COMMON AND RARE VARIANTS OF THE
RENNIN-ANGIOTENSIN SYSTEM AND
THEIR RELATION TO ANTIHYPERTENSIVE
DRUG RESPONSES

Tuula Hannila-Handelberg

Academic dissertation

To be publicly discussed with the permission of the Faculty of Medicine, University of Helsinki, in the Auditorium of the Department of Oncology, Helsinki University Central Hospital, Haartmaninkatu 4, on December 11th 2009 at 12 noon.

Helsinki 2009
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ISBN 978-952-10-5869-1 (PDF)
http://ethesis.helsinki.fi
Yliopistopaino
Helsinki 2009
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ABBREVIATIONS

11βHSD2 11β-hydroxysteroid dehydrogenase type II
ABP ambulatory blood pressure
ACE angiotensin converting enzyme
ADD1 α-adducin gene
AGT angiotensinogen
AGTR1 angiotensin II type I receptor
AME apparent mineralocorticoid excess
Ang I angiotensin I
Ang II angiotensin II
Ang III angiotensin III
Ang IV angiotensin IV
Ang (1-7) angiotensin (1-7)
Ang (1-9) angiotensin (1-9)
BP blood pressure
BMI body mass index
cDNA complementary DNA
CCT captopril challenge test
ENaC epithelial sodium channel
FH-II familial hyperaldosteronism type II
GRA glucocorticoid-remediable aldosteronism
GWA genome-wide association
MR mineralocorticoid receptor
NCC thiazide-sensitive Na+/Cl- cotransporter
Nedd4-2 neural precursor cell expressed, developmentally down-regulated 4-2
OBP office blood pressure
PCR polymerase chain reaction
PRA plasma renin activity
RAS renin-angiotensin system
RT-PCR reverse transcription-PCR
WCE white coat effect
WNK lysine deficient serine-threonine protein kinase (With No K = lysine)

In addition, standard one-letter and three-letter abbreviations are used for nucleotides and amino acids.
ABSTRACT

Most of the diseases affecting public health, like hypertension, are multifactorial by etiology. Hypertension is influenced by genetic, lifestyle, and environmental factors. Estimation of the influence of genes to the risk of essential hypertension varies from 30 to 50%. It is plausible that in most of the cases susceptibility to hypertension is determined by the action of more than one gene. Although the exact molecular mechanism underlying essential hypertension remains obscure, several monogenic forms of hypertension have been identified. Documented forms of monogenic forms of hypertension include Liddle’s syndrome, glucocorticoid-remediable aldosteronism and apparent mineralocorticoid excess, which result in increased reabsorption of sodium in the kidneys with subsequent increase of blood pressure (BP). Since common genetic variations may predict, not only to susceptibility to hypertension, but also response to antihypertensive drug therapy, pharmacogenetic approaches may provide useful markers in finding relations between candidate genes and phenotypes of hypertension.

The aim of this study was to identify genetic mutations and polymorphisms contributing to human hypertension, and examine their relationships to intermediate phenotypes of hypertension, such as BP responses to antihypertensive drugs or biochemical laboratory values.

Two groups of patients were investigated in the present study. The first group was collected from the database of patients investigated in the Hypertension Outpatient Ward, Helsinki University Central Hospital, and consisted of 399 subjects considered to have essential hypertension. Secondary forms of hypertension have been excluded. Frequencies of the mutant or variant alleles were compared with those in two reference groups, healthy blood donors (n = 301) and normotensive males (n = 175). The second group of subjects with hypertension was collected prospectively. Altogether 313 male subjects were screened for the study. The study subjects underwent a protocol lasting eight months, including four one-month drug treatment periods with antihypertensive medications (thiazide diuretic, β-blocker, calcium channel antagonist, and an angiotensin II receptor antagonist). A total of 208 subjects completed the study. Each
drug treatment period was preceded by one-month placebo period. BP responses and laboratory values were related to polymorphisms of several candidate genes of the renin-angiotensin system (RAS). In addition, two patients with typical features of Liddle’s syndrome were screened for mutations in kidney epithelial sodium channel (ENaC) subunits.

Two novel mutations causing Liddle’s syndrome were identified. The first mutation identified located in the β-subunit of ENaC affecting the PY motif. The second mutation found located in the γ-subunit, constituting the first identified Liddle mutation locating in the extracellular domain. This mutation showed 2-fold increase in channel activity in vitro. Three gene variants, of which two are novel, were identified in ENaC subunits. The prevalence of the variants was three times higher in hypertensive patients (9%) than in reference groups (3%). The variant carriers had increased daily urinary potassium excretion rate in relation to their renin levels compared with controls suggesting increased ENaC activity, although in vitro they did not show increased channel activity.

Of the common polymorphisms of the RAS studied, angiotensin II receptor type I (AGTR1) 1166 A/C polymorphism was associated with modest changes in RAS activity. Thus, patients homozygous for the C allele tended to have increased aldosterone and decreased renin levels. In vitro functional studies using transfected HEK293 cells provided additional evidence that the AGTR1 1166 C allele may be associated with increased expression of the AGTR1. Common polymorphisms of the α-adducin (ADD1 Gly460Trp) and the RAS (AGTR1 1166 A/C, ACE I/D and AGT Met235Thr) genes did not significantly predict BP responses to one-month monotherapies with hydrochlorothiazide, bisoprolol, amlodipin, or losartan.

In conclusion, two novel mutations of ENaC subunits causing Liddle’s syndrome were identified. In addition, three common ENaC polymorphisms were shown to be associated with occurrence of essential hypertension, but their exact functional and clinical consequences remain to be explored. The AGTR1 1166 C allele may modify the endocrine phenotype of hypertensive patients, when present in homozygous form. Certain widely studied polymorphisms of the ACE, angiotensinogen, AGTR1 and α-
adducin genes did not significantly affect responses to a thiazide, β-blocker, calcium channel antagonist, and angiotensin II receptor antagonist.
INTRODUCTION

Hypertension affects about 25% of adult population in industrialised societies (Lifton et al. 2001). It constitutes one of the major risk factors for ischemic heart disease, stroke, and end stage renal disease, and shortens predicted life-expectancy (Gong, Hubner 2006). Hypertension causes morbidity and mortality, and because of its high prevalence, it also presents national health as well as economical burden (Kearney et al. 2005). Of five million Finns, 500 000 people taking antihypertensive medication are subsidised by the Social Insurance Institution of Finland in the upper compensation class (www.kela.fi).

Based on epidemiological studies, systolic blood pressure (BP) tends to rise until the age of 80 years, whereas diastolic BP rises only until the age of 50 to 60 years, after which it starts to decline (Franklin et al. 1997). In majority of cases, the etiology for high BP is unknown, and the disorder is called as essential hypertension. In the remaining 5 to 10% of cases, pathophysiological link to hypertension can be identified (secondary hypertension). Common causes for secondary hypertension are obstructive sleep apnoea, primary hyperaldosteronism, renal artery stenosis or renal parenchymal diseases. Less common causes are pheochromocytoma, Cushing’s syndrome, hyperparathyreoidism, aortic coarctation, or intracranial tumor (Calhoun et al. 2008).

Essential hypertension is a multifactorial disease determined by environmental influences, including excessive salt intake, obesity, psychosocial stress, physical inactivity or alcohol, and genetic factors (Materson 2007). Estimates of genetic components on hypertension range from 30 to 50% (Romano-Spica et al. 2003). Evidence derived from family studies has shown greater concordance of BP in biological siblings than adoptive siblings living in the same household (Rice et al. 1989). Twin studies have also documented higher degree of correlation among monozygous twins in comparison with dizygous twins, or biological siblings (Williams et al. 1990). However, until now no definitive gene alteration causing susceptibility to common essential hypertension has been identified. In contrast, molecular genetic studies have identified several genes causing Mendelian forms of hypertension,
providing new insights into mechanisms regulating BP (Lifton et al. 2001, Lifton 1996). However, monogenic diseases causing hypertension seem to be very rare, and less than 0.1% of population is estimated to carry such a mutation (Rossier, Schild 2008).

A difficulty in finding genes affected in essential hypertension results from the polygenic nature of inheritance, interactions of multiple genes regulating BP, interaction of genes and environmental factors, differences in demographic features as well as age-dependent penetrance (Risch, Merikangas 1996). In addition, the simple phenotyping into hypertension and normotension may result in missclassifications. One approach to study genes involved in essential hypertension is to phenotype subjects according to biochemical laboratory values and responses to antihypertensive drug therapy, in order to sharpen the subphenotyping of the disease (Turner et al. 2001).

Identification of genes responsible for hypertension may provide new diagnostic tools as well as etiological classification of hypertension, and provide new targets for therapeutic interventions in the future. The main purpose of this study was to identify genes causing susceptibility to hypertension by exploring relations between genetic mutations and variants, and clinical characteristics, such as laboratory variables and BP responses to antihypertensive drugs, in hypertensive patients.
REVIEW OF THE LITERATURE

1. Blood pressure and hypertension

1.1 Definition of blood pressure and high blood pressure

BP is a continuous trait varying during each cardiac cycle. Arterial BP is the pressure inside the large arterial vessels. It is controlled by cardiac output and peripheral resistance. When the left ventricle of the heart contracts to eject blood to large arteries, the highest pressure inside the vessels is systolic, and the lowest pressure just before systole is called diastolic BP (Guyton 1991).

Hypertension is chronically elevated BP. Definition of high BP is greater than the upper range of accepted normal. Based on epidemiological studies, it can be defined as the level above of which therapeutic interventions have been shown clinical benefits to reduce the risk of endpoints (Lifton et al. 2001). There has been a downward direction towards the determination between normal BP level and hypertension. According to the latest report of the European Society of Hypertension and the European Society of Cardiology, BP can be classified in categories (Mancia et al. 2007). Optimal adult values for systolic and diastolic BP levels in office measurements are < 120/80 mmHg, normal values are between 120-129/80-84, and high normal 130-139/85-89. Grade I hypertension includes levels between 140-159/90-99, grade II 160-179/100-109, and grade III ≥ 180/110 for systolic and diastolic BP values. Office BP (OBP) values of 140/90 mmHg correspond to home BP level of 135/85 mmHg, and to average 24-hour ambulatory BP (ABP) level of 135/85 mmHg, average daytime and night-time values being 140/90 and 125/75, respectively (www.kaypahoito.fi). According to the Finnish Society for Hypertension, individual’s BP level is determined as the average of at least four measurements recorded in separate visits, where a mean of two measurements is included (www.kaypahoito.fi). The diagnosis of hypertension is defined when the average systolic BP rises over level 140 mmHg, or diastolic over 90 mmHg constantly.
1.2 Prevalence of hypertension

Hypertension affects about 25% of adult population in western societies (Lifton et al. 2001). It has been estimated that almost 60 million individuals in the USA and one billion worldwide are affected (Hajjar, Kotchen 2003). There are also racial differences in the nature and prevalence of hypertension (Pratt et al. 2002). In Finland, antihypertensive medication of over half a million people was subsidised in the upper compensation class by the Social Insurance Institution of Finland in 2007 (www.kela.fi). In addition, there were also subjects on antihypertensive mediation subsidised in the lower compensation class, and these cases are not included in statistics. During a 20-year follow-up from 1982 to 2002, BP level has decreased in the Finnish population (Kastarinen et al. 2006). In 2002, the prevalence of hypertension in working population was estimated to reach about 50% among males and 30% among females. BP level was higher in males than in females. From 2002 to 2007, previous down-ward trend in prevalence of hypertension has been slower or even elevated (Kastarinen et al. 2009). In international comparison, BP level is still high in Finnish population, when six European countries, including England, Germany, Italy, Spain, Sweden, and Canada and the USA, were compared (Wolf-Maier et al. 2003). Thus, the prevalence of hypertension was the second highest in Finland (49%), after Germany (55%). The average of the European prevalence of hypertension was 44% compared with the prevalence numbers of 28% in North-America.

1.3 Blood pressure regulation

The purpose of BP is to maintain tissue perfusion with oxygen and nutrients. Under normal circumstances, arterial BP deviates 10 to 15% from its usual level (Guyton 1991). The body has several mechanisms to maintain BP within optimal level. The main two systems are the central nervous system, by adjusting the diameter of blood vessels and the heart rate, and the kidneys, by regulating electrolyte and water homeostasis. The central nervous system controls circulatory system mainly with autonomous nervous system, which effects are mediated by adrenaline and noradrenaline. Electrolyte and
water balance and long-term regulation of BP is mainly controlled by the renin-angiotensin system (RAS) (Guyton 1991). Electrolyte homeostasis is regulated by sodium transporters along nephrons including Na+/H+ exchangers in the proximal tubule, Na+/K+2Cl- cotransporters in the thick ascending limb of Henle, Na+/Cl-cotransporters in distal convoluted tubule, and epithelial sodium channel (ENaC) in the distal tubule and collecting duct (Su, Menon 2001). Figure 1 provides a simplified summary on the mechanisms of BP regulation.

Figure 1. Mechanisms of arterial blood pressure regulation
RAS = renin-angiotensin system, ANP = atrial natriuretic peptide, NO = nitric oxide, Dash line = negative feedback (adapted from Cowley 2006).
1.4 Blood pressure measurement

The Finnish Society for Hypertension has established guidelines for the treatment of elevated BP, including the instructions of BP measurement. According to the guidelines, the diagnosis of elevated BP and the decision to start antihypertensive drug therapy should be based on duplicate measurements made at least on four different occasions (www.kaypahoito.fi).

OBP measurement is in routine use in evaluating BP level, measured by a doctor or a nurse. At least two measurements spaced by 1 to 2 minutes per visit are recommended (Mancia et al. 2007). Self-measurement of BP at home is alternative for OBP measurement. Its advantages are easiness to put in practice, possibility to carry out recordings on different days and for a longer follow-up period, and avoidance of significant white coat effect (WCE). Home measurements may more accurately predict cardiovascular events than OBP measurements (Mancia et al. 2007). In addition, OBP measurements may over-estimate BP levels and under-estimate the control with antihypertensive medication, compared with self-made measurements at home, which usually give lower values than OBP measurements. Therefore, experts recommended using home measurements as an important aid in clinical practise (Niiranen et al. 2006a).

24-hour ABP measurements provide additional information of daytime and night-time average BP levels. ABP is usually lower than OBP. ABP measurement is recommended in cases when there is a large variability in OBP measurements during the same or different visits, when there is an inconsistency between OBP and home measurements, or when there is a suspicion of resistance to drug treatment or occurrence of hypotensive episodes (Mancia et al. 2007). Especially, night-time BP values have prognostic value. Thus, non-dippers (night-time BP decrease is blunted) have been reported to have a greater prevalence of organ damages (Mancia et al. 2007). ABP also predicts better organ damages than OBP. In adjustment of antihypertensive drug treatment, ABP monitoring and home measurements were comparable, when using the same BP target (Niiranen et al. 2006b).
2. Renin-angiotensin system and sodium homeostasis

2.1 Renin-angiotensin system

Sodium balance and long-term BP level is mainly regulated by the RAS. In the cascade of the RAS, the limiting factor under physiological conditions is renin, first discovered in 1898 by Finnish scientist Robert Tigerstedt (Fyhrquist, Saijonmaa 2008). Renin is an enzyme secreted by the juxtaglomerular cells in the kidneys (Corvol, Jeunemaitre 1997). Factors regulating renin secretion are intrarenal BP (at the juxtaglomerular apparatus), sodium concentration in the renal tubules (at macula densa), or activation of sympathetic nerves in the kidneys. Hypotension, hyponatremia, stenosis in renal artery and increased activity of sympathetic nervous system activate renin excretion. An upright posture as well as morning time likewise stimulate renin secretion (Nomura et al. 1992). The renin substrate angiotensinogen (AGT) synthesized by the liver is cleaved by renin to angiotensin I (Ang I). Ang I is converted to vasoactive peptide angiotensin II (Ang II) by angiotensin converting enzyme (ACE). The effects of Ang II are mediated by angiotensin II type 1 (AGTR1) and type 2 receptors. AGTR1 mediates vasoconstriction, thirst, release of vasopressin, and aldosterone in adrenal cortex. Ang II has also been shown to be involved in inflammatory process including atherosclerosis and ageing (Fyhrquist, Saijonmaa 2008). Ang II type 2 receptors generally mediate opposing effects compared with those mediated by AGTR1, including vasodilatation and release of nitric oxidase. Aldosterone is a steroid hormone secreted by the zona glomerulosa of the adrenal cortex, regulating sodium and potassium balance. It binds to mineralocorticoid receptor (MR) and activates the ENaC in the distal tubule and collecting duct cells of the kidneys, resulting in increased sodium and subsequent water reabsorption (Lee et al. 2000).

Classical RAS has expanded after the identification of new angiotensins (Ruiz-Ortega et al. 2007). Angiotensin III (Ang III) generated from Ang II exerts its actions similar to those of Ang II. Angiotensin IV (Ang IV) is formed from Ang III, and has been considered to mediate vasodilatatory effects via insulin-related amino peptidase receptors (IRAP). Angiotensin (1-9) (Ang (1-9)) can be formed from Ang I.
Angiotensin (1-7) (Ang (1-7)) is generated from Ang II or from Ang I. Ang (1-7) has been found to have actions opposing those of Ang II by binding to the mas receptor which mediates vasodilating effects. Ang (1-7) may have cardiovascular protective effects by regulating BP. Homologous to ACE is enzyme ACE II that degrades Ang II to Ang (1-7) and converts Ang I to Ang (1-9). Renin or prorenin binds to specific receptor, which is thought to potentiate the effects of renin by increasing the conversion rate of Ang I to Ang II. In addition to circulating RAS, there is a local tissue RAS in most tissues and organs (Fyhrquist, Saijonmaa 2008). Simplified view of the RAS pathway is shown in Figure 2.
2.2 Epithelial sodium channel

ENaC expression has been documented in several organs, including lungs, salivary glands, sweat glands, colon, and the kidneys (Snyder 2005). In the kidneys, ENaC is located in the distal collecting tubule, being the last step in regulating sodium balance in humans. ENaC is activated by several hormones, particularly aldosterone, vasopressin, and insulin (Snyder 2005). The channel is composed of α-, β- and γ-subunits. Each subunit contains two transmembrane domains, an extracellular loop spanning the plasma membrane twice, and carboxy (COOH) and amino (NH₂) termini in the cytoplasm. All three subunits are co-localised in the apical membrane of the distal collecting duct (Canessa et al. 1994). For normal channel regulation, all three subunits are required (Schild et al. 1996). The channel expression on the cell surface is controlled by an intracellular enzyme called Nedd4-2, which belongs to a Nedd4 ubiquitin ligase enzyme family (Rotin 2008). Nedd4-2 has a compatible WW (tryptophan-tryptophan) binding site with the proline-rich amino acid sequence of the carboxy terminus of ENaC, called PY motif. The interaction between the WW domain of Nedd4-2 and the PY motif of ENaC is essential for the inhibition of ENaC activity, resulting in internalisation and degradation of ENaC (Gormley et al. 2003). The disruption of PY motif prevents its interaction with Nedd4-2, and thus, ENaC remains activated on the cell surface, instead of its normal inactivation by internalisation. The proposed stoichiometry of ENaC, 1α:1β:1γ, is based on the structure of chicken acid-sensitive ion channel ASIC, which belongs to the same ion channel family (Figure 3) (Jasti et al. 2007). ENaC subunits are also expressed in the cardiovascular system where they may function as mechanosensors and chemosensors (Drummond et al. 2008).
These three subunits of ENaC are encoded by three separate genes. The gene coding for \( \alpha \)-subunit (SCNN1A) is located on chromosome 12p13 (Iwai et al. 2001). The genes coding for the \( \beta \)-subunit (SCNN1B) and \( \gamma \)-subunit (SCNN1G) are co-localised on chromosome 16p12-p13 (Voilley et al. 1995). Several mutations in the carboxy terminal domain that truncate or change the DNA sequence of the last exon in the \( \beta \)-ENaC or \( \gamma \)-ENaC subunits affecting the PY motif, have been found to be responsible for Liddle's syndrome, a rare form of autosomal dominant hypertension (Shimkets et al. 1994, Hansson et al. 1995a). Instead, a number of mutations in the amino terminal end and extracellular loop in either \( \alpha \)-, \( \beta \)- or \( \gamma \)-ENaC subunits were found to result in pseudohypoaldosternism type I, a severe salt wasting and hypotensive disease (Chang et al. 1996, Strautnieks et al. 1996). Mutations located in the different ENaC subunits are summarised in Figure 4.
3. Strategies to approach genes involved in hypertension

Two main strategies to map genetic variants influencing the risk of hypertension are linkage and association analyses, which can be applied in candidate gene and genome-wide studies (Binder 2007). Recently, genome-wide scans have occupied an important role, like in all complex diseases. Pharmacogenetic approach entails evaluation of genetic targets in relation to individual’s drug responses (Turner et al. 2001).

The selection of candidate genes has been based on understanding of the pathophysiologic role of the encoded proteins in BP regulation (Cowley 2006). Candidate gene association studies compare the frequencies of polymorphic alleles between unrelated affected patients and non-affected healthy controls (cases vs. controls) (Risch, Merikangas 1996). Many candidate genes have been components of the RAS (Lifton et al. 2001).

Genome-wide linkage analysis can be applied in families with multiple affected family members. In genome-wide linkage studies, hundreds of polymorphic markers are
genotyped across the genome (Binder 2007) to identify markers that segregate more often than expected in affected family members. Linkage analysis gives likelihood ratios (logarithm of the odds, LODs) for the association of chromosomal regions with the disease (Lander, Schork 1994). Suggestive linkage for hypertension has been identified for almost all chromosomes (Binder 2007, Samani 2003). However, the results have been difficult to replicate in other studies (Binder 2007, Samani 2003). Even large-scale searches for genetic variants predisposing to essential hypertension have failed to demonstrate definite linkage to any chromosomal loci (Koivukoski et al. 2004, Liu et al. 2004, Rice et al. 2006, Wu et al. 2006). In contrast, genome-wide linkage analysis has been applied successfully for single-gene disorders, such as Liddle’s syndrome (Shimkets et al. 1994). A genome-wide scan study comprising of hypertensive individuals from the Finnish Twin Cohort provided evidence of chromosomal locus 3q as the best contributor to human essential hypertension (Perola et al. 2000).

The completion of the Human Genome Project and HapMap Project have made possible to execute large-scale genome-wide association (GWA) studies (Huang et al. 2009). A GWA study is an approach to find genetic variations (i.e. single nucleotide polymorphisms) in association with a particular disease or trait of interest. Large number of genetic variants are genotyped and then analysed for trait or disease association in GWA studies. Previously, two GWA studies on hypertension could not provide reliable evidence of genetic association on hypertension (Wellcome Trust Case Control Consortium 2007, Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research et al. 2007). A subsequent replication study of the top 6 single nucleotide polymorphisms from the former study, failed to show plausible variants for hypertension (Ehret et al. 2008). In contrast, quite recently novel hypertension targets have been published among patients with young-onset hypertension in Taiwanese population (Yang et al. 2009). Two GWA studies including participants of European ancestry found several variants with evidence of association with BP and hypertension (Newton-Cheh et al. 2009, Levy et al. 2009). However, each variant in original (Newton-Cheh et al. 2009) and replicate analysis
(Levy et al. 2009) explains only 0.05 to 0.10% of total BP variation, and 1 mmHg for systolic and 0.5 mmHg for diastolic BP per variant allele.

4. Hypertension due to single-gene abnormalities

Mendelian or monogenic forms of hypertension are caused by mutations in single genes (for reviews, see Lifton et al. 2001, Staessen et al. 2003, Stowasser, Gordon 2006). These include Liddle’s syndrome, apparent mineralocorticoid excess (AME), glucocorticoid-remediable aldosteronism (GRA), Gordon’s syndrome, familial hyperaldosteronism type II (FH II), specific form of hypertension exacerbated by pregnancy, and autosomal dominant hypertension with brachydactyly. Table 1 lists monogenic forms of hypertension and hypotension, as reviewed by Staessen et al. (2003). Most of them affect directly or indirectly the distal nephron, resulting in sodium retention, and thereby hypertension (Lifton et al. 2001). Less-severe mutations linked to monogenic forms of hypotension may be expected to be protective against the development of hypertension (Rossier, Schild 2008).
Table 1. Monogenic forms of hypertension and hypotension characterized at the molecular level

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</tr>
<tr>
<td>excess (AME)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoid-removable</td>
<td>autosomal dominant</td>
<td>CYP11B1 and CYP11B2, fusion gene</td>
<td>( \text{renin, aldosterone, hypokalemia} )</td>
</tr>
<tr>
<td>aldosteronism (GRA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gordon’s syndrome</td>
<td>autosomal dominant</td>
<td>WNK1 and WNK4</td>
<td>( \text{renin, aldosterone, hypokalemia} )</td>
</tr>
<tr>
<td>HT exacerbated by pregnancy</td>
<td>autosomal dominant</td>
<td>MR</td>
<td>( \text{renin, aldosterone, hypokalemia} )</td>
</tr>
<tr>
<td>HT with brachydactyly</td>
<td>autosomal dominant</td>
<td>Mapped to chromosome 12</td>
<td>( \text{short fingers, renin and aldosterone} )</td>
</tr>
<tr>
<td>Mutations in peroxisome</td>
<td>autosomal dominant</td>
<td>PPARG</td>
<td>( \text{insulin resistance, diabetes mellitus,} )</td>
</tr>
<tr>
<td>proliferator-activated receptor-( \gamma )</td>
<td></td>
<td></td>
<td>( \text{hypertension} )</td>
</tr>
<tr>
<td>Familial hyperaldosteronism</td>
<td>autosomal dominant</td>
<td>mapped to chromosome 7p22</td>
<td>( \text{renin, aldosterone} )</td>
</tr>
<tr>
<td>type II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Congenital adrenal hyperplasia (CAH)*</td>
<td>autosomal recessive</td>
<td>CYP21A2, CYP11B1, CYP17</td>
<td>( \text{renin, aldosterone, hypokalemia} )</td>
</tr>
<tr>
<td>Familial glucocorticoid</td>
<td>autosomal dominant /</td>
<td>GR</td>
<td>( \text{renin, aldosterone, hypokalemia} )</td>
</tr>
<tr>
<td>resistance*</td>
<td>recessive</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hypotension</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA 1*</td>
<td>autosomal recessive</td>
<td>( \alpha^-), ( \beta^-) and ( \gamma^-)ENaC (inactivation)</td>
<td>( \text{aldosterone, hyperkalemia} )</td>
</tr>
<tr>
<td>PHA 1*</td>
<td>autosomal dominant</td>
<td>MR</td>
<td>( \text{aldosterone, hyperkalemia} )</td>
</tr>
<tr>
<td>Bartter's syndrome</td>
<td>autosomal recessive</td>
<td>NKCC2, ROMK, CLCNKB</td>
<td>( \text{renin, aldosterone} )</td>
</tr>
<tr>
<td>Gitelman's syndrome</td>
<td>autosomal recessive</td>
<td>NCC</td>
<td>( \text{renin, aldosterone, hypokalemia} )</td>
</tr>
</tbody>
</table>

HT = hypertension, GR = glucocorticoid receptor, PHA = pseudohypoaldosteronism, NKCC2 = Na+K+Cl− cotransporter, ROMK = renal outer medullary potassium channel, CLCNKB = kidney specific chloride channel, NCC = Na+Cl− cotransporter.

* typical age of onset in infancy. (Modified from Staessen et al. 2003)

4.1 Liddle’s syndrome

The first report of patients with this syndrome was described in 1963 by the American physician Grant Liddle who examined siblings with early-onset hypertension. Typical manifestations along with hypertension were low plasma renin, low aldosterone, hypokalemic alkalosis, and diminished urinary excretion of aldosterone (Liddle et al. 1963). The female index patient had 18 affected family members. Her BP did not
respond to spironolactone. She was later found to develop end-stage renal disease, and after renal transplantation her BP was normalised (Botero-Velez et al. 1994).

Liddle’s syndrome is inherited according to an autosomal dominant model. Linkage analyses localised the gene causing Liddle’s syndrome to chromosome 16p12 (Shimkets et al. 1994). In 1994, Shimkets et al. (1994) were the first to demonstrate mutations in the β-subunit of the ENaC gene in five different kindreds with Liddle’s syndrome. The first kindred was the original one described by Liddle in 1963 (Liddle et al. 1963). The functional significance of this mutation in vitro was elucidated by Schild et al. (1995). The mutated β-ENaC gene in combination with the normal α-ENaC and γ-ENaC genes resulted in increased amiloride-sensitive sodium current, compared with three normal subunits when expressed in Xenopus oocytes. In 1995, Hansson et al. described the first Liddle mutation in the γ-ENaC gene (Hansson et al. 1995a). Until now, and excluding the data of the present study, molecular genetic surveys of patients with Liddle’s syndrome have identified altogether 14 mutations in the β-ENaC and three in the γ-ENaC genes. The mutations either delete the PY motif in the β-subunit truncating the last 34 to 76 amino acids from of the C-terminal domain (Shimkets et al. 1994, Jeunemaitre et al. 1997, Jackson et al. 1998, Inoue et al. 1998b, Melander et al. 1998, Kyuma et al. 2001, Nakano et al. 2002) or γ-subunit (Hansson et al. 1995a, Yamashita et al. 2001, Wang et al. 2007), or change the sequence of the PY motif in the β-subunit (Yamashita et al. 2001, Hansson et al. 1995b, Tamura et al. 1996, Uehara et al. 1998, Inoue et al. 1998a, Gao et al. 2001, Furuhashi et al. 2005, Freundlich, Ludwig 2005, Ciechanowicz et al. 2005, Wang et al. 2006, Rossi et al. 2008, Sawathiparnich et al. 2009) (for summary, see Table 2). No mutations of the α-subunit have been shown to cause Liddle’s syndrome.

Mutations identified remove or disturb the sequence of the proline-rich PY motif of the cytoplasmic carboxy termini. This alteration prevents the interaction of the PY motif with the cytoplasmic enzyme Nedd4-2, which results in increased activity of ENaC, increased sodium and subsequent water reabsorption, increased potassium excretion, and inhibition of the RAS with low renin and low aldosterone levels in patients with Liddle’s syndrome. The increased activity of ENaC is based on increased expression of
ENaC on cell surface, by preventing its interaction to Nedd4-2, thus increasing sodium ion transport of ENaC (Knight et al. 2006).

Patients with Liddle’s syndrome respond favourably to treatment with amiloride or triamterene, which both are ENaC blockers, in combination with low-salt diet. According to the case report, also renal transplantation from healthy organ donor can cure hypertension and hypokalemia (Botero-Velez et al. 1994). Spironolactone, which is aldosterone antagonist, is ineffective.

Table 2. Mutations of the subunits of the epithelial sodium channel (ENaC) causing Liddle's syndrome. Reports from other studies.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nucleotide change</th>
<th>Ethnicity of the index case</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg564stop **</td>
<td>C→T</td>
<td>North-American</td>
<td>Shimkets et al. 1994*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Swedish</td>
<td>Melander et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Japanese</td>
<td>Kyuma et al. 2001</td>
</tr>
<tr>
<td>Qln589stop</td>
<td>C→T</td>
<td>North-American?</td>
<td>Shimkets et al. 1994</td>
</tr>
<tr>
<td>Thr592Fr</td>
<td>C insertion</td>
<td>North-American?</td>
<td>Shimkets et al. 1994</td>
</tr>
<tr>
<td>Arg595Fr</td>
<td>C deletion</td>
<td>North-American?</td>
<td>Shimkets et al. 1994</td>
</tr>
<tr>
<td>579del32</td>
<td>Deletion of 32 nucleotides</td>
<td>Portuguese</td>
<td>Jeunemaitre et al. 1997*</td>
</tr>
<tr>
<td>Arg597Fr</td>
<td>C insertion</td>
<td>Japanese</td>
<td>Inoue et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>British</td>
<td>Jackson et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Japanese</td>
<td>Nakano et al. 2001</td>
</tr>
<tr>
<td>β-ENaC / PY motif deletion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro615Ser</td>
<td>C→T</td>
<td>Japanese</td>
<td>Inoue et al. 1998*</td>
</tr>
<tr>
<td>Pro616Leu</td>
<td>C→T</td>
<td>African-American</td>
<td>Hansson et al. 1995*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Japanese</td>
<td>Uehara et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chinese</td>
<td>Gao et al. 2001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Japanese</td>
<td>Yamashita et al. 2001</td>
</tr>
<tr>
<td>Pro616Ser</td>
<td>C→T</td>
<td>Japanese</td>
<td>Uehara et al. 1998</td>
</tr>
<tr>
<td>Pro616Arg</td>
<td>C→G</td>
<td>Japanese</td>
<td>Furuhashi et al. 2005*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Czech</td>
<td>Ciechanowicz et al. 2005</td>
</tr>
<tr>
<td>Tyr618His</td>
<td>T→C</td>
<td>Japanese</td>
<td>Tamura et al. 1996*</td>
</tr>
<tr>
<td>Pro616His</td>
<td>C→A</td>
<td>Afro-Haitian</td>
<td>Freundlich et al. 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chinese</td>
<td>Wang et al. 2006</td>
</tr>
<tr>
<td>Pro617Leu</td>
<td>C→T</td>
<td>Italian</td>
<td>Rossi et al. 2008*</td>
</tr>
<tr>
<td>Pro615His</td>
<td>C→A</td>
<td>Thai</td>
<td>Sawathiparnich et al. 2009</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp574stop</td>
<td>G→A</td>
<td>Japanese</td>
<td>Hansson et al. 1995*</td>
</tr>
<tr>
<td>Trp576stop</td>
<td>G→A</td>
<td>Japanese</td>
<td>Yamashita et al. 2001</td>
</tr>
<tr>
<td>Glu583Fr</td>
<td>del of AGCTC</td>
<td>Chinese</td>
<td>Wang et al. 2007</td>
</tr>
</tbody>
</table>

*Functional significance tested in Xenopus oocytes
**original Liddle case
4.2 Syndrome of apparent mineralocorticoid excess

AME is an autosomal recessive disease first described in the 1970’s (New et al. 1977, Wilson et al. 2001b). Clinical manifestations are similar to those seen in Liddle’s syndrome, including hypertension with low plasma potassium, low renin and low aldosterone levels. The diagnosis has been based on elevated urinary cortisol to cortisone ratio in hypertensive patients, including hypokalemia, low renin and aldosterone, and metabolic alkalosis (Quinkler et al. 2004).

Cortisol, which is secreted from the zona fasciculata of the adrenal cortex, has the same affinity as aldosterone to MR in vitro, but in vivo aldosterone has more potent affinity. In cells expressing MR, cortisol is metabolised to inactive cortisone by the enzyme 11β-betahydroxysteroid dehydrogenase type II (11βHSD2), thus protecting MR from cortisol present in ca. 100-fold higher concentrations in plasma (Wilson et al. 2001b). In AME, the defect is caused by homozygous or compound heterozygous mutations in the gene encoding 11βHSD2 (Mune et al. 1995, Dave-Sharma et al. 1998). Mutations result in decreased enzymatic activity of 11βHSD2, and the excess of cortisol activates MR, resulting in increased activation of ENaC (Figure 2). Thus, reabsorption of sodium and water are increased resulting in raised BP. The 11βHSD2 gene has been located to human chromosome 16q22 (Agarwal et al. 1995, Krozowski et al. 1995). Over 30 different mutations in exons 2-5 have been reported, comprising missense mutations, deletions and insertions (Quinkler et al. 2004). In principle, heterozygous subjects have normal phenotype, but it has been suggested that heterozygous state might predispose to essential hypertension. Even in subjects homozygous for the mutation causing AME, phenotype may vary widely (Morineau et al. 2006). There is evidence that reduced 11βHSD2 activity might be a factor in a subset of patients with essential hypertension (Soro et al. 1995). In Japanese population, rare missense mutations in the 11βHSD2 gene were not related to essential hypertension (Kamide et al. 2006). When studying compound heterozygous AME patients, Lavery et al. (2003) found that the heterozygous parents of the patients often presented with features of essential hypertension. An experimental study with the mouse model of AME hypothesises that
abnormal renal dysfunction through imbalance in electrolyte levels promotes a cascade to chronic hypertension (Bailey et al. 2008).

AME can be treated by reducing endogenous production of cortisol with dexamethasone, MR antagonist spironolactone, ENaC inhibitors, and thiazidic diuretics to reduce hypercalciuria (Wilson et al. 2001b). Kidney transplantation has also been reported to cure AME (Palermo et al. 1998).

4.2.1 Liquorice syndrome

An acquired form of AME is liquorice syndrome, which may result from chronic ingestion of large amounts of liquorice products (Palermo et al. 2004). Liquorice can be separated from the roots of *Glycyrrhiza glabra*. Due to its sweet taste, it has been applied in various products, such as chocolate, chewing gum and ice cream. Liquorice contains glycyrrhetinic acid, which is an inhibitor of 11βHSD2. Inhibition leads to a condition reminiscent of AME, including hypokalemia, suppressed plasma renin and aldosterone as well as hypertension. The effect of liquorice to BP and electrolytes is reversible (Stewart et al. 1987). Like AME, liquorice syndrome also responds to spironolactone (Palermo et al. 2004).

4.3 Glucocorticoid-remediable aldosteronism

GRA, also called familial hyperaldosteronism type I, was first described in 1966 (Sutherland et al. 1966). It is inherited in an autosomal dominant fashion. Clinical characteristics vary from mild to severe hypertension with hypokalemia, low renin and increased aldosterone levels. Patients may be misdiagnosed to have primary aldosteronism. The diagnosis of GRA is based on elevated 18-oxocortisol, but genetic testing is also available (McMahon, Dluhy 2004).
The molecular hallmark underlying GRA is a mutation in chromosome 8, which contains the genes \textit{CYP11B1}, coding for 11\(\beta\)-hydroxylase, and \textit{CYP11B2}, coding for aldosterone synthase. An unequal crossover at meiosis produces a fusion gene derived from these two genes (Lifton et al. 1992). Normally, 11\(\beta\)-hydroxylase catalyses the conversion of 11-deoxycorticol to cortisol in adrenal cortex, and aldosterone synthase converts corticosterone to aldosterone. Aldosterone is usually stimulated by Ang II. In GRA, the fusion gene, which has aldosterone syntase activity, results in ectopic aldosterone overproduction from zona fasciculata, instead of zona glomerulosa, and under the regulation of ACTH, is not suppressed as would be under the regulation of Ang II. This leads to upregulation of ENaC, increased sodium and water reabsorption, and hypertension with low renin but increased aldosterone (McMahon, Dluhy 2004).

Patients with GRA can be treated with administration of glucocorticoid analogues such as dexamethasone, which suppress ACTH secretion by negative feedback, and they also respond to spironolactone (and eplerenone), which are mineralocorticoid receptor antagonists. Amiloride and triamterene may also be effective by blocking ENaC in the distal nephron (McMahon, Dluhy 2004, Dluhy, Lifton 1999).

4.4 Familial hyperaldosteronism type II

FH-II is a rare form of primary aldosteronism initially described in patients, whose condition resembled GRA, but was not suppressed by a dexamethasone challenge test, as distinct from GRA (Stowasser et al. 1992). Transmission of phenotype has followed an autosomal dominant model of inheritance in 15 families, but remained unclear in other 24 families (Sukor et al. 2008). The underlying genetic defect has been localised to chromosome 7p22, although the exact genetic mechanism has not been elucidated (Sukor et al. 2008, Lafferty et al. 2000). Hypertension in FH-II is responsive to spironolactone, but not to glucocorticoids (Garovic et al. 2006).
4.5 Gordon’s syndrome

Gordon’s syndrome, or pseudohypoaldosteronism type II, or familial hypertension with hyperkalemia, was originally described in 1970 by Richard Gordon (Gordon 1986). In Finland, this syndrome was described in the 80’s (Soppi et al. 1986). It follows an autosomal dominant form of inheritance, and is clinically characterised by hypertension with hyperkalemia, low renin and normal or elevated aldosterone level, and hyperchloremia with metabolic acidosis (Wilson et al. 2001a).

Gordon’s syndrome is caused by mutations in genes coding for serine-threonine protein kinases (WNK) 1 (WNK1) and 4 (WNK4), which are located in chromosomes 12 and 17, respectively. Both WNK kinases are expressed in distal convoluted tubule and collecting duct in the kidneys. Wild-type WNK4 inhibits the activity of the thiazide-sensitive Na+/Cl− cotransporter (NCC). A missense mutation in the WNK4 gene increases the activity of NCC leading to increased reabsorption of Na+ and Cl−. Wild-type WNK1 inhibits WNK4. WNK1 mutations in Gordon’s syndrome increase WNK1 expression, and release WNK4-mediated inhibition of NCC, but they may also activate ENaC (Huang et al. 2008).

Patients with the WNK4 mutations respond well to treatment with thiazide diuretics. In contrast, patients with the WNK1 mutation are apparently not as sensitive as those carrying WNK4 mutation to thiazide diuretics (Huang et al. 2008).

4.6 Hypertension exacerbated by pregnancy

Hypertension exacerbated by pregnancy is an autosomal dominantly inherited disease, identified initially in a hypertensive 15-year old boy with low serum renin and aldosterone (Geller et al. 2000). Molecular studies showed that the underlying cause of the disease was a missense mutation in the gene coding for MR (Ser810Leu), resulting in increased activation of MR, increased sodium reabsorption, volume expansion, and hypertension (Geller et al. 2000). All affected family members were heterozygous for
the mutation. The term for the syndrome may be misleading, because it is not limited to females, although hypertension typically worsened during pregnancy in those carrying the mutation. It has been proposed that mutated MR is more sensitive to nonmineralocorticoid steroid hormones such as progesterone, the concentrations of which are particularly high during pregnancy. The MR antagonist spironolactone can also activate the mutated receptor, and thereby paradoxically worsen hypertension. In addition, a previous study indicated that cortisone, with no affinity for the wild-type MR, may activate the mutated MR (810Leu), thus providing possible explanation for hypertension in affected males and non-pregnant females (Rafestin-Oblin et al. 2003). The Ser810Leu mutation has not been described in any other but the original kindred. The treatment of this condition is the delivery of the fetus which results in reduction in progesterone levels. (Garovic et al. 2006). Recently, dihydropyridine class calcium channel antagonists have been reported to inhibit aldosterone-induced activation of the MR (Dietz et al. 2008).

4.7 Autosomal dominant hypertension with brachydactyly

In addition to monogenic forms of hypertension affecting renal salt reabsorption, one additional Mendelian form of hypertension, associated with brachydactyly, has been described. The syndrome was first identified in members of a Turkish family in 1973, and it was noticed to inherit in autosomal dominant model (Bilginturan et al. 1973). It has been mapped to chromosome 12 (Schuster et al. 1996). The gene or genes responsible for the syndrome are not known, but genetic rearrangements in the short arm of chromosome 12 may be involved (Bähring et al. 2008). Also sporadic cases of this syndrome have been reported (Litwin et al. 2003, Derbent et al. 2006). Typical clinical characteristics are severe hypertension with short fingers and vascular or neurovascular anomalies. Biochemical markers for the RAS are normal. In affected subjects, stroke under the age of 50 years has been a typical cause of death (Schuster et al. 1996). Treatment consists of multi-drug therapy (Luft 2003).
5. Genes of the renin-angiotensin system and essential hypertension

5.1 Common genetic polymorphisms of the renin-angiotensin system

Genetic variation of the RAS was initially associated with human essential hypertension in 1992 by Jeunemaitre et al. Previously, the AGT gene was localised to human chromosome 1q. Jeunemaitre et al. identified several polymorphisms in the AGT gene, and a variant substituting threonine for methionine at codon 235 (Met235Thr) was found to be significantly associated with human hypertension (Jeunemaitre et al. 1992). Subsequently, also controversial results of the associations between the Thr allele and hypertension have been published. In the meta-analysis by Staessen et al. (Staessen et al. 1999a), the AGT 235Thr allele was associated with hypertension in Caucasians but not in Blacks or Asians. Another meta-analysis (Sethi et al. 2003) showed that the Thr allele was associated with hypertension both in Caucasian and Asian populations dose-dependently. In contrast to previous meta-analyses, the Thr allele was associated with decreased risk of hypertension in German population with 1300 subjects (Mondry et al. 2005). Finnish linkage and association studies do not support the hypothesis of the AGT 235Thr polymorphism having a role in the pathogenesis of essential hypertension (Kiema et al. 1996, Kainulainen et al. 1999). Plasma AGT has been shown to be higher in subjects with the AGT 235Thr allele (Jeunemaitre et al. 1992, Jeunemaitre et al. 1993). The frequency of the Thr allele has varied in ethnic groups as well as in men and women. It has been reported to be more common in African and Asian than in Caucasian populations. The AGT 235Thr polymorphism is in almost complete linkage disequilibrium with the -6 G/A (an adenine instead of guanine) promoter polymorphism of the AGT gene (Inoue et al. 1997). This promoter polymorphism is functional one, since the A allele was associated with increased rate of AGT transcription, which could result in increase in plasma AGT in the subjects with the 235Thr allele. While there is no evidence that the Thr variant directly affects the function or metabolism of the AGT gene, it could mediate predisposition to hypertension in an unknown way (Corvol, Jeunemaitre 1997).
Another polymorphism of the AGT gene in promoter region is a substitution of A for G at position –217 (AGT -217 G/A). The –217 A variant has been found to associate with hypertension in Taiwanese population (Wu et al. 2003) and African-Americans but not in Caucasians (Jain et al. 2002). The frequency of the –217 A allele was significantly increased in African-American hypertensive subjects in comparison with normotensive controls. There is some evidence that the variant -217 A is related to higher transcriptional activity of the AGT gene (Wu et al. 2003, Jain et al. 2002). In the meta-analysis comprising 1400 subjects, the -217 A allele was associated with the increased risk of hypertension (Pereira et al. 2007).

ACE has a key role in catalysing the reaction of Ang I to vasoactive Ang II. The ACE gene has been localised to chromosome 17q. The ACE gene contains an insertion/deletion polymorphism depending on the presence (I) or absence (D) of a 287-bp DNA fragment in intron 16 of the ACE gene (Rigat et al. 1990). The mutant D allele has been associated with hypertension in males (O'Donnell et al. 1998, Fornage et al. 1998, Higaki et al. 2000), and related to elevated plasma ACE constantly (Rigat et al. 1990, Tiret et al. 1992, Todd et al. 1995, Mondorf et al. 1998). Plasma ACE has been found to rise with the increased number of the D allele in Caucasians. Since the ACE I/D variation is not believed to have direct effects on ACE expression or function (Pereira et al. 2006), the mechanism, how the ACE I/D polymorphism might influence to serum ACE activity, is unclear. According to a meta-analysis of 23 studies, the D allele was not associated with hypertension (Staessen et al. 1997). However, in a subgroup analysis, the DD homozygosity was associated with the increased risk of hypertension in Asian population and in women. In German population, the ACE I/D polymorphism did not predict the presence or severity of hypertension (Mondry et al. 2005). Finnish studies have failed to demonstrate a correlation of the ACE I/D polymorphism and BP (Kiema et al. 1996, Kainulainen et al. 1999).

The AGTR1 is a receptor that mediates vasoconstrictory effects of Ang II. The AGTR1 gene has been localised in human chromosome 3q. The AGTR1 gene has a polymorphism where cytosine is substituted for adenine at position 1166 (1166 A/C) in
3’ untranslated region (UTR) (Bonnardeaux et al. 1994). The AGTR1 1166 A/C polymorphism has been associated with hypertension in several (Kainulainen et al. 1999, Bonnardeaux et al. 1994, Hingorani et al. 1995, Wang et al. 1997) but not in all studies (Schmidt et al. 1997, Takami et al. 1998). The A allele was even more frequent in hypertensive subjects than in normotensive controls (Castellano et al. 2003). A meta-analysis of 38 studies found no firm association between the AGTR1 1166 A/C polymorphism and hypertension (Mottl et al. 2008). However, there were a lot of methodological problems and the studies included proved to be extremely heterogeneous, which made any definitive conclusions impossible.

The frequency of the C allele was shown to be 28% in hypertensive subjects and 16% in normotensive controls in a relatively small association study in the Finnish population (Kainulainen et al. 1999). A genome-wide scan suggested that the chromosome 3q locus, also encompassing the AGTR1 locus, was the most important contributor to essential hypertension in a Finnish linkage study carried out in non-identical twins (Perola et al. 2000). Collectively and with only limited data available, the AGTR1 1166 A/C polymorphism at present remains the most reasonable candidate as a genetic marker in essential hypertension in Finnish population.

5.2. Epithelial sodium channel in essential hypertension

The pathophysiological role of ENaC mutations has been well documented in Liddle’s syndrome. In families with Liddle’s syndrome, some of the affected adult family members presented with milder phenotype, mild hypertension and mild hypokalemia (Shimkets et al. 1994, Findling et al. 1997). This raised a question that some patients with essential hypertension may have a defect in ENaC. In subsequent studies, no linkage could be demonstrated between hypertension and ENaC in black Caribbeans (Munroe et al. 1998). In contrast, Wong et al. found in Australian white population a linkage between systolic, but not diastolic, BP and chromosome 16q12, where the β-ENaC and γ-ENaC genes are located (Wong et al. 1999). Chang et al. failed to demonstrate any mutations in ENaC in an unselected group of Japanese patients with
essential hypertension (Chang, Fujita 1996). Most of the variants identified in the subunits of ENaC have been missense mutations and none of them affected directly the PY motif at the C terminus, which is changed or deleted in Liddle’s syndrome (Hummler 2003). In addition, only few studies have provided functional testing in vitro in order to define the pathophysiological significance of the novel polymorphisms.

5.2.1 β-ENaC

A genetic variant resulting in substitution of threonine for methionine at amino acid 594, Thr594Met, was identified in African Americans (Su et al. 1996). Although it was not initially linked to hypertension in black individuals, subsequent studies in London black people suggested a positive association of this variant and hypertension (Baker et al. 1998, Dong et al. 2001). Plasma renin was lower both in hypertensive and normotensive subjects with the Thr594Met variant than in participants without the variant (Baker et al. 1998). However, Persu et al. failed to demonstrate increased channel activity in vitro (Persu et al. 1998). The same study group also identified six other rare single nucleotide polymorphisms in the β-ENaC gene (Persu et al. 1998).

Highest (1.3-1.5 fold) increase in channel activity in Xenopus oocyte system was shown for the variant β-ENaC Gly589Ser, which was found in one female subject with low plasma renin and aldosterone levels as well as mild hypokalemia. The same polymorphism was identified in a hypertensive Swedish male patient with normal potassium level (Melander et al. 1998). None of the 186 controls, consisting of Finnish and Swedish individuals, had this variant. Functional testing of the variant was not performed in the latter study (Melander et al. 1998). Rayner et al. identified a β-ENaC missense mutation (Arg563Gln) in a South-African black population (Rayner et al. 2003). This mutation was strongly associated with low renin, low aldosterone and hypokalemia, but even in those carrying the mutant allele, only a minority showed typical characteristics of Liddle’s syndrome. Functional characterization of the variant was not carried out. The Arg563Gln polymorphism has also been related to increased risk of pre-eclampsia (Dhanjal et al. 2006). When studying Chilean patients with essential hypertension and normotensive controls, Gonzales et al. (2007) identified a polymorphic guanidine-thymidine short-tandem-repeat polymorphism in intron 8 of the
β-ENaC gene. Plasma renin levels decreased with the length of the short-tandem repeats, suggesting an association of the polymorphic region and low-renin hypertension.

5.2.2 γ-ENaC

In the promoter region of the γ-ENaC gene, the polymorphism G(-173)A was associated with BP in Japanese population (Iwai et al. 2001). The variant showed 2.5-fold reduction in promoter activity. In contrast, this polymorphism was not associated with BP in an Australian population sample (Morris et al. 2001). Persu et al. found no association between the γ-ENaC gene and hypertension (Persu et al. 1999). In addition to common polymorphisms (Thr387Cys, Thr474Cys, and Cys549Thr), two rare heterozygous mutations, 594insPro and Arg631His were identified by Persu et al. (1999). Both mutations were related to low plasma renin. However, when expressed in Xenopus oocyte system, no significant sodium current compared with the normal constructs was shown (Persu et al. 1999). To create a maximal contrast of genetic differences, unrelated subjects from the highest and lowest deciles for systolic BP were collected from a large Australian cohort of general population (Busst et al. 2007). Three of 26 identified single nucleotide polymorphisms locating in intron 5 and 6 were associated with systolic BP, showing evidence of the γ-ENaC gene participating in the determination of systolic BP.

5.2.3 α-ENaC

In the promoter region of the α-ENaC gene, the A allele of the G(-946)A polymorphism was associated with increased risk of hypertension, with 1.5-fold increase in promoter activity (Iwai et al. 2002). The α-ENaC Thr663Ala variant associated with normotension both in white and black populations, thus acting as protective allele against hypertension (Ambrosius et al. 1999). The α-ENaC Thr663Ala did not affect sodium channel activity in in vitro studies. In two subsequent studies, the Thr allele was
associated with increased channel activity and was thus suggested to contribute to variation of BP levels (Samaha et al. 2004, Tong et al. 2006).

5.3 α-adducin

Adducin is a membrane cytoskelon protein consisting of an α-subunit with either a β- or a γ-subunit (Matsuoka et al. 2000). Subunits are encoded by three different genes, ADD1 (α), ADD2 (β) and ADD3 (γ) (Matsuoka et al. 2000). Adducin is involved in the formation of actin-spectrin lattice, actin polymerization, and cell signal transduction, including interaction with Na-K-ATPase (Manunta et al. 2007).

The α-adducin gene was first characterized in Milan Hypertensive strain of rats, which represents an animal model of salt-sensitive hypertension (Bianchi et al. 1994). A point mutation Phe316Tyr of ADD1 in rats was associated with hypertension (Bianchi et al. 1994) and altered cellular homeostasis with increased Na+/K+ pump (Tripodi et al. 1996). Adducin protein has a very high degree of homology between rats and humans (94%) (Barlassina et al. 2000b). A subsequent study resulted in identification of polymorphism, Gly460Trp, in human ADD1 (Cusi et al. 1997). This first linkage and case-control study demonstrated an association between the Trp allele and hypertension (Cusi et al. 1997). Several subsequent studies have confirmed the association between the 460Trp allele of ADD1 and hypertension (Castellano et al. 1997, Iwai et al. 1997, Barlassina et al. 2000a) (for review, see Manunta et al. 2007, Bianchi et al. 2005). This association was not confirmed by other studies (Ishikawa et al. 1998, Kamitani et al. 1998, Kato et al. 1998). Patients carrying the Trp allele had lower plasma renin activity (PRA) in comparison to GlyGly homozygotes (Barlassina et al. 2000b, Cusi et al. 1997, Glorioso et al. 1999), and those with low-renin hypertension had higher BP level in the presence of the Trp allele (Cusi et al. 1997, Mulatero et al. 2002, Sugimoto et al. 2002). The Gly460Trp polymorphism has also been related to sodium sensitivity (Cusi et al. 1997, Manunta et al. 1999).
Adducin has been suggested to affect BP through the interaction with Na-K-ATPase. The Trp allele of the ADD1 Gly460Trp polymorphism was associated with higher affinity of Na-K-ATPase resulting in increased sodium reabsorption in the kidneys, and subsequently, low-renin hypertension (Bianchi et al. 1994, Castellano et al. 1997).

6. Pharmacogenetics of antihypertensive drugs in hypertension

Occurrence of hypertension is associated with increased risks of cardiovascular and kidney disease. These risks can be alleviated by effective antihypertensive drug therapy (Mancia et al. 2007). However, less than one-third of hypertensive individuals achieve an adequate BP level (Kaplan, Opie 2006, Varis et al. 2009, Kastarinen et al. 2009). One factor influencing successful BP control is the difficulty in predicting the efficacy of antihypertensive drugs in an individual patient. It is customary for clinicians to use a “trial-and-error” method based on national recommendations, but this approach is far from ideal.

Pharmacogenetics is the study of genetically determined responses to therapeutic drugs (Arnett et al. 2006). Candidate genes for pharmacogenetic studies influencing inter-individual responses to BP are especially those coding for biochemical or hormone components of BP regulation, enzymes of drug metabolism and drug transporters (Schwartz, Turner 2004). Ideally, individual genotype could be used to tailor individual’s BP treatment. Similarly, clinical chemical variables such as serum renin levels have been sought for as predictors for responsiveness of antihypertensive drugs (Preston et al. 1998, Blumenfeld, Laragh 2001). Although monogenic conditions of hypertension account only for a very small portion of BP variation, they provide targets for individualised drug treatment. The treatment of patients with Liddle’s syndrome with ENaC antagonists amiloride or triamterene constitutes an example of individualised drug treatment of severe human hypertension (Lifton et al. 2001).
The majority of the gene-drug interaction studies have investigated BP responses to drug treatments in relation to the polymorphisms of the RAS. Most of the studies have evaluated drug responses after treatment periods lasting from weeks to months, and only few studies have evaluated the acute response of antihypertensive drugs. The results of the polymorphisms of the RAS in relation to responses to different antihypertensive medications have been inconsistent. Table 3 provides a summary of pharmacogenetic studies on the genes of the RAS.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Polymorphism</th>
<th>Antihypertensive drug</th>
<th>Duration of treatment</th>
<th>Association</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACE</strong></td>
<td>I/D</td>
<td>Benazepril</td>
<td>2 months</td>
<td>DD genotype associated with greater SBP and DBP responses</td>
<td>Li et al. 2003</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Fosinopril</td>
<td>6 months</td>
<td>DD genotype associated with greater SBP and DBP responses</td>
<td>Stavroulakis et al. 2000</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Enalapril</td>
<td>6 months</td>
<td>I allele associated with greater SBP and DBP responses</td>
<td>Haas et al. 1998</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Imidapril</td>
<td>6 weeks</td>
<td>II genotype associated with greater DBP response</td>
<td>Ohmichi et al. 1997</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Captopril, lisinopril</td>
<td>6 weeks</td>
<td>I allele associated with greater mean ABP response, lisinopril no differences among genotypes</td>
<td>O’Toole et al. 1998</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Irbesartan, atenolol</td>
<td>3 months</td>
<td>II genotype associated with greater DBP response for irbesartan, no differences for atenolol</td>
<td>Kurland et al. 2001</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Hydrochlorothiazide</td>
<td>4 weeks</td>
<td>II women greater SBP and DBP response, DD men greater SBP and DBP response</td>
<td>Schwartz et al. 2002</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Atenol, lisinopril, nifedipine</td>
<td>4 weeks</td>
<td>No differences among genotypes</td>
<td>Dudley et al. 1996</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Captopril, enalapril, lisinopril, perindopril</td>
<td>4 weeks</td>
<td>No differences among genotypes</td>
<td>Hingorani et al. 1995</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Perindopril</td>
<td>4 weeks</td>
<td>No differences among genotypes</td>
<td>Harrap et al. 2003</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Imidapril, benazepril</td>
<td>6 weeks</td>
<td>No differences among genotypes</td>
<td>Yu et al. 2003</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Lisinopril, amlodipine, doxazocin, chlorthalidone</td>
<td>6 months</td>
<td>No differences among genotypes</td>
<td>Arnett et al. 2005</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Enalapril</td>
<td>12 months</td>
<td>No differences among genotypes</td>
<td>Sasaki et al. 1996</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Telmisartan</td>
<td>12 months</td>
<td>No differences among genotypes</td>
<td>Redon et al. 2005</td>
</tr>
<tr>
<td></td>
<td>I/D</td>
<td>Diuretics, β blockers, Ca antagonists, ACE inhibitors</td>
<td>10 years</td>
<td>No differences among genotypes</td>
<td>Schelleman et al. 2006a</td>
</tr>
<tr>
<td><strong>AGT</strong></td>
<td>Met 235Thr</td>
<td>Captopril, enalapril, lisinopril, perindopril</td>
<td>4 weeks</td>
<td>Thr allele associated with greater SBP and DBP responses</td>
<td>Hingorani et al. 1995</td>
</tr>
<tr>
<td></td>
<td>Met 235Thr</td>
<td>Atenol, lisinopril, nifedipine</td>
<td>4 weeks</td>
<td>No differences among genotypes</td>
<td>Dudley et al. 1996</td>
</tr>
<tr>
<td></td>
<td>Met 235Thr</td>
<td>Diuretics, β blockers, Ca antagonists, ACE inhibitors</td>
<td>10 years</td>
<td>No differences among genotypes</td>
<td>Schelleman et al. 2006b</td>
</tr>
<tr>
<td></td>
<td>Met 235Thr</td>
<td>Irbesartan, atenolol</td>
<td>3 months</td>
<td>Thr allele associated with greater SBP response for atenolol, no differences for irbesartan</td>
<td>Kurland et al. 2004</td>
</tr>
<tr>
<td></td>
<td>Met 235Thr</td>
<td>Irbesartan, atenolol</td>
<td>3 months</td>
<td>No differences among genotypes</td>
<td>Kurland et al. 2001</td>
</tr>
<tr>
<td>Gene</td>
<td>Polymorphism</td>
<td>Antihypertensive drug</td>
<td>Duration of treatment</td>
<td>Association</td>
<td>Reference</td>
</tr>
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<td>-------</td>
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<td>-------------------------------</td>
</tr>
<tr>
<td>AGT</td>
<td>217 G/A</td>
<td>Enalapril, lisinopril</td>
<td>2 months</td>
<td>G allele associated with greater SBP and DBP response</td>
<td>Woodiwiss et al. 2006</td>
</tr>
<tr>
<td>AGTR1</td>
<td>1166 A/C</td>
<td>Perindopril, nitrendipine</td>
<td>2 months</td>
<td>C allele associated with greater SBP and DBP responses for perindopril, no difference for nitrendipine</td>
<td>Benetos et al. 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Captopril, enalapril, lisinopril, perindopril</td>
<td>4 weeks</td>
<td>No differences among genotypes</td>
<td>Hingorani et al. 1995</td>
</tr>
<tr>
<td></td>
<td>1166 A/C</td>
<td>Irbesartan, atenolol</td>
<td>3 months</td>
<td>No differences among genotypes</td>
<td>Kurland et al. 2001</td>
</tr>
<tr>
<td></td>
<td>1166 A/C</td>
<td>Telmisartan</td>
<td>12 months</td>
<td>No differences among genotypes</td>
<td>Redon et al. 2005</td>
</tr>
</tbody>
</table>

A single dose effect

| AGTR1 | 1166 A/C     | Active metabolite of losartan                | 90 min               | CC subjects showed blunted BP response during high salt diet                | Spiering et al. 2005          |
|       | 1166 A/C     | Losartan                                      | 3 h                  | C allele associated with greater BP response                                | Miller et al. 1999            |
| ACE   | I/D          | Captopril                                     | 1 h                  | No differences among genotypes                                             | Nakono et al. 1997            |
|       | I/D          | Captopril                                     | 1 h                  | No differences among genotypes                                             | Mondorf et al. 1998           |
|       | I/D          | Enalapril                                     | 6h                   | No differences among genotypes                                             | Todd et al. 1995              |
|       | I/D          | Enalapril (30 min, intravenous infusion)      | 10h                  | II genotype associated with greater mean BP response                       | Ueda et al. 1998              |
| AGT   | Met235Thr    | Captopril                                     | 1 h                  | No differences among genotypes                                             | Mondorf et al. 1998           |

SBP = systolic blood pressure, DBP = diastolic blood pressure, ABP = ambulatory blood pressure
In three Italian studies, the *ADD1* 460Trp allele was associated with increased BP response to diuretic treatment compared with the 460Gly allele. The first study was performed by Cusi et al. (1997), who showed that the 460Trp allele was associated with markedly increased BP reduction to hydrochlorothiazide treatment compared with the 460Gly allele in 58 hypertensive subjects. A subsequent study (Glorioso et al. 1999) confirmed the previous results in 143 hypertensive patients. Sciarrone et al. (2003) repeated these results, but also showed that hypertensive patients carrying at least one *ACE* I allele and one *ADD1* 460Trp allele had the best mean BP response to hydrochlorothiazide treatment compared with the combined group of the DD genotypes of the *ACE* I/D and GlyGly of the *ADD1* 460 polymorphisms. These results have not been replicated in non-Hispanic African Americans and non-Hispanic white adults (Turner et al. 2003), or in a cohort of Dutch patients (Schelleman et al. 2006b).

The largest pharmacogenetic study executed is the GenHAT (The Genetics of Hypertension Associated Treatment) study (Arnett et al. 2002). The purpose of the GenHAT study was to genotype hypertension-related gene polymorphisms, including *ACE* I/D, *AGTR1* 1166 A/C, and *ADD1* Gly460Trp, and to investigate gene-treatment interactions to the outcomes, coronary heart disease and BP reduction. The GenHAT Study was primarily a clinical end-point study, where antihypertensive treatment was titrated according to BP values. 37 000 study participants were randomised to four treatment groups of antihypertensive medication, chlorthalidone (thiazide-like diuretic), amlodipine, lisinopril or doxazosin (α-receptor blocker). The study results showed that the *ACE* I/D genotypes did not predict responses to any of the study medications (Arnett et al. 2005). There were no differences between the *ADD1* 460Trp allele carriers and non-carriers in responses to six months therapy with different drugs to systolic BP, whereas the reduction in diastolic BP was slightly smaller (0.3 mmHg) in the Trp allele carriers than non-carriers (Davis et al. 2007).

In addition to the polymorphisms of the RAS genes and *ADD1*, other genes including genetic polymorphisms for endothelial nitric oxide synthase, the α-subunit of G protein and β-adrenoceptor antagonists, have been investigated in responses to antihypertensive
AIMS OF THE STUDY

The present study was conducted in order to relate the occurrence of common and rare variants of the RAS to the presence of hypertension itself and to its phenotypic characteristics, especially to the activity of the RAS and to the antihypertensive response of drugs known to inhibit this system.

The detailed aims were as follows:

1. To identify and characterise mutations of the subunits of ENaC in hypertensive patients with low plasma renin and aldosterone levels.

2. To search for common variants of ENaC subunits in patients with essential hypertension and to study their relation to the circulating renin and aldosterone levels.

3. To assess the relation of some common genetic variants of the RAS to the activity of the RAS and acute ACE inhibition.

4. To examine the relation between common genetic variations of the RAS / α-adducin and response to four different types of antihypertensive monotherapies.
SUBJECTS AND METHODS

1. Patients

1.1 Patients with low-renin hypertension (Study I)

In Study I, two unrelated Finnish patients with treatment–resistant hypertension suspected to have Liddle’s syndrome were examined at the Hypertension Outpatient Departments of the University Hospitals of Helsinki and Turku. Both patients fulfilled the following characteristics: early-onset of hypertension, normal body mass index (BMI), low serum potassium and low serum renin (Table 4). The patients were examined for the mutations of the exon 13 of the β-ENaC or γ-ENaC genes. The first-degree family members of the patients (probands) were also invited to give blood samples for molecular studies of the ENaC mutations.

1.2 Patients with treatment-resistant hypertension (Studies I-III)

Hypertensive patients were collected from the database of the Hypertension Outpatient Ward, Helsinki University Central Hospital. Initially, they were referred to hospital because of moderate-to-severe hypertension, suspicion of secondary forms of hypertension, or hypertension resistant to drug treatment. Case records of all consecutive 615 patients with hypertension were reviewed. Those 598 individuals, whose addresses were available, were sent a letter with request to donate a blood sample for genetic studies on hypertension. A total of 399 individuals (183 males and 216 females) responded and provided venous blood samples for DNA analysis.

The previous clinical history of the 399 patients was evaluated, which resulted in the exclusion of 52 subjects. The reasons for the exclusion were: clinical records were missing or insufficient in four cases, 22 subjects were considered as normotensive, and 26 turned out have a secondary form of hypertension (see Study II). The remaining 347
patients (186 females and 161 males, mean age 49.3 years, SD±10.0) comprised the cohort of patients with essential hypertension in Study II.

Antihypertensive drug treatment had been in use in 283 (82%) of the patients (diuretics, 19%; β-blocking agents, 35%; calcium-channel blockers, 21%; ACE-inhibitors, 33%; angiotensin receptor antagonists, 1%). At least two concomitant drugs were used by 24% of the patients.

Several examinations were performed to exclude renovascular hypertension. First, most of the patients (298 of the final cohort of 347 patients) underwent a test for the responsiveness of serum aldosterone level and PRA to postural change (Study II). The test was carried out at the inpatient ward in 220 cases and at the outpatient ward in 78 cases. In addition, urine samples for determination of daily (24-hour) excretion of potassium and sodium were collected, and analysed in 262 patients without potassium supplementation. Also blood samples were taken for determination of serum creatinine, uric acid, cholesterol, potassium and sodium, and blood glucose. Second, one to three days later of the postural stimulation test, a captopril challenge test (CCT) was performed. The test was carried out in a total of 313-315 patients (Studies II-III), and was performed at the inpatient ward in 229-231 cases and at the outpatient ward in 84 cases. To avoid the distracting effect of drug therapy on renin and aldosterone levels in the postural test and CCT, the study patients had been advised to stop using the current antihypertensive medication and hormone substitution, estrogens and spironolactone for at least four weeks, diuretics and prostaglandin inhibitors for two weeks and β-adrenergic antagonists and ACE inhibitors for one week before the tests. The only antihypertensive agents permitted at the time of the tests were calcium channel antagonists to prevent severe rise of BP levels, because they are known to have no or only minor effects on blood renin and aldosterone levels (Seifarth et al. 2002).

The DNA samples of 27 patients with the lowest PRA and aldosterone values during the CCT were screened for variants in exons 13 of the β- and γ-ENaC genes. The DNA samples of 347 patients with essential hypertension were genotyped for the variants identified (Study II).
A total of 315 hypertensive patients underwent the CCT, and they were included in the Study III, in which common genetic variants of the RAS were screened for.

1.3 Hypertensive patients in the pharmacogenetic study (the GENRES Study) (Studies III-IV)

In Study IV, Finnish white men aged 35 to 60 years with hypertension were recruited with newspaper advertisement. An invitation was directed to those with antihypertensive medication or previously measured (on separate visits) three diastolic BP readings ≥ 95 mmHg.

At the first study visit, the inclusion and exclusion criteria were evaluated, relevant medical history was recorded, and physical examination was performed. Exclusion from the study was based on the following criteria: three or more antihypertensive drugs in use, secondary form of hypertension, drug-treated diabetes mellitus, congestive heart failure, coronary heart disease, cerebrovascular disease, kidney disease (blood creatinine concentration > 115 µmol/l), obstructive pulmonary disease, a disease treated with corticosteroids, clinically significant liver disease, abuse of drugs or alcohol, and BMI ≥ 32 kg/m². The first study subject was recruited in October, 1999, and the last subject completed the study in February, 2004. The objective was to get together minimum 192 men with hypertension, based on power calculations. A total of 313 subjects were altogether screened for the study.

The study was designed as a randomised, double-blind, placebo-controlled, single-centre, cross-over study (Figure 5). The duration of the actual pharmacogenetic study was altogether eight months (32 weeks). It started with a run-in placebo period, before which possible antihypertensive drug therapy, used by 81% of the study subjects, was stopped. At the end of the first placebo period, blood samples for renin, aldosterone, glucose, creatinine, blood cell counts, and serum electrolytes were taken. A 24-hour urine collection was performed for the measurement of urinary electrolytes.
The first placebo period was followed by four treatment periods in randomised order with one of the four antihypertensive drugs, each separated by a placebo period. Each period lasted for four weeks. Randomisation for all possible 24 drug sequences was done after the first placebo period. The antihypertensive drugs included in the study were angiotensin II receptor antagonist (losartan 50 mg; Cozaar, Merck & Co.), β-adrenergic antagonist (bisoprolol 5 mg; Emconcor, Merck KGaA), calcium channel blocker (amlodipine 5 mg; Norvasc, Pfizer), and a thiazide diuretic (hydrochlorothiazide 25 mg; Hydrex semi, Orion Pharma). All preparations, including placebo, were packed in identical gelatin capsules, and were taken once daily, in the morning.

BP measurements were carried out as OBP and ABP measurements after each placebo and treatment periods (ie. before and after active drug treatment periods). The measurements took place between 7.30 and 11 a.m. on the same day of the week and within a time-interval of 2 hours for each subject. For OBP measurements, three measurements were taken with 1-minute intervals after a 30-minute rest in a sitting position, using a semi-automated device (Omron M4, Omron Healthcare, Japan), and the mean of the last two measurements was used in the analyses. ABP measurements were done with a devise with a position sensor (Diasys Integra, Novacor, France). Directions to continue normal life with the exception of strong physical activity were given before BP recordings. BP readings were taken every 15 minutes (upright position) or 30 minutes (supine position). Daytime was determined from 7 a.m. to 10 p.m., and night-time from 10 p.m. to 7 a.m. For the ABP recording to be accepted, ≥ 15 daytime
and ≥ 7 night-time readings were required. The mean 24-hour ABP level was calculated as the mean of the daytime (weight: 15/24) and night-time (weight: 9/24) values.

Of the 313 subjects screened for the study, 244 subjects completed at least one placebo period and 211 subjects went through all four placebo periods. At least one active drug period was completed by 233 subjects, and all four drug periods by 208 subjects. Withdrawals occurred to 105 subjects during the study (see Hiltunen et al. 2007). The reasons for withdrawal were: BP > 200/120 mmHg (n = 12), aortic dilatation (n = 7), significant left ventricular hypertrophy (n = 1), and previous myocardial infarction (n = 2) detected in echocardiography, angina pectoris (n = 3), atrial fibrillation (n = 4), asthma (n = 2), normotension (n = 7), non-compliance (n = 8), kidney disease (n = 4), various medical causes (n = 6), and personal reasons (n = 49). Subjects who had stopped the study were included in the analyses of those treatment periods they had gone through. During the study, subject safety was confirmed with BP measurements and electrocardiogram after each period, laboratory tests, and echocardiography during the first placebo period, as well as offering a possibility to contact to the research physician at any time.

Altogether 244 subjects genotyped for the AGTR1 1166 A/C polymorphism were also included for the replicate analyses of the activity of the RAS in Study III.

2. Reference groups for allelic frequencies

2.1 Normotensive males (Studies I-III)

For the estimation of the allele and genotype frequencies, DNA samples were collected from control populations. Normotensive males (Studies I-III) were selected from the participants in the Alpha-Tocopherol, Beta Carotene (ATBC) study (1994). DNA samples were available from 70% of the original subjects recruited for this cancer prevention trial. Subjects who fulfilled the following criteria were selected: no known
hypertensive disorder, no antihypertensive drugs ever in use, systolic and diastolic BP values ≤ 128 and ≤ 84 mmHg, respectively, at each BP measurement, repeated five times at 1-year intervals. The final group comprised 175 males whose mean systolic BP was 114.9 (SD±5.4) mmHg and diastolic BP 73.7 (SD±4.3) during the 5-year follow-up. There was no access to the clinical data of the subjects.

2.2 Blood donors (Studies I-II)

Another control population with collected DNA samples consisted of 301 randomly selected healthy blood donors aged 40 to 50 years (mean, 45 years) visiting the Finnish Red Cross Blood Transfusion Service (Studies I-II). BP measurements were not recorded from blood donors. Complications of hypertension and use of more than one antihypertensive drug were contraindications for blood donation.

3. Ethical consideration

The local Ethics Review Committee of the Department of Medicine, Helsinki University Central Hospital approved all the studies. Study IV was also approved by the National Agency for Medicines of Finland. All patients and controls gave their written informed consent before the study-related activities.

4. Clinical tests of the renin-angiotensin system

The activity of the RAS was evaluated with two clinical function tests (Studies II-III), initially designed for the exclusion of renovascular hypertension, the CCT and postural test. In the study cohort of patients with essential hypertension, the effect of the polymorphisms of the ENaC (Study II) and RAS (Study III) genes was evaluated in
relation to PRA and aldosterone levels. Both PRA and aldosterone were assayed radioimmunologically (Medix Ltd, Espoo, Finland).

4.1 Captopril challenge test

The CCT was based on the administration of ACE inhibitor, captoril, which suppresses the production of Ang II and aldosterone and by feedback mechanism raises renin secretion (Muller et al. 1986). Patients with renovascular hypertension have an accentuated renin response to captopril stimulation. In essential or renovascular hypertension, aldosterone levels are suppressed, whereas in primary hyperaldosteronism, aldosterone levels remain elevated. In this study, the CCT was started by sitting for at least 30 minutes, followed by oral administration of 50 mg captopril. BP was measured at 15-minute intervals for 2 hours. Blood samples for the determination of PRA and serum aldosterone concentration were drawn immediately before and 60 minutes after captopril administration.

4.2 Postural stimulation test

The postural stimulation test evaluates the influence of postural change to plasma renin and aldosterone. The RAS is normally activated in response to postural change. The upright posture increases plasma aldosterone level in aldosterone-producing adenoma and idiopathic hyperaldosteronism (Nomura et al. 1992). In this test, the first blood sample for the determination of PRA and serum aldosterone concentration was taken after at least 60 minutes of rest in supine position. The second blood sample was taken after 2 hours of upright position.
5. Molecular genetic studies

5.1 Molecular methods

The molecular methods are described in more detail in original Studies I-IV.

DNA was amplified by polymerase chain reaction (PCR) in varying conditions (Studies I-IV). To determine the nucleotide sequence, the purified DNA products were sequenced with the dye terminator cycle-sequencing procedure and the ABI Prism 377 automatic DNA sequencer (Perkin Elmer), and the sequences were analysed with Sequencher software (Gene Codes, Ann Arbor, MI, USA). Mutations or polymorphisms were detected with specific detection methods among relatives, hypertensive patients and control individuals. Genotyping was mainly performed using PCR amplification followed by restriction enzyme digestion and electrophoretic separation of the cleavage products on agarose or polyacrylamide gel.

5.1 Functional studies

The properties of the ENaC mutations and variants on channel function were studied in vitro in Xenopus oocytes using electrophysiological techniques described by Schild et al. (1995). Site-directed mutagenesis was used to create a construct for elucidating the functional activity of the mutation. Mutations were introduced into the corresponding wild-type complementary DNA (cDNA). For transfection, healthy stage V and VI Xenopus oocytes were injected with mRNAs coding for wild-type and mutated human ENaC gene. In Study I, human α- and β-ENaC wild-types were injected together with either γ-ENaC wild-type or γ-ENaC Asn530Ser mutant. In Study II, α- and β-ENaC wild-types were injected together with either γ-ENaC wild-type or γ-ENaC Val546Ile, or α- and γ-ENaC wild-types together with either the β-ENaC wild-type or the mutant β-ENaC Gly589Ser. ENaC activity was assessed by measurement of the amiloride-sensitive current using two-electrode voltage clamp technique, where a membrane
potential recording electrode and a current delivering electrode are implanted into the oocytes (Schild et al. 1995).

In order to study a possible splicing error, the $\beta$-ENaC splice site i12-17 C→T variant was studied by reverse transcription-PCR (RT-PCR). RNA was isolated from human lymphocytes of two heterozygous carriers of the variant and one non-carrier of the hypertensive patients. cDNA was prepared from RNA followed by PCR. The amplified PCR products of the mutant and wild-type alleles were separated on gel-electrophoresis and thereafter also sequenced to exclude the presence of splicing error. Possible splicing differences of the wild-type and mutant allele were also evaluated in silico.

To elucidate the functional impact of the AGTR1 1166 A/C polymorphism, three luciferase constructs were created (see Study III). The mutant AGTR1 was generated by site-directed mutagenesis. Thereafter, constructs and control plasmid were transfected into HEK293 cells. After 24 hours, luciferase activity was measured with Dual Luciferase Assay System (Promega, Madison, USA).

6. Statistical analyses

A $\chi^2$ test or Ficher’s exact test, if expected frequency in any cell was less than five, were used for the frequency analysis of each genotype or alleles between hypertensives and controls. SPSS software (versions 11.0-15.0, SPSS Inc., Chicago, IL) was used in statistical analyses. Normality of distributions of the variables was analysed using skewness, kurtosis and Kolmigorow-Smirnow tests. For normally distributed variables, the data are presented as mean (±SD or SE), and parametric tests, a $t$-test or linear regression analysis, were used. Levene’s test was used to confirm the homogeneity of variances before the use of the $t$-test. For non-normally distributed variables, the data are presented as median (interquartile range), and statistical significance was analysed using non-parametric tests. Kruskal-Wallis test or Joncheere-Terpsta trend test were used in the comparison between three different groups, and Mann-Whitney test between
two groups. In Studies III-IV, significant findings were subjected to multivariate analyses using the General Linear Model procedure of SPSS. Non-normally distributed variables were transformed by the Blom method to normalised ranks for multivariate analyses. Explanatory variables included genotype, age, BMI, gender, use of calcium channel blocker or potassium substitution during the CCT, examination place, duration of hypertension, earlier use of antihypertensive medication, earlier use of a thiazide diuretic, daily urinary excretion of sodium and potassium, and corresponding BP level on placebo periods.

Aldosterone and renin were non-normally distributed, and Mann-Whitney test was used in statistical analyses. When covariates were included in the analyses, ANCOVA with ranks or logarithm-transformed values of the variables was used. Logistic regression was used to obtain age and gender adjusted odds ratios for hypertension in the ENaC variant carriers in comparison with non-carriers. Because of the relatively small sizes of the variant groups, the analyses were primarily performed with all variant groups combined. The variant groups were also compared separately with the wild-type ENaC group (Study II). BP responses to captopril were derived by calculating the greatest reduction in systolic and diastolic BP between baseline and after 60 minutes of captopril administration (Study III). Unpaired student t-test was used in the comparison of functional expression in the two groups (Study I) and differences in luciferase activities (Study III).

In Study IV, the BP level during the placebo periods was determined as the mean of all placebo periods. BP response to a study drug was the difference between BP level after drug treatment period and the mean of the placebo recordings. The ABP responses were the primary efficacy variables, since ABP recordings showed better repeatability than OBP recordings during placebo periods (Hiltunen et al. 2007). The genotypes were used as explanatory variables in the analyses. WCE for systolic and diastolic BP was determined as the difference between OBP and daytime ABP levels during placebo period. For secondary analysis, rare homozygotes of the AGTR1 1166 and ADD1 460 were pooled with heterozygotes (AC+CC and GlyTrp+TrpTrp). The additive effect of putative risk alleles (I for ACE I/D and Trp for ADD1 Gly/Trp) was also estimated.
RESULTS

1. Rare mutations in the ENaC subunits (Study I)

1.1 Mutations identified in the ENaC β- and γ-subunits

In Study I, DNA samples of the two patients suspected to have Liddle’s syndrome were screened for mutations in exons 13 of the β- and γ-subunits of ENaC. Two novel mutations were identified. The mutation in the Proband 1 was caused by a G insertion after codon 600 in exon 13 of the β-ENaC gene resulting in a frameshift (β-ENaC Thr601→Frmshft) and is predicted to create a premature stop codon deleting the PY motif of the β-ENaC gene. In Proband 2, a point mutation in exon 13 of the γ-ENaC gene, predicted to result in substitution of serine for asparagine at codon 530 (γ-ENaC Asn530Ser) in the extracellular domain, was identified. Both probands were heterozygous for the mutations.

The β-ENaC Thr601→Frmshft mutation was detected in Proband 1’s two children and her mother. Its occurrence was not examined in other hypertensive patients or controls. The γ-ENaC Asn530Ser mutation was screened for in all available family members of Proband 2, and in addition in hypertensive patients (n = 399), healthy blood donors (n = 291) and normotensive males (n = 175). The mutation was found in Proband’s mother, in one of the healthy blood donors, in one normotensive male, but not in the 399 hypertensive patients.

1.2 Clinical characteristics of the patients with Liddle’s syndrome

Proband 1 was a female with normal BMI. She was known to be normotensive until the age of 19 when her BP level rose to a level of 190/120 mmHg. In clinical examination, there were no signs of secondary form of hypertension, but laboratory tests showed low
plasma renin, low serum potassium, and almost undetectable daily urinary aldosterone excretion (Table 4). Serum aldosterone concentration was not determined. Her two children, aged nine and 14 years at the time of the study and carrying the same mutation (β-ENaC Thr601→Frmshift), were measured BP levels 120/60 and 128/70 mmHg, respectively. The Proband’s mother was also the carrier of the β-ENaC mutation and was on hypertensive medication. The Proband’s father has shown elevated systolic BP levels, but he was not a carrier of the mutation.

Proband 2 was a male with no known previous diseases and with normal BMI. At the age of 25 years he was reported BP values up to 180/120 mmHg. His laboratory tests revealed low PRA, low serum aldosterone concentration and hypokalemia on several occasions. Daily urinary aldosterone level was very low (Table 4). Cardiac ultrasound showed left ventricular hypertrophy with septal thickness of 12-15 mm. The Proband’s mother carried the same γ-ENaC Asn530Ser mutation. She was diagnosed to have hypertension at the age of 40 years. Her laboratory tests revealed low to normal plasma potassium and low plasma renin levels. The Proband’s father was not examined.

| Table 4. Clinical characteristics of patients with Liddle mutations |
|----------------------|----------------------|----------------------|----------------------|
| Variable             | Proband 1            | Proband 2            | Normal values        |
| Mutation             | β-ENaC Thr601→Frmshift | γ-ENaC Asn530Ser     |                       |
| Gender               | Female               | Male                 |                       |
| Age (years)          |                      |                      |                       |
| at onset of hypertension | 19                   | 25                   | 20-25                |
| at the time of diagnosis | 34                   | 28                   |                       |
| BMI (kg/m²)          | 18                   | 20.5                 | 20-25                |
| Blood pressure (mmHg) |                      |                      | 3.7-5.3              |
| without amiloride/triamterene | 190/120             | 180/120              |                       |
| with amiloride/triamterene | 130/95               | 120/88               |                       |
| Serum potassium      |                      |                      |                       |
| before treatment     | 2.4                  | 3.0                  |                       |
| after treatment      | 3.8                  | 4.7                  |                       |
| PRA (μg/l per h)     | 0.6                  | 0.1                  | 2-5                  |
| Serum aldosterone (pmol/l) | ND                 | 158                 | 260-1000             |
| dU-aldo (nmol/l)     | 0.55                 | 1.0                  | <50                  |

BMI = body mass index, PRA = plasma renin activity, dU-aldo = 24 hour urinary aldosterone secretion, ND = not determined
2. Common genetic variants of the ENaC subunits (Study II)

2.1 Genetic variants identified in the β-ENaC and γ-ENaC subunits

At first, 27 DNA samples of 399 hypertensive patients with the lowest renin and aldosterone levels in the CCT were screened for the mutations in exons 13 of the β- and γ-ENaC genes. Three different types of variants were found in altogether six individuals. The first variant in the β-ENaC gene revealed a nucleotide substitution of A for G, predicted to result in a substitution of serine for glycine in codon 589 (β-ENaC Gly589Ser). This variant has been described previously (Melander et al. 1998, Persu et al. 1998). The second variant was a novel G to C substitution at codon 546 in the γ-ENaC gene, predicted to cause a substitution of isoleucine for valine (γ-ENaC Val546Ile). The third variant was a novel substitution of T for C in intron 12 of the β-ENaC gene, 17 bases before exon 13 (β-ENaC i12-17CT).

The three variants were then genotyped in the whole hypertensive patient group (n = 347), normotensive males (n = 175) and healthy blood donors (n = 301). Altogether 32 carriers (including the six original cases described above) were identified among the hypertensive patients. Of these, there were 16 subjects carrying the variant β-ENaC i12-17CT, eight carrying the β-ENaC Gly589Ser, and eight carrying the γ-ENaC Val546Ile variant. The mutations in intron 12 and at codon 589 of β-ENaC were also present in two normotensive males and three blood donors, and the mutation at codon 546 in γ-ENaC in one normotensive male and three blood donors (Table 5). All variants were present in a heterozygous form, with no homozygous or compound heterozygous individuals present in any group. The prevalence of the variants was three times higher among hypertensives (9%) compared with normotensive males (3%) or blood donors (3%) (p < 0.01). When the frequency of each individual variant in hypertensive patients was compared with the combined control groups, the β-ENaC i12-17CT variant was found to occur more often in hypertensive patients (5%) than in the combined control groups (1%) (p = 0.001), while the frequencies of the β-ENaC Gly589Ser and γ-ENaC
Val546Ile alleles in hypertensive patients did not differ from those in the combined control groups.

Table 5. Variants identified in the β- and γ-ENaC subunits and their carrier frequencies in hypertensives and controls

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>β-ENaC n (%)</th>
<th>β-ENaC Gly589Ser n (%)</th>
<th>γ-ENaC Val546Ile n (%)</th>
<th>All n (%)</th>
<th>Non-carriers n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension (347)</td>
<td>16 (4.6)</td>
<td>8 (2.3)</td>
<td>8 (2.3)</td>
<td>32 (9.2)</td>
<td>315 (90.8)</td>
</tr>
<tr>
<td>Normotensive males (175)</td>
<td>2 (1.1)</td>
<td>2 (1.1)</td>
<td>1 (0.6)</td>
<td>5 (2.9)</td>
<td>170 (97.1)</td>
</tr>
<tr>
<td>Blood donors (301)</td>
<td>3 (1.0)</td>
<td>3 (1.0)</td>
<td>3 (1.0)</td>
<td>9 (3.0)</td>
<td>292 (97.0)</td>
</tr>
</tbody>
</table>

2.2. Demographic data

When the clinical characteristics of the 347 hypertensive patients according to their ENaC variant carrier status were compared, the carriers of the ENaC variants did not differ from non-carriers for age, BMI, chemical laboratory values, potassium substitution, prevalence of cerebrovascular disorder, or gestational hypertension.

2.3 The activity of the RAS in relation to the ENaC variants

Associations of the ENaC variants with circulating renin and aldosterone were evaluated with two function tests, a postural test and CCT. First, 298 patients had undergone the postural test for responsiveness of renin and aldosterone. Due to small samples sizes, the variant carriers were primarily combined. The median values of PRA and aldosterone did not differ statistically significantly between variant carriers and non-carriers. The median values of PRA in variant carriers compared with non-carriers were 0.7 µg/l/h and 0.8 in the supine position, and 1.5 and 1.9 in the upright position, and for aldosterone 368 pmol/l and 369 in the supine, and 761 and 936 in the upright position, respectively (Study II, Table 3).
In the CCT, PRA and aldosterone concentration were measured before and 60 minutes after the administration of the ACE inhibitor captopril. No statistically significant differences were found in renin and aldosterone levels according to ENaC variant status (Study II, Table 3). Some evidence of lower renin response among the ENaC variant carriers compared to non-carriers in the CCT was seen, but the difference was not statistically significant (p = 0.087).

2.4 Serum and urinary electrolytes in relation to the ENaC variants

The influence of the ENaC variants was also evaluated in relation to sodium and potassium homeostasis. Serum sodium and potassium levels and their daily urinary excretions did not differ between the combined group of ENaC variants and non-carriers. When daily urinary excretion of potassium was related to PRA (dU-K/PRA) measured during the postural test, the median ratios in carriers and non-carriers were 56 and 38 in the upright position (p = 0.034), and the corresponding median values for the mean of supine and upright PRA were 74 and 51 (p = 0.048). Analyses of daily urinary potassium in relation to plasma aldosterone (dU-K/aldosterone supine, dU-K/aldosterone upright, dU-K/aldosterone mean of supine and upright) demonstrated higher ratios in the variant carriers compared with the non-carriers in females and non-significantly in the combined group of males and females, but not in males. Collectively, hypertensive patients carrying ENaC variants tended to excrete increased amounts of potassium in relation to prevailing plasma renin and aldosterone.

3. Some genetic variants of the RAS (Study III)

3.1 Clinical characteristics of hypertensive patients

Clinical characteristics of the study subjects (n = 315) are presented in Study III (Study III, Table 1).
3.2 Genotype and allele frequencies of the variants of the RAS

In order to evaluate possible associations of candidate gene variants with the activity of the RAS, four known polymorphisms in the RAS (*AGTR1* 1166 A/C, *ACE* I/D, *AGT* Met235Thr and *AGT* −217 G/A) were genotyped in hypertensive patients and normotensive males. They were shown to follow the Hardy-Weinberg equilibrium. Genotype frequencies are summarised in Table 6. The genotype or allele distributions of the polymorphisms studied did not differ statistically significantly between patients and normotensive male controls. The presence of the A allele at AGT −217 was always associated with the presence of the Thr allele of the *AGT* Met235Thr polymorphism in hypertensive patients and controls, suggesting a tight linkage of these loci.

<table>
<thead>
<tr>
<th>Gene polymorphism</th>
<th>All Females (n=314-315)</th>
<th>Males (n=169-170)</th>
<th>Normotensive males (n=172-175)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AGTR1</em> 1166 A/C</td>
<td>AA 197</td>
<td>107</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>AC 110</td>
<td>61</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>CC 8</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>MAF 0.20</td>
<td>0.19</td>
<td>0.21</td>
</tr>
<tr>
<td><em>ACE</em> I/D</td>
<td>II 73</td>
<td>42</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>ID 163</td>
<td>91</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>DD 79</td>
<td>37</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>MAF 0.49</td>
<td>0.49</td>
<td>0.46</td>
</tr>
<tr>
<td><em>AGT</em> Met235Thr</td>
<td>MM 108</td>
<td>48</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>MT 152</td>
<td>88</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>TT 55</td>
<td>34</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>MAF 0.42</td>
<td>0.46</td>
<td>0.37</td>
</tr>
<tr>
<td><em>AGT</em> −217 G/A</td>
<td>GG 253</td>
<td>136</td>
<td>117</td>
</tr>
<tr>
<td></td>
<td>GA 56</td>
<td>32</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>AA 5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>MAF 0.11</td>
<td>0.10</td>
<td>0.11</td>
</tr>
</tbody>
</table>

MAF = minor allele frequency
3.3 The activity of the RAS and acute response to ACE inhibition

3.3.1 Renin and aldosterone levels before and after captopril in the different genotypes of the RAS

The results of the analyses of PRA and aldosterone levels and their responses to the administration of captopril are illustrated in Figure 6A and 6B.

A

Figure 6. Plasma renin activity and serum aldosterone values in captopril challenge test in different AGTR1 1166 (A) and ACE I/C, AGT Met235Thr and AGT -217 G/A (B) genotype groups. Boxes indicate median and interquartile range. P values from multivariate analyses.
Hypertensive patients with the \textit{AGTR1} 1166 CC genotype tended to have the lowest PRA levels (Figure 6A). The response (CCT 60 min minus CCT 0 min) of PRA to the CCT was also lowest in the CC patients \((p = 0.16\) for AA vs AC vs CC in Kruskal-Wallis test; \(p = 0.07\) for AA vs CC in Mann-Whitney test; \(p = 0.047\) for AA vs CC in multivariate analysis). Patients with the \textit{AGTR1} 1166 CC genotype had the highest levels of serum aldosterone at baseline \((p = 0.04\) for AA vs AC vs CC in Kruskal-Wallis test, \(p = 0.02\) for AA vs CC in both Mann-Whitney test and multivariate analysis) and after 60 min of captopril administration \((p = 0.03\) and 0.01, respectively). In addition, the aldosterone responses of the CC patients tended to decline more markedly after captopril administration \((p = 0.11\) for AA vs CC in multivariate analysis). No statistically significant differences in PRA or aldosterone levels were seen between the different \textit{ACE} I/D genotypes or the \textit{AGT} Met235Thr genotypes (Figure 6B). Some suggestive \textit{AGT} -217 genotype-related differences in PRA and aldosterone values could be seen (Figure 6B). Compared with the GG homozygotes, the GA group had lower PRA values \((p = 0.02\) in both Kruskal-Wallis test and GG vs GA comparison). The five AA homozygotes did not, however, follow the trend, suggesting the possibility of chance.

3.3.2 Replication of \textit{AGTR1} 1166 CC-related results in a cohort of hypertensive males (the GENRES Study)

As the study suggested an association between the \textit{AGTR1} CC genotype and low renin as well high aldosterone levels, the results were also tested in patients included the GENRES Study (Study IV). The levels of PRA, serum aldosterone and aldosterone to PRA ratio followed a pattern similar to the subjects with treatment-resistant hypertension. However, the two-sided p values were nonsignificant (Figure 7). Then the results from these two study cohorts (patients with treatment-resistant hypertension and patients in the GENRES Study) were combined. The p values from this analysis were 0.3 for PRA, 0.007 for serum aldosterone and 0.04 for aldosterone to PRA ratio (Figure 7). In addition, Fisher’s method was used to combine the p values of comparisons between the AA and CC genotype groups at baseline and obtained p values of 0.2, 0.003
and 0.02, respectively. These findings on serum aldosterone and renin suggest a recessive effect of the C allele.

Figure 7. Plasma renin activity and serum aldosterone concentrations and their ratio at baseline in the different AGTR1 1166 genotype groups in A. subjects with treatment-resistant hypertension, B. subjects of the GENRES Study, and C. in the combined (A + B) group. Boxes indicate median and interquartile range.
3.4 Serum and urinary electrolytes in relation to the polymorphisms of the RAS

Serum and urinary electrolytes were also analysed according to the different polymorphisms of the RAS including AGTRI 1166 A/C, ACE I/D, AGT Met235Thr and AGT –217 G/A. No significant differences in serum and urinary electrolytes could be seen.

4. Common genetic variants of the RAS / ADD1 in the pharmacogenetic study (Study IV)

4.1 Clinical baseline characteristics and blood pressure responses to antihypertensive medication

Clinical baseline characteristics of the study subjects in the pharmacogenetic GENRES Study (n = 233) and their OBP and ABP responses to amlodipine, bisoprolol, hydrochlorothiazide and losartan are presented in original article (Study IV, Table 1). Laboratory tests were taken after the first placebo run-in period. There were no significant differences between the genotypes of the ADD1 Gly460Trp, AGTRI 1166 A/C, ACE I/D and AGT Met235Thr polymorphisms, and age, BMI, duration of hypertension, or placebo BP levels.

4.2 Genotype and allele frequencies

The genotype frequencies of the ADD1 Gly460Trp, AGTRI 1166 A/C, ACE I/D and AGT Met235Thr polymorphisms are summarised in Table 7. The genotype distributions were in agreement with the Hardy-Weinberg equilibrium. The C allele of the AGTRI
polymorphism proved to be more common in patients with hypertension in Study III (allele frequency, 0.20) compared with GENRES patients (0.15, p = 0.02).

Table 7. Genotype numbers and their frequencies (% in parenthesis) in hypertensive male patients in the GENRES study.

<table>
<thead>
<tr>
<th>Gene polymorphism</th>
<th>n=233</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADD1 Gly469Trp</td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>134 (58)</td>
</tr>
<tr>
<td>GW</td>
<td>85 (36)</td>
</tr>
<tr>
<td>WW</td>
<td>14 (6)</td>
</tr>
<tr>
<td>MAF</td>
<td>0.24</td>
</tr>
<tr>
<td>AGTR1 1166 A/C</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>170 (62)</td>
</tr>
<tr>
<td>AC</td>
<td>57 (35)</td>
</tr>
<tr>
<td>CC</td>
<td>6 (3)</td>
</tr>
<tr>
<td>MAF</td>
<td>0.15</td>
</tr>
<tr>
<td>ACE I/D</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>42 (18)</td>
</tr>
<tr>
<td>ID</td>
<td>119 (51)</td>
</tr>
<tr>
<td>DD</td>
<td>72 (31)</td>
</tr>
<tr>
<td>MAF</td>
<td>0.44</td>
</tr>
<tr>
<td>AGT Met235Thr</td>
<td></td>
</tr>
<tr>
<td>MM</td>
<td>88 (38)</td>
</tr>
<tr>
<td>MT</td>
<td>103 (44)</td>
</tr>
<tr>
<td>TT</td>
<td>42 (18)</td>
</tr>
<tr>
<td>MAF</td>
<td>0.40</td>
</tr>
</tbody>
</table>

MAF = minor allele frequency

4.3 Biochemical laboratory values in relation to the different genotypes

The association of the selected gene polymorphisms with PRA and plasma aldosterone levels as well as serum and urinary electrolytes was also explored. The median PRA and aldosterone values for the AGTR1 1166 A/C polymorphism are reported in Study III (Figure 7). The trends towards lower PRA and higher aldosterone levels in the CC genotype were not statistically significant. Serum sodium levels tended to increase with the number of the ADD1 460Trp alleles; the median values were 142 mmol/l for the GlyGly, 142 for GlyTrp and 143 for TrpTrp genotype (p = 0.02 in Joncheere-Terpstra test). The presence of the AGT 235Thr allele showed a borderline association with lower PRA values, with median values of 1.2 for the MetMet, 1.2 for MetThr and 0.7 μg/l/h.
for ThrThr genotypes (p = 0.07 in Joncheere-Terpstra test). The ACE I/D polymorphism was not related to PRA or aldosterone levels, or serum and urinary electrolytes.

4.4 White coat effect

WCE and OBP and ABP levels from placebo periods are shown in original article (Study IV, Table 2). OBP levels were systematically higher than ABP levels, as was expected. Systolic WCE tended to increase with the number of the Trp allele of the ADD1 Gly460Trp polymorphism and the Thr allele of the AGT Met235Thr polymorphism (p = 0.03 for the ADD1 460Trp and, p = 0.01 for the AGT 235Thr, in Joncheere-Terpsta test). These associations were statistically significant also in multivariate analysis (p = 0.04 for the ADD1 Gly460Trp, and p = 0.03 for the AGT Met235Thr). This was a surprising result, which also may represent a chance finding. There are no previous reports on genetic associations on WCE. Diastolic WCE was not significantly associated with any of the studied polymorphisms.

5. Genetic mutations and variants and their blood pressure responses to antihypertensive drugs

5.1 Responses to ENaC blockers (Study I)

The Proband 1 with the β-ENaC Thr601→Frmshft mutation was treated with triamterene 50 mg daily. Her BP level decreased from 190/120 to around 130/95 mmHg. Her serum potassium level increased from 2.4 to normal, 3.8 mmol/l (Table 4). The Proband’s mother became normotensive based on ambulatory BP recordings after one month treatment with amiloride 10 mg daily. Her mean daytime BP dropped from 174/90 to 141/82 mmHg, and night-time from 152/74 to 130/73 mmHg. In addition, serum potassium was normalised from 3.3 to 4.0 mmol/l.
OBP measurements of the Proband 2 with the \(\gamma\)-ENaC Asn530Ser mutation revealed BP level of 180/120 mmHg. Three months treatment with triamterene 50 mg + amlodipine 10 mg daily reduced his ABP from a mean daytime level 140/95 to 126/83 mmHg (Figure 8). Upon treatment, his serum potassium level increased from 3.0 up to 4.7 mmol/l (Table 4). The Proband’s mother was diagnosed to have hypertension at the age of 40 years. After treatment with \(\beta\)-blocker atenolol, her BP remained at 140/90 mmHg, but with the combination of atenolol 50 mg, hydrochlorothiazide 25 mg and amiloride 2.5 mg daily, her BP level dropped to 110/80 mmHg.

![Figure 8. Ambulatory blood pressure measurement of the Proband 2. Treatment with amlodipine alone (upper) and in combination with ENaC blocker triamterene (lower).](image)

5.2 Blood pressure responses to acute ACE inhibition (Study III)

Baseline plasma renin levels correlated positively to systolic and diastolic BP responses during the CCT (ie. the higher PRA, the larger BP reduction). In contrast, BP responses upon the CCT, analysed according to the \(AGTR1\) 1166 A/C, \(ACE\) I/D or \(AGT\) Met235Thr or -217 G/A polymorphisms, showed no statistical significant differences in relation to the genotypes.
5.3 Pharmacogenetic effects of antihypertensive drugs (Study IV)

The impact of the genetic variants of the RAS and ADD1 on BP responses to antihypertensive drug therapy was evaluated. All the detailed results of ABP and OBP responses from drug treatment periods are shown in original article (Study IV, Tables 3 and 4, and Supplementary Information Tables 5 and 6).

The variant ADD1 460Trp allele was associated with a blunted systolic ABP response to hydrochlorothiazide (p = 0.01 in Joncheere-Terpsta test). In multivariate analysis, when adjusted for earlier use of antihypertensive medication or a thiazide diuretic, the difference remained significant (p = 0.03). Combination of the ADD1 460Trp allele carriers into only one group (GlyTrp and TrpTrp), as in most of the earlier studies, gave corresponding results (data not shown). When only subjects without earlier diuretic treatment (n = 109 for the GlyGly, 64 for GlyTrp and 11 for TrpTrp genotypes) were taken to the analyses, the results remained similar. When subjects were further restricted to the 44 without any earlier antihypertensive treatment (n = 28 for the GlyGly, 14 for GlyTrp and 2 for TrpTrp genotypes), there was no difference in BP responses to hydrochlorothiazide between the ADD1 genotype groups. The ADD1 460Trp allele was not significantly associated with diastolic ABP responses, or systolic and diastolic OBP responses, to hydrochlorothiazide treatment. The ADD1 460Trp allele was also associated with lower systolic ABP response to bisoprolol in univariate analysis (p = 0.03 in Joncheere-Terpsta test), but in multivariate analysis the difference was not statistically significant (p = 0.09). The ADD1 460 polymorphism did not associate with BP responses to amlodipine or losartan.

The AGT 235Thr allele was associated with slightly but not significantly lower ABP responses to losartan (p = 0.06 for systolic and 0.09 for diastolic BP). In multivariate analysis, the difference was also significant (p = 0.04 for systolic ABP response). The AGT 235 genotypes did not associate with ABP or OBP responses to HCT, bisoprolol and amlodipine.
The ACE I/D and AGTR1 1166 A/C polymorphisms were not associated with BP responses to any of the study drugs. When the combination of the AGTR1 1166 AC and CC genotypes were compared with the AA genotypes, no difference emerged between these two groups.

In addition, upon search for possible gene-gene interactions of the combined ACE and ADD1 genotypes associated with BP responses to any of the study drugs, no significant results were revealed by linear regression analysis. Further, when subjects with the combination of the ACE DD and ADD1 GlyGly genotype (n = 37-40) were compared against subjects with the combination of the ACE II and ADD1 GlyTrp+TrpTrp genotype (n = 14-18 in different drugs) as suggested by Sciarrone et al. (2003), there were no statistically significant differences in placebo BP levels or BP responses to the drugs studied.

6. Functional studies

6.1 Mutations of Liddle’s syndrome (Study I)

All previously described mutations associated with Liddle’s syndrome have been point or frameshift mutations located in the intracellular domain and affecting the PY motif of the β- or γ-ENaC genes. Such mutated channels have been shown to increase the amiloride-sensitive sodium current compared with wild-type channels (Table 2) (Hansson et al. 1995a, Schild et al. 1995, Jeunemaitre et al. 1997, Hansson et al. 1995b, Tamura et al. 1996, Inoue et al. 1998a, Furuhashi et al. 2005, Rossi et al. 2008). Functional experiments with the β-ENaC Thr601→Frmshft were not considered to be necessary, since it is a typical Liddle’s syndrome mutation causing removal of the PY motif. In contrast, it was important to elucidate the functional consequences of the γ-ENaC Asn530Ser mutation. When expressed in Xenopus oocytes, the mutant construct showed 2-fold increase in the amiloride sensitive sodium-current compared with the wild-type channel. To distinguish whether the increased activity of mutated ENaC was
caused by an increased number of the mutated channels on the cell surface or an increased flux of ions through the channels, the expression was also tested with a specific FLAG-anti-FLAG recognition method, which reflects the expression of the number of channels on the cell surface (Firsov et al. 1996). The cell surface expression did not differ between the wild-type and mutant ENaC suggesting that the higher activity of the mutant ENaC is due to higher flux of sodium ions, and not a higher number of ion channels on the cell membrane.

6.2 ENaC variants (Study II)

The β-ENaC Gly589Ser and γ-ENaC Val547Ile were expressed in Xenopus oocyte expression system, using a protocol similar to that in Study I. These mutations did not affect ENaC channel activity significantly, when compared with the wild-type channel, as measured by the amiloride-sensitive sodium current. This suggests that neither of these mutations have functional consequences, at least to the extent detectable by this type of in vitro system. However, there was a borderline increase in the sodium current for the β-ENaC Gly589Ser variant compared with the wild-type subunit.

Intronic mutations do not change the DNA coding sequence, but they may affect regulation of gene expression or may be involved in the mRNA splicing process. In order to examine the potential effect of the intronal mutation β-ENaC i12-17 on mRNA splicing, cDNA was made from RNA fraction prepared from human lymphocytes of two heterozygous carriers and one non-carrier of the mutation. Regardless the primers used in RT-PCR, permitting the identification of a possible failure to splice the intron 12, cDNA sequence analysis showed only normally spliced mRNA in the β-ENaC i12-17CT carriers. Thus, no evidence of a splicing defect could be demonstrated.
6.3 Functional test of the AGTR1 gene (Study III)

The effect of the AGTR1 1166 A/C polymorphism on AGTR1 gene expression was studied in an indirect way, using a reporter plasmid construct (luciferase) linked to the 3’-UTR of the AGTR1 mRNA. Three different plasmids were transfected into HEK293 cells: the first containing the luciferase coding region only, the second containing the luciferase coding region with AGTR1 mRNA 3’UTR with 1166A, and the third containing the luciferase coding region with the mRNA 3’UTR with AGTR1 1166C. The AGTR1 1166C polymorphism increased the luciferase activity by about 2-fold compared with AGTR1 1166A (p < 0.05). These data suggest that the CC genotype may be associated with increased AGTR1 expression through elevated mRNA levels.
DISCUSSION

1. Challenges in collecting study participants

Since probably only less than half of BP variation in population level is accounted for by genetic factors, and the rest by environmental factors, it is important to characterise hypertension study participants properly for the interest of phenotype (Kurtz, Spence 1993). Subjects with secondary hypertension and other disturbing factors should be excluded from a cohort of patients with essential hypertension. Previously, it has been estimated that primary aldosteronism accounts for up to 20% of patients with treatment-resistant hypertension (Calhoun et al. 2008). In this respect, 13% of subjects with treatment-resistant hypertension donated DNA samples for genetic studies were excluded from genotype-phenotype association analyses. On the other hand, phenotype of patients with Liddle’s syndrome may vary notably even in family members which can leave possible candidates outside molecular studies (Botero-Velez et al. 1994, Jeunemaitre et al. 1997). In this study (Study I), both probands had typical clinical features of Liddle’s syndrome, which made exhilarating to collect their DNA samples for molecular studies.

The cohort of treatment-resistant hypertensive patients was collected retrospectively from the database of patients visited in the Hypertension Outpatients Ward, Helsinki University Central Hospital (Studies I-III). Such study cases may represent extremity of population, but on the other hand, sample collection from clinical databases creates a picture of how clinical methods really work in multiform type of patients in practice. At present, CCT has been replaced by non-invasive radiological techniques, and its diagnostic value in the exclusion of renovascular hypertension is limited (Lenz et al. 1999). However, it may provide data of renin and aldosterone levels in a single patient, and in special cases radiological techniques cannot be used. It can be also postulated that the CCT could serve as a valuable model as a challenge test to reveal hidden genetic influences on the activity of the RAS.
In this study, the CCT and postural test were carefully performed and were made in the same protocol for each patient. Antihypertensive medication was withdrawn adequately to avoid distracting effects to biochemical test variables, and only calcium channel blockers were allowed. Circadian rhythms, which may affect plasma renin and aldosterone (Lamarre-Cliche et al. 2005), had also been considered, because the tests were done at mornings. Only food and salt intake were not controlled or restricted.

The pharmacogenetic GENRES Study was a randomised, prospective, double-blind single-centre study. BP responses were examined with four different antihypertensive drugs, including angiotensin II receptor antagonist losartan 50 mg, β-adrenergic antagonist bisoprolol 5 mg, calcium channel blocker amlodipine 5 mg, and diuretic hydrochlorothiazide 25 mg daily, after one month monotherapy. The drugs covered the four main classes of antihypertensive agents, and the doses of each group were sufficient and tolerable. They did not represent equipotent doses, since the study design was not directed to compare their pharmacologic potency. The study was validated by confirming the expected association between PRA and BP responses to different classes of antihypertensive drugs (Suonsyrjä et al. 2008), which agrees well with previous studies (Preston et al. 1998). Phenotype of the participants was also specified with certain exclusion criteria, including diabetes, cardiovascular or kidney disease or severe pulmonary disease. Gender-specific dimorphism is a typical feature in human hypertension, as males tend to have higher BP than age-matched premenopausal females (Dubey et al. 2002). Accordingly, participants were limited only to males to reduce intrinsic and extrinsic hormonal factors, including menstrual cycle, to the variation of BP. Study inclusion criteria assumed that subjects were already on antihypertensive medication (ie. the diagnosis of hypertension was done prior to the study) or three diastolic BP recordings ≥ 95 mmHg were measured on separate visits previously. High BP phenotype was also assured by OBP and ABP measurement after the first placebo period. If patient revealed normotensive according to the recordings, he was excluded from the study. Each study participant went through eight months lasting study protocol. It is possible that living conditions and life style have changed from the beginning to the end of the study affecting BP levels. However, patients visited every four week in the study laboratory. In this regard, measurements resemble those made in
normal clinical practice. Especially, placebo-BP levels were quite accurate, because they were based on four separate periods in most of the study subjects, which also reduces random effects in drug responses.

Reference groups, randomly chosen blood donors and normotensive males, accompanied the analyses of allele distributions. In principle, even though blood donors are healthy individuals, they are allowed to have one medication or one drug-combination product for treatment of hypertension. This data was not available, since the study protocol was not designed to disclose health information of the controls. This is a limitation of the study, and as such may hide possible allelic associations between patients and controls.

2. Variation of results in different studies

Polygenic etiology makes studies on genes involved in hypertension extremely challenging which is reflected in inconsistency of published results. Divergent data may result from differences in the study design, sample sizes, and choice of the study populations which may show differences in age, race, sex, and BMI, as well as use of control groups. Diagnosis of hypertension may have been based on a single point estimate of BP, and controls may have been collected even from different populations as study cases (Mottl et al. 2008), which may cause erroneous results, since allele frequencies often vary from population to another (Corvol, Jeunemaitre 1997). Most of the association studies have examined single nucleotide polymorphisms. On the other hand, combinations of polymorphisms may lead to results different from analysis of single nucleotide polymorphisms (van Rossum et al. 2005). The ABP data increases the relevance of the results, as they are more reproducible (Hiltunen et al 2007, Bottini et al 1992) and predict better cardiovascular morbidity than OBP measurements (Staessen et al. 1999b, Verdecchia 2000), but is less frequently available.
3. Liddle’s syndrome and epithelial sodium channel

In this study, two previously unpublished mutations, one in the β-ENaC (β-ENaC Thr601→Pnmshift) and the other in the γ-ENaC (γ-ENaC Asn530Ser) genes, causing Liddle’s syndrome were found. The γ-ENaC Asn530Ser mutation is the first mutation found in the extracellular domain of an ENaC gene among patients with typical Liddle’s syndrome like symptoms. Importantly, it does not affect the PY motif of the γ-ENaC. These ENaC mutations in Liddle’s syndrome are the first ones that have been described at the molecular level in Finland. In addition to these, 17 Liddle additional mutations have been reported in literature (for summary, see Table 2). Of these, 14 were located in the β-ENaC gene and three in the γ-ENaC gene. Nationalities among the carriers of the mutations have varied from Swedish, Czech, British, Italian, Portuguese, to Japanese, American, Afro-American, Afro-Haitian and Chinese (Table 2).

Liddle’s syndrome appears to be rare, at least if judged on the basis of molecular documentation. It is possible, however, that not all novel types of mutations will be published today. On the other hand, Liddle’s syndrome may often remain undiagnosed. The carriers of the mutations have been hypertensive, but not necessarily hypokalemic (Findling et al. 1997, Freundlich et al. 2005). Typically, the syndrome becomes diagnosed in young adulthood, although the youngest index case was four years of age at the time of molecular studies (Freundlich, Ludwig 2005). Routine molecular diagnostics of Liddle’s syndrome is hardly available in any center. Liddle’s syndrome may be present even when a positive family history of hypertension is lacking, since sporadic cases have been reported (Nakano et al. 2002, Yamashita et al. 2001, Wang et al. 2007, Hansson et al. 1995b, Uehara et al. 1998). Clinical suspicion of Liddle’s syndrome, with or without family history but with typical laboratory values, could justify an empiric treatment with ENaC antagonists triamterene or amiloride, which in these patients may lower BP levels as a monotherapy.
4. Epithelial sodium channel and essential hypertension

Three variants in the ENaC gene were found in this study, including \( \beta\)-ENaC Gly589Ser, \( \gamma\)-ENaC Val546Ile, and \( \beta\)-ENaC i12-17CT. All of them were present in heterozygous state, and no homozygous or compound heterozygous individual was identified. The prevalence of the variants was 9% in patients with hypertension, which was three times higher than the corresponding prevalences in two control populations. Patients with variants showed increased urinary potassium rate in relation to their renin levels, suggesting the possibility of increased mineralocorticoid effect. The \( \beta\)-ENaC Gly589Ser turned out to be previously reported in two single patients, one in France (Persu et al. 1998) and the other in Sweden (Melander et al. 1998). When in the present study the \( \beta\)-ENaC Gly589Ser and \( \gamma\)-ENaC Val546Ile were subjected to Xenopus oocyte system to test the functional significance of the polymorphisms \textit{in vitro}, no increased channel activity of the mutated ENaC could be shown, when compared with the wild-type channel. However, there was a borderline increase in sodium current for the \( \beta\)-ENaC Gly589Ser variant compared with the wild-type. Previously, this substitution was reported to result in a significant 1.3 to 1.5-fold increase in the activity of the channel compared with the wild-type (Persu et al. 1998). It is possible that functional testing of the \( \beta\)-ENaC Gly589Ser in \textit{Xenopus} oocytes is not sensitive enough to detect subtle alterations in channel activity and may thus not perfectly mirror the situation \textit{in vivo}. As to the intronal mutation \( \beta\)-ENaC i12-17CT, no clear evidence for alteration in mRNA splicing could be obtained. It remains to be investigated whether this rare variant is in linkage disequilibrium with some other DNA alteration, possibly associated with increase in \( \beta\)-ENaC activity.

The results on the three ENaC variants identified have not yet been replicated by other investigators. Because ENaC has a critical role in sodium reabsorption and its relation to Liddle’s syndrome has been unequivocally demonstrated, it can be seen as good candidate gene for essential hypertension, too. However, currently there is no certain evidence that the ENaC variants cause susceptibility to essential hypertension or modify its phenotype.
Hypertensive individuals carrying the ENaC variants tended to have increased urinary potassium excretion rate in relation to prevailing plasma renin and aldosterone levels in comparison with non-carriers. Especially, daily urinary potassium in relation to plasma aldosterone demonstrated higher ratios in the variant carriers compared with non-carriers in females, but not in males. This may be explained by sexual dimorphism of human hypertension (Dubey et al. 2002).

5. Polymorphisms of the RAS and essential hypertension

5.1 Association of RAS gene polymorphisms with hypertension

The first candidate gene associated with human hypertension was the AGT gene (Jeunemaitre et al. 1992). Afterwards, the results of the genes in the RAS associated with human hypertension have been divergent which may have resulted from differences in ethnic background, age and gender of the study subjects, and size of the patient and control groups. In this study, the distribution of the polymorphisms of the RAS studied, including AGTR1 1166 A/C, ACE I/D or AGT Met235Thr or -217 G/A, did not differ between hypertensive subjects comprising both males and females, and normotensive male controls. Even if only hypertensive males from the patient cohort (Study III) were taken to the analysis, no differences in allele frequencies between patients and controls were found.

5.2 Association of RAS gene polymorphisms with the activity of RAS in essential hypertension

In this study, there was a tendency to elevated plasma aldosterone and diminished plasma renin levels in association with the AGTR1 1166 C allele when present in homozygous form. In contrast, there were no significant differences in PRA and aldosterone levels with the ACE I/D or AGT Met235Thr or -217 G/A polymorphisms.
This assumption was supported by the data coming from two independent groups of hypertensive subjects. However, the C allele was uncommon and the number of homozygous subjects was small even when two hypertensive cohorts were combined. In addition, there was a large inter-individual variation in renin and aldosterone levels. There is only limited data from earlier studies for comparison. In 29 Dutch hypertensive patients, there were no differences in responses in renin and aldosterone to the intravenous active metabolite of losartan, AGTR1 antagonist, between the AA and CC genotypes of the AGTR1 1166 A/C polymorphism, during a low-salt diet (Spiering et al. 2005). Baseline renin and aldosterone levels did not differ in these two genotype groups, in contrast to the present study (Spiering et al. 2005). In another previous study, 66 healthy normotensive subjects were divided into subgroups according to the presence or absence of the C allele, as a result of two genotype groups, the AA and combined AC + CC groups. The C allele was associated with higher aldosterone levels at baseline, and two and three hours post-dose, after a single dose losartan. In addition, aldosterone response for losartan was increased in the C allele carriers compared with non-carriers (Miller et al. 1999). The polymorphisms ACE I/D (Mondorf et al. 1998, Nakano et al. 1997) or the AGT Met235Thr (Mondorf et al. 1998) did not predict better PRA responses to ACE inhibitor captopril in patients with essential hypertension, which findings are reminiscent of the results of the present study.

Cardiovascular effects of Ang II are mediated via AGTR1, resulting in vasoconstriction and aldosterone production, as well as BP rise. Subsequently, renin secretion is inhibited by negative feedback mechanism. The AGTR1 gene has been associated with essential hypertension (Bonnerdeux et al. 1994). However, there were no differences between the genotype groups of the AGTR1 1166 A/C polymorphism with respect to renin or aldosterone responses to Ang II (Spiering et al. 2000). In contrast, functional in vitro testing suggested that the 1166 C allele might be associated with increased AGTR1 mRNA expression and, therefore, predispose to increased Ang II effects (Study III). It is of interest that two previous studies have reported that the presence of the 1166 C allele attenuates binding of the regulatory microRNA-155 to AGTR1 3’-UTR, which in turn may result in increased expression of AGTR1 (Martin et al 2007, Sethupathy et al 2007). This effect seems to be inherited along the C allele as a recessive trait.
6. Pharmacogenetic effects

Studies on the relation between polymorphisms of the RAS and ADD1 genes and responses to different antihypertensive medications have mostly resulted in inconsisted findings. Most of the studies have suffered from small sample sizes, retrospective study designs, and lack of randomization. In many cases, pharmacogenetic studies were not designed as randomized clinical trials but have merely been observational in nature (Schelleman et al. 2006b). In the present study, attempts to identify pharmacogenetic effects were designed in two ways. First, the effect of a single dose of captopril on BP (and plasma renin and aldosterone) responses were evaluated in relation to polymorphism of the RAS in hypertensive patients. This short-term study took advantage of a previous prospective clinical study in which this drug challenge test was originally used to evaluate its value in diagnosis of renovascular hypertension. Second, genetic variants of the RAS and ADD1 were prospectively evaluated in relation to long-term responses of four different antihypertensive drugs, using a system in which each monotherapy period lasted for one month with one month’s intervening placebo periods.

6.1 The relation of the RAS gene polymorphisms to blood pressure lowering during short-term ACE inhibition

No significant differences in responses to BP to a single dose of oral captopril between the different genotype groups of the polymorphisms of the RAS including AGTR1 1166 A/C, ACE I/D or AGT Met235Thr or -217 G/A were shown in this study. Systolic BP response was mildly but non-significantly blunted in the AGTR1 1166 CC patients compared with the AA and AC patients.

The effects of a single-dose ACE inhibitor or AGTR1 blocker on BP and the variants of the RAS have been evaluated in a few previous studies. Nakano et al. (1997) found no difference in responses to BP after one hour of a single 50 mg dose of oral captopril.
between the three *ACE* I/D genotypes in Japanese patients with essential hypertension. Todd et al. (1995) reported that the I/D polymorphisms did not predict the responses to BP after a single 10 mg dose of enalapril in 27 normotensive males in a one day follow-up. Mondorf et al. (1998) investigated the effect of 50 mg dose of oral captopril on BP level, and found greater, but non-significant, BP response in DD carriers of the *ACE* polymorphism compared with the II carriers after captopril. In contrast, no pharmacogenetic *AGT* Met235Thr–related effects were found (Mondorf et al. 1998). In the study of Miller et al. (1999), a single dose of losartan, an angiotensin receptor antagonist, decreased mean arterial pressure and increased the aldosterone response in the combined *AGTR1* 1166 AC+CC group, while it did not influence significantly these parameters in the AA group. Their findings are similar to our data on the CC carriers, showing higher aldosterone values than the AA or AC carriers. Spiering et al. (2005) found no differences in responses of BP after AGTR1 blockade with active metabolite of losartan between the AA and CC genotypes of the *AGTR1* 1166 A/C polymorphism during low salt diet in 29 Dutch essential hypertensive patients. Instead, during high salt diet, the BP response was blunted in the CC carriers compared with the AA carriers. The investigators suggested that in order to see true differences between AA and CC patients, the studies should be carried out under low salt conditions, when the RAS in turned on. The acute gene-drug effects of the *AGT* -217 G/A polymorphism on BP responses have not been reported previously. There is one earlier study of two months treatment of ACE inhibitors, wherein subjects were divided into the AA and GA+AA genotype groups of the *AGT* -217 G/A polymorphism in 194 hypertensive patients of African ancestry (Woodiwiss et al. 2006). The G allele was associated with increased BP as well PRA responses after treatment. Overall, there are no conclusive and well-replicated data on the relation between various RAS gene variants and response to ACE inhibitor treatment.
6.2 The genotypes of the RAS and ADD1 in relation to blood pressure responses in the pharmacogenetic GENRES Study

The relationships between genetic variants of the RAS and ADD1 and one month use of losartan, bisoprolol, amlodipin and hydrochlorothiazide on BP responses were evaluated (Study IV). The AGT 235Thr allele was non-significantly associated with blunted BP responses to losartan compared with the Met allele. In contrast, the AGTR1 1166 A/C and ACE I/D polymorphisms were not associated with BP responses to any of the study drugs.

Some of the previous studies investigated the effect of the AGT Met235Thr polymorphisms, showed better BP response associated with the AGT 235Thr allele with ACE inhibitors (Hingorani et al. 1995), or β-blockers (Kurland et al. 2004), while some other studies found no gene-drug interaction with β-blockers, ACE inhibitors, calcium channel antagonist, or angiotensin receptor blockers on BP response between the AGT Met235Thr genotypes (Kurland et al. 2001, Dudley et al. 1996, Schelleman et al. 2006b, Kurland et al. 2004). The results of our study are in line with these studies, indicating no distinct evidence for an association between the AGT Met235Thr genotypes and BP response with different antihypertensive drugs.

The C allele of the AGTR1 1166 A/C polymorphism has been associated with increased response to AGTR1 antagonist (Benetos et al. 1996). Though the AGTR1 1166 A/C polymorphism has been associated with hypertension (Bonnardeaux et al. 1994), no gene-drug interaction was found between the AGTR1 1166 A/C polymorphism and AGTR1 antagonist, ACE inhibitor, β-blocker or calcium channel antagonist (Hingorani et al. 1995, Kurland et al. 2001, Redon et al. 2005, Benetos et al. 1996). The present study could not, either, show any associations of the AGTR1 1166 A/C polymorphism in responses to BP. It is still possible that patients homozygous for the AGTR1 C allele comprise a pharmacogenetically distinct group. The GENRES Study contained only six individuals with this genotype, which may hamper definite conclusions on their responses. Moreover, the novel data of the present study, showing that the CC genotype is characterized by increased aldosterone to renin ratio, render aldosterone antagonists
as attractive candidates in this patient group. Clearly, studies in which the effectiveness of spironolactone and/or related drugs are compared according to different AGTR1 genotypes are warranted.

In previous pharmacogenetic studies, the ACE I/D polymorphisms have been associated with antihypertensive drug responses with conflicting results. Some studies have shown no difference in BP responses between the ACE I/D genotypes to different drugs (Hingorani et al. 1995, Dudley et al. 1996, Harrap et al. 2003, Yu et al. 2003, Arnett et al. 2005, Sasaki et al. 1996, Redon et al. 2005, Schelleman et al. 2006a), or better BP response was observed to diuretics, ACE inhibitors and AGTR1 antagonists associated with the I allele (Haas et al. 1998, Ohmichi et al. 1997, O’Toole et al. 1998, Kurland et al. 2001, Schwartz et al. 2002, Sciarrone et al. 2003) or DD genotype (Li et al. 2003, Stavroulakis et al. 2000). Collectively, there appears no consistent evidence for an association between ACE genotypes and antihypertensive drug responses.

The presence of the ADD1 460Trp allele did not predict better BP response to hydrochlorothiazide contrary to some previous reports (Cusi et al. 1997, Glorioso et al. 1999, Sciarrone et al. 2003). In contrast, the Trp allele was associated with blunted BP response to hydrochlorothiazide (Study IV). In previous studies from Italy, the ADD1 460Trp allele predicted better response to diuretics (Cusi et al. 1997, Glorioso et al. 1999, Sciarrone et al. 2003). On the contrary, these findings were not reproduced in some other studies (Schelleman et al. 2006b, Turner et al. 2003). According to the very large GenHAT study (Davis et al. 2007), the Trp allele of the ADD1 460 polymorphism did not seem to be a useful clinical marker for BP response to diuretics (chlorthalidone), or any other antihypertensive drugs, amlodipine, lisinoprol or doxazosin, which data are in harmony with those of the present study. Quite recently, Bianchi et al. (2009) took notice of controversial findings in the pharmacogenetic studies of ADD1 and hydrochlorothiazide treatment, emphasizing differences in study designs. Factors, such as too short wash-out period to remove the effect of the previous antihypertensive medication, or switch to diuretic treatment from previous treatment without any wash-out, or other confounding effects including diuretic treatment given in combination
therapy, were suggested. The role of the ADD1 Gly460Trp polymorphism is still under continuous debate.
CONCLUSIONS

Two novel mutations causing Liddle’s syndrome, a monogenic form of hypertension, could be identified in the present study. One of the mutations (γ-ENaC Asn530Ser) is the first, and thus far the only reported, to localize in the extracellular portion of the ENaC and to show an increased channel activity at the same time. Monogenic forms of hypertension are probably rare in general population, but they may be also underdiagnosed. Identification of such mutations may require contribution by a research laboratory specialised to molecular genetics. On the other hand, in clinical practice it may be worth of trying to treat patients with typical clinical manifestation of Liddle’s syndrome empirically with an ENaC antagonist, even when a DNA study is not feasible or does not identify a mutation.

Three different variants in the ENaC genes were found, the β-ENaC Gly589Ser, γ-ENaC Val547Ile and β-ENaC i12-17. The γ-ENaC Val546Ile and β-ENaC i12-17 are novel. The prevalence of the variants was 9% in patients with hypertension, which was three times higher than in normotensive males and randomly selected blood donors. Patients with the variant ENaC subunits showed increased urinary potassium rate in relation to their renin levels. Functional studies in vitro did not provide direct evidence for the assumption that the variants are associated with increased ENaC channel activity, but the in vitro systems used may not be sensitive enough for this purpose. Although the β- and γ-subunits of ENaC have been linked to monogenic Liddle’s syndrome, there is yet no conclusive evidence for a role of the ENaC genes in human essential hypertension.

The cardiovascular effects of Ang II are mainly mediated by AGTR1. The AGTR1 1166 A/C polymorphism was previously suggested to be associated with hypertension and/or its complications. As a novel finding, the present study demonstrates an association between increased plasma aldosterone to renin ratio and AGTR1 CC genotype, present in approximately 3% of hypertensive individuals, suggesting that this genotype determines an endocrine subphenotype. Further studies are needed to explore the possibility that this genotype may indicate a particular sensitivity to certain drugs.
There was a large inter-individual variation in responses to four main classes of antihypertensive drugs, including an angiotensin II receptor antagonist, a β-adrenergic antagonist, a calcium channel blocker, and a thiazide diuretic. No association of the studied polymorphisms of the RAS genes, AGT, ACE, AGTR1 and ADD1 with BP responses could be demonstrated. Nor could this study replicate some of the previous gene-drug interactions of positive findings. These results do not exclude the possibility that the genes tested, or some other genes implicated in regulation of BP, contain alterations that may be associated with antihypertensive drug effects. However, known polymorphisms of the RAS cannot be used in choosing antihypertensive drugs in essential hypertension for the present.
ACKNOWLEDGEMENTS

This work was carried out during the years 2000-2009 in the laboratory of Professor Kimmo Kontula at the Department of Medicine, University of Helsinki, and Research Program of Molecular Medicine, Biomedicum Helsinki. I wish to express my sincere gratitude to all those people who made this project possible and contributed to the study. Especially, I warmly thank:

Professors Reijo Tilvis and Olavi Ylikorkala, the former and present heads of the Institute of Clinical Medicine, and Professors Kimmo Kontula and Vuokko Kinnula, the former and present heads of the Department of Medicine, for providing excellent research facilities.

My supervisors, Professor Kimmo Kontula, who introduced me the interesting field of genetics of hypertension and suggested the topic to this study, and Timo Hiltunen, MD, PhD, who has guided me through the research project and offered valuable help in computers and statistical programs. I am deeply grateful for Kimmo’s and Timo’s continuous support.

Professor Eero Mervaala and Docent Olavi Ukkola for evaluating the manuscript and for their valuable comments.

Professor Ville Valtonen, the head of the Division of Infectious Diseases at the Helsinki University Central Hospital for his constant support during the study, and in addition in guidance in infectious diseases. I am also thankful for facilities the Clinic of Infectious Diseases has provided for writing process.

Docent Ilkka Tikkanen, who suggested me research work in the field of hypertension just when I had arrived to Helsinki to continue my specialisation in internal medicine.


All the present and former workers in Kimmo’s laboratory: Susanna Saarinen, Hanna Nieminen, Saara Nyqvist, Ilse Paetau, Tarja Pajunen, Tuula Soppela, Sirpa Stick and Jaana Valkeapää for
excellent technical help, and secretaries Raija Selivuo and Minna Ollikainen for help in many practical matters.

Mari Kaunisto and Maija Wessman for pleasant company at coffee breaks.

All the patients who have participated in this study.

Docent Asko Järvinen for organising research periods as well as supportive discussions, and all the colleagues and workmates, especially Inka Aho, Anneli Harjupää, Katarina Kainulainen, Pia Kivelä, Inka Liesmaa, Heikki Repo and Jussi Sutinen, for creating supportive atmosphere where to work, as well as professional and non-professional discussions.

My friends for sharing non-scientific conversations.

My parents for supporting and believing in me for all these years, and my parents-in-law for providing help in childcare for so many times.

Finally, my dear husband Jari, for being the most important support, and our daughters Anna and Aino, who have brought so much joy and happiness to my life.

This study was financially supported by grants from the Aarne Koskelo Foundation, the Biomedicum Helsinki Foundation, the Finnish Medical Foundation, the Research Foundation of Orion Corporation, the Sigrid Juselius Foundation, the Special State Share of the Helsinki University Central Hospital, and the Research Funds of the University of Helsinki.

Espoo, November 2009

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