57-72.

Epstein MP & Satten GA 2003 Inference on haplotype effects in case-control studies using unphased genotype data. *Am.J.Hum.Genet.* **73** 1316-1329.

Espiner EA & Donald RA 1980 Aldosterone regulation in primary aldosteronism: influence of salt balance, posture and ACTH. *Clin.Endocrinol.(Oxf)* **12** 277-286.

Fagard R, Brguljan J, Staessen J, Thijs L, Derom C, Thomis M & Vlietinck R 1995 Heritability of conventional and ambulatory blood pressures. A study in twins. *Hypertension* **26** 919-924.

Falkner B & Kushner H 1990 Effect of chronic sodium loading on cardiovascular response in young blacks and whites. *Hypertension* **15** 36-43.

Fan R & Knapp M 2003 Genome association studies of complex diseases by case-control designs. *Am.J.Hum.Genet.* **72** 850-868.

Fardella CE, Mosso L, Gomez-Sanchez C, Cortes P, Soto J, Gomez L, Pinto M, Huete A, Oestreicher E, Foradori A & Montero J 2000 Primary hyperaldosteronism in essential hypertensives: prevalence, biochemical profile, and molecular biology. *J.Clin.Endocrinol.Metab* **85** 1863-1867.

Fardella CE, Rodriguez H, Montero J, Zhang G, Vignolo P, Rojas A, Villarroel L & Miller WL 1996 Genetic variation in P450c11AS in Chilean patients with low renin hypertension. *J.Clin.Endocrinol.Metab* **81** 4347-4351.

Fava C, Burri P, Almgren P, Groop L, Hulthen UL & Melander O 2004 Heritability of ambulatory and office blood pressure phenotypes in Swedish families. *J.Hypertens.* **22** 1717-1721.

Fejes-Toth G & Naray-Fejes-Toth A 2007 Early aldosterone-regulated genes in cardiomyocytes: clues to cardiac remodeling? *Endocrinology* **148** 1502-1510.

Felder RA, Sanada H, Xu J, Yu PY, Wang Z, Watanabe H, Asico LD, Wang W, Zheng S, Yamaguchi I, Williams SM, Gainer J, Brown NJ, Hazen-Martin D, Wong LJ, Robillard JE, Carey RM, Eisner GM & Jose PA 2002 G protein-coupled receptor kinase 4 gene variants in human essential hypertension. *Proc.Natl.Acad.Sci.U.S.A* **99** 3872-3877.

Ferrari P, Shaw SG, Nicod J, Saner E & Nussberger J 2004 Active renin versus plasma renin activity to define aldosterone-to-renin ratio for primary aldosteronism. *J.Hypertens.* **22** 377-381.

Ferriss JB, Beevers DG, Brown JJ, Davies DL, Fraser R, Lever AF, Mason P, Neville AM & Robertson JI 1978 Clinical, biochemical and pathological features of low-renin ("primary") hyperaldosteronism. *Am.Heart J.* **95** 375-388.

Ferriss JB, Brown JJ, Fraser R, Haywood E, Davies DL, Kay AW, Lever AF, Robertson JI, Owen K & Peart WS 1975 Results of adrenal surgery in patients with hypertension, aldosterone excess, and low plasma renin concentration. *Br.Med.J.* **1** 135-138.

Ferriss JB, Brown JJ, Fraser R, Kay AW, Neville AM, O'Muircheartaigh IG, Robertson JI, Symington T & Lever AF 1970 Hypertension with aldosterone excess and low plasma-renin: preoperative distinction between patients with and without adrenocortical tumour. *Lancet* **2** 995-1000.

Feuk L, Carson AR & Scherer SW 2006 Structural variation in the human genome. *Nat.Rev.Genet.* **7** 85-97.

Fiad TM, Cunningham SK, Hayes FJ & McKenna TJ 1997 Effects of nifedipine treatment on the renin-angiotensin-aldosterone axis. *J.Clin.Endocrinol.Metab* **82** 457-460.

Fiebeler A, Schmidt F, Muller DN, Park JK, Dechend R, Bieringer M, Shagdarsuren E, Breu V, Haller H & Luft FC 2001 Mineralocorticoid receptor affects AP-1 and nuclear factor-kappab activation in angiotensin II-induced cardiac injury. *Hypertension* **37** 787-793.

Fisher ND, Gleason RE, Moore TJ, Williams GH & Hollenberg NK 1994 Regulation of aldosterone secretion in hypertensive blacks. *Hypertension* **23** 179-184.

Fisher ND, Hurwitz S, Jeunemaitre X, Hopkins PN, Hollenberg NK & Williams GH 2002 Familial aggregation of low-renin hypertension. *Hypertension* **39** 914-918.

Fishman LM, Kuchel O, Liddle GW, Michelakis AM, Gordon RD & Chick WT 1968 Incidence of primary aldosteronism uncomplicated "essential" hypertension. A prospective study with elevated aldosterone secretion and suppressed plasma renin activity used as diagnostic criteria. *JAMA* **205** 497-502.

Follenius M, Krauth MO, Saini J & Brandenberger G 1992 Effect of awakening on aldosterone. *J.Endocrinol.Invest* **15** 475-478.

Francalacci P, Bertranpetit J, Calafell F & Underhill PA 1996 Sequence diversity of the control region of mitochondrial DNA in Tuscany and its implications for the peopling of Europe. *Am.J.Phys.Anthropol.* **100** 443-460.

Fraser R, Beretta-Piccoli C, Brown JJ, Cumming AM, Lever AF, Mason PA, Morton JJ & Robertson JI 1981 Response of aldosterone and 18-hydroxycorticosterone to angiotensin II in normal subjects and patients with essential hypertension, Conn's syndrome, and nontumorous hyperaldosteronism. *Hypertension* **3** I87-I92.

Fredlund P, Saltman S & Catt KJ 1975 Aldosterone production by isolated adrenal glomerulosa cells: stimulation by physiological concentrations of angiotensin II. *Endocrinology* **97** 1577-1586.

Freel EM & Connell JM 2004 Mechanisms of hypertension: the expanding role of aldosterone. *J.Am.Soc.Nephrol.* **15** 1993-2001.

Freel EM, Ingram M, Friel EC, Fraser R, Brown M, Samani NJ, Caulfield M, Munroe P, Farrall M, Webster J, Clayton D, Dominiczak AF, Davies E & Connell JM 2007 Phenotypic consequences of variation across the aldosterone synthase and 11-beta hydroxylase locus in a hypertensive cohort: data from the MRC BRIGHT Study. *Clin.Endocrinol.(Oxf)* **67** 832-838.

Freel EM, Ingram M, Wallace AM, White A, Fraser R, Davies E & Connell JM 2008 Effect of variation in *CYP11B1* and *CYP11B2* on corticosteroid phenotype and hypothalamic-pituitary-adrenal axis activity in hypertensive and normotensive subjects. *Clin.Endocrinol.(Oxf)* **68** 700-706.

Funder J & Myles K 1996 Exclusion of corticosterone from epithelial mineralocorticoid receptors is insufficient for selectivity of aldosterone action: in vivo binding studies. *Endocrinology* **137** 5264-5268.

Funder JW 2009 Reconsidering the roles of the mineralocorticoid receptor. *Hypertension* **53** 286-290.

Funder JW, Carey RM, Fardella C, Gomez-Sanchez CE, Mantero F, Stowasser M, Young WF, Jr. & Montori VM 2008 Case detection, diagnosis, and treatment of patients with primary aldosteronism: an endocrine society clinical practice guideline. *J.Clin.Endocrinol.Metab* **93** 3266-3281.

Funder JW, Pearce PT, Smith R & Smith AI 1988 Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. *Science* **242** 583-585.

Fyhrquist F & Saijonmaa O 2008 Renin-angiotensin system revisited. *J.Intern.Med.* **264** 224-236.

Galen FX, Guyenne TT, Devaux C, Auzan C, Corvol P & Menard J 1979 Direct radioimmunoassay of human renin. *J.Clin.Endocrinol.Metab* **48** 1041-1043.

Ganapathipillai S, Laval G, Hoffmann IS, Castejon AM, Nicod J, Dick B, Frey FJ, Frey BM, Cubeddu LX & Ferrari P 2005 *CYP11B2-CYP11B1* haplotypes associated with decreased 11 beta-hydroxylase activity. *J.Clin.Endocrinol.Metab* **90** 1220-1225.

Ganguly A & Hampton T 1985 Calcium-dependence of serotonin-mediated aldosterone secretion and differential effects of calcium antagonists. *Life Sci.* **36** 1459-1464.

Ganguly A, Melada GA, Luetscher JA & Dowdy AJ 1973 Control of plasma aldosterone in primary aldosteronism: distinction between adenoma and hyperplasia. *J.Clin.Endocrinol.Metab* **37** 765-775.

Ganguly A & Weinberger MH 1979 Low renin hypertension: a current review of definitions and controversies. *Am.Heart J.* **98** 642-652.

Garovic VD, Hilliard AA & Turner ST 2006 Monogenic forms of low-renin hypertension. *Nat.Clin.Pract.Nephrol.* **2** 624-630.

Garty H & Palmer LG 1997 Epithelial sodium channels: function, structure, and regulation. *Physiol Rev.* **77** 359-396.

Gaukrodger N, Mayosi BM, Imrie H, Avery P, Baker M, Connell JMC, Watkins H, Farrall M & Keavney B 2005 A rare variant of the leptin gene has large effects on blood pressure and carotid intima-medial thickness: a study of 1428 individuals in 248 families. *Journal of Medical Genetics* **42** 474-478.

Gavras H, Oliver JA & Cannon PJ 1976 Interrelations of renin, angiotensin II, and sodium in hypertension and renal failure. *Annu.Rev.Med.* **27** 485-521.

Ge D, Zhu H, Huang Y, Treiber FA, Harshfield GA, Snieder H & Dong Y 2007 Multilocus analyses of Renin-Angiotensin-aldosterone system gene variants on blood pressure at rest and during behavioral stress in young normotensive subjects. *Hypertension* **49** 107-112.

Geerling JC, Engeland WC, Kawata M & Loewy AD 2006 Aldosterone target neurons in the nucleus tractus solitarius drive sodium appetite. *J.Neurosci.* **26** 411-417.

Geley S, Johrer K, Peter M, Denner K, Bernhardt R, Sippell WG & Kofler R 1995 Amino acid substitution R384P in aldosterone synthase causes corticosterone methyloxidase type I deficiency. *J.Clin.Endocrinol.Metab* **80** 424-429.

Geley S, Kapelari K, Johrer K, Peter M, Glatzl J, Vierhapper H, Schwarz S, Helmberg A, Sippell WG, White PC & Kofler R 1996 *CYP11B1* mutations causing congenital adrenal hyperplasia due to 11 beta-hydroxylase deficiency. *J.Clin.Endocrinol.Metab* **81** 2896-2901.

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

Genest J, Lemieux G, Davignon A, Koiw E, Nowaczynski W & Steyermark P 1956 Human arterial hypertension: a state of mild chronic hyperaldosteronism? *Science* **123** 503-505.

George JM, Wright L, Bell NH & Bartter FC 1970 The syndrome of primary aldosteronism. *Am.J.Med.* **48** 343-356.

Giacche M, Vuagnat A, Hunt SC, Hopkins PN, Fisher ND, Azizi M, Corvol P, Williams GH & Jeunemaitre X 2000 Aldosterone stimulation by angiotensin II : influence of gender, plasma renin, and familial resemblance. *Hypertension* **35** 710-716.

Giacchetti G, Ronconi V, Lucarelli G, Boscaro M & Mantero F 2006 Analysis of screening and confirmatory tests in the diagnosis of primary aldosteronism: need for a standardized protocol. *J.Hypertens.* **24** 737-745.

Giardina E, Pietrangeli I, Martinez-Labarga C, Martone C, de Angelis F, Spinella A, De Stefano G, Rickards O & Novelli G 2008 Haplotypes in *SLC24A5* Gene as Ancestry Informative Markers in Different Populations. *Curr.Genomics* **9** 110-114.

Gigante B, Rubattu S, Russo R, Porcellini A, Enea I, De Paolis P, Savoia C, Natale A, Piras O & Volpe M 1997 Opposite feedback control of renin and aldosterone biosynthesis in the adrenal cortex by angiotensin II AT1-subtype receptors. *Hypertension* **30** 563-568.

Goldenberg I, Moss AJ, Ryan D, McNitt S, Eberly SW & Zareba W 2006 Polymorphism in the angiotensinogen gene, hypertension, and ethnic differences in the risk of recurrent coronary events. *Hypertension* **48** 693-699.

Goldstone R, Horton R, Carlson EJ & Hsueh WA 1983 Reciprocal changes in active and inactive renin after converting enzyme inhibition in normal man. *J.Clin.Endocrinol.Metab* **56** 264-268.

Gomez-Sanchez EP 1986 Intracerebroventricular infusion of aldosterone induces hypertension in rats. *Endocrinology* **118** 819-823.

Gomez-Sanchez EP, Fort CM & Gomez-Sanchez CE 1990 Intracerebroventricular infusion of RU28318 blocks aldosterone-salt hypertension. *Am.J.Physiol* **258** E482-E484.

Gong M & Hubner N 2006 Molecular genetics of human hypertension. *Clin.Sci.(Lond)* **110** 315-326.

Goodfriend TL, Egan BM & Kelley DE 1998 Aldosterone in obesity. *Endocr.Res.* **24** 789-796.

Gordon RD, Laragh JH & Funder JW 2005 Low renin hypertensive states: perspectives, unsolved problems, future research. *Trends Endocrinol.Metab* **16** 108-113.

Gordon RD, Stowasser M, Tunny TJ, Klemm SA & Rutherford JC 1994 High incidence of primary aldosteronism in 199 patients referred with hypertension. *Clin.Exp.Pharmacol.Physiol* **21** 315-318.

Gordon RD, Wolfe LK, Island DP & Liddle GW 1966 A diurnal rhythm in plasma renin activity in man. *J.Clin.Invest* **45** 1587-1592.

Gould AB & Green D 1971 Kinetics of the human renin and human substrate reaction. *Cardiovasc.Res.* **5** 86-89.

Gower DB 1975 Catabolism and excretion of steroids. In *Biochemistry of Steroid Hormones*, pp 149-184. Ed HLJ Makin. London: Blackwell Scientific Publications.

Gradman AH, Schmieder RE, Lins RL, Nussberger J, Chiang Y & Bedigian MP 2005 Aliskiren, a novel orally effective renin inhibitor, provides dose-dependent antihypertensive efficacy and placebo-like tolerability in hypertensive patients. *Circulation* **111** 1012-1018.

Grassi G, Arenare F, Pieruzzi F, Brambilla G & Mancia G 2009 Sympathetic activation in cardiovascular and renal disease. *J.Nephrol.* **22** 190-195.

Grassi G, Quarti-Trevano F, Dell'oro R & Mancia G 2008 Essential hypertension and the sympathetic nervous system. *Neurol.Sci.* **29 Suppl 1** S33-S36.

Gregory TR 2004 Insertion-deletion biases and the evolution of genome size. *Gene* **324** 15-34.

Grim CE 1975 Editorial: Low renin "essential" hypertension; a variant of classic primary aldosteronism? *Arch.Intern.Med.* **135** 347-350.

Grossmann C, Krug AW, Freudinger R, Mildenberger S, Voelker K & Gekle M 2007 Aldosterone-induced EGFR expression: interaction between the human mineralocorticoid receptor and the human EGFR promoter. *Am.J.Physiol Endocrinol.Metab* **292** E1790-E1800.

Guillon G, Trueba M, Joubert D, Grazzini E, Chouinard L, Cote M, Payet MD, Manzoni O, Barberis C, Robert M & . 1995 Vasopressin stimulates steroid secretion in human adrenal glands: comparison with angiotensin-II effect. *Endocrinology* **136** 1285-1295.

Gunnells JC, Jr. & McGuffin WL, Jr. 1975 Low renin hypertension. *Annu.Rev.Med.* **26** 259-275.

Gunnells JC, Jr., McGuffin WL, Jr., Robinson RR, Grim CE, Wells S, Silver D & Glenn JF 1970 Hypertension, adrenal abnormalities, and alterations in plasma renin activity. *Ann.Intern.Med.* **73** 901-911.

Guyton AC, Coleman TG & Granger HJ 1972 Circulation: overall regulation. *Annu.Rev.Physiol* **34** 13-46.

Guzman B, Cormand B, Ribases M, Gonzalez-Nunez D, Botey A & Poch E 2006 Implication of chromosome 18 in hypertension by sibling pair and association analyses: putative involvement of the RKHD2 gene. *Hypertension* **48** 883-891.

Gwynne JT & Strauss JF, III 1982 The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocr.Rev.* **3** 299-329.

Haber E, Koerner T, Page LB, Kliman B & Purnode A 1969 Application of a radioimmunoassay for angiotensin I to the physiologic measurements of plasma renin activity in normal human subjects. *J.Clin.Endocrinol.Metab* **29** 1349-1355.

Hajjar I & Kotchen T 2003 Regional variations of blood pressure in the United States are associated with regional variations in dietary intakes: the NHANES-III data. *J.Nutr.* **133** 211-214.

Halperin E & Stephan DA 2009 SNP imputation in association studies. *Nat.Biotechnol.* **27** 349-351.

Hamilton BP, Zadik Z, Edwin CM, Hamilton JH & Kowarski AA 1979 Effect of adrenal suppression with dexamethasone in essential hypertension. *J.Clin.Endocrinol.Metab* **48** 848-853.

Hampf M, Dao NT, Hoan NT & Bernhardt R 2001 Unequal crossing-over between aldosterone synthase and 11beta-hydroxylase genes causes congenital adrenal hyperplasia. *J.Clin.Endocrinol.Metab* **86** 4445-4452.

Hanchard N, Elzein A, Trafford C, Rockett K, Pinder M, Jallow M, Harding R, Kwiatkowski D & McKenzie C 2007 Classical sickle beta-globin haplotypes exhibit a high degree of long-range haplotype similarity in African and Afro-Caribbean populations. *BMC.Genet.* **8** 52.

Hanneman RL 1996 Intersalt: hypertension rise with age revisited. *BMJ* **312** 1283-1284.

Hansson JH, Nelson-Williams C, Suzuki H, Schild L, Shimkets R, Lu Y, Canessa C, Iwasaki T, Rossier B & Lifton RP 1995a Hypertension caused by a truncated epithelial sodium channel gamma subunit: genetic heterogeneity of Liddle syndrome. *Nat.Genet.* **11** 76-82.

Hansson JH, Schild L, Lu Y, Wilson TA, Gautschi I, Shimkets R, Nelson-Williams C, Rossier BC & Lifton RP 1995b A de novo missense mutation of the beta subunit of the epithelial sodium channel causes hypertension and Liddle syndrome, identifying a proline-rich segment critical for regulation of channel activity. *Proc.Natl.Acad.Sci.U.S.A* **92** 11495-11499.

Hansson L, Hedner T, Lund-Johansen P, Kjeldsen SE, Lindholm LH, Syvertsen JO, Lanke J, de Faire U, Dahlof B & Karlberg BE 2000 Randomised trial of effects of calcium antagonists compared with diuretics and beta-blockers on cardiovascular morbidity and mortality in hypertension: the Nordic Diltiazem (NORDIL) study. *Lancet* **356** 359-365.

Hartman D, Sagnella GA, Chesters CA & Macgregor GA 2004 Direct renin assay and plasma renin activity assay compared. *Clin.Chem.* **50** 2159-2161.

Harvey BJ, Alzamora R, Stubbs AK, Irnaten M, McEneaney V & Thomas W 2008 Rapid responses to aldosterone in the kidney and colon. *J.Steroid Biochem.Mol.Biol.* **108** 310-317.

Harvey PJ, Wing LM, Savage J & Molloy D 1999 The effects of different types and doses of oestrogen replacement therapy on clinic and ambulatory blood pressure and the renin-angiotensin system in normotensive postmenopausal women. *J.Hypertens.* **17** 405-411.

Hautanena A, Lankinen L, Kupari M, Janne OA, Adlercreutz H, Nikkila H & White PC 1998 Associations between aldosterone synthase gene polymorphism and the adrenocortical function in males. *J.Intern.Med.* **244** 11-18.

Havlik RJ, Garrison RJ, Feinleib M, Kannel WB, Castelli WP & McNamara PM 1979 Blood pressure aggregation in families. *Am.J.Epidemiol.* **110** 304-312.

He FJ & Macgregor GA 2004 Effect of longer-term modest salt reduction on blood pressure. *Cochrane.Database.Syst.Rev.* CD004937.

Hedrick PW 2007 Balancing selection. *Curr.Biol.* **17** R230-R231.

Heller S, Linhart A, Jindra A, Jachymova M, Horky K, Peleska J, Hlubocka Z, Umnerova V & Aschermann M 2004 Association of -344/T/C aldosterone synthase polymorphism (*CYP11B2*) with left ventricular structure and humoral parameters in young normotensive men. *Blood Press* **13** 158-163.

Henderson SO, Haiman CA & Mack W 2004 Multiple Polymorphisms in the renin-angiotensin-aldosterone system (ACE, CYP11B2, AGTR1) and their contribution to hypertension in African Americans and Latinos in the multiethnic cohort. *Am.J.Med.Sci.* **328** 266-273.

Higaki J, Ogihara T, Imai N, Kumahara Y, Hontani S, Nishiura M, Ogawa H, Hirose S & Murakami K 1984 A new sensitive direct radioimmunoassay for human plasma renin and its clinical application. *J.Lab Clin.Med.* **104** 947-954.

Higaki J, Ogihara T & Ogawa M 1989 Proteolytic activation of aldosterone biosynthesis in rabbit adrenal capsular cells by alpha-chymotrypsin. *Methods Find.Exp.Clin.Pharmacol.* **11** 555-558.

Himathongkam T, Dluhy RG & Williams GH 1975 Potassium-aldosterone-renin interrelationships. *J.Clin.Endocrinol.Metab* **41** 153-159.

Himmelmann A, Bergbrant A, Svensson A, Hansson L & Aurell M 1996 Remikiren (Ro 42-5892)--an orally active renin inhibitor in essential hypertension. Effects on blood pressure and the renin-angiotensin-aldosterone system. *Am.J.Hypertens.* **9** 517-522.

Hiramatsu K, Yamada T, Yukimura Y, Komiya I, Ichikawa K, Ishihara M, Nagata H & Izumiyama T 1981 A screening test to identify aldosterone-producing adenoma by measuring plasma renin activity. Results in hypertensive patients. *Arch.Intern.Med.* **141** 1589-1593.

Hirschhorn JN & Daly MJ 2005 Genome-wide association studies for common diseases and complex traits. *Nat.Rev.Genet.* **6** 95-108.

Hollenberg NK, Chenitz WR, Adams DF & Williams GH 1974 Reciprocal influence of salt intake on adrenal glomerulosa and renal vascular responses to angiotensin II in normal man. *J.Clin.Invest* **54** 34-42.

Honda M, Nowaczynski W, Guthrie GP, Jr., Messerli FH, Tolis G, Kuchel O & Genest J 1977 Response of several adrenal steroids to ACTH stimulation in essential hypertension. *J.Clin.Endocrinol.Metab* **44** 264-272.

Hooper L, Bartlett C, Davey SG & Ebrahim S 2002 Systematic review of long term effects of advice to reduce dietary salt in adults. *BMJ* **325** 628.

Horisberger JD 1998 Amiloride-sensitive Na channels. *Curr.Opin.Cell Biol.* **10** 443-449.

Horisberger JD, Lemas V, Kraehenbuhl JP & Rossier BC 1991 Structure-function relationship of Na,K-ATPase. *Annu.Rev.Physiol* **53** 565-584.

Horn PS & Pesce AJ 2003 Reference intervals: an update. *Clin.Chim.Acta* **334** 5-23.

Hottenga JJ, Whitfield JB, Posthuma D, Willemsen G, de Geus EJ, Martin NG & Boomsma DI 2007 Genome-wide scan for blood pressure in Australian and Dutch subjects suggests linkage at 5P, 14Q, and 17P. *Hypertension* **49** 832-838.

Hunt SC, Hasstedt SJ, Kuida H, Stults BM, Hopkins PN & Williams RR 1989 Genetic heritability and common environmental components of resting and stressed blood pressures, lipids, and body mass index in Utah pedigrees and twins. *Am.J.Epidemiol.* **129** 625-638.

Hunt SC, Xin Y, Wu LL, Cawthon RM, Coon H, Hasstedt SJ & Hopkins PN 2006 Sodium bicarbonate cotransporter polymorphisms are associated with baseline and 10-year follow-up blood pressures. *Hypertension* **47** 532-536.

Ichikawa J, Sumino H, Ichikawa S & Ozaki M 2006 Different effects of transdermal and oral hormone replacement therapy on the renin-angiotensin system, plasma bradykinin level, and blood pressure of normotensive postmenopausal women. *Am.J.Hypertens.* **19** 744-749.

Illingworth DR, Kenny TA & Orwoll ES 1982 Adrenal function in heterozygous and homozygous hypobetalipoproteinemia. *J.Clin.Endocrinol.Metab* **54** 27-33.

Imrie H, Freel M, Mayosi BM, Davies E, Fraser R, Ingram M, Cordell HJ, Farrall M, Avery PJ, Watkins H, Keavney B & Connell JM 2006 Association between aldosterone production and variation in the 11beta-hydroxylase (*CYP11B1*) gene. *J.Clin.Endocrinol.Metab* **91** 5051-5056.

Imtiaz F, Savilahti E, Sarnesto A, Trabzuni D, Al Kahtani K, Kagevi I, Rashed MS, Meyer BF & Jarvela I 2007 The T/G 13915 variant upstream of the lactase gene (LCT) is the founder allele of lactase persistence in an urban Saudi population. *J.Med.Genet.* **44** e89.

Inagami T, Mizuno K, Naruse M, Nakamaru M, Naruse K, Hoffman LH & McKenzie JC 1989 Active and inactive renin in the adrenal. *Am.J.Hypertens.* **2** 311-319.

Inglis GC, Ingram MC, Holloway CD, Swan L, Birnie D, Hillis WS, Davies E, Fraser R & Connell JM 1999 Familial pattern of corticosteroids and their metabolism in adult human subjects--the Scottish Adult Twin Study. *J.Clin.Endocrinol.Metab* **84** 4132-4137.

Ingram CJ, Elamin MF, Mulcare CA, Weale ME, Tarekegn A, Raga TO, Bekele E, Elamin FM, Thomas MG, Bradman N & Swallow DM 2007 A novel polymorphism associated with lactose tolerance in Africa: multiple causes for lactase persistence? *Hum.Genet.* **120** 779-788.

Ingram CJ, Mulcare CA, Itan Y, Thomas MG & Swallow DM 2009 Lactose digestion and the evolutionary genetics of lactase persistence. *Hum.Genet.* **124** 579-591.

Ioannidis J & Lau J 2001 Evolution of treatment effects over time: empirical insight from recursive cumulative metaanalyses. *Proc.Natl.Acad.Sci.U.S.A* **98** 831-836.

Ioannidis JP, Contopoulos-Ioannidis DG & Lau J 1999 Recursive cumulative meta-analysis: a diagnostic for the evolution of total randomized evidence from group and individual patient data. *J.Clin.Epidemiol.* **52** 281-291.

Ioannidis JP, Ntzani EE, Trikalinos TA & Contopoulos-Ioannidis DG 2001 Replication validity of genetic association studies. *Nat.Genet.* **29** 306-309.

Iwai N, Kajimoto K, Tomoike H & Takashima N 2007 Polymorphism of *CYP11B2* determines salt sensitivity in Japanese. *Hypertension* **49** 825-831.

James GD, Sealey JE, Muller F, Alderman M, Madhavan S & Laragh JH 1986 Renin Relationship to Sex, Race and Age in A Normotensive Population. *Journal of Hypertension* **4** S387-S389.

James VH, Tunbridge RD & Wilson GA 1976 Studies on the control of aldosterone secretion in man. *J.Steroid Biochem.* **7** 941-948.

Jansen PM, Danser AH, Imholz BP & van den Meiracker AH 2009 Aldosterone-receptor antagonism in hypertension. *J.Hypertens.* **27** 680-691.

Jeske YW, So A, Kelemen L, Sukor N, Willys C, Bulmer B, Gordon RD, Duffy D & Stowasser M 2008 Examination of chromosome 7p22 candidate genes RBaK, PMS2 and GNA12 in familial hyperaldosteronism type II. *Clin.Exp.Pharmacol.Physiol* **35** 380-385.

Ji W, Foo JN, O'Roak BJ, Zhao H, Larson MG, Simon DB, Newton-Cheh C, State MW, Levy D & Lifton RP 2008 Rare independent mutations in renal salt handling genes contribute to blood pressure variation. *Nat.Genet.* **40** 592-599.

Joehrer K, Geley S, Strasser-Wozak EM, Azziz R, Wollmann HA, Schmitt K, Kofler R & White PC 1997 CYP11B1 mutations causing non-classic adrenal hyperplasia due to 11 beta-hydroxylase deficiency. *Hum.Mol.Genet.* **6** 1829-1834.

Johar S, Cave AC, Narayanapanicker A, Grieve DJ & Shah AM 2006 Aldosterone mediates angiotensin II-induced interstitial cardiac fibrosis via a Nox2-containing NADPH oxidase. *FASEB J.* **20** 1546-1548.

Jones CA 2003 Hypertension and renal dysfunction: NHANES III. *J.Am.Soc.Nephrol.* **14** S71-S75.

Jorde LB, Watkins WS & Bamshad MJ 2001 Population genomics: a bridge from evolutionary history to genetic medicine.

Jowett TP & Slater JD 1977 Development of radioimmunoassays for the measurement of aldosterone in unprocessed plasma and simple plasma extracts. *Clin.Chim.Acta* **80** 435-446.

Kaneshiro Y, Ichihara A, Takemitsu T, Sakoda M, Suzuki F, Nakagawa T, Hayashi M & Inagami T 2006 Increased expression of cyclooxygenase-2 in the renal cortex of human prorenin receptor gene-transgenic rats. *Kidney Int.* **70** 641-646.

Kang AK, Duncan JA, Cattran DC, Floras JS, Lai V, Scholey JW & Miller JA 2001 Effect of oral contraceptives on the renin angiotensin system and renal function. *Am.J.Physiol Regul.Integr.Comp Physiol* **280** R807-R813.

Kannel WB 1990 Influence of multiple risk factors on the hazard of hypertension. *J.Cardiovasc.Pharmacol.* **16 Suppl 5** S53-S57.

Kaplan NM 1967a Hypokalemia in the hypertensive patient, with observations on the incidence of primary aldosteronism. *Ann.Intern.Med.* **66** 1079-1090.

Kaplan NM 1967b The steroid content of adrenal adenomas and measurements of aldosterone production in patients with essential hypertension and primary aldosteronism. *J.Clin.Invest* **46** 728-734.

Kaplan NM 1994 *Clinical Hypertension*. New Delhi: Waverly International.

Kaplan NM 2001 Cautions over the current epidemic of primary aldosteronism. *Lancet* **357** 953-954.

Kaplan NM 2004 The current epidemic of primary aldosteronism: causes and consequences. *J.Hypertens.* **22** 863-869.

Kaplan NM 2007 Is there an unrecognized epidemic of primary aldosteronism? Con. *Hypertension* **50** 454-458.

Kaplan NM & Opie LH 2006 Controversies in hypertension. *Lancet* **367** 168-176.

Karin M 1998 New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? *Cell* **93** 487-490.

Karpac J, Ostwald D, Bui S, Hunnewell P, Shankar M & Hochgeschwender U 2005 Development, maintenance, and function of the adrenal gland in early postnatal proopiomelanocortin-null mutant mice. *Endocrinology* **146** 2555-2562.

Kater CE & Biglieri EG 1994 Disorders of steroid 17 alpha-hydroxylase deficiency. *Endocrinol.Metab Clin.North Am.* **23** 341-357.

Kathiresan S, Larson MG, Benjamin EJ, Corey D, Murabito JM, Fox CS, Wilson PW, Rifai N, Meigs JB, Ricken G, Lifton RP, Levy D & Vasan RS 2005 Clinical and genetic correlates of serum aldosterone in the community: the Framingham Heart Study. *Am.J.Hypertens.* **18** 657-665.

Katz FH, Romfh P & Smith JA 1975 Diurnal variation of plasma aldosterone, cortisol and renin activity in supine man. *J.Clin.Endocrinol.Metab* **40** 125-134.

Kayes-Wandover KM & White PC 2000 Steroidogenic enzyme gene expression in the human heart. *J.Clin.Endocrinol.Metab* **85** 2519-2525.

Ke X & Cardon LR 2003 Efficient selective screening of haplotype tag SNPs. *Bioinformatics.* **19** 287-288.

Ke X, Hunt S, Tapper W, Lawrence R, Stavrides G, Ghori J, Whittaker P, Collins A, Morris AP, Bentley D, Cardon LR & Deloukas P 2004 The impact of SNP density on fine-scale patterns of linkage disequilibrium. *Hum.Mol.Genet.* **13** 577-588.

Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK & He J 2005 Global burden of hypertension: analysis of worldwide data. *Lancet* **365** 217-223.

Keavney B, Mayosi B, Gaukrodger N, Imrie H, Baker M, Fraser R, Ingram M, Watkins H, Farrall M, Davies E & Connell J 2005 Genetic variation at the locus encompassing 11-beta hydroxylase and aldosterone synthase accounts for heritability in cortisol precursor (11-deoxycortisol) urinary metabolite excretion. *J.Clin.Endocrinol.Metab* **90** 1072-1077.

Kemendy AE, Kleyman TR & Eaton DC 1992 Aldosterone alters the open probability of amiloride-blockable sodium channels in A6 epithelia. *Am.J.Physiol* **263** C825-C837.

Kennedy BP, Rao F, Botiglieri T, Sharma S, Lillie EO, Ziegler MG & O'Connor DT 2005 Contributions of the sympathetic nervous system, glutathione, body mass and gender to blood pressure increase with normal aging: influence of heredity. *J.Hum.Hypertens.* **19** 951-969.

Kennon B, Ingram MC, Friel EC, Anderson NH, MacKenzie SM, Davies E, Shakerdi L, Wallace AM, Fraser R & Connell JM 2004 Aldosterone synthase gene variation and adrenocortical response to sodium status, angiotensin II and ACTH in normal male subjects. *Clin.Endocrinol.(Oxf)* **61** 174-181.

Khaw KT, Bingham S, Welch A, Luben R, O'Brien E, Wareham N & Day N 2004 Blood pressure and urinary sodium in men and women: the Norfolk Cohort of the European Prospective Investigation into Cancer (EPIC-Norfolk). *Am.J.Clin.Nutr.* **80** 1397-1403.

Kidambi S, Kotchen JM, Grim CE, Raff H, Mao J, Singh RJ & Kotchen TA 2007 Association of adrenal steroids with hypertension and the metabolic syndrome in blacks. *Hypertension* **49** 704-711.

Kifor I, Moore TJ, Fallo F, Sperling E, Chiou CY, Menachery A & Williams GH 1991 Potassium-stimulated angiotensin release from superfused adrenal capsules and enzymatically dispersed cells of the zona glomerulosa. *Endocrinology* **129** 823-831.

Kigoshi T, Morimoto S & Takeda R 1980 Role of the renin-angiotensin system in enhancing the aldosterone response to adenocorticotropin during acute sodium depletion in normal subjects. *J.Clin.Endocrinol.Metab* **51** 1102-1106.

Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB & Knepper MA 1998 The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proc.Natl.Acad.Sci.U.S.A* **95** 14552-14557.

Kisch ES, Dluhy RG & Williams GH 1976 Enhanced aldosterone response to angiotensin II in human hypertension. *Circ.Res.* **38** 502-505.

Klein K & Henk W 1963 [Clinical experimental studies on the influence of aldosterone on hemodynamics and blod coagulation.]. *Z.Kreislaufforsch.* **52** 40-53.

Komiya I, Yamada T, Aizawa T, Takasu N, Niwa A, Maruyama Y & Ogawa A 1991 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** 99-110.

Komiya I, Yamada T, Takara M, Asawa T, Shimabukuro M, Nishimori T & Takasu N 2000 Lys(173)Arg and -344T/C variants of CYP11B2 in Japanese patients with low-renin hypertension. *Hypertension* **35** 699-703.

Komlosi P, Fintha A & Bell PD 2004 Current mechanisms of macula densa cell signalling. *Acta Physiol Scand.* **181** 463-469.

Kotchen TA, Kotchen JM, Grim CE, George V, Kaldunski ML, Cowley AW, Hamet P & Chelius TH 2000a Genetic determinants of hypertension: identification of candidate phenotypes. *Hypertension* **36** 7-13.

Kotchen TA, Kotchen JM, Grim CE, George V, Kaldunski ML, Cowley AW, Hamet P & Chelius TH 2000b Genetic determinants of hypertension: identification of candidate phenotypes. *Hypertension* **36** 7-13.

Kotchen TA, Piering AW, Cowley AW, Grim CE, Gaudet D, Hamet P, Kaldunski ML, Kotchen JM & Roman RJ 2000c Glomerular hyperfiltration in hypertensive African Americans. *Hypertension* **35** 822-826.

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

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

Krop M & Danser AH 2008 Circulating versus tissue renin-angiotensin system: on the origin of (pro)renin. *Curr.Hypertens.Rep.* **10** 112-118.

Kumar NN, Benjafield AV, Lin RC, Wang WY, Stowasser M & Morris BJ 2003 Haplotype analysis of aldosterone synthase gene (*CYP11B2*) polymorphisms shows association with essential hypertension. *J.Hypertens.* **21** 1331-1337.

Kupari M, Hautanen A, Lankinen L, Koskinen P, Virolainen J, Nikkila H & White PC 1998 Associations between human aldosterone synthase (*CYP11B2*) gene polymorphisms and left ventricular size, mass, and function. *Circulation* **97** 569-575.

Kupper N, Willemsen G, Riese H, Posthuma D, Boomsma DI & de Geus EJ 2005 Heritability of daytime ambulatory blood pressure in an extended twin design. *Hypertension* **45** 80-85.

Kurtz I 1998 Molecular pathogenesis of Bartter's and Gitelman's syndromes. *Kidney Int.* **54** 1396-1410.

Lafferty AR, Torpy DJ, Stowasser M, Taymans SE, Lin JP, Huggard P, Gordon RD & Stratakis CA 2000 A novel genetic locus for low renin hypertension: familial hyperaldosteronism type II maps to chromosome 7 (7p22). *J.Med.Genet.* **37** 831-835.

Langer B, Grima M, Coquard C, Bader AM, Schlaeder G & Imbs JL 1998 Plasma active renin, angiotensin I, and angiotensin II during pregnancy and in preeclampsia. *Obstet.Gynecol.* **91** 196-202.

Laragh JH 1973 Vasoconstriction-volume analysis for understanding and treating hypertension: the use of renin and aldosterone profiles. *Am.J.Med.* **55** 261-274.

Lau NC, Lim LP, Weinstein EG & Bartel DP 2001 An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* **294** 858-862.

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

Leckie BJ & McGhee NK 1980 Reversible activation-inactivation of renin in human plasma. *Nature* **288** 702-705.

Lenzini L, Seccia TM, Aldighieri E, Belloni AS, Bernante P, Giuliani L, Nussdorfer GG, Pessina AC & Rossi GP 2007 Heterogeneity of aldosterone-producing adenomas revealed by a whole transcriptome analysis. *Hypertension* **50** 1106-1113.

Levy D, Larson MG, Benjamin EJ, Newton-Cheh C, Wang TJ, Hwang SJ, Vasan RS & Mitchell GF 2007 Framingham Heart Study 100K Project: genome-wide associations for blood pressure and arterial stiffness. *BMC.Med.Genet.* **8 Suppl 1** S3.

Lewontin RC & Krakauer J 1973 Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. *Genetics* **74** 175-195.

Li A, Tedde R, Krozowski ZS, Pala A, Li KX, Shackleton CH, Mantero F, Palermo M & Stewart PM 1998 Molecular basis for hypertension in the "type II variant" of apparent mineralocorticoid excess. *Am.J.Hum.Genet.* **63** 370-379.

Lieberman S & Lin YY 2001 Reflections on sterol sidechain cleavage process catalyzed by cytochrome P450(scc). *J.Steroid Biochem.Mol.Biol.* **78** 1-14.

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

Lifton RP, Dluhy RG, Powers M, Rich GM, Gutkin M, Fallo F, Gill JR, Jr., Feld L, Ganguly A, Laidlaw JC & . 1992b Hereditary hypertension caused by chimaeric gene duplications and ectopic expression of aldosterone synthase. *Nat.Genet.* **2** 66-74.

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

Lifton RP, Hopkins PN, Williams RR, Hollenberg NK, Williams GH & Dluhy RG 1989 Evidence for heritability of non-modulating essential hypertension. *Hypertension* **13** 884-889.

Lillie EO, Mahata M, Khandrika S, Rao F, Bundey RA, Wen G, Chen Y, Taupenot L, Smith DW, Mahata SK, Ziegler MG, Cockburn M, Schork NJ & O'Connor DT 2007 Heredity of endothelin secretion: human twin studies reveal the influence of polymorphism at the chromogranin A locus, a novel determinant of endothelial function. *Circulation* **115** 2282-2291.

Lillie EO & O'Connor DT 2006 Early phenotypic changes in hypertension: a role for the autonomic nervous system and heredity. *Hypertension* **47** 331-333.

Lim PO, Dow E, Brennan G, Jung RT & MacDonald TM 2000 High prevalence of primary aldosteronism in the Tayside hypertension clinic population. *J.Hum.Hypertens.* **14** 311-315.

Lim PO, Jung RT & MacDonald TM 2002a Is aldosterone the missing link in refractory hypertension?: aldosterone-to-renin ratio as a marker of inappropriate aldosterone activity. *J.Hum.Hypertens.* **16** 153-158.

Lim PO, MacDonald TM, Holloway C, Friel E, Anderson NH, Dow E, Jung RT, Davies E, Fraser R & Connell JM 2002b Variation at the aldosterone synthase (*CYP11B2*) locus contributes to hypertension in subjects with a raised aldosterone-to-renin ratio. *J.Clin.Endocrinol.Metab* **87** 4398-4402.

Lim PO, Struthers AD & MacDonald TM 2002c The neurohormonal natural history of essential hypertension: towards primary or tertiary aldosteronism? *J.Hypertens.* **20** 11-15.

Lin D, Sugawara T, Strauss JF, III, Clark BJ, Stocco DM, Saenger P, Rogol A & Miller WL 1995 Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science* **267** 1828-1831.

Linhart KB & Majzoub JA 2008 Pomc knockout mice have secondary hyperaldosteronism despite an absence of adrenocorticotropin. *Endocrinology* **149** 681-686.

Liu J, Papasian C & Deng HW 2007 Incorporating single-locus tests into haplotype cladistic analysis in case-control studies. *PLoS.Genet.* **3** e46.

Loh KC, Koay ES, Khaw MC, Emmanuel SC & Young WF, Jr. 2000 Prevalence of primary aldosteronism among Asian hypertensive patients in Singapore. *J.Clin.Endocrinol.Metab* **85** 2854-2859.

Longini IM, Jr., Higgins MW, Hinton PC, Moll PP & Keller JB 1984 Environmental and genetic sources of familial aggregation of blood pressure in Tecumseh, Michigan. *Am.J.Epidemiol.* **120** 131-144.

Losel R, Schultz A & Wehling M 2004 A quick glance at rapid aldosterone action. *Mol.Cell Endocrinol.* **217** 137-141.

Lovas K, McFarlane I, Nguyen HH, Curran S, Schwabe J, Halsall D, Bernhardt R, Wallace AM & Chatterjee VK 2009 A novel *CYP11B2* gene mutation in an Asian family with aldosterone synthase deficiency. *J.Clin.Endocrinol.Metab* **94** 914-919.

Luetscher JA, Kraemer FB & Wilson DM 1989 Prorenin and vascular complications of diabetes. *Am.J.Hypertens.* **2** 382-386.

Luft FC, Rankin LI, Bloch R, Weyman AE, Willis LR, Murray RH, Grim CE & Weinberger MH 1979 Cardiovascular and humoral responses to extremes of sodium intake in normal black and white men. *Circulation* **60** 697-706.

Luft FC, Weinberger MH, Fineberg NS, Miller JZ & Grim CE 1987 Effects of age on renal sodium homeostasis and its relevance to sodium sensitivity. *Am.J.Med.* **82** 9-15.

Lumbers ER 1971 Activation of renin in human amniotic fluid by low pH. *Enzymologia.* **40** 329-336.

Luna A & Nicodemus KK 2007 snp.plotter: an R-based SNP/haplotype association and linkage disequilibrium plotting package. *Bioinformatics.* **23** 774-776.

Lupski JR 1998 Genomic disorders: structural features of the genome can lead to DNA rearrangements and human disease traits. *Trends Genet.* **14** 417-422.

MacKenzie SM, Huda SS, Sattar N, Fraser R, Connell JM & Davies E 2008 Depot-specific steroidogenic gene transcription in human adipose tissue. *Clin.Endocrinol.(Oxf)* **69** 848-854.

Madeddu P, Emanueli C & El Dahr S 2007 Mechanisms of disease: the tissue kallikrein-kinin system in hypertension and vascular remodeling. *Nat.Clin.Pract.Nephrol.* **3** 208-221.

Mastroberardino L, Spindler B, Forster I, Loffing J, Assandri R, May A & Verrey F 1998 Ras pathway activates epithelial Na+ channel and decreases its surface expression in Xenopus oocytes. *Mol.Biol.Cell* **9** 3417-3427.

Matsubara M, Omori F, Fujita S, Metoki H, Kikuya M, Fujiwara T, Araki T & Imai Y 2001 Haplotypes of aldosterone synthase (*CYP11B2*) gene in the general population of Japan: the Ohasama study. *Clin.Exp.Hypertens.* **23** 603-610.

Matsubara M, Sato T, Nishimura T, Suzuki M, Kikuya M, Metoki H, Michimata M, Tsuji I, Ogihara T & Imai Y 2004 *CYP11B2* polymorphisms and home blood pressure in a population-based cohort in Japanese: the Ohasama study. *Hypertens.Res.* **27** 1-6.

Matsukawa N, Nonaka Y, Higaki J, Nagano M, Mikami H, Ogihara T & Okamoto M 1993 Dahl's salt-resistant normotensive rat has mutations in cytochrome P450(11 beta), but the salt-sensitive hypertensive rat does not. *J.Biol.Chem.* **268** 9117-9121.

Mayes D, Furuyama S, Kem DC & Nugent CA 1970 A radioimmunoassay for plasma aldosterone. *J.Clin.Endocrinol.Metab* **30** 682-685.

McAllister JM & Hornsby PJ 1988 Dual regulation of 3 beta-hydroxysteroid dehydrogenase, 17 alpha-hydroxylase, and dehydroepiandrosterone sulfotransferase by adenosine 3',5'-monophosphate and activators of protein kinase C in cultured human adrenocortical cells. *Endocrinology* **122** 2012-2018.

McAreavey D, Murray GD, Lever AF & Robertson JI 1983 Similarity of idiopathic aldosteronism and essential hypertension. A statistical comparison. *Hypertension* **5** 116-121.

McDougall JG, Butkus A, Coghlan JP, Denton DA, Muller J, Oddie CJ, Robinson PM & Scoggins BA 1980 Biosynthetic ad morphological evidence for inhibition of aldosterone production following administration of ACTH to sheep. *Acta Endocrinol.(Copenh)* **94** 559-570.

McKenzie CA, Abecasis GR, Keavney B, Forrester T, Ratcliffe PJ, Julier C, Connell JM, Bennett F, McFarlane-Anderson N, Lathrop GM & Cardon LR 2001 Trans-ethnic fine mapping of a quantitative trait locus for circulating angiotensin I-converting enzyme (ACE). *Hum.Mol.Genet.* **10** 1077-1084.

McKenzie CA, Julier C, Forrester T, McFarlane-Anderson N, Keavney B, Lathrop GM, Ratcliffe PJ & Farrall M 1995 Segregation and linkage analysis of serum angiotensin I-converting enzyme levels: evidence for two quantitative-trait loci. *Am.J.Hum.Genet.* **57** 1426-1435.

McVean GA, Myers SR, Hunt S, Deloukas P, Bentley DR & Donnelly P 2004 The fine-scale structure of recombination rate variation in the human genome. *Science* **304** 581-584.

Menachery A, Braley LM, Kifor I, Gleason R & Williams GH 1991 Dissociation in plasma renin and adrenal ANG II and aldosterone responses to sodium restriction in rats. *Am.J.Physiol* **261** E487-E494.

Menard J, Bews J & Heusser C 1984 A multirange ELISA for the measurement of plasma renin in humans and primates. *J.Hypertens.Suppl* **2** S275-S278.

Meneton P, Galan P, Bertrais S, Heudes D, Hercberg S & Menard J 2008 High plasma aldosterone and low renin predict blood pressure increase and hypertension in middle-aged Caucasian populations. *J.Hum.Hypertens.* **22** 550-558.

Merke DP & Bornstein SR 2005 Congenital adrenal hyperplasia. *Lancet* **365** 2125-2136.

Michelakis AM, Yoshida H & Dormois JC 1975 Plasma renin activity and plasma aldosterone during the normal menstrual cycle. *Am.J.Obstet.Gynecol.* **123** 724-726.

Millar JA, Leckie BJ, Morton JJ, Jordan J & Tree M 1980 A microassay for active and total renin concentration in human plasma based on antibody trapping. *Clin.Chim.Acta* **101** 5-15.

Miller JZ, Daugherty SA, Weinberger MH, Grim CE, Christian JC & Lang CL 1983 Blood pressure response to dietary sodium restriction in normotensive adults. *Hypertension* **5** 790-795.

Miller WL 1987 Structure of genes encoding steroidogenic enzymes. *J.Steroid Biochem.* **27** 759-766.

Miller WL 1988 Molecular biology of steroid hormone synthesis. *Endocr.Rev.* **9** 295-318.

Mitsuuchi Y, Kawamoto T, Miyahara K, Ulick S, Morton DH, Naiki Y, Kuribayashi I, Toda K, Hara T, Orii T & . 1993 Congenitally defective aldosterone biosynthesis in humans: inactivation of the P-450C18 gene (*CYP11B2*) due to nucleotide deletion in CMO I deficient patients. *Biochem.Biophys.Res.Commun.* **190** 864-869.

Miura R, Nakamura K, Miura D, Miura A, Hisamatsu K, Kajiya M, Hashimoto K, Nagase S, Morita H, Fukushima KK, Emori T, Ishihara K & Ohe T 2006 Aldosterone synthesis and cytokine production in human peripheral blood mononuclear cells. *J.Pharmacol.Sci.* **102** 288-295.

Mizuno Y, Yoshimura M, Yasue H, Sakamoto T, Ogawa H, Kugiyama K, Harada E, Nakayama M, Nakamura S, Ito T, Shimasaki Y, Saito Y & Nakao K 2001 Aldosterone production is activated in failing ventricle in humans. *Circulation* **103** 72-77.

Moneva MH & Gomez-Sanchez CE 2002 Pathophysiology of adrenal hypertension. *Semin.Nephrol.* **22** 44-53.

Mongeau JG, Biron P & Sing CF 1986 The influence of genetics and household environment upon the variability of normal blood pressure: the Montreal Adoption Survey. *Clin.Exp.Hypertens.A* **8** 653-660.

Montori VM, Schwartz GL, Chapman AB, Boerwinkle E & Turner ST 2001 Validity of the aldosterone-renin ratio used to screen for primary aldosteronism. *Mayo Clin.Proc.* **76** 877-882.

Montori VM & Young WF, Jr. 2002 Use of plasma aldosterone concentration-to-plasma renin activity ratio as a screening test for primary aldosteronism. A systematic review of the literature. *Endocrinol.Metab Clin.North Am.* **31** 619-32, xi.

Morganti A, Pelizzola D, Mantero F, Gazzano G, Opocher G & Piffanelli A 1995 Immunoradiometric versus enzymatic renin assay: results of the Italian Multicenter Comparative Study. Italian Multicenter Study for Standardization of Renin Measurement. *J.Hypertens.* **13** 19-26.

Mornet E, Dupont J, Vitek A & White PC 1989 Characterization of two genes encoding human steroid 11 beta-hydroxylase (P-450(11) beta). *J.Biol.Chem.* **264** 20961-20967.

Morris RW & Kaplan NL 2002 On the advantage of haplotype analysis in the presence of multiple disease susceptibility alleles. *Genet.Epidemiol.* **23** 221-233.

Morton NE & Collins A 1998 Tests and estimates of allelic association in complex inheritance. *Proc.Natl.Acad.Sci.U.S.A* **95** 11389-11393.

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

Moura AM & Worcel M 1984 Direct action of aldosterone on transmembrane 22Na efflux from arterial smooth muscle. Rapid and delayed effects. *Hypertension* **6** 425-430.

Mulatero P 2008 A new form of hereditary primary aldosteronism: familial hyperaldosteronism type III. *J.Clin.Endocrinol.Metab* **93** 2972-2974.

Mulatero P, Dluhy RG, Giacchetti G, Boscaro M, Veglio F & Stewart PM 2005 Diagnosis of primary aldosteronism: from screening to subtype differentiation. *Trends Endocrinol.Metab* **16** 114-119.

Mulatero P, Rabbia F, Milan A, Paglieri C, Morello F, Chiandussi L & Veglio F 2002a Drug effects on aldosterone/plasma renin activity ratio in primary aldosteronism. *Hypertension* **40** 897-902.

Mulatero P, Schiavone D, Fallo F, Rabbia F, Pilon C, Chiandussi L, Pascoe L & Veglio F 2000 *CYP11B2* gene polymorphisms in idiopathic hyperaldosteronism. *Hypertension* **35** 694-698.

Mulatero P, Stowasser M, Loh KC, Fardella CE, Gordon RD, Mosso L, Gomez-Sanchez CE, Veglio F & Young WF, Jr. 2004 Increased diagnosis of primary aldosteronism, including surgically correctable forms, in centers from five continents. *J.Clin.Endocrinol.Metab* **89** 1045-1050.

Mulatero P, Veglio F, Pilon C, Rabbia F, Zocchi C, Limone P, Boscaro M, Sonino N & Fallo F 1998 Diagnosis of glucocorticoid-remediable aldosteronism in primary aldosteronism: aldosterone response to dexamethasone and long polymerase chain reaction for chimeric gene. *J.Clin.Endocrinol.Metab* **83** 2573-2575.

Mulatero P, Verhovez A, Morello F & Veglio F 2007 Diagnosis and treatment of low-renin hypertension. *Clin.Endocrinol.(Oxf)* **67** 324-334.

Mulatero P, Williams TA, Milan A, Paglieri C, Rabbia F, Fallo F & Veglio F 2002b Blood pressure in patients with primary aldosteronism is influenced by bradykinin B(2) receptor and alpha-adducin gene polymorphisms. *J.Clin.Endocrinol.Metab* **87** 3337-3343.

Muller J 1995 Aldosterone: the minority hormone of the adrenal cortex. *Steroids* **60** 2-9.

Mune T & White PC 1996 Apparent mineralocorticoid excess: genotype is correlated with biochemical phenotype. *Hypertension* **27** 1193-1199.

Munroe PB, Wallace C, Xue MZ, Marcano AC, Dobson RJ, Onipinla AK, Burke B, Gungadoo J, Newhouse SJ, Pembroke J, Brown M, Dominiczak AF, Samani NJ, Lathrop M, Connell J, Webster J, Clayton D, Farrall M, Mein CA & Caulfield M 2006 Increased support for linkage of a novel locus on chromosome 5q13 for essential hypertension in the British Genetics of Hypertension Study. *Hypertension* **48** 105-111.

Musch MW, Lucioni A & Chang EB 2008 Aldosterone regulation of intestinal Na absorption involves SGK-mediated changes in NHE3 and Na^+ pump activity. *Am.J.Physiol Gastrointest.Liver Physiol* **295** G909-G919.

Naftilan AJ, Zuo WM, Inglefinger J, Ryan TJ, Jr., Pratt RE & Dzau VJ 1991 Localization and differential regulation of angiotensinogen mRNA expression in the vessel wall. *J.Clin.Invest* **87** 1300-1311.

Nagai Y, Miyata K, Sun GP, Rahman M, Kimura S, Miyatake A, Kiyomoto H, Kohno M, Abe Y, Yoshizumi M & Nishiyama A 2005 Aldosterone stimulates collagen gene expression and synthesis via activation of ERK1/2 in rat renal fibroblasts. *Hypertension* **46** 1039-1045.

Nakamura Y, Suzuki S, Suzuki T, Ono K, Miura I, Satoh F, Moriya T, Saito H, Yamada S, Ito S & Sasano H 2006 MDM2: a novel mineralocorticoid-responsive gene involved in aldosterone-induced human vascular structural remodeling. *Am.J.Pathol.* **169** 362-371.

Naray-Fejes-Toth A, Canessa C, Cleaveland ES, Aldrich G & Fejes-Toth G 1999 sgk is an aldosterone-induced kinase in the renal collecting duct. Effects on epithelial na+ channels. *J.Biol.Chem.* **274** 16973-16978.

Nebert DW & Russell DW 2002 Clinical importance of the cytochromes P450. *Lancet* **360** 1155-1162.

Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM & Nebert DW 2004 Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* **14** 1-18.

Neri G, De Toni R, Tortorella C, Rebuffat P, Bova S, Cargnelli G, Petrelli L, Spinazzi R & Nussdorfer GG 2006 Ouabain chronic infusion enhances the growth and steroidogenic capacity of rat adrenal zona glomerulosa: the possible involvement of the endothelin system. *Int.J.Mol.Med.* **18** 315-319.

Neville AM 1978 The nodular adrenal. *Invest Cell Pathol.* **1** 99-111.

New MI 2003 Inborn errors of adrenal steroidogenesis. *Mol.Cell Endocrinol.* **211** 75-83.

Newhouse SJ, Wallace C, Dobson R, Mein C, Pembroke J, Farrall M, Clayton D, Brown M, Samani N, Dominiczak A, Connell JM, Webster J, Lathrop GM, Caulfield M & Munroe PB 2005 Haplotypes of the WNK1 gene associate with blood pressure variation in a severely hypertensive population from the British Genetics of Hypertension study. *Hum.Mol.Genet.* **14** 1805-1814.

Newton-Cheh C, Guo CY, Gona P, Larson MG, Benjamin EJ, Wang TJ, Kathiresan S, O'Donnell CJ, Musone SL, Camargo AL, Drake JA, Levy D, Hirschhorn JN & Vasan RS 2007 Clinical and genetic correlates of aldosterone-to-renin ratio and relations to blood pressure in a community sample. *Hypertension* **49** 846-856.

Newton-Cheh C & Hirschhorn JN 2005 Genetic association studies of complex traits: design and analysis issues. *Mutat.Res.* **573** 54-69.

Newton-Cheh C, Johnson T, Gateva V, Tobin MD, Bochud M, Coin L, Najjar SS, Zhao JH, Heath SC, Eyheramendy S, Papadakis K, Voight BF, Scott LJ, Zhang F, Farrall M, Tanaka T, Wallace C, Chambers JC, Khaw KT, Nilsson P, van der HP, Polidoro S, Grobbee DE, Onland-Moret NC, Bots ML, Wain LV, Elliott KS, Teumer A, Luan J, Lucas G, Kuusisto J, Burton PR, Hadley D, McArdle WL, Brown M, Dominiczak A, Newhouse SJ, Samani NJ, Webster J, Zeggini E, Beckmann JS, Bergmann S, Lim N, Song K, Vollenweider P, Waeber G, Waterworth DM, Yuan X, Groop L, Orho-Melander M, Allione A, Di Gregorio A, Guarrera S, Panico S, Ricceri F, Romanazzi V, Sacerdote C, Vineis P, Barroso I, Sandhu MS, Luben RN, Crawford GJ, Jousilahti P, Perola M, Boehnke M, Bonnycastle LL, Collins FS, Jackson AU, Mohlke KL, Stringham HM, Valle TT, Willer CJ, Bergman RN, Morken MA, Doring A, Gieger C, Illig T, Meitinger T, Org E, Pfeufer A, Wichmann HE, Kathiresan S, Marrugat J, O'Donnell CJ, Schwartz SM, Siscovick DS, Subirana I, Freimer NB, Hartikainen AL, McCarthy MI, O'Reilly PF, Peltonen L, Pouta A, de Jong PE, Snieder H, van Gilst WH, Clarke R, Goel A, Hamsten A, Peden JF, Seedorf U, Syvanen AC, Tognoni G, Lakatta EG, Sanna S, Scheet P, Schlessinger D, Scuteri A, Dorr M, Ernst F, Felix SB, Homuth G, Lorbeer R, Reffelmann T, Rettig R, Volker U, Galan P, Gut IG, Hercberg S, Lathrop GM, Zelenika D, Deloukas P, Soranzo N, Williams FM, Zhai G, Salomaa V, Laakso M, Elosua R, Forouhi NG, Volzke H, Uiterwaal CS, van der Schouw YT, Numans ME, Matullo G, Navis G, Berglund G, Bingham SA, Kooner JS, Connell JM, Bandinelli S, Ferrucci L, Watkins H, Spector TD, Tuomilehto J, Altshuler D, Strachan DP, Laan M, Meneton P, Wareham NJ, Uda M, Jarvelin MR, Mooser V, Melander O, Loos RJ, Elliott P, Abecasis GR, Caulfield M & Munroe PB 2009a Genome-wide association study identifies eight loci associated with blood pressure. *Nat.Genet.* **41** 666-676.

Newton-Cheh C, Larson MG, Vasan RS, Levy D, Bloch KD, Surti A, Guiducci C, Kathiresan S, Benjamin EJ, Struck J, Morgenthaler NG, Bergmann A, Blankenberg S, Kee F, Nilsson P, Yin X, Peltonen L, Vartiainen E, Salomaa V, Hirschhorn JN, Melander O & Wang TJ 2009b Association of common variants in NPPA and NPPB with circulating natriuretic peptides and blood pressure. *Nat.Genet.* **41** 348-353.

Nguyen G & Contrepas A 2008 The (pro)renin receptors. *J.Mol.Med.* **86** 643-646.

Nguyen HH, Hannemann F, Hartmann MF, Wudy SA & Bernhardt R 2008 Aldosterone synthase deficiency caused by a homozygous L451F mutation in the *CYP11B2* gene. *Mol.Genet.Metab* **93** 458-467.

Nicod J, Bruhin D, Auer L, Vogt B, Frey FJ & Ferrari P 2003 A biallelic gene polymorphism of *CYP11B2* predicts increased aldosterone to renin ratio in selected hypertensive patients. *J.Clin.Endocrinol.Metab* **88** 2495-2500.

Nogueira EF, Xing Y, Morris CA & Rainey WE 2009 Role of angiotensin II-induced rapid response genes in the regulation of enzymes needed for aldosterone synthesis. *J.Mol.Endocrinol.* **42** 319-330.

Nomoto S, Massa G, Mitani F, Ishimura Y, Miyahara K, Toda K, Nagano I, Yamashiro T, Ogoshi S, Fukata J, Onishi S, Hashimoto K, Doi Y, Imura H & Shizuta Y 1997 CMO I deficiency caused by a point mutation in exon 8 of the human *CYP11B2* gene encoding steroid 18-hydroxylase (P450C18). *Biochem.Biophys.Res.Commun.* **234** 382-385.

Nonaka Y, Fujii T, Kagawa N, Waterman MR, Takemori H & Okamoto M 1998 Structure/function relationship of CYP11B1 associated with Dahl's salt-resistant rats--expression of rat CYP11B1 and CYP11B2 in Escherichia coli. *Eur.J.Biochem.* **258** 869-878.

Nonaka Y, Takemori H, Halder SK, Sun T, Ohta M, Hatano O, Takakusu A & Okamoto M 1995 Frog cytochrome P-450 (11 beta,aldo), a single enzyme involved in the final steps of glucocorticoid and mineralocorticoid biosynthesis. *Eur.J.Biochem.* **229** 249-256.

Nussberger J, Waeber B, Brunner HR, Burris JF & Vetter W 1984 Highly sensitive microassay for aldosterone in unextracted plasma: comparison with two other methods. *J.Lab Clin.Med.* **104** 789-796.

O'Brien E, Barton J, Nussberger J, Mulcahy D, Jensen C, Dicker P & Stanton A 2007 Aliskiren reduces blood pressure and suppresses plasma renin activity in combination with a thiazide diuretic, an angiotensin-converting enzyme inhibitor, or an angiotensin receptor blocker. *Hypertension* **49** 276-284.

O'Connell JR & Weeks DE 1998 PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am.J.Hum.Genet.* **63** 259-266.

Oelkers W 1985 Prolonged ACTH infusion suppresses aldosterone secretion in spite of high renin activity. *Acta Endocrinol.(Copenh)* **108** 91-97.

Oelkers W, Boelke T & Bahr V 1988 Dose-response relationships between plasma adrenocorticotropin (ACTH), cortisol, aldosterone, and 18-hydroxycorticosterone after injection of ACTH-(1-39) or human corticotropin-releasing hormone in man. *J.Clin.Endocrinol.Metab* **66** 181-186.

Oelkers W, Brown JJ, Fraser R, Lever AF, Morton JJ & Robertson JI 1974 Sensitization of the adrenal cortex to angiotensin II in sodium-deplete man. *Circ.Res.* **40** 69-77.

Oelkers W, Diederich S & Bahr V 1992 Diagnosis and therapy surveillance in Addison's disease: rapid adrenocorticotropin (ACTH) test and measurement of plasma ACTH, renin activity, and aldosterone. *J.Clin.Endocrinol.Metab* **75** 259-264.

Ogihara T, Hata T, Maruyama A, Mikami H, Nakamaru M, Mandai T & Kumahara Y 1979 Studies on the renin-angiotensin-aldosterone system in elderly hypertensive patients with an angiotensin II antagonist. *Clin.Sci.(Lond)* **57** 461-463.

Ogishima T, Suzuki H, Hata J, Mitani F & Ishimura Y 1992 Zone-specific expression of aldosterone synthase cytochrome P-450 and cytochrome P-45011 beta in rat adrenal cortex: histochemical basis for the functional zonation. *Endocrinology* **130** 2971-2977.

Okamoto M & Nonaka Y 1992 Molecular biology of rat steroid 11 beta-hydroxylase [P450(11 beta)] and aldosterone synthase [P450(11 beta, aldo)]. *J.Steroid Biochem.Mol.Biol.* **41** 415-419.

Olivieri O, Ciacciarelli A, Signorelli D, Pizzolo F, Guarini P, Pavan C, Corgnati A, Falcone S, Corrocher R, Micchi A, Cressoni C & Blengio G 2004 Aldosterone to Renin ratio in a primary care setting: the Bussolengo study. *J.Clin.Endocrinol.Metab* **89** 4221-4226.

Padfield PL 2002 Primary aldosteronism, a common entity? the myth persists. *J.Hum.Hypertens.* **16** 159-162.

Padfield PL 2003 Prevalence and role of a raised aldosterone to renin ratio in the diagnosis of primary aldosteronism: a debate on the scientific logic of the use of the ratio in practice. *Clin.Endocrinol.(Oxf)* **59** 422-426.

Padfield PL, Brown JJ, Davies D, Fraser R, Lever AF, Morton JJ & Robertson JI 1981 The myth of idiopathic hyperaldosteronism. *Lancet* **2** 83-84.

Padfield PL, Brown JJ, Lever AF, Schalekamp MA, Beevers DG, Davies DL, Robertson JI & Tree M 1975 Is low-renin hypertension a stage in the development of essential hypertension or a diagnostic entity? *Lancet* **1** 548-550.

Padmanabhan S, Melander O, Hastie C, Menni C, Delles C, Connell JM & Dominiczak AF 2008 Hypertension and genome-wide association studies: combining high fidelity phenotyping and hypercontrols. *J.Hypertens.* **26** 1275-1281.

Page RD 1996 TreeView: an application to display phylogenetic trees on personal computers. *Comput.Appl.Biosci.* **12** 357-358.

Paillard F, Chansel D, Brand E, Benetos A, Thomas F, Czekalski S, Ardaillou R & Soubrier F 1999 Genotype-phenotype relationships for the renin-angiotensin-aldosterone system in a normal population. *Hypertension* **34** 423-429.

Palmer LG & Frindt G 2000 Aldosterone and potassium secretion by the cortical collecting duct. *Kidney Int.* **57** 1324-1328.

Pascoe L, Curnow KM, Slutsker L, Connell JM, Speiser PW, New MI & White PC 1992a Glucocorticoid-suppressible hyperaldosteronism results from hybrid genes created by unequal crossovers between *CYP11B1* and *CYP11B2*. *Proc.Natl.Acad.Sci.U.S.A* **89** 8327-8331.

Pascoe L, Curnow KM, Slutsker L, Rosler A & White PC 1992b Mutations in the human CYP11B2 (aldosterone synthase) gene causing corticosterone methyloxidase II deficiency. *Proc.Natl.Acad.Sci.U.S.A* **89** 4996-5000.

Patel S, Steeds R, Channer K & Samani NJ 2000 Analysis of promoter region polymorphism in the aldosterone synthase gene (*CYP11B2*) as a risk factor for myocardial infarction. *Am.J.Hypertens.* **13** 134-139.

Paul M, Wagner J & Dzau VJ 1993 Gene expression of the renin-angiotensin system in human tissues. Quantitative analysis by the polymerase chain reaction. *J.Clin.Invest* **91** 2058-2064.

Payne AH & Hales DB 2004 Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr.Rev.* **25** 947-970.

Penner SB, Campbell NR, Chockalingam A, Zarnke K & Van Vliet B 2007 Dietary sodium and cardiovascular outcomes: a rational approach. *Can.J.Cardiol.* **23** 567-572.

Penning TM 1997 Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr.Rev.* **18** 281-305.

Perschel FH, Schemer R, Seiler L, Reincke M, Deinum J, Maser-Gluth C, Mechelhoff D, Tauber R & Diederich S 2004 Rapid screening test for primary hyperaldosteronism: ratio of plasma aldosterone to renin concentration determined by fully automated chemiluminescence immunoassays. *Clin.Chem.* **50** 1650-1655.

Peter M, Fawaz L, Drop SL, Visser HK & Sippell WG 1997 Hereditary defect in biosynthesis of aldosterone: aldosterone synthase deficiency 1964-1997. *J.Clin.Endocrinol.Metab* **82** 3525-3528.

Pettersson ME, Kurland CG & Berg OG 2009 Deletion rate evolution and its effect on genome size and coding density. *Mol.Biol.Evol.* **26** 1421-1430.

Pezzi V, Mathis JM, Rainey WE & Carr BR 2003 Profiling transcript levels for steroidogenic enzymes in fetal tissues. *J.Steroid Biochem.Mol.Biol.* **87** 181-189.

Pham-Huu-Trung MT, Bogyo A, de Smitter N & Girard F 1985 Effects of proopiomelanocortin peptides and angiotensin II on steroidogenesis in isolated aldosteronoma cells. *J.Clin.Endocrinol.Metab* **61** 467-471.

Pilbrow AP, Palmer BR, Frampton CM, Yandle TG, Troughton RW, Campbell E, Skelton L, Lainchbury JG, Richards AM & Cameron VA 2007 Angiotensinogen M235T and T174M gene polymorphisms in combination doubles the risk of mortality in heart failure. *Hypertension* **49** 322-327.

Plagnol V & Wall JD 2006 Possible ancestral structure in human populations. *PLoS.Genet.* **2** e105.

Pojoga L, Gautier S, Blanc H, Guyene TT, Poirier O, Cambien F & Benetos A 1998 Genetic determination of plasma aldosterone levels in essential hypertension. *Am.J.Hypertens.* **11** 856-860.

Portaluppi F, Vergnani L, Manfredini R & Fersini C 1996 Endocrine mechanisms of blood pressure rhythms. *Ann.N.Y.Acad.Sci.* **783** 113-131.

Portrat-Doyen S, Tourniaire J, Richard O, Mulatero P, Aupetit-Faisant B, Curnow KM, Pascoe L & Morel Y 1998 Isolated aldosterone synthase deficiency caused by simultaneous E198D and V386A mutations in the *CYP11B2* gene. *J.Clin.Endocrinol.Metab* **83** 4156-4161.

Poulsen K, Sancho J & Haber E 1974 A simplified radioimmunoassay for plasma aldosterone employing an antibody of unique specificity. *Clin.Immunol.Immunopathol.* **2** 373-380.

Pratt JH, Dale SL & Melby JC 1976 The effect of administered ACTH on aldosterone metabolism and secretion. *J.Clin.Endocrinol.Metab* **42** 355-360.

Pratt JH, Jones JJ, Miller JZ, Wagner MA & Fineberg NS 1989 Racial differences in aldosterone excretion and plasma aldosterone concentrations in children. *N.Engl.J.Med.* **321** 1152-1157.

Pratt JH, Rebhun JF, Zhou L, Ambrosius WT, Newman SA, Gomez-Sanchez CE & Mayes DF 1999 Levels of mineralocorticoids in whites and blacks. *Hypertension* **34** 315-319.

Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA & Reich D 2006 Principal components analysis corrects for stratification in genome-wide association studies. *Nat.Genet.* **38** 904-909.

Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ & Sham PC 2007 PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am.J.Hum.Genet.* **81** 559-575.

Qin W, Rudolph AE, Bond BR, Rocha R, Blomme EA, Goellner JJ, Funder JW & McMahon EG 2003 Transgenic model of aldosterone-driven cardiac hypertrophy and heart failure. *Circ.Res.* **93** 69-76.

Rajput C, Makhijani K, Norboo T, Afrin F, Sharma M, Pasha ST & Pasha MA 2005 *CYP11B2* gene polymorphisms and hypertension in highlanders accustomed to high salt intake. *J.Hypertens.* **23** 79-86.

Rao F, Wessel J, Wen G, Zhang L, Rana BK, Kennedy BP, Greenwood TA, Salem RM, Chen Y, Khandrika S, Hamilton BA, Smith DW, Holstein-Rathlou NH, Ziegler MG, Schork NJ & O'Connor DT 2007 Renal albumin excretion: twin studies identify influences of heredity, environment, and adrenergic pathway polymorphism. *Hypertension* **49** 1015-1031.

Redgrave J, Rabinowe S, Hollenberg NK & Williams GH 1985 Correction of abnormal renal blood flow response to angiotensin II by converting enzyme inhibition in essential hypertensives. *J.Clin.Invest* **75** 1285-1290.

Reich DE, Cargill M, Bolk S, Ireland J, Sabeti PC, Richter DJ, Lavery T, Kouyoumjian R, Farhadian SF, Ward R & Lander ES 2001 Linkage disequilibrium in the human genome. *Nature* **411** 199-204.

Reichardt HM, Kaestner KH, Wessely O, Gass P, Schmid W & Schutz G 1998 Analysis of glucocorticoid signalling by gene targeting. *J.Steroid Biochem.Mol.Biol.* **65** 111-115.

Reynolds RM, Walker BR, Phillips DI, Dennison EM, Fraser R, MacKenzie SM, Davies E & Connell JM 2009 Programming of hypertension: associations of plasma aldosterone in adult men and women with birthweight, cortisol, and blood pressure. *Hypertension* **53** 932-936.

Rice GI, Jones AL, Grant PJ, Carter AM, Turner AJ & Hooper NM 2006 Circulating activities of angiotensin-converting enzyme, its homolog, angiotensin-converting enzyme 2, and neprilysin in a family study. *Hypertension* **48** 914-920.

Rice T, Vogler GP, Perusse L, Bouchard C & Rao DC 1989 Cardiovascular risk factors in a French Canadian population: resolution of genetic and familial environmental effects on blood pressure using twins, adoptees, and extensive information on environmental correlates. *Genet.Epidemiol.* **6** 571-588.

Richards AM, Nicholls MG, Espiner EA, Ikram H, Cullens M & Hinton D 1986 Diurnal patterns of blood pressure, heart rate and vasoactive hormones in normal man. *Clin.Exp.Hypertens.A* **8** 153-166.

Rocha R, Rudolph AE, Frierdich GE, Nachowiak DA, Kekec BK, Blomme EA, McMahon EG & Delyani JA 2002 Aldosterone induces a vascular inflammatory phenotype in the rat heart. *Am.J.Physiol Heart Circ.Physiol* **283** H1802-H1810.

Rogerson FM, Brennan FE & Fuller PJ 2004 Mineralocorticoid receptor binding, structure and function. *Mol.Cell Endocrinol.* **217** 203-212.

Romagni P, Rossi F, Guerrini L, Quirini C & Santiemma V 2003 Aldosterone induces contraction of the resistance arteries in man. *Atherosclerosis* **166** 345-349.

Rosler A 1984 The natural history of salt-wasting disorders of adrenal and renal origin. *J.Clin.Endocrinol.Metab* **59** 689-700.

Rossi E, Regolisti G, Perazzoli F, Negro A, Davoli S, Nicoli D, Sani C & Casali B 2001 -344C/T polymorphism of CYP11B2 gene in Italian patients with idiopathic low renin hypertension. *Am.J.Hypertens.* **14** 934-941.

Rossi GP, Belfiore A, Bernini G, Fabris B, Caridi G, Ferri C, Giacchetti G, Letizia C, Maccario M, Mannelli M, Palumbo G, Patalano A, Rizzoni D, Rossi E, Pessina AC & Mantero F 2008 Body mass index predicts plasma aldosterone concentrations in overweight-obese primary hypertensive patients. *J.Clin.Endocrinol.Metab* **93** 2566-2571.

Rossi GP, Bernini G, Caliumi C, Desideri G, Fabris B, Ferri C, Ganzaroli C, Giacchetti G, Letizia C, Maccario M, Mallamaci F, Mannelli M, Mattarello MJ, Moretti A, Palumbo G, Parenti G, Porteri E, Semplicini A, Rizzoni D, Rossi E, Boscaro M, Pessina AC & Mantero F 2006 A prospective study of the prevalence of primary aldosteronism in 1,125 hypertensive patients. *J.Am.Coll.Cardiol.* **48** 2293-2300.

Rotin D 2000 Regulation of the epithelial sodium channel (ENaC) by accessory proteins. *Curr.Opin.Nephrol.Hypertens.* **9** 529-534.

Rumsby G, Skinner C, Lee HA & Honour JW 1993 Combined 17 alpha-hydroxylase/17,20-lyase deficiency caused by heterozygous stop codons in the cytochrome P450 17 alpha-hydroxylase gene. *Clin.Endocrinol.(Oxf)* **39** 483-485.

Russo CJ, Melista E, Cui J, DeStefano AL, Bakris GL, Manolis AJ, Gavras H & Baldwin CT 2005 Association of NEDD4L ubiquitin ligase with essential hypertension. *Hypertension* **46** 488-491.

Sabeti PC, Reich DE, Higgins JM, Levine HZ, Richter DJ, Schaffner SF, Gabriel SB, Platko JV, Patterson NJ, McDonald GJ, Ackerman HC, Campbell SJ, Altshuler D, Cooper R, Kwiatkowski D, Ward R & Lander ES 2002 Detecting recent positive selection in the human genome from haplotype structure. *Nature* **419** 832-837.

Sagnella GA 2001 Why is plasma renin activity lower in populations of African origin? *J.Hum.Hypertens.* **15** 17-25.

San Cristobal P, de los HP, Ponce-Coria J, Moreno E & Gamba G 2008 WNK kinases, renal ion transport and hypertension. *Am.J.Nephrol.* **28** 860-870.

Sanz-Rosa D, Cediel E, de las HN, Miana M, Balfagon G, Lahera V & Cachofeiro V 2005 Participation of aldosterone in the vascular inflammatory response of spontaneously hypertensive rats: role of the NFkappaB/IkappaB system. *J.Hypertens.* **23** 1167-1172.

Saris JJ, 't Hoen PA, Garrelds IM, Dekkers DH, den Dunnen JT, Lamers JM & Jan Danser AH 2006 Prorenin induces intracellular signaling in cardiomyocytes independently of angiotensin II. *Hypertension* **48** 564-571.

Schambelan M, Brust NL, Chang BC, Slater KL & Biglieri EG 1976 Circadian rhythm and effect of posture on plasma aldosterone concentration in primary aldosteronism. *J.Clin.Endocrinol.Metab* **43** 115-131.

Schunkert H, Danser AHJ, Hense HW, Derkx FHM, Kurzinger S & Riegger GAJ 1997 Effects of estrogen replacement therapy on the renin-angiotensin system in postmenopausal women. *Circulation* **95** 39-45.

Schunkert H, Hengstenberg C, Holmer SR, Broeckel U, Luchner A, Muscholl MW, Kurzinger S, Doring A, Hense HW & Riegger GA 1999a Lack of association between a polymorphism of the aldosterone synthase gene and left ventricular structure. *Circulation* **99** 2255-2260.

Schunkert H, Hense HW, Andus T, Riegger GA & Straub RH 1999b Relation between dehydroepiandrosterone sulfate and blood pressure levels in a population-based sample. *Am.J.Hypertens.* **12** 1140-1143.

Schunkert H, Ingelfinger JR, Hirsch AT, Tang SS, Litwin SE, Talsness CE & Dzau VJ 1992 Evidence for tissue-specific activation of renal angiotensinogen mRNA expression in chronic stable experimental heart failure. *J.Clin.Invest* **90** 1523-1529.

Schwartz GL, Chapman AB, Boerwinkle E, Kisabeth RM & Turner ST 2002 Screening for primary aldosteronism: implications of an increased plasma aldosterone/renin ratio. *Clin.Chem.* **48** 1919-1923.

Schwartz GL & Turner ST 2005 Screening for primary aldosteronism in essential hypertension: diagnostic accuracy of the ratio of plasma aldosterone concentration to plasma renin activity. *Clin.Chem.* **51** 386-394.

Sealey JE 1991 Plasma renin activity and plasma prorenin assays. *Clin.Chem.* **37** 1811-1819.

Sealey JE, Atlas SA & Laragh JH 1980 Prorenin and other large molecular weight forms of renin. *Endocr.Rev.* **1** 365-391.

Sealey JE, Gordon RD & Mantero F 2005 Plasma renin and aldosterone measurements in low renin hypertensive states. *Trends Endocrinol.Metab* **16** 86-91.

Sealey JE, Itskovitzeldor J, Rubattu S, James GD, August P, Thaler I, Levron J & Laragh JH 1994 Estradiol-Related and Progesterone-Related Increases in the Renin-Aldosterone System - Studies During Ovarian Stimulation and Early-Pregnancy. *Journal of Clinical Endocrinology and Metabolism* **79** 258-264.

Sealey JE & Laragh JH 1975 Radioimmunoassay of plasma renin activity. *Semin.Nucl.Med.* **5** 189-202.

Sealey JE & Laragh JH 1996 Renin and prorenin: advances and declines in methodology. *Clin.Chem.* **42** 993-994.

Sealey JE, Trenkwalder P, Gahnem F, Catanzaro D & Laragh JH 1995 Plasma renin methodology: inadequate sensitivity and accuracy of direct renin assay for clinical applications compared with the traditional enzymatic plasma renin activity assay. *J.Hypertens.* **13** 27-30.

Seasholtz TM, Wessel J, Rao F, Rana BK, Khandrika S, Kennedy BP, Lillie EO, Ziegler MG, Smith DW, Schork NJ, Brown JH & O'Connor DT 2006 Rho kinase polymorphism influences blood pressure and systemic vascular resistance in human twins: role of heredity. *Hypertension* **47** 937-947.

Seely EW, Conlin PR, Brent GA & Dluhy RG 1989 Adrenocorticotropin stimulation of aldosterone: prolonged continuous versus pulsatile infusion. *J.Clin.Endocrinol.Metab* **69** 1028-1032.

Seifarth C, Trenkel S, Schobel H, Hahn EG & Hensen J 2002 Influence of antihypertensive medication on aldosterone and renin concentration in the differential diagnosis of essential hypertension and primary aldosteronism. *Clin.Endocrinol.(Oxf)* **57** 457-465.

Seifert C & Oelkers W 1981 Aldosterone response to sodium deprivation and angiotensin II in patients with hypopituitarism. *Acta Endocrinol.(Copenh)* **96** 361-369.

Semple PF, Nicholls MG, Tree M & Fraser R 1978 [des-Asp1]angiotensin II in the dog: blood levels and effect on aldosterone. *Endocrinology* **103** 1476-1482.

Sequeira SJ, Loughlin T, Cunningham S, Culliton MT, Hannon S, Heffernan A & McKenna TJ 1986 Evaluation of an aldosterone radioimmunoassay: the renin-angiotensin-aldosterone axis as a function of sex and age. *Ann.Clin.Biochem.* **23 (Pt 1)** 65-75.

Sethupathy P, Borel C, Gagnebin M, Grant GR, Deutsch S, Elton TS, Hatzigeorgiou AG & Antonarakis SE 2007 Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3' untranslated region: a mechanism for functional single-nucleotide polymorphisms related to phenotypes. *Am.J.Hum.Genet.* **81** 405-413.

Sever PS, Gradman AH & Azizi M 2009 Managing cardiovascular and renal risk: the potential of direct renin inhibition. *J.Renin.Angiotensin.Aldosterone.Syst.* **10** 65-76.

Sever PS, Peart WS, Meade TW, Davies IB & Gordon D 1979 Ethnic differences in blood pressure with observations on noradrenaline and renin. 1. A working population. *Clin.Exp.Hypertens.* **1** 733-744.

Sever PS & Poulter NR 1989 A hypothesis for the pathogenesis of essential hypertension: the initiating factors. *J.Hypertens.Suppl* **7** S9-12.

Shade RE & Grim CE 1975 Suppression of renin and aldosterone by small amounts of DOCA in normal man. *J.Clin.Endocrinol.Metab* **40** 652-658.

Sharma KK, Lindqvist A, Zhou XJ, Auchus RJ, Penning TM & Andersson S 2006 Deoxycorticosterone inactivation by AKR1C3 in human mineralocorticoid target tissues. *Mol.Cell Endocrinol.* **248** 79-86.

Shih PA & O'Connor DT 2008 Hereditary determinants of human hypertension: strategies in the setting of genetic complexity. *Hypertension* **51** 1456-1464.

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

Shinzawa K, Ishibashi S, Murakoshi M, Watanabe K, Kominami S, Kawahara A & Takemori S 1988 Relationship between zonal distribution of microsomal cytochrome P-450s (P-450(17)alpha,lyase and P-450C21) and steroidogenic activities in guinea-pig adrenal cortex. *J.Endocrinol.* **119** 191-200.

Sim MK & Qui XS 2003 Angiotensins in plasma of hypertensive rats and human. *Regul.Pept.* **111** 179-182.

Simard J, Durocher F, Mebarki F, Turgeon C, Sanchez R, Labrie Y, Couet J, Trudel C, Rheaume E, Morel Y, Luu-The V & Labrie F 1996 Molecular biology and genetics of the 3 beta-hydroxysteroid dehydrogenase/delta5-delta4 isomerase gene family. *J.Endocrinol.* **150 Suppl** S189-S207.

Simon D, Hartmann DJ, Badouaille G, Caillot G, Guyenne TT, Corvol P, Pau B & Marchand J 1992 Two-site direct immunoassay specific for active renin. *Clin.Chem.* **38** 1959-1962.

Sinclair AM, Isles CG, Brown I, Cameron H, Murray GD & Robertson JW 1987 Secondary hypertension in a blood pressure clinic. *Arch.Intern.Med.* **147** 1289-1293.

Siragy HM 2004 AT1 and AT2 receptor in the kidney: role in health and disease. *Semin.Nephrol.* **24** 93-100.

Sirianni R, Sirianni R, Carr BR, Pezzi V & Rainey WE 2001 A role for src tyrosine kinase in regulating adrenal aldosterone production. *J.Mol.Endocrinol.* **26** 207-215.

Skinner SL 1967 Improved assay methods for renin "concentration" and "activity" in human plasma. Methods using selective denaturation of renin substrate. *Circ.Res.* **20** 391-402.

Slattery ML, Bishop DT, French TK, Hunt SC, Meikle AW & Williams RR 1988 Lifestyle and blood pressure levels in male twins in Utah. *Genet.Epidemiol.* **5** 277-287.

Slight SH, Joseph J, Ganjam VK & Weber KT 1999 Extra-adrenal mineralocorticoids and cardiovascular tissue. *J.Mol.Cell Cardiol.* **31** 1175-1184.

Smith GD & Phillips AN 1996 Inflation in epidemiology: "the proof and measurement of association between two things" revisited. *BMJ* **312** 1659-1661.

Soejima M & Koda Y 2007 Population differences of two coding SNPs in pigmentation-related genes *SLC24A5* and *SLC45A2*. *Int.J.Legal Med.* **121** 36-39.

Somekawa S, Imagawa K, Naya N, Takemoto Y, Onoue K, Okayama S, Takeda Y, Kawata H, Horii M, Nakajima T, Uemura S, Mochizuki N & Saito Y 2009 Regulation of aldosterone and cortisol production by the transcriptional repressor neuron restrictive silencer factor. *Endocrinology* **150** 3110-3117.

Sookoian S, Gianotti TF, Gonzalez CD & Pirola CJ 2007 Association of the C-344T aldosterone synthase gene variant with essential hypertension: a meta-analysis. *J.Hypertens.* **25** 5-13.

Soundararajan R, Zhang TT, Wang J, Vandewalle A & Pearce D 2005 A novel role for glucocorticoid-induced leucine zipper protein in epithelial sodium channel-mediated sodium transport. *J.Biol.Chem.* **280** 39970-39981.

Spark RF, Dale SL, Kahn PC & Melby JC 1969 Activation of aldosterone secretion in primary aldosteronism. *J.Clin.Invest* **48** 96-104.

Sparkes RS, Klisak I & Miller WL 1991 Regional mapping of genes encoding human steroidogenic enzymes: P450scc to 15q23-q24, adrenodoxin to 11q22; adrenodoxin reductase to 17q24-q25; and P450c17 to 10q24-q25. *DNA Cell Biol.* **10** 359-365.

Spat A & Hunyady L 2004 Control of aldosterone secretion: a model for convergence in cellular signaling pathways. *Physiol Rev.* **84** 489-539.

Spindler B & Verrey F 1999 Aldosterone action: induction of p21(ras) and fra-2 and transcription-independent decrease in myc, jun, and fos. *Am.J.Physiol* **276** C1154-C1161.

Staessen JA, Li Y & Thijs L 2007 Meta-analysis of blood pressure and the *CYP11B2* polymorphism highlights the need for better designed studies. *J.Hypertens.* **25** 37-39.

Staessen JA, Wang JG, Brand E, Barlassina C, Birkenhager WH, Herrmann SM, Fagard R, Tizzoni L & Bianchi G 2001 Effects of three candidate genes on prevalence and incidence of hypertension in a Caucasian population. *J.Hypertens.* **19** 1349-1358.

Stamler J, Rose G, Stamler R, Elliott P, Dyer A & Marmot M 1989 INTERSALT study findings. Public health and medical care implications. *Hypertension* **14** 570-577.

Stephens JC, Schneider JA, Tanguay DA, Choi J, Acharya T, Stanley SE, Jiang R, Messer CJ, Chew A, Han JH, Duan J, Carr JL, Lee MS, Koshy B, Kumar AM, Zhang G, Newell WR, Windemuth A, Xu C, Kalbfleisch TS, Shaner SL, Arnold K, Schulz V, Drysdale CM, Nandabalan K, Judson RS, Ruano G & Vovis GF 2001 Haplotype variation and linkage disequilibrium in 313 human genes. *Science* **293** 489-493.

Stewart PM, Corrie JE, Shackleton CH & Edwards CR 1988 Syndrome of apparent mineralocorticoid excess. A defect in the cortisol-cortisone shuttle. *J.Clin.Invest* **82** 340-349.

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

Stewart PM, Wallace AM, Valentino R, Burt D, Shackleton CH & Edwards CR 1987 Mineralocorticoid activity of liquorice: 11-beta-hydroxysteroid dehydrogenase deficiency comes of age. *Lancet* **2** 821-824.

Stocco DM 2001 StAR protein and the regulation of steroid hormone biosynthesis. *Annu.Rev.Physiol* **63** 193-213.

Stockand JD 2002 New ideas about aldosterone signaling in epithelia. *Am.J.Physiol Renal Physiol* **282** F559-F576.

Stockand JD & Meszaros JG 2003 Aldosterone stimulates proliferation of cardiac fibroblasts by activating Ki-RasA and MAPK1/2 signaling. *Am.J.Physiol Heart Circ.Physiol* **284** H176-H184.

Stowasser M & Gordon RD 2001 Familial hyperaldosteronism. *J.Steroid Biochem.Mol.Biol.* **78** 215-229.

Stowasser M & Gordon RD 2004 Primary aldosteronism--careful investigation is essential and rewarding. *Mol.Cell Endocrinol.* **217** 33-39.

Stowasser M & Gordon RD 2006 Monogenic mineralocorticoid hypertension. *Best.Pract.Res.Clin.Endocrinol.Metab* **20** 401-420.

Stowasser M, Gordon RD, Rutherford JC, Nikwan NZ, Daunt N & Slater GJ 2001 Diagnosis and management of primary aldosteronism. *J.Renin.Angiotensin.Aldosterone.Syst.* **2** 156-169.

Strachan T & Read AP 2004 *Human molecular genetics 3*. London: Garland Science.

Sturm RA 2009 Molecular genetics of human pigmentation diversity. *Hum.Mol.Genet.* **18** R9-17.

Sugiyama T, Yoshimoto T, Hirono Y, Suzuki N, Sakurada M, Tsuchiya K, Minami I, Iwashima F, Sakai H, Tateno T, Sato R & Hirata Y 2005a Aldosterone increases osteopontin gene expression in rat endothelial cells. *Biochem.Biophys.Res.Commun.* **336** 163-167.

Sugiyama T, Yoshimoto T, Tsuchiya K, Gochou N, Hirono Y, Tateno T, Fukai N, Shichiri M & Hirata Y 2005b Aldosterone induces angiotensin converting enzyme gene expression via a JAK2-dependent pathway in rat endothelial cells. *Endocrinology* **146** 3900-3906.

Sun Y, Zhang J, Lu L, Chen SS, Quinn MT & Weber KT 2002 Aldosterone-induced inflammation in the rat heart : role of oxidative stress. *Am.J.Pathol.* **161** 1773-1781.

Sutherland DJ, Ruse JL & Laidlaw JC 1966 Hypertension, increased aldosterone secretion and low plasma renin activity relieved by dexamethasone. *Can.Med.Assoc.J.* **95** 1109-1119.

Suzuki S, Uchida D, Koide H, Suyama K, Shibata T, Yoshida T, Tanaka T, Noguchi Y, Saito Y & Tatsuno I 2008 A possible association between aldosterone response to vasopressin and circadian change of aldosterone in the patients with aldosterone-producing adenoma. *Peptides* **29** 2225-2231.

Takeda R, Miyamori I, Ikeda M, Koshida H, Takeda Y, Yasuhara S, Morise T & Takimoto H 1984 Circadian rhythm of plasma aldosterone and time dependent alterations of aldosterone regulators. *J.Steroid Biochem.* **20** 321-323.

Takeda Y, Miyamori I, Yoneda T, Hatakeyama H, Inaba S, Furukawa K, Mabuchi H & Takeda R 1996 Regulation of aldosterone synthase in human vascular endothelial cells by angiotensin II and adrenocorticotropin. *J.Clin.Endocrinol.Metab* **81** 2797-2800.

Tamaki S, Iwai N, Tsujita Y & Kinoshita M 1999 Genetic polymorphism of CYP11B2 gene and hypertension in Japanese. *Hypertension* **33** 266-270.

Tanabe A, Naruse M, Takagi S, Tsuchiya K, Imaki T & Takano K 2003 Variability in the renin/aldosterone profile under random and standardized sampling conditions in primary aldosteronism. *J.Clin.Endocrinol.Metab* **88** 2489-2494.

Tanahashi H, Mune T, Takahashi Y, Isaji M, Suwa T, Morita H, Yamakita N, Yasuda K, Deguchi T, White PC & Takeda J 2005 Association of Lys173Arg polymorphism with CYP11B2 expression in normal adrenal glands and aldosterone-producing adenomas. *J.Clin.Endocrinol.Metab* **90** 6226-6231.

Taylor PJ, Cooper DP, Gordon RD & Stowasser M 2009 Measurement of aldosterone in human plasma by semiautomated HPLC-tandem mass spectrometry. *Clin.Chem.* **55** 1155-1162.

Tillmann HC, Schumacher B, Yasenyev O, Junker M, Christ M, Feuring M & Wehling M 2002 Acute effects of aldosterone on intracardiac monophasic action potentials. *Int.J.Cardiol.* **84** 33-39.

Tishkoff SA, Reed FA, Ranciaro A, Voight BF, Babbitt CC, Silverman JS, Powell K, Mortensen HM, Hirbo JB, Osman M, Ibrahim M, Omar SA, Lema G, Nyambo TB, Ghori J, Bumpstead S, Pritchard JK, Wray GA & Deloukas P 2007 Convergent adaptation of human lactase persistence in Africa and Europe. *Nat.Genet.* **39** 31-40.

Tobin MD, Sheehan NA, Scurrah KJ & Burton PR 2005 Adjusting for treatment effects in studies of quantitative traits: antihypertensive therapy and systolic blood pressure. *Stat.Med.* **24** 2911-2935.

Touyz RM 2004 Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension* **44** 248-252.

Tsujita Y, Iwai N, Katsuya T, Higaki J, Ogihara T, Tamaki S, Kinoshita M, Mannami T, Ogata J & Baba S 2001 Lack of association between genetic polymorphism of *CYP11B2* and hypertension in Japanese: the Suita Study. *Hypertens.Res.* **24** 105-109.

Tsukada K, Ishimitsu T, Teranishi M, Saitoh M, Yoshii M, Inada H, Ohta S, Akashi M, Minami J, Ono H, Ohrui M & Matsuoka H 2002 Positive association of CYP11B2 gene polymorphism with genetic predisposition to essential hypertension. *J.Hum.Hypertens.* **16** 789-793.

Tsunoda K, Abe K, Goto T, Yasujima M, Sato M, Omata K, Seino M & Yoshinaga K 1986 Effect of age on the renin-angiotensin-aldosterone system in normal subjects: simultaneous measurement of active and inactive renin, renin substrate, and aldosterone in plasma. *J.Clin.Endocrinol.Metab* **62** 384-389.

Tsutamoto T, Sakai H, Tanaka T, Fujii M, Yamamoto T, Wada A, Ohnishi M & Horie M 2007 Comparison of active renin concentration and plasma renin activity as a prognostic predictor in patients with heart failure. *Circ.J.* **71** 915-921.

Tsutamoto T, Wada A, Maeda K, Mabuchi N, Hayashi M, Tsutsui T, Ohnishi M, Sawaki M, Fujii M, Matsumoto T, Horie H, Sugimoto Y & Kinoshita M 2000 Transcardiac extraction of circulating endothelin-1 across the failing heart. *Am.J.Cardiol.* **86** 524-528.

Tucci JR, Espiner EA, Jagger PI, Pauk GL & Lauler DP 1967 ACTH stimulation of aldosterone secretion in normal subjects and in patients with chronic adrenocortical insufficiency. *J.Clin.Endocrinol.Metab* **27** 568-575.

Tuck ML, Dluhy RG & Williams GH 1975 Sequential responses of the renin-angiotensin-aldosterone axis to acute postural change: effect of dietary sodium. *J.Lab Clin.Med.* **86** 754-763.

Ulick S 1976 Diagnosis and nomenclature of the disorders of the terminal portion of the aldosterone biosynthetic pathway. *J.Clin.Endocrinol.Metab* **43** 92-96.

Ulick S, Wang JZ, Blumenfeld JD & Pickering TG 1992a Cortisol inactivation overload: a mechanism of mineralocorticoid hypertension in the ectopic adrenocorticotropin syndrome. *J.Clin.Endocrinol.Metab* **74** 963-967.

Ulick S, Wang JZ & Morton DH 1992b The biochemical phenotypes of two inborn errors in the biosynthesis of aldosterone. *J.Clin.Endocrinol.Metab* **74** 1415-1420.

Ulmer PS & Meikle AW 2000 Sample requirements for plasma renin activity and immunoreactive renin. *Clin.Chem.* **46** 1442-1444.

Unger N, Lopez S, I, Pitt C, Walz MK, Philipp T, Mann K & Petersenn S 2004 Comparison of active renin concentration and plasma renin activity for the diagnosis of primary hyperaldosteronism in patients with an adrenal mass. *Eur.J.Endocrinol.* **150** 517-523.

Vaandrager AB, Hogema BM & de Jonge HR 2005 Molecular properties and biological functions of cGMP-dependent protein kinase II. *Front Biosci.* **10** 2150-2164.

Vasan RS, Evans JC, Larson MG, Wilson PW, Meigs JB, Rifai N, Benjamin EJ & Levy D 2004 Serum aldosterone and the incidence of hypertension in nonhypertensive persons. *N.Engl.J.Med.* **351** 33-41.

Verrelli BC, McDonald JH, Argyropoulos G, Destro-Bisol G, Froment A, Drousiotou A, Lefranc G, Helal AN, Loiselet J & Tishkoff SA 2002 Evidence for balancing selection from nucleotide sequence analyses of human G6PD. *Am.J.Hum.Genet.* **71** 1112-1128.

Verrey F, Fakitsas P, Adam G & Staub O 2008 Early transcriptional control of ENaC (de)ubiquitylation by aldosterone. *Kidney Int.* **73** 691-696.

Victora CG, Adair L, Fall C, Hallal PC, Martorell R, Richter L & Sachdev HS 2008 Maternal and child undernutrition: consequences for adult health and human capital. *Lancet* **371** 340-357.

Viengchareun S, Le Menuet D, Martinerie L, Munier M, Pascual-Le Tallec L & Lombes M 2007 The mineralocorticoid receptor: insights into its molecular and (patho)physiological biology. *Nucl.Recept.Signal.* **5** e012.

Vollmer WM, Sacks FM, Ard J, Appel LJ, Bray GA, Simons-Morton DG, Conlin PR, Svetkey LP, Erlinger TP, Moore TJ & Karanja N 2001 Effects of diet and sodium intake on blood pressure: subgroup analysis of the DASH-sodium trial. *Ann.Intern.Med.* **135** 1019-1028.

Wagner C, Jensen BL, Kramer BK & Kurtz A 1998 Control of the renal renin system by local factors. *Kidney Int.Suppl* **67** S78-S83.

Wagner MJ, Ge Y, Siciliano M & Wells DE 1991 A hybrid cell mapping panel for regional localization of probes to human chromosome 8. *Genomics* **10** 114-125.

Wahl M, Whalley ET, Unterberg A, Schilling L, Parsons AA, Baethmann A & Young AR 1996 Vasomotor and permeability effects of bradykinin in the cerebral microcirculation. *Immunopharmacology* **33** 257-263.

Wald H, Goldstein O, Asher C, Yagil Y & Garty H 1996 Aldosterone induction and epithelial distribution of CHIF. *Am.J.Physiol* **271** F322-F329.

Walker BR, Campbell JC, Fraser R, Stewart PM & Edwards CR 1992 Mineralocorticoid excess and inhibition of 11 beta-hydroxysteroid dehydrogenase in patients with ectopic ACTH syndrome. *Clin.Endocrinol.(Oxf)* **37** 483-492.

Ward R 1990 Familial aggregation and genetic epidemiology of blood pressure. In *Hypertension: pathophysiology, diagnosis and management*, pp 81-100. Eds JH Laragh & BM Brenner. New York: Raven Press.

Warnock DG 2001 Liddle syndrome: genetics and mechanisms of Na+ channel defects. *Am.J.Med.Sci.* **322** 302-307.

Waterman MR 1995 A rising StAR: an essential role in cholesterol transport. *Science* **267** 1780-1781.

Webster MK, Goya L, Ge Y, Maiyar AC & Firestone GL 1993 Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. *Mol.Cell Biol.* **13** 2031-2040.

Wehling M, Spes CH, Win N, Janson CP, Schmidt BM, Theisen K & Christ M 1998 Rapid cardiovascular action of aldosterone in man. *J.Clin.Endocrinol.Metab* **83** 3517-3522.

Weidmann P, Beretta-Piccoli C, Ziegler WH, Keusch G, Gluck Z & Reubi FC 1978 Age versus urinary sodium for judging renin, aldosterone, and catecholamine levels: studies in normal subjects and patients with essential hypertension. *Kidney Int.* **14** 619-628.

Weinberger MH & Fineberg NS 1991 Sodium and volume sensitivity of blood pressure. Age and pressure change over time. *Hypertension* **18** 67-71.

Weinberger MH, Grim CE, Hollifield JW, Kem DC, Ganguly A, Kramer NJ, Yune HY, Wellman H & Donohue JP 1979 Primary aldosteronism: diagnosis, localization, and treatment. *Ann.Intern.Med.* **90** 386-395.

Weinberger MH, Kem DC, Gomez-Sanchez C, Kramer NJ, Martin BT & Nugent CA 1975 The effect of dexamethasone on the control of plasma aldosterone concentration in normal recumbent man. *J.Lab Clin.Med.* **85** 957-967.

Weinberger MH, Kramer NJ, Grim CE & Petersen LP 1977 The effect of posture and saline loading on plasma renin activity and aldosterone concentration in pregnant, non-pregnant and estrogen-treated women. *J.Clin.Endocrinol.Metab* **44** 69-77.

Weinberger MH, Miller JZ, Luft FC, Grim CE & Fineberg NS 1986 Definitions and characteristics of sodium sensitivity and blood pressure resistance. *Hypertension* **8** II127-II134.

Weir BS 1996 *Genetic Data Analysis II*. Massachusetts: Sinauer Associates, Inc.

Wessel J, Moratorio G, Rao F, Mahata M, Zhang L, Greene W, Rana BK, Kennedy BP, Khandrika S, Huang P, Lillie EO, Shih PA, Smith DW, Wen G, Hamilton BA, Ziegler MG, Witztum JL, Schork NJ, Schmid-Schonbein GW & O'Connor DT 2007 C-reactive protein, an 'intermediate phenotype' for inflammation: human twin studies reveal heritability, association with blood pressure and the metabolic syndrome, and the influence of common polymorphism at catecholaminergic/beta-adrenergic pathway loci. *J.Hypertens.* **25** 329-343.

White PC & Slutsker L 1995 Haplotype analysis of *CYP11B2*. *Endocrine Research* **21** 437-442.

White PC 1994 Disorders of aldosterone biosynthesis and action. *N.Engl.J.Med.* **331** 250-258.

White PC 2004 Aldosterone synthase deficiency and related disorders. *Mol.Cell Endocrinol.* **217** 81-87.

White PC, Curnow KM & Pascoe L 1994 Disorders of steroid 11 beta-hydroxylase isozymes. *Endocr.Rev.* **15** 421-438.

White PC, Mune T & Agarwal AK 1997 11 beta-Hydroxysteroid dehydrogenase and the syndrome of apparent mineralocorticoid excess. *Endocr.Rev.* **18** 135-156.

White PC, New MI & Dupont B 1986 Structure of human steroid 21-hydroxylase genes. *Proc.Natl.Acad.Sci.U.S.A* **83** 5111-5115.

White PC & Speiser PW 1994 Steroid 11 beta-hydroxylase deficiency and related disorders. *Endocrinol.Metab Clin.North Am.* **23** 325-339.

White PC & Speiser PW 2000 Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocr.Rev.* **21** 245-291.

Whitworth JA, Mangos GJ & Kelly JJ 2000 Cushing, cortisol, and cardiovascular disease. *Hypertension* **36** 912-916.

Whitworth JA, Saines D, Thatcher R, Butkus A & Scoggins BA 1983 Blood pressure and metabolic effects of ACTH in normotensive and hypertensive man. *Clin.Exp.Hypertens.A* **5** 501-522.

Wielputz MO, Lee IH, Dinudom A, Boulkroun S, Farman N, Cook DI, Korbmacher C & Rauh R 2007 (NDRG2) stimulates amiloride-sensitive Na+ currents in Xenopus laevis oocytes and fisher rat thyroid cells. *J.Biol.Chem.* **282** 28264-28273.

Williams GH 2005 Aldosterone biosynthesis, regulation, and classical mechanism of action. *Heart Fail.Rev.* **10** 7-13.

Williams GH & Dluhy RG 2005 Disorders of the adrenal cortex. In *Harrison's Principles of Internal Medicine*, edn 16, Eds DL Kasper, E Braunwald, AS Fauci, SL Hauser, DL Longo & JL Jameson. New York: McGraw-Hill.

Williams GH, Dluhy RG, Lifton RP, Moore TJ, Gleason R, Williams R, Hunt SC, Hopkins PN & Hollenberg NK 1992 Non-modulation as an intermediate phenotype in essential hypertension. *Hypertension* **20** 788-796.

Williams GH, Rose LI, Dluhy RG, Dingman JF & Lauler DP 1971 Aldosterone response to sodium restriction and ACTH stimulation in panhypopituitarism. *J.Clin.Endocrinol.Metab* **32** 27-35.

Williams TA, Mulatero P, Bosio M, Lewicka S, Palermo M, Veglio F & Armanini D 2004 A particular phenotype in a girl with aldosterone synthase deficiency. *J.Clin.Endocrinol.Metab* **89** 3168-3172.

Wilson FH, Hariri A, Farhi A, Zhao H, Petersen KF, Toka HR, Nelson-Williams C, Raja KM, Kashgarian M, Shulman GI, Scheinman SJ & Lifton RP 2004 A cluster of metabolic defects caused by mutation in a mitochondrial tRNA. *Science* **306** 1190-1194.

Wisgerhof M & Brown RD 1978 Increased adrenal sensitivity to angiotensin II in low-renin essential hypertension. *J.Clin.Invest* **61** 1456-1462.

Wisgerhof M, Carpenter PC & Brown RD 1978 Increased adrenal sensitivity to angiotensin II in idiopathic hyperaldosteronism. *J.Clin.Endocrinol.Metab* **47** 938-943.

Wolffe AP & Matzke MA 1999 Epigenetics: regulation through repression. *Science* **286** 481-486.

Wong S, Brennan FE, Young MJ, Fuller PJ & Cole TJ 2007 A direct effect of aldosterone on endothelin-1 gene expression in vivo. *Endocrinology* **148** 1511-1517.

Woodhead J & Weeks I 1985 Chemiluminescence immunoassay. *Pure and Appl.Chem.* **57** 523-529.

Workman RJ, Sussman CR, Burkitt DW & Liddle GW 1979 Circulating levels of angiotensin I measured by radioimmunoassay in hypertensive subjects. *J.Lab Clin.Med.* **93** 847-856.

Yamamoto N, Yasue H, Mizuno Y, Yoshimura M, Fujii H, Nakayama M, Harada E, Nakamura S, Ito T & Ogawa H 2002 Aldosterone is produced from ventricles in patients with essential hypertension. *Hypertension* **39** 958-962.

Yanase T 1995 17 alpha-Hydroxylase/17,20-lyase defects. *J.Steroid Biochem.Mol.Biol.* **53** 153-157.

Yanase T, Simpson ER & Waterman MR 1991 17 alpha-hydroxylase/17,20-lyase deficiency: from clinical investigation to molecular definition. *Endocr.Rev.* **12** 91-108.

Yaswen L, Diehl N, Brennan MB & Hochgeschwender U 1999 Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat.Med.* **5** 1066-1070.

Yee KM & Struthers AD 1998 Aldosterone blunts the baroreflex response in man. *Clin.Sci.(Lond)* **95** 687-692.

Young DB 1988 Quantitative analysis of aldosterone's role in potassium regulation. *Am.J.Physiol* **255** F811-F822.

Young M, Fullerton M, Dilley R & Funder J 1994a Mineralocorticoids, hypertension, and cardiac fibrosis. *J.Clin.Invest* **93** 2578-2583.

Young M, Fullerton M, Dilley R & Funder J 1994b Mineralocorticoids, hypertension, and cardiac fibrosis. *J.Clin.Invest* **93** 2578-2583.

Young M, Head G & Funder J 1995 Determinants of cardiac fibrosis in experimental hypermineralocorticoid states. *Am.J.Physiol* **269** E657-E662.

Young MJ & Funder JW 1996 The renin-angiotensin-aldosterone system in experimental mineralocorticoid-salt-induced cardiac fibrosis. *Am.J.Physiol* **271** E883-E888.

Young MJ, Moussa L, Dilley R & Funder JW 2003 Early inflammatory responses in experimental cardiac hypertrophy and fibrosis: effects of 11 beta-hydroxysteroid dehydrogenase inactivation. *Endocrinology* **144** 1121-1125.

Young WF 2007 Primary aldosteronism: renaissance of a syndrome. *Clin.Endocrinol.(Oxf)* **66** 607-618.

Yu L, Romero DG, Gomez-Sanchez CE & Gomez-Sanchez EP 2002 Steroidogenic enzyme gene expression in the human brain. *Mol.Cell Endocrinol.* **190** 9-17.

Yuan J, Jia R & Bao Y 2007 Aldosterone up-regulates production of plasminogen activator inhibitor-1 by renal mesangial cells. *J.Biochem.Mol.Biol.* **40** 180-188.

Zeng ZP, Naruse M, Guan BJ, Naruse K, Sun ML, Zang MF, Demura H & Shi YF 1992 Endothelin stimulates aldosterone secretion in vitro from normal adrenocortical tissue, but not adenoma tissue, in primary aldosteronism. *J.Clin.Endocrinol.Metab* **74** 874-878.

Zhang G, Rodriguez H, Fardella CE, Harris DA & Miller WL 1995 Mutation T318M in the CYP11B2 gene encoding P450c11AS (aldosterone synthase) causes corticosterone methyl oxidase II deficiency. *Am.J.Hum.Genet.* **57** 1037-1043.

Zhang MZ, Yao B, Fang X, Wang S, Smith JP & Harris RC 2009 Intrarenal dopaminergic system regulates renin expression. *Hypertension* **53** 564-570.

Zhu H, Sagnella GA, Dong Y, Miller MA, Onipinla A, Markandu ND & Macgregor GA 2003a Contrasting associations between aldosterone synthase gene polymorphisms and essential hypertension in blacks and in whites. *J.Hypertens.* **21** 87-95.

Zhu X, Chang YP, Yan D, Weder A, Cooper R, Luke A, Kan D & Chakravarti A 2003b Associations between hypertension and genes in the renin-angiotensin system. *Hypertension* **41** 1027-1034.

Zhu X, McKenzie CA, Forrester T, Nickerson DA, Broeckel U, Schunkert H, Doering A, Jacob HJ, Cooper RS & Rieder MJ 2000 Localization of a small genomic region associated with elevated ACE. *Am.J.Hum.Genet.* **67** 1144-1153.

Zhu X, Yan D, Cooper RS, Luke A, Ikeda MA, Chang YP, Weder A & Chakravarti A 2003c Linkage disequilibrium and haplotype diversity in the genes of the renin-angiotensin system: findings from the family blood pressure program. *Genome Res.* **13** 173-181.

Ziera T, Irlbacher H, Fromm A, Latouche C, Krug SM, Fromm M, Jaisser F & Borden SA 2009 Cnksr3 is a direct mineralocorticoid receptor target gene and plays a key role in the regulation of the epithelial sodium channel. *FASEB J.*

Zusman RM 1987 Effects of converting-enzyme inhibitors on the renin-angiotensin-aldosterone, bradykinin, and arachidonic acid-prostaglandin systems: correlation of chemical structure and biologic activity. *Am.J.Kidney Dis.* **10** 13-23.