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Blood pressure genetics: from candidate genes to genome-wide association studies

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LIST OF ORIGINAL PUBLICATIONS

I **Sõber S**, Org E, Kepp K, Juhanson P, Eyheramendy S, Gieger C, Lichtner P, Klopp N, Veldre G, Viigimaa M, Döring A; Kooperative Gesundheitsforschung in der Region Augsburg Study, Putku M, Kelgo P; HYPertension in ESTonia Study, Shaw-Hawkins S, Howard P, Onipinla A, Dobson RJ, Newhouse SJ, Brown M, Dominiczak A, Connell J, Samani N, Farrall M; MRC British Genetics of Hypertension Study, Caulfield MJ, Munroe PB, Illig T, Wichmann HE, Meitinger T, Laan M. (2009). Targeting 160 candidate genes for blood pressure regulation with a genome-wide genotyping array. PLoS One, 4(6):e6034.

II **Sõber S**, Laan M, Annilo T. (2010). MicroRNAs miR-124 and miR-135a are potential regulators of the mineralocorticoid receptor gene (NR3C2) expression. Biochem Biophys Res Commun, 391(1):727–32.

III Org E, Eyheramendy S, Juhanson P, Gieger C, Lichtner P, Klopp N, Veldre G, Döring A, Viigimaa M, **Sõber S**, Tomberg K, Eckstein G; KORA, Kelgo P, Rebane T, Shaw-Hawkins S, Howard P, Onipinla A, Dobson RJ, Newhouse SJ, Brown M, Dominiczak A, Connell J, Samani N, Farrall M; BRIGHT, Caulfield MJ, Munroe PB, Illig T, Wichmann HE, Meitinger T, Laan M. (2009). Genome-wide scan identifies CDH13 as a novel susceptibility locus contributing to blood pressure determination in two European populations. Hum Mol Genet. 15;18(12):2288–96.

In addition, unpublished data collected in the framework of the ICBP consortium (submitted for publication) is used.

Author's contributions:

Ref. I – participated in experimental design, performed *in silico* experiments, data analysis and contributed to manuscript preparation.

Ref. II – participated in experimental design, conducted experiments and data analysis and drafted the initial manuscript.

Ref. III – contributed to statistical analyses of the data.

LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
ADHD	attention deficit hyperactivity disorder
BMI	body mass index
bp	base pair
BP	blood pressure
CEU	Utah residents with Northern and Western European ancestry from the CEPH collection
CMV	cytomegalovirus
DBP	diastolic blood pressure
GWA	genome-wide association
GWAS	GWA study
HTN	hypertension
kb	kilobase (1000 base pairs)
KORA	Kooperative Gesundheitsforschung in der Region Augsburg
Mb	megabase (1000,000 base pairs)
LD	linkage disequilibrium
MAF	minor allele frequency
miRNA, miR	microRNA
mmHg	millimeters of mercury (~ 133 Pa)
Pa	pascal
qPCR	quantitative polymerase chain reaction
QTL	quantitative trait locus
RAAS	renin-angiotensin-aldosterone system
RISC	RNA-induced silencing complex
SBP	systolic blood pressure
SHR	spontaneously hypertensive rats
SNP	single nucleotide polymorphism
UTR	untranslated region
WTCCC	Wellcome trust case-control consortium

INTRODUCTION

Blood is essential for the survival of all vertebrates and many other animals. Its most important function is the transport of oxygen to target tissues and subsequent removal of waste products. The flow of blood through the circulatory system is maintained by a blood pressure gradient that is generated by the pumping action of the heart. Blood pressure is regulated by multiple interacting pathways that operate in different tissues and at different time scales to maintain adequate oxygenation of target tissues. The blood pressure of a single individual may vary substantially due to several factors including physical activity, health and psychological state. There is also considerable inter-individual variation, a significant proportion (30–60%) of which has been attributed to genetic factors (Harrap 1994). As a result of the large number of blood pressure regulating pathways and genes as well as the large influence of external environment, it is considered a genetically complex trait.

Blood pressure has proven to be one of the most difficult traits to study in human genetics. Until recently, the genetic causes of blood pressure variation in the general population remained largely impenetrable by the available genetic methods. To this day, despite a wealth of information on the physiology of blood pressure regulation and considerable interest due to the substantial public health burden associated with hypertension, the genetic basis of inter-individual variation in general human populations remains poorly understood.

The greatest insights into the genetic mechanisms underlying blood pressure regulation have come from studies of rare familial forms of blood pressure dysregulation (Lifton et al. 2001). In recent years, advances in genome-wide genotyping technology have made large-scale association studies viable. The first attempts in this field, the Framingham heart study (Levy et al. 2007) and Wellcome Trust Case Control Consortium study (WTCCC 2007) met with limited success. However, subsequent larger studies by CHARGE (Levy et al. 2009) and Global BPGen (Newton-Cheh et al. 2009) consortia proved that the concept of genome wide association studies can be successful in untangling the genetics of blood pressure regulation. Several loci attained unequivocal statistical significance in these studies and a number of the discovered associations can be robustly replicated across different populations.

Interestingly, the results of these studies are in some discord with the previous knowledge as very few of the previously known candidate genes are highlighted in these studies and several key genes in blood pressure regulating pathways appear not to contribute to the genetic variability. Also, the explained proportion of the genetic component of blood pressure variability remains small. This highlights the need for alternative approaches to identify the genes involved in these pathways. These alternatives should include a combination of molecular, biochemical and bioinformatic methods. Such integrative approach has already proven successful in ascertaining the networks and interactions involved in many biological processes, including blood pressure regulation.

The purpose of this thesis is to explore several aspects of the previously known blood pressure candidate genes using contemporary methods. The first part of the literature overview of this thesis focuses on the current knowledge of the genetics of human blood pressure regulation. The second part will give a short summary of the present understanding on microRNA based gene regulation and its known functions in the regulation of human metabolism, especially in blood pressure homeostasis. In the experimental part of this work the genetic associations with blood pressure were mapped in blood pressure candidate genes and haplotype analyses were performed, also the importance of lifestyle factors was assessed. The genes were also subjected to bioinformatic analysis of the microRNA based gene regulation system, which was followed by wet-lab experimental validation of the findings. As a complementary approach, data from a genome wide association study for blood pressure traits in European populations was analyzed.

1. LITERATURE REVIEW

1.1. Blood pressure

In large organisms oxygen and nutrients cannot reach target tissues simply by passive diffusion. This necessitates an active delivery system, which is composed of a vascular system where blood is circulated actively by the pumping of the heart. The pressure of blood in the vascular system is higher than ambient pressure in order to keep the blood vessels from collapsing and avoid components of the external environment from entering the blood stream through wounds (Anderson 1993). In addition to the static pressure, there is an actively maintained gradient of blood pressure that keeps the blood flowing from the ventricles of the heart and arteries through the capillaries into veins and ultimately back to the atria of the heart (Witzlieb 1987). In physiological terms this dynamic component of blood pressure is defined as the product of cardiac output and total peripheral resistance.

Historically, William Harvey proposed the first description of circulating blood in 1616. He disproved the hitherto undisputed belief, dating back to ancient Greek philosophers Hippocrates and Galen, that blood was continuously produced by the heart and then distributed to the body through the vessels (Harvey 2001). The end of the nineteenth century was especially pivotal in blood pressure research as physiological studies resulted in the discoveries of several blood pressure regulating substances: adrenalin (Oliver and Schafer 1895) and vasopressin (Oliver and Schafer 1895) in 1895 followed by renin in 1898 (Basso and Terragno 2001). Concurrently, Riva-Rocci reported his modified sphygmomanometer that in conjunction with the work of Nikolai Korotkoff, who published his discovery of the Korotkoff sounds in 1905, forms the basis of the most convenient and widely used method for measuring blood pressure to this day. In humans the measurements are usually taken from the upper arm and expressed in millimeters of mercury (mmHg = ~133 Pa). Two measurements are recorded – systolic blood pressure (SBP) represents the highest peak in pressure during the contraction of the heart and diastolic blood pressure (DBP) corresponds to the lowest pressure while the heart relaxes.

1.1.1. Mechanisms of blood pressure regulation

Blood pressure is regulated by several interacting mechanisms (**Figure 1**). These processes can be divided by their nature and purposes into local or global as well as long-term vs. short-term regulation pathways (Witzlieb 1987). It has to be kept in mind that this division is somewhat artificial and the blood pressure of each individual is ultimately determined by a complex interplay and cumulative action of all blood pressure regulating mechanisms. The fact that many antihypertensive medications target ‘short-term’ regulation pathways (e.g. the renin-angiotensin system) indicates that alterations in any blood pressure adjustment pathway can affect long-term blood pressure and hypertension.

The main physiological mechanism for maintaining long-term blood pressure centers on the kidney and affects blood volume. Kidneys reduce blood volume by diverting water from blood to urine. An increase in blood volume causes a rise in mean cardiovascular pressure that in turn increases cardiac output and therefore augments blood flow to the kidneys. This in turn leads to increased urinary output and a consequent reduction in blood volume (Anderson 1993). This mechanism is affected directly by hormones aldosterone (Fardella and Miller 1996) and vasopressin (Aoyagi et al. 2009) that increase salt retention and decrease urinary output. Also, the atrial natriuretic peptide induces diuresis in response to excessive workload to the heart (Potter et al. 2009). Pharmacologically, this pathway can be affected by diuretics (Duarte and Cooper-DeHoff 2010). Another central regulator of both short- and long-term blood pressure is the autonomous nervous system, particularly the sympathetic branch (Joyner et al. 2008). It mediates the baroreflex mechanism and stress responses via epinephrine and norepinephrine secretion, which causes vasoconstriction and increased heart rate (Grassi et al. 2008). α- and β-blockers can be used for pharmacological interference with this mechanism.

A critical pathway in the blood pressure regulating network is the renin-angiotensin system. This pathway is involved in acute responses to hypovolemic shock, but also maintains constant basal activation to control blood pressure homeostasis. The pathway involves stepwise conversion of angiotensinogen into angiotensin II through consecutive cleavage by renin (released from the kidneys) and angiotensin converting enzyme (ACE; released from the kidneys and lungs) (Witzlieb 1987). Angiotensin II is a powerful vasoconstrictor (rapid response) and also the strongest activator of aldosterone synthesis in the adrenal cortex (delayed, long-term response) (Fardella and Miller 1996). This intimate coupling with aldosterone release illustrates the interconnected nature of blood pressure regulating networks and is the reason why these two mechanisms are often referred to as the renin-angiotensin-aldosterone system (RAAS). The role of locally acting blood pressure control mechanisms is to distribute blood to the target tissues and organs according to their metabolic needs. These processes do not affect blood volume, instead they increase vascular compliance and reduce vascular resistance to blood flow. These processes employ vasorelaxing messenger molecules to cause increased blood flow to specific vascular beds. Several metabolically important substances have direct effect on the vascular tone. These include O_2, lactic acid, pyruvate and also ATP, ADP, AMP (Witzlieb 1987). Some gases, including NO, CO (Boehning and Snyder 2003) and H_2S (Yang et al. 2008) can also function as blood pressure regulating messenger molecules. Blood flow to some organs with wildly fluctuating requirements like skin and the gastrointestinal system are regulated by the kinin-kallikrein system. This system is present in both tissues and plasma and involves the cleavage of kininogen into active kinins (kallidin and bradykinin) by the kallikrein peptidase (Campbell 2001). The effects of vasorelaxants are counteracted by locally acting vasoconstricting substances. These include serotonin (Witzlieb 1987) and endothelins (Agapitov and Haynes 2002).

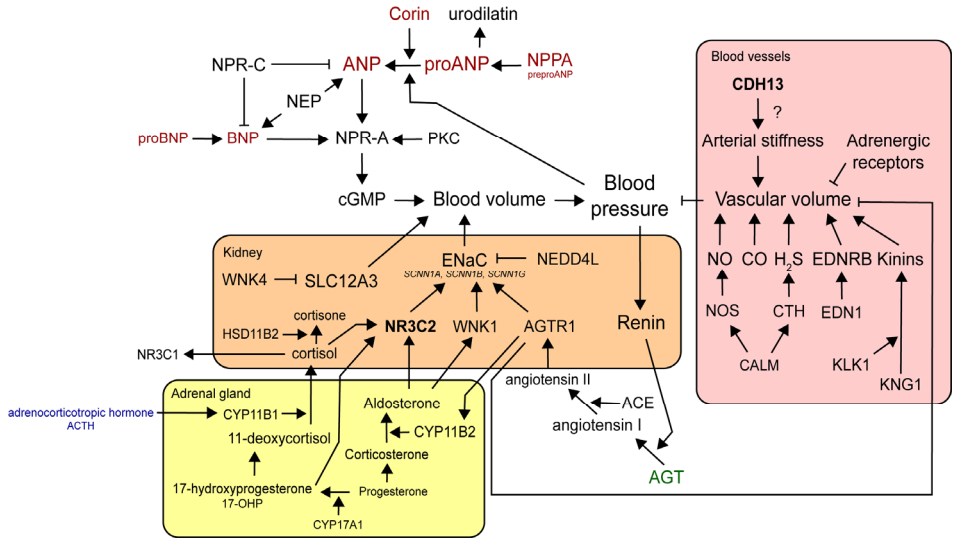

Figure 1. Blood pressure regulating organs and networks.
NPR-C – natriuretic peptide C receptor; ANP – atrial natriuretic peptide; NPPA – atrial natriuretic peptide precursor; NEP – neutral endopeptidase; BNP – brain natriuretic peptide; NPR-A – ANP receptor; PKC – protein kinase C; CDH13 – cadherin-13; NO – nitric oxide; CO – carbon monoxide; EDNRB – endothelin receptor B; NOS – nitric oxide synthase; CTH – cystathionine gamma-lyase; EDN1 – endothelin; CALM – calmodulin; KLK1 – kallikrein; KNG1 – kininogen; WNK4 – serine/threonine-protein kinase WNK4; SLC12A3 – renal thiazide-sensitive sodium-chloride cotransporter; ENaC – epithelial sodium channel; NEDD4L – E3 ubiquitin-protein ligase NEDD4-like; NR3C2 – mineralocorticoid receptor; WNK1 – serine/threonine-protein kinase WNK1; AGTR1 – angiotensin receptor 1; HSD11B2 – corticosteroid 11-beta-dehydrogenase; NR3C1 – glucocorticoid receptor; CYP11B2 – aldosterone synthase; CYP11B1 – cytochrome P450 11B1; ACE – angiotensin converting enzyme; AGT – angiotensinogen; CYP17A1 – steroid 17-alpha-hydroxylase/17,20 lyase. Colors indicate involved organs: red – heart; blue – brain; green – liver.

1.2. Diagnosis of hypertension and its treatment

Hypertension is a common pathological condition characterized by elevated blood pressure levels. It affects over 25% of adults in developed countries (>35% in Europe) (Wolf-Maier et al. 2003) and is a common cause of morbidity and mortality around the world. Hypertension is the most prevalent cause of coronary artery disease, cardiac infarction and stroke. It is classified as essential (primary) or secondary. Secondary hypertension is caused by a known underlying primary condition, usually diabetes or kidney problems. Primary hypertension is more prevalent but it has no clearly identifiable cause. The actual blood pressure levels used to diagnose hypertension by medical practitioners have changed over the years. Currently, hypertension is usually diagnosed in

adult individuals with SBP ≥ 140 mmHg or DBP ≥ 90 mmHg (Mancia et al. 2007) (**Table 1**).

Several treatments are available to lower blood pressure. Usually, physicians recommend lifestyle changes as the first course of action, but multiple effective pharmacological treatments are widely used. Several classes of drugs are available for treatment. The most commonly prescribed drugs include diuretics, ACE inhibitors, calcium antagonists, angiotensin receptor antagonists and β-blockers (Mancia et al. 2009). However, monotherapy with any single class of antihypertensive drugs often fails in a given patient (Mancia et al. 2007). In addition, in a significant fraction of patients (>20%) effective BP control is not achieved despite receiving >3 antihypertensive medications (Sarafidis and Bakris 2008). This highlights the need for the development of new antihypertensive medications and improvement of the general understanding of BP regulation, as currently the aetiology of hypertension remains unknown in the majority of cases. This lack of understanding of the causes of hypertension often leads to a time-consuming and painstaking trial and error process to find the suitable treatment regimen for the patient (Mancia et al. 2009).

Although the causes of essential hypertension are unknown, several etiological contributing factors have been identified. The risk of developing hypertension is influenced by both genetic and environmental factors (**Figure 2**). Sex and age of the individuals affect the risk of hypertension as males are known to have higher blood pressure levels compared to women and systolic blood pressure increases with age. In addition several modifiable behavioral risk factors exist. These include obesity, high salt intake, alcohol consumption, smoking and lack of exercise (Carretero and Oparil 2000).

The reverse condition to hypertension is hypotension. It is characterized by lower than normal blood pressure and is often accompanied by low mood, dizziness and fainting (De Buyzere et al. 1998). Acute cases of secondary hypotension can cause seizures and shock. Usually the condition is relatively mild and asymptomatic. Several causes are known, including a number of genetic disorders (Lifton et al. 2001).

Table 1. European Society of Hypertension guidelines for classification and diagnosis of hypertension (Mancia et al. 2007).

Category	**SBP**		**DBP**
Optimal	< 120	and	< 80
Normal	120–129	and/or	80–84
High normal	130–139	and/or	85–89
Grade 1 hypertension*	140–159	and/or	90–99
Grade 2 hypertension	160–179	and/or	100–109
Grade 3 hypertension	≥ 180	and/or	≥ 110
Isolated systolic hypertension	≥ 140	and	< 90

* – Antihypertensive treatment is recommended starting from grade 1 hypertension.

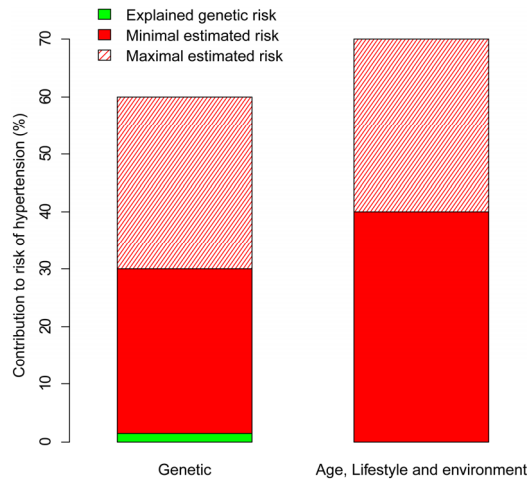

Figure 2. Schematic representation of relative contributions of genetic and environmental risk factors for developing hypertension (Ehret 2010) (Org et. al. unpublished data).

1.3. Blood pressure genetics

Since the beginnings of modern genetics a strict division has existed between studies of monogenic traits with Mendelian inheritance and the common continuous characteristics of all individuals (like height, weight or blood pressure). Monogenic traits are affected only by a single gene, which made them much easier to study and explain their inheritance. The latter category of conditions was classified as polygenic, complex traits (Motulsky 1997). Many genes that often interact with one another in unpredictable ways and may also be affected by the environment influence these traits. This separation existed largely due to methodological shortcomings and a truly unified approach to genetic studies has become experimentally feasible only in the recent years. Blood pressure has been known to be a complex, polygenic trait since the 1950s (Ehret 2010). The heritability of blood pressure has been estimated in family studies at 30–60% (Harrap 1994), with different studies giving varying results (**Figure 2**) (De Faire 1978).

1.3.1. Linkage studies of blood pressure regulation

Historically, the first methods for finding genes responsible for blood pressure regulation without prior suspected candidate loci were based on tracking the transmission of genetic markers in families (Lifton 1996). These methods rely on linkage of genomic regions in close proximity. Usually, closely situated genomic regions are transmitted together from parents to children, unless a meiotic crossing-over event occurs between them. The likelihood of such an event increases with the distance between the regions. This allows for tracking of the

segregation of genomic regions and phenotypes of interest in closely related individuals without requiring genotyping of very large numbers of markers.

1.3.1.1. Mendelian forms of blood pressure dysregulation

During the course of several decades a large number of genes involved in blood pressure regulation have been uncovered in this manner. Most of these findings have come from studies into rare Mendelian forms of hyper- and hypotension (Lifton 1996). These conditions are transmitted through the affected families in accordance with the laws of Mendelian segregation and the responsible genes are traceable by linkage-based methods. Eighteen genes that cause Mendelian forms of hypertension or hypotension have been uncovered so far (**Table 2**). These genes can affect the blood pressure of the patients to a large extent and often different variants of the same gene can lead to either increased or decreased blood pressure depending on whether the gene is deactivated or abnormally activated by the mutation (**Table 2**). In all cases of blood pressure affecting syndromes with Mendelian inheritance, the mechanism of disease has proven to be disturbed reabsorbtion of salt in the distal nephron (Lifton et al. 2001). This finding is in accordance with the central role of the kidney in maintaining long term blood pressure homeostasis (Guyton 1991). The pathology may be caused directly by impaired function of the ion-transport channels responsible for salt reabsorption (Recessive pseudohypoaldosteronism type 1, Liddle's syndrome, Gitelman's syndrome, Bartter syndrome) and their control mechanisms in the kidney (Apparent mineralocorticoid excess, Dominant pseudohypoaldosteronism type 1, Hypertension exacerbated in pregnancy, Gordon's syndrome). In other cases the disease is brought on indirectly by aberrant synthesis of aldosterone, the major regulator of renal salt reabsorption (Aldosterone synthase deficiency, Glucocorticoid remediable aldosteronism, Adrenal hyperplasia) (Vehaskari 2009).

The genes and molecular mechanisms behind some forms of monogenic hyper- and hypotension remain to be identified. Two forms of Gordon's syndrome have been described that are not caused by the known mutations in WNK kinases (Disse-Nicodeme et al. 2001). Hypertension with brachydactyly has been mapped to a region on the short arm of chromosome 12, but the underlying genes are unknown. Also Familial hyperaldosteronism type 2, possibly the most common form of monogenic hypertension (Stowasser and Gordon 2004), has been mapped to a 5 Mb region on chromosome 7p22, but the responsible gene has not yet been conclusively identified (Jeske et al. 2008; Carss et al. 2010). Traditionally, the contribution of monogenic forms of blood pressure dysregulation to the genetic component of blood pressure determination in the general population has been considered insignificant due to the rarity of these conditions (Lifton 1996). However, some of the syndromes including Liddle's syndrome and Familial hypertension type 2 have proven to be more common than previously thought (Stowasser and Gordon 2004; Vehaskari 2009) and

should therefore be considered in the clinical diagnosis of hypertension, especially in children and younger individuals. The high prevalence of hyperaldosteronism in hypertensive adult individuals (~10%) (Fardella et al. 2000; Vehaskari 2009) may point to the unappreciated role of monogenic hypertension in a significant fraction of patients. Currently many cases of monogenic hypertension may remain undiagnosed owing to cryptic familial segregation due to incomplete penetrance and the fact that the carriers of these syndromes often do not exhibit as drastic blood pressure deviations as previously thought (Vehaskari 2009).

Table 2. Genes containing mutations responsible for monogenic forms of hypertension or hypotension.

	Blood pressure			
Gene	**LoF***	**GoF****	**Syndrome**	**References**
CYP11B2	↓	↑	Aldosterone synthase deficiency, Glucocorticoid-remediable aldosteronism	(Mitsuuchi et al. 1992), (Lifton et al. 1992)
HSD11B2	↑	–	Apparent mineralocorticoid excess	(Mune et al. 1995)
SCNN1B	↓	↑	Recessive PHAI***, Liddle's syndrome	(Chang et al. 1996), (Shimkets et al. 1994)
SCNN1G	↓	↑	Recessive PHAI, Liddle's syndrome	(Chang et al. 1996), (Hansson et al. 1995)
SCNN1A	↓	–	Recessive PHAI	(Chang et al. 1996)
SLC12A3	↓	–	Gitelman's syndrome	(Simon et al. 1996)
NR3C2	↓	↑	Dominant PHAI, Hypertension exacerbated in pregnancy	(Geller et al. 1998), (Geller et al. 2000)
SLC12A1	↓	–	Bartter Syndrome type 1	(Simon et al. 1996)
KCNJ1	↓	–	Bartter Syndrome type 2	(Simon et al. 1996)
CLCNKB	↓	–	Bartter Syndrome type 3	(Simon et al. 1997)
BSND	↓	–	Bartter Syndrome type 4	(Zaffanello et al. 2006)
CASR	–	↓	Bartter Syndrome type 5	(Vezzoli et al. 2006)
WNK4	↑	–	Gordon's syndrome	(Wilson et al. 2001)
WNK1	–	↑	Gordon's syndrome	(Wilson et al. 2001)
CYP11B1	↑	–	Congenital adrenal hyperplasia	(White et al. 1991)
CYP17A1	↑	–	Congenital adrenal hyperplasia	(Kagimoto et al. 1988)
CYP21A2	↓	–	Congenital adrenal hyperplasia type 1	(Speiser and White 2003)
NR3C1	↑	–	Familial glucocorticoid resistance	(Hurley et al. 1991)

* Loss of function mutations;
** Gain of Function mutations;
*** PHAI – Pseudohypoaldosteronism type I;
Arrows indicate whether the mutations cause elevated (up) or decreased (down) blood pressure. Dashes indicate that no mutations of that category have been described.

1.3.1.2. Linkage studies of blood pressure variation in the general population

Attempts to extend linkage studies to uncover the genetic determinants of blood pressure variation in general populations were largely unsuccessful. The first such studies were targeted to candidate genes with known physiological functions in blood volume regulation or other pathways related to vascular functions. Over a hundred linkage studies focusing on 26 candidate genes have been published with conflicting and inconclusive results (Cowley 2006). Only the genes encoding angiotensinogen (*AGT*) and adducin (*ADD1* and *ADD2*) demonstrate consistent linkage to blood pressure phenotypes in some hypertensive groups (Agarwal et al. 2005).

With the increased number of suitable genetic markers (mostly microsatellites and SNPs) becoming available in the mid-1990s it became possible to perform linkage studies without defining specific target regions or genes on the genome (Kato and Julier 1999). From 1996 to 2006 over 30 genome-wide linkage scans were performed and at least one linkage peak was reported in every human chromosome (Cowley 2006).

Meta-analyses of the reported linkage results point to regions on chromosomes 17p11, 2p14 and 3p14 showing most consistent linkage to blood pressure traits (Koivukoski et al. 2004; Wu et al. 2006). Also, the reported linkage peaks cover large chromosomal regions and usually contain tens to hundreds of genes. This makes pinpointing the actual causal variants very difficult and only a few of the genes responsible for linkage signals have been identified. So far only the linkage peak on chromosome 5q23–5q35 has been successfully fine mapped to *FGF1* gene (Tomaszewski et al. 2007). In general, these studies were very hard to replicate and most of the discovered linkage peaks did not reach strict statistical significance levels after correction for multiple testing (Charchar et al. 2008). Both genome-wide and candidate gene based linkage studies of hypertension in the general population suffered from the lack of power of the linkage approach to identify the causative loci for non-mendelian, multigenic traits (Risch and Merikangas 1996).

1.3.2. Animal models in blood pressure research

Development of adequate animal models is highly beneficial for the study of any disease or phenotype. Some of the first insights into blood pressure regulation were gained from studies on rabbits and dogs (Basso and Terragno 2001). Currently, rodent models are most widely used for the study of blood pressure physiology and genetics. Several genetically homogenous hypertensive and hypotensive rat strains have been developed by selective breeding and subsequent inbreeding (Lerman et al. 2005). The most common strains in current use include spontaneously hypertensive rats (SHR) and Dahl salt-sensitive rats. Hypertensive and hypotensive strains of mice also exist, but are not widely used (Rapp 2000).

Such rat strains have been used extensively for linkage mapping of genomic regions affecting blood pressure and over 270 QTLs have been identified (Cowley 2006). These studies benefit from the genetic homogeneity of the inbred rat strains and the possibility of obtaining informative crosses between rat strains. However, the process is laborious and requires large numbers of animals to attain the statistical significance needed to define multiple linkage peaks. Also, the identified regions are large and need to be narrowed down extensively by the painstaking process of producing consomic and congenic rat strains through meticulous breeding and marker-assisted selection processes (Rapp 2000). Consequently, only a few of the genes underlying rat QTLs have been identified. So far, variants in *Cyp11b2* (Aldosterone synthase) (Rapp and Dahl 1976) and *Cd36* (Delles et al. 2009) have been implicated in underlying rat blood pressure QTLs.

Transgenic mouse models with reduced or increased expression of potential blood pressure affecting genes have proven to be effective for candidate gene confirmation and detailed analysis of the phenotypes resulting from aberrant gene function. Although most of these studies are confirmatory and only strengthen the previous evidence for the role of the gene under study, some revealing discoveries have underlined the redundancies present in the blood pressure regulating networks. For example, the disruption of adrenergic pathways does not produce long-term alterations in blood pressure in the studied rodent models (Lerman et al. 2005) and the knockout of the potent vasoconstrictor endothelin actually causes elevation of blood pressure in mice (Kurihara et al. 1994).

It has to be kept in mind that the results from animal models cannot be guaranteed to apply for humans since the marked differences in body size, posture, diet etc. are likely to result in differences in blood pressure physiology between species. The nocturnal lifestyle of these animals has to be taken into account as well. Even in the circumstances where the physiology is comparable, genetic differences may result in situations where a gene that causes BP variation between rodent lines may not contain informative polymorphism in humans (Delles et al. 2009).

1.3.3. Association studies of blood pressure regulation

Linkage studies have not revealed any major linkage peaks present for blood pressure traits (Rafiq et al. 2010). This indicated that the hope that variation in only a few genes is responsible for the majority of inter-individual blood pressure variation (oligogenic hypothesis) is unfounded and in fact many genes and loci are involved. Traditional linkage studies are unsuited for untangling loci involved in this type of inheritance and association studies were proposed as an alternative approach (Risch and Merikangas 1996). Instead of looking for cosegregation of markers with the studied phenotype in families like in the case

of linkage studies, association studies look for correlations of marker genotypes with the phenotype of interest in samples of unrelated individuals.

Blood pressure is a classical continuous trait (with continuous distribution from low to high values) (**Figure 3**) and therefore requires the use of appropriate analysis methods to perform association testing. As an alternative approach, binary analysis methods can be used if the study sample is divided into cases and controls according to the presence of hypertension. This has the benefits of allowing the use of analysis methods applicable only to binary traits, but also has the disadvantages of discarding some of the relevant quantitative data and requires setting relatively arbitrary cutoffs to define hypertension. This may pose problems in comparing the results of different studies.

Binary trait analysis also avoids the issue of individuals receiving antihypertensive treatment as they can be automatically categorized as cases. This issue is a significant problem in quantitative analysis of blood pressure because a marked proportion of hypertensive individuals are receiving blood pressure lowering medications that obscure their true blood pressure values. Currently there are two ways to deal with this problem. The simplest method is to exclude the individuals receiving antihypertensive treatment. This is a straightforward method, but has the significant disadvantage of omitting the most informative individuals from the study since the people with highest blood pressures are also the most likely to be treated for hypertension. The currently preferred method is interpolation of untreated systolic and diastolic blood pressure values based on the measurements from treated individuals (Tobin et al. 2005). The prevalent practice is to add 15 mmHg to the systolic and 10 mmHg to the diastolic blood pressures of treated individuals before analysis. This method has the advantage of preserving treated individuals in the analysis. However, as it amounts to adding fairly arbitrary weight constants to increase the importance of a significant proportion of individuals in the analysis, it makes the estimated effect sizes unreliable.

A large number of association studies focusing on polymorphisms in blood pressure candidate genes have been performed. Majority of these studies were each focused on a few marker polymorphisms in selected candidate loci. The results of these efforts were inconsistent and most genes have both positive and negative association results reported in the literature (**Supplementary table 1**). Consequently, no firm conclusions can be drawn from these studies regarding the involvement of the polymorphisms in any gene in blood pressure regulation. This inconsistency in study results can to some extent be attributed to differences in the studied samples resulting from variations in phenotyping criteria and differences in genetic as well life-style and environmental circumstances of different populations.

As association studies lose power with decreasing minor allele frequencies of the studied markers, the research of rare blood pressure affecting variants has only begun recently, facilitated by the development of novel analytical methods and collection of large population samples. The studies carried out thus far show that rare variation in blood pressure candidate genes can partially account for blood pressure variation at the population level (Ji et al. 2008; Feng and Zhu 2010).

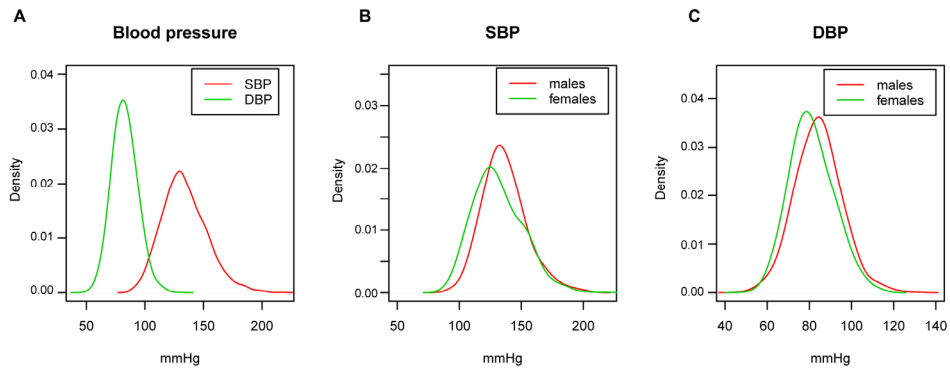

Figure 3. – Distribution of systolic and diastolic blood pressures in 1642 individuals from South-German KORA (Kooperative Gesundheitsforschung in der Region Augsburg) S3 cohort sample.

1.3.3.1. Genome wide association studies of blood pressure traits

Technological advances in the field of genome-wide genotyping and large-scale association studies have made genotyping of hundreds of thousands to millions of marker positions over the genome possible. The first attempts in this field by Wellcome Trust Case Control Consortium (WTCCC) (WTCCC 2007) and the Framingham Heart Study (Levy et al. 2007) failed to identify any markers significantly associated with blood pressure. Subsequent attempts to replicate the top markers identified by the WTCCC study also failed (Ehret et al. 2008; Hong et al. 2009). These initial frustrating setbacks were followed by concentrated efforts by many research groups that resulted in large collaborative studies by CHARGE (Levy et al. 2009) and Global BPGen (Newton-Cheh et al. 2009) consortia. These studies finally produced the first unequivocal evidence of genetic variants affecting blood pressure in the general population. So far, 11 genome wide association studies for blood pressure traits have been published (including the study reported in this thesis) and common variants in over a dozen genes have unequivocal support for involvement in blood pressure determination in the general population (**Table 3**).

Currently populations of European origin have been studied most thoroughly, but Korean (Cho et al. 2009), Chinese (Yang et al. 2009), African-American (Adeyemo et al. 2009) and Japanese (Takeuchi et al. 2009; Hiura et al. 2010) populations have been studied as well. These studies, both targeted and genome-wide, have shown that blood pressure is one of the most difficult traits to study. The observed associations have proven to be hard to replicate and the associations discovered in different studies rarely overlap. So far only four loci contain associations that have gained support in more than one study: *ATP2B1*, *CYP17A1* and *SH2B3* (**Table 3**). Notably, all three of these genes exhibit pleiotropic effects and have been associated with other phenotypes in addition to blood pressure (Fodinger et al. 2000; Hunt et al. 2008; Montgomery et al.

2008). The effects of the detected genetic variants are minuscule, around 1 mmHg for SBP and 0.5 mmHg for DBP and therefore require large sample sizes to detect.

Even the largest GWA studies performed so far (with sample sizes in tens of thousands of individuals) appear to be underpowered to detect most of the blood pressure affecting genetic variants. Consequently, the associations so far discovered can only explain a small fraction of the total heritable component of blood pressure variation (< 3%). Also, the association study methodology suffers from the very phenomenon that makes these studies possible: in the vast majority of cases the actual causal genetic variant is not genotyped, but is detected by proxy SNPs in linkage disequilibrium with the causal polymorphism. Therefore, the genetic variation causing the association lies in a region delimited by the local extent of LD around the detected proxy SNP. These regions can be large and contain several genes, making it hard to localize, identify and unravel the underlying physiology.

It is also notable that only two previously known blood pressure candidate genes: *CYP17A1* and *MTHFR* have been detected by GWAS and the majority of the findings are novel genes with no known link to blood pressure affecting mechanisms. The lack of previous candidate genes amongst the findings indicates a previously unappreciated extent of the blood pressure regulating networks and the elucidation of the roles of these genes in blood pressure regulation will undoubtedly prove to be a challenge.

Table 3. Published genome wide association studies for blood pressure traits.

Study	Statistically significant loci	Reference
Wellcome Trust Case Control Consortium	None	(WTCCC 2007)
Framingham Heart Study	None	(Levy et al. 2007)
AFDS	*STK39*	(Wang et al. 2009)
KARE	***ATP2B1***	(Cho et al. 2009)
Yang et al.	None in single locus analysis	(Yang et al. 2009)
KORA	*CDH13*	(Org et al. 2009)*
Global BPGen	***CYP17A1***, *CYP1A2, FGF5,* ***SH2B3***, *MTHFR, c10orf107, ZNF652, PLCD3*	(Newton-Cheh et al. 2009)
CHARGE	***ATP2B1***, *C18orf1,* ***SH2B3***, *ATXN2,* ***CYP17A1***, *PLEKHA7, ULK4, CACNB2, TBX3-TBX5, CSK-ULK3*	(Levy et al. 2009)
HUFS	*PMS1, SLC24A4, YWHA7, IPO7, CACANA1H*	(Adeyemo et al. 2009)
Takeuchi et al.	None in GWAS	(Takeuchi et al. 2009)
Suita Study	*CCBE1* in GWAS; not replicated	(Hiura et al. 2010)

* – Study is included in this thesis

Genes with reported associations in more than one study are indicated in bold.

1.4. microRNAs and their involvement in blood pressure regulation

MicroRNAs (miRNAs) are small regulatory single-stranded RNA molecules. They are 20 – 25 nucleotides in length and function by reducing the expression of specific target genes. This method of gene silencing was initially discovered in *C. elegans*, but the mechanism has proven to be prevalent in most multicellular animals (Bartel 2009). miRNAs are endogenous in origin and are transcribed from their specific genes. They are processed in multiple stages from longer precursor RNA molecules into mature miRNAs.

The miRNA genes are transcribed by RNA polymerase II (like protein coding genes) and the primary transcript (pri-miRNA) may be hundreds of kbs long. The pri-miRNA contains a stem-loop structure (~70 nucleotides in length) that is cleaved off by the microprocessor complex (composed of enzymes Drosha and Pasha). The resulting shorter hairpin molecules, named pre-miRNAs, are subsequently transported out of the nucleus into the cytoplasm by Exportin-5. In the cytoplasm, the pre-miRNAs are further cleaved by the RNase III enzyme Dicer. In this step, the loop part of the pre-miRNA hairpin is removed, leaving a short double-stranded miRNA:miRNA* duplex. One strand from this duplex is then included in the RNA Induced Silencing Complex (RISC). Once included in this complex, the miRNA becomes functional and operates by targeting the RISC to mRNA molecules with specific target sites. miRNA binding sites are usually located in the 3'UTR of the target mRNA.

miRNAs can reduce the translation levels from the targeted mRNA but can also cause its degradation (Guo et al. 2010). Currently, more than a thousand miRNAs have been identified in humans (Kozomara and Griffiths-Jones 2011). miRNAs have been found to target a large proportion of genes in mammals and they participate in almost all cellular processes, including proliferation, differentiation and cell death. The miRNA system has been acknowledged as one of the major gene expression regulating mechanisms alongside the transcription factors. Several algorithms have been developed to predict the binding sites of miRNAs in the 3'UTRs of genes (John et al. 2004; Krek et al. 2005; Lewis et al. 2005). Prediction algorithms rely mainly on the strict requirement of complementarity between the target mRNA and the miRNA "seed" region, a short stretch of 6–8 nucleotides near the 5' end of the miRNA. Due to the limited length of the seed region, this complementarity is not sufficient to achieve adequate specificity of predictions and most algorithms typically employ additional criteria to find reliable predictions. These criteria may include conservation of the target regions in several species and additional complementarity in the 3' part of the miRNA. As a rule, however, these methods still suffer from limited accuracy and large numbers of false positive predictions.

1.4.1. miRNAs in homeostasis

miRNAs have been mostly implicated in developmental processes and in cell cycle control. It has been hypothesized that in the adult organisms the primary role of miRNAs lies in the determination of cellular identity. miRNAs exhibit pervasive dysregulation in cancerous states (Volinia et al. 2010). The evidence for miRNA involvement in quotidian metabolic processes is less prevalent. Nevertheless, miRNAs have also been implicated in the regulation of several endocrine processes. For example, miR-18 and miR-124 are known to regulate the glucocorticoid receptor *NR3C1* and influence the glucocorticoid responsiveness of the brain (Vreugdenhil et al. 2009). miR-133a has been shown to reduce insulin production in the pancreas in response to chronically elevated glucose levels (Fred et al. 2010). miRNA expression profiles have been determined for several tissues and organs relevant to blood pressure regulation (including kidneys, heart and brain), highlighting several miRNAs expressed specifically in those tissues. Expression profile of miRNAs has been investigated in Dahl salt-sensitive rats. It was shown that miRNA expression profile in the kidneys and heart of rats on normal and high-salt diet did not differ, indicating that salt loading does not have a significant effect on miRNA expression (Naraba and Iwai 2005). On the other hand, miRNAs are known to influence blood pressure by targeting the angiotensin II receptor 1 (Sethupathy et al. 2007). Interestingly, the miRNA binding site in the 3'UTR of the *AGTR1* gene is polymorphic and affects the blood pressure levels in the general population (Ceolotto et al. 2010). These studies represent an interesting example of reverse genetics uncovering a genetic variant underlying complex human disease.

2. AIMS OF THE STUDY

The purpose of this study was to improve the understanding of genetic mechanisms of blood pressure regulation using three broad strategies:

1) Analysis of functional blood pressure candidate genes. This study explored the role of common polymorphisms in blood pressure variation:
   - How well are the blood pressure candidate genes covered with common genetic variants on Affymetrix 500k genome-wide genotyping chips?
   - Do functional candidate genes contain variants that exhibit association with blood pressure traits?
   - Are the associations identified in the discovery population replicable in other study samples and populations?
   - How do life-style factors affect or modify the detection of genetic associations?
   - Does haplotype-based analysis combining multiple markers have advantages over single marker tests in detecting associations in blood pressure candidate genes?

2) Characterization of the role of miRNAs in blood pressure determination by:
   - Performing *in silico* identification of potential miRNA interactions with blood pressure candidate genes.
   - Experimentally verifying the most promising predicted miRNA-gene interactions.

3) Application of genome wide association study (GWAS) approach. The study aimed to investigate the role of GWAS and meta-analysis in identifying novel genes associated with blood pressure variation by contributing to:
   - A GWAS conducted in the German population sample (KORA S3) followed by meta-analysis with replication samples from other European populations.
   - An analysis of the replicability of reported genetic associations from large meta-analysis (CHARGE and Global BPGen) in individual study samples from Germany (KORA S3).

3. RESULTS

3.1. Part I – Candidate gene based studies

3.1.1. Association study of blood pressure candidate genes (Ref I)

Over many years, researchers have gathered a wealth of information regarding the genes involved in various aspects of blood pressure regulation. In the current study, 160 genes with previously published evidence for involvement in blood pressure regulation were selected (**Supplementary Table 1**).

The majority of these genes were selected based on physiological evidence of involvement in blood pressure regulation. This includes genes that are known to affect blood pressure directly as well as genes belonging to relevant regulatory pathways. Also, genes with less direct evidence that have a plausible theoretical connection to blood pressure regulation are included in this category. A number of genes have genetic evidence to support their inclusion in the list of candidate genes. Sixteen genes are implicated in monogenic forms of hyper- and hypotension and several genes were included due to proximity to reported linkage peaks. 26 genes were included based on results from animal models (mainly knockout mice) or involvement in blood pressure related phenotypes (diabetes, cardiovascular disease or metabolic syndrome). The evidence for blood pressure regulating roles for some of these genes is irrefutable while the evidence for some other genes may be of more speculative nature.

Many of these genes have been previously targeted by association studies (n = 79; 49%) (**Supplementary table 1**). The number of reported studies per gene varies (range: 1–31) with *ACE* and *AGT* having received most attention (31 and 30 reported association studies respectively)

3.1.1.1. Samples and data collections used in the study

In the current study, the genetic associations in these genes were explored in four sample sets representing three European populations (**Figure 4**) (**Table 4**). All populations are of European ancestry and exhibit low levels of stratification. The KORA S3 and S4 populations have been collected and genotyped in the framework of the KORA (Kooperative Gesundheitsforschung in der Region Augsburg) study, which aims to identify the genetic causes of several common diseases. The HYPEST cohort is an Estonian sample collected with the specific aim of studying blood pressure genetics in Estonians. The BRIGHT study is a British family based case-control sample of hypertensive individuals. The first phase of the work detailed in this thesis employed the genome wide genotyping data from the KORA S3 cohort. Data were collected using the Affymetrix 500k genotyping chips, but only a subset of this data were utilized in current work. Namely, only SNPs from the 160 blood pressure candidate gene loci and their

10 kb flanking regions were extracted from this dataset. This analysis was carried out as an evidence-based targeted study of previously identified blood pressure candidate genes, meaning that the data for the majority of the genotyped markers were discarded in this study and only markers in the selected target regions were analyzed. The rationale behind this approach is that the best associations identified in GWA studies so far rarely include previously known candidate genes. This raises questions about the validity of the candidate genes in determining population wide differences in blood pressure. Also, the reduction in the number of studied markers helps to pinpoint smaller association signals by reducing the multiple testing problem.

Table 4. Demographics of the studied population samples.

	KORA S3	**KORA S4**	**HYPEST**	**BRIGHT**
individuals*	1644	1830	1297	4908
individuals in quantitative analysis**	1017	1551	1070	0
cases/controls	364/596	447/1119	596/650	2401/1969
SBP (mean/SD)	133.8/19.0	129.2/18.9	139.6/18.4	141.5/23.3
DBP (mean/SD)	82.7/10.7	81.6/10.7	86.3/11.1	86.8/12.8
sex (male/female)	813/831	906/924	447/850	1938/2970
age (mean/SD)	55.6/7.0	52.7/9.0	45.4/13.7	58.3/10.1
BMI (mean/SD)	27.7/4.2	25.6/2.7	26.2/3.9	26.6/3.8

* Number of individuals in the sample.
** Individuals without diabetes or antihypertensive medication

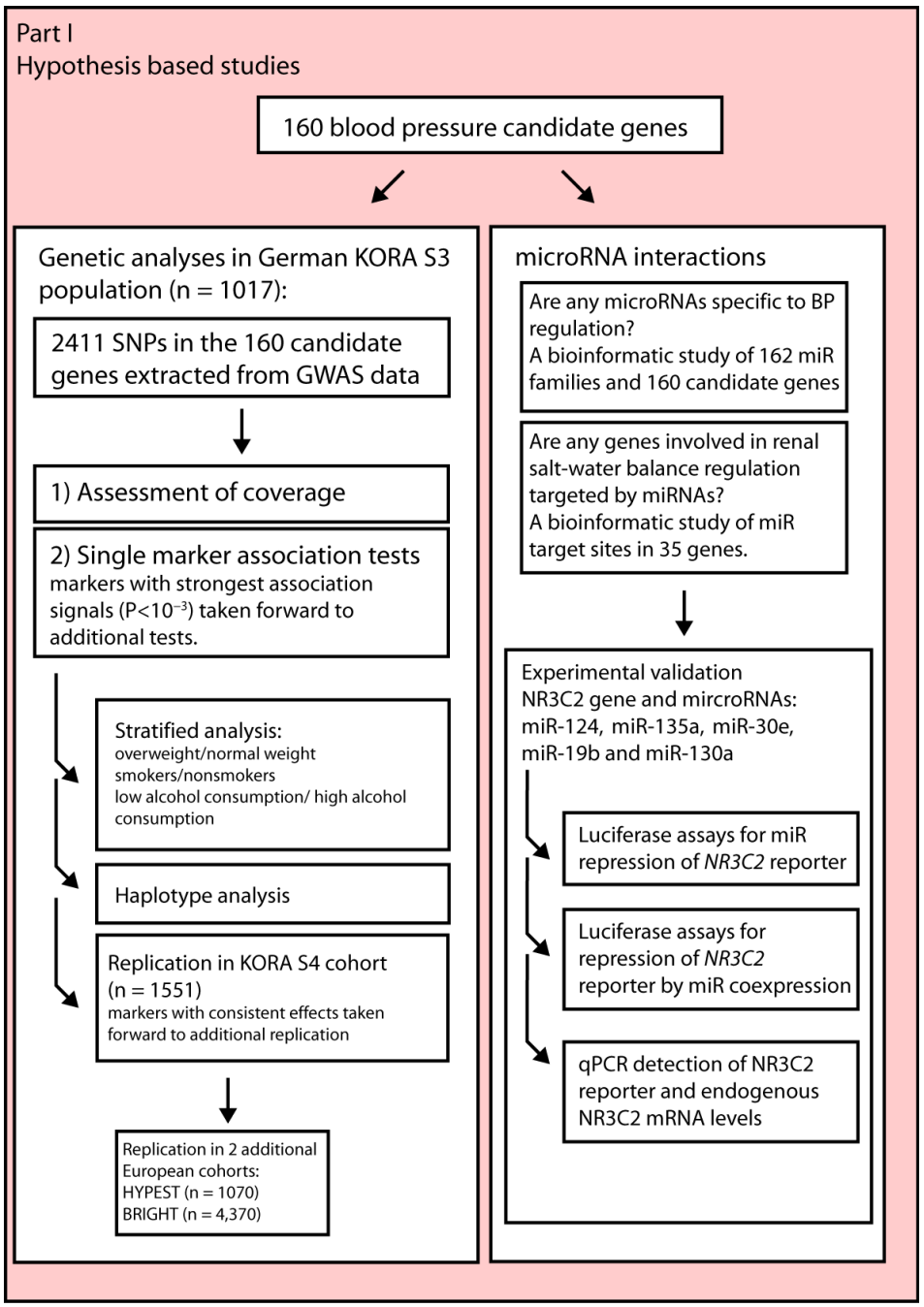

Figure 4. Schematic representation of the conducted studies. SBP – systolic blood pressure; DBP – diastolic blood pressure; HTN – hypertension; BP – blood pressure; miR –microRNA; *NR3C2* – mineralocorticoid receptor.

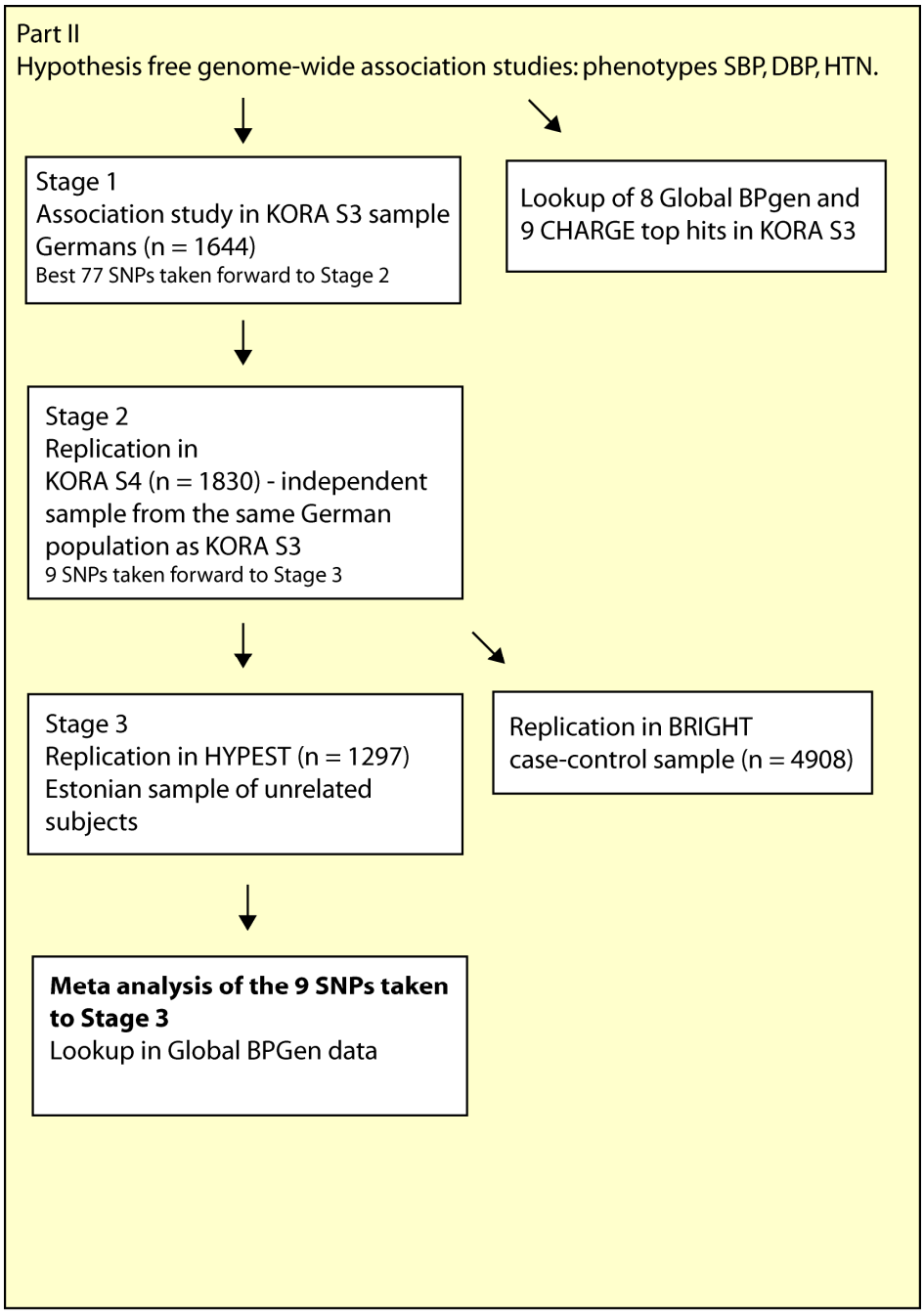

Figure 4 (continued).

3.1.1.2. Common variation in blood pressure candidate genes is unevenly captured by SNPs on Affymetrix 500K array

First, the coverage of common genetic variation in the candidate genes (**Supplementary Table 1**) was assessed. The selected candidate regions (genic region ±10 kb) cover a total of 11.4 Mb. The studied set contained both genes with very short lengths like *NPPC* (0.8 kb) and very long genes like *CACNA1C* (645 kb) (**Ref I, Figure 2**). The average length of the studied genes was 51.1 kb. 2411 SNPs passed quality control criteria in these regions in the KORA S3 dataset. Of these, 20% were located in the flanking regions of the genes, 64% were intronic and 16% exonic. Among the 387 exonic SNPs, 21 were nonsynonymous (**Figure 5A**). On average, there were 15.2 genotyped SNPs per gene region (range 0 to 168) with genotyped SNP densities ranging from 0 to 0.6 markers/kb. The average distance between markers was 6.5 kb. 11 genes (*ADRB3*, *AVP*, *AVPR1B*, *GIPR*, *HP*, *INS*, *SLC5A2*, *SLC9A5*, *TBXA2R*, *TH* and *VEGFB*) did not contain any genotyped SNPs and had to be excluded from subsequent analyses. A significant fraction of the studied SNPs in our dataset were rare polymorphisms. A total of 571 SNPs had low minor allele frequencies (< 5%) and 107 of these were monomorphic (**Figure 5B**). The minor allele frequencies of the rest of the SNPs were fairly uniformly distributed from 0.05 to 0.5 as expected.

Hapmap data were used to assess the coverage of common variation (MAF ≥ 0.05) by the genotyped SNPs (**Figure 5C**). Coverage was estimated as the percentage of SNPs in Hapmap phase II (release 23) CEU population in target regions that were tagged by genotyped Affymetrix 500K SNPs with $r^2 \geq 0.8$. 16 (10%) of the studied genes did not contain common genetic variation according to Hapmap data. Of the remaining 144 genes 47 were poorly tagged with < 50% of the common variation captured, 15 of these genes were not tagged at all. Overall the coverage of the studied genes ranged from 0% to 100% and on average, 52.5% of the Hapmap common variation per gene is captured by the genotyped SNPs. Newer genotyping chips like Affymetrix 6.0 array and Illumina 1M-Duo, with larger amounts of genotyped markers offer better coverage of these genes, but some gaps still remain (**Figure 5C**). It is clear that the coverage over different candidate genes varies substantially with a significant proportion of the genes being inadequately tagged. However, 97 genes are tagged well, with coverage over 50% and in 14 genes all of the common variation is captured on the Affymetrix 500K genotyping chip.

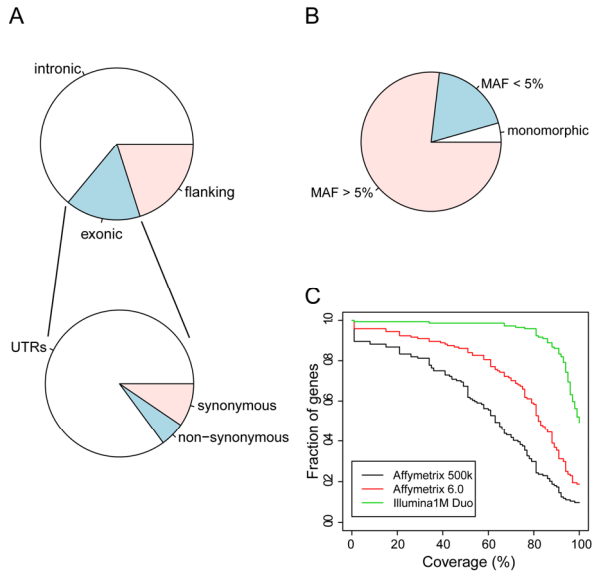

Figure 5. Distribution and coverage of 2411 Affymetrix 500k SNPs in blood pressure candidate loci. A) Functional category of SNPs. UTR – untranslated region; flanking – within 10 kb from candidate gene. B) Minor allele frequency (MAF) distribution of SNPs. C) Coverage of common hapmap variation on Affymetrix 500k and contemporary genotyping arrays. Calculated coverage for Affymetrix 6.0 and Illumina1M Duo arrays is theoretical and assumes 100% genotyping efficiency. Coverage is defined as percentage of Hapmap release 22 SNPs tagged with with $r^2 \geq 0.8$ by Affymetrix SNPs.

3.1.1.3. Single marker association landscape in the KORA S3 population

Association tests were performed for 2,304 polymorphic markers located in 149 blood pressure candidate genes. Associations were tested with both hypertension (HTN) and SBP/DBP as quantitative traits. Individuals taking blood pressure lowering medication were excluded from quantitative trait tests with SBP and DBP.

Associations with hypertension were performed using the Cochran-Armitage trend test (1 degree of freedom) and logistic regression for multivariate analysis. Systolic and diastolic blood pressures both with and without covariates were tested with linear regressions with age and sex of the individuals used as covariates. All markers that passed quality control criteria (call rate ≥ 93%, Hardy-Weinberg equilibrium $P \geq 0.001$) were included in the analysis.

Majority of the studied loci (81%) did not reveal any evidence of association with blood pressure traits in the KORA S3 population and only 12 of the tested markers in 7 genes (*ADRA2A, LEP, LEPR, PTGER3, SLC2A1, SLC4A2, SLC8A1*) showed associations close to significance ($P < 10^{-3}$). The strongest associations were detected in *LEP* and *LEPR* genes with DBP (rs10954174; $P = 5.2\times10^{-5}$) and SBP (rs10889553; $P=4.5\times10^{-5}$) respectively. None of the detected

P values were significant after Bonferroni correction ($\alpha = 0.05/2304 = 2.17\times10^{-5}$) and power analysis indicated that the KORA S3 sample was underpowered to detect associations with rare markers (MAF<0.1) and small effects (<3 mmHg for SBP) (**Ref I, Figure 3**).

Replication was performed for one marker from each of the 7 genes that exhibited association signals in the KORA S3 cohort. The first round of replication was performed in the KORA S4 sample from the same Southern-German population as the KORA S3. None of these markers retained the significance level exhibited in the discovery sample and only 4 of the markers had association P values below 0.1. Two of these SNPs (rs10889553 and rs11195419) had consistent effects across both populations and were studied further in the Estonian HYPEST cohort and the BRIGHT case-control sample from UK. None of the SNPs were successfully replicated in these populations. However, the strength of the association signal between rs10889553 and DBP was increased in a joint meta-analysis of KORA S3 and the replication resources (**Ref I, Table 3**).

3.1.1.4. Effects of lifestyle factors on genetic associations in blood pressure candidate genes

It is known that a number of life-style parameters can influence blood pressure. Obesity is usually accompanied by high blood pressure (Kotsis et al. 2010) and it has been demonstrated that smoking and alcohol consumption contribute to the risk of developing hypertension. In addition, the lack of physical exercise and high salt intake are known to cause elevated blood pressure. The seven genetic associations that were taken to the replication steps were studied for dependence on BMI, smoking habits and alcohol consumption, as data were available for these variables in the studied cohorts. The BRIGHT cohort did not have data on smoking habits and alcohol consumption and was therefore not usable for studying these factors. In order to determine whether obesity, smoking habits and alcohol intake affect the previously identified genetic associations, the individuals in KORA S3 cohort and the replication samples were stratified based on these criteria. The individuals in the studied samples were divided into normal weight ($BMI < 25\ kg/m^2$) and overweight ($BMI \geq 25\ kg/m^2$) groups. Based on smoking, individuals were stratified into non-smokers and smokers (current and ex-smokers). According to alcohol intake individuals were grouped as low consumers (< 20 g ethanol per day) and high consumers (≥ 20g/day). After stratification associations were retested in each group.

Five SNPs out of seven did not exhibit any interaction with the selected life-style factors and the associations remained largely consistent across the studied groups (**Ref I, Supplementary tables 5–7**). The association of marker rs11195419 in *ADRA2A* (Alpha 2A adrenergic receptor) with hypertension was only observed in smokers ($P=3.66\times10^{-3}$) (**Figure 6C**) and the overweight group ($P=8.0\times10^{-3}$) (**Figure 6B**) while the individuals with normal weight (P=0.78) and non-smokers (P=0.57) appeared to be unaffected (**Ref I, Table 3**). The associations with rs10889553 in *LEPR* (Leptin receptor) with SBP and DBP were detected only in

the normal weight subgroup (**Figure 6A**). In addition, associations with DBP and HTN were observed among smokers and high alcohol consumers while non-smokers and low alcohol consumers exhibited no association (P > 0.05) (**Ref I, Table 3**). With the exception of the effect of rs10889553 in normal weight individuals the effects of genotype carrier status appeared to be exacerbated in individuals with unhealthy life-style.

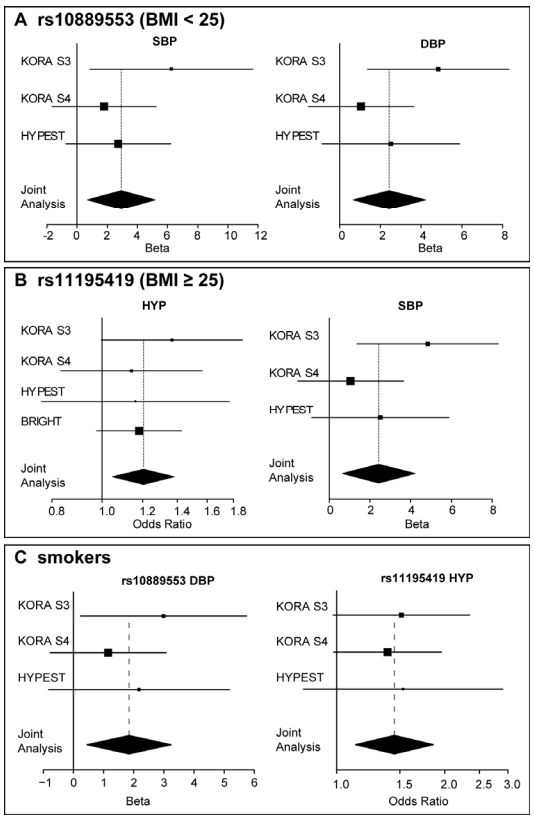

Figure 6. SNP effects in meta-analysis stratified by lifestyle factors. Forest plots showing the effect sizes of the association of rs10889553 and rs11195419 with blood pressure in subjects stratified according to their Body Mass Index, BMI (<25 kg/m^2 normal weight; ≥25 kg/m^2 overweight) and smoking habits. Effects in individual populations KORA S3, KORA S4, HYPEST and BRIGHT are indicated by solid rectangles with sizes proportional to 95% confidence interval (horizontal lines). Solid diamonds represent 95% confidence intervals in meta-analysis using inverse variance method with fixed effects. Effects of association of (A) rs10889553 (Leptin receptor, LEPR) with systolic (SBP) and diastolic (DBP) blood pressure in normal weight population; (B) rs11195419 (Alpha-2A-Adrenergic Receptor, ADRA2A) in with hypertension (HYP) and SBP in overweight population; (C) rs10889553 with DBP and rs11195419 with HYP among smokers, including smokers at the recruitment and/or previous regular smokers during their lifetime.

3.1.1.5. Haplotype analysis of the genes with strongest single marker associations

It has been proposed that haplotype studies are more sensitive than single marker analyses, especially for rarer variants, as they incorporate information from multiple markers (de Bakker et al. 2005). Therefore, the seven genes with previously detected single marker associations were subjected to haplotype analysis in KORA S3 sample. The analysis was carried out using a regression based method that implicitly performs phasing and accounts for uncertainty in haplotype inference implemented in WHAP (version 2.09) software (Purcell et al. 2007). Tests were performed for haplotypes of length one (as comparison to single SNP tests), three, five and eight SNPs in a sliding window along the genes. No haplotype effects were observed in *ADRA2A, LEP, SLC2A1, SLC4A2*, and *SLC8A1* genes as single marker tests gave lowest P values in these genes (**Ref I, Table 4**).

Two genes, *LEPR* (**Figure 7A**) and *PTGER3* (**Figure 7B**), exhibited evidence of haplotype contribution to the associations. In the *LEPR* locus the responsible haplotype is composed of 5 SNPs (rs1887285, rs17097182, rs10889553, rs970467 and rs9436746) and is located in 5' region of the gene. The risk haplotype in this locus was TATCA and the strongest associations were observed with hypertension (logistic regression $P=1.7\times10^{-4}$) and SBP ($P=2.8\times10^{-4}$). Carriers of the less common CTCCC haplotype had lower SBP and reduced risk of hypertension.

In *PTGER3* the best-supported sliding window size was three SNPs long and the top region was also located near the start of the gene. The highlighted region contains three common haplotypes composed of five SNPs (rs2206344, rs3765894, rs34885906, rs2744918 and rs2268062). The haplotype ATAAA was associated with hypertension (logistic regression $P=1.6\times10^{-5}$) with other haplotypes having protective effects.

The candidate gene centric study carried out in this thesis indicates that the coverage of the genes of interest on the chosen genotyping platform may be a serious issue on arrays of moderate marker density. Two potential associations of blood pressure traits with *LEPR* and *ADRA2A* are also higlighted, but these remain tentative and require further confirmation.

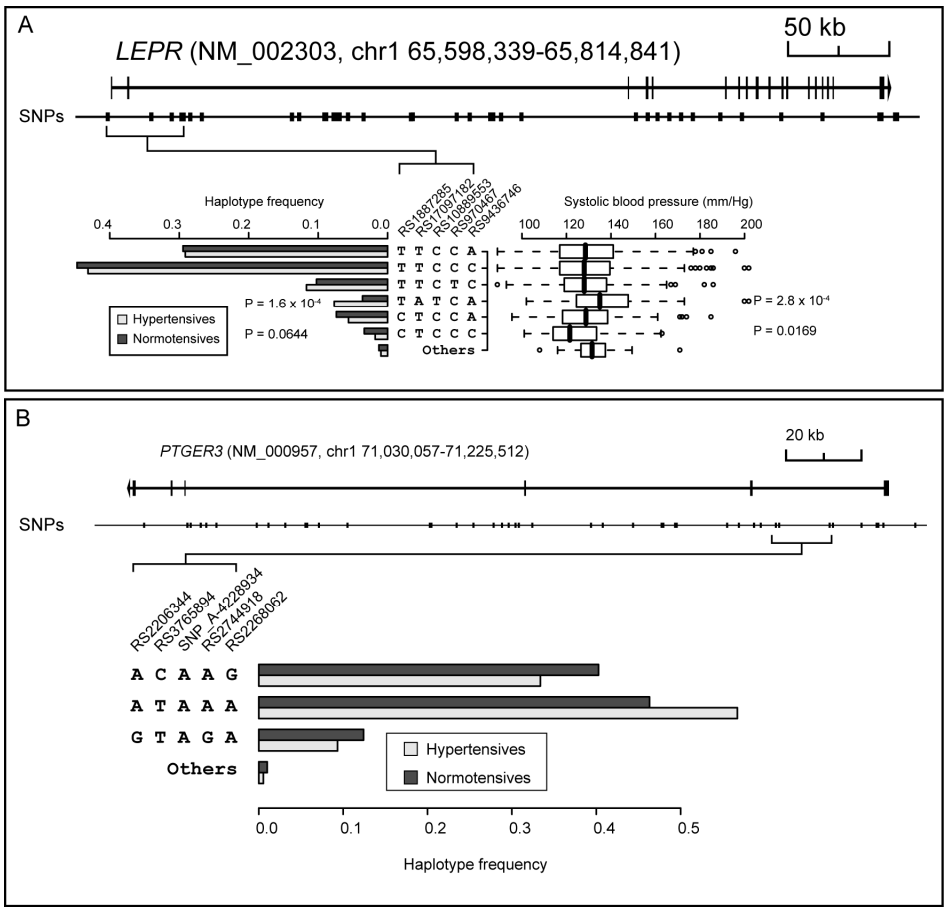

Figure 7. Haplotype association analyses. Exon-intron structures of the genes are shown at the top of the figures along with SNPs on the Affymetrix 500k array. Bottom half of the figures shows the major haplotypes and their distribution in the studied samples. A) *LEPR,* B) *PTGER3*.

3.1.2. miRNA regulation of blood pressure candidate genes (Ref II and unpublished data)

As genetic analyses are only capable of identifying genes responsible for differences in blood pressure between individuals due to DNA variations but not necessarily the genes that are physiologically most important, complementary avenues of research have to be explored for a complete understanding of the blood pressure controlling mechanisms. MiRNAs have emerged as a major layer of gene expression control and in mammals virtually all cellular and metabolic processes are believed to be influenced by them to some extent (Bartel 2009). The role of miRNAs in blood pressure regulation remains poorly understood, but several miRNAs are preferentially expressed in tissues responsible

for BP regulation (e.g. kidneys) and mir-155 is known to regulate the angiotensin II type 1 receptor, a critical component of arterial BP regulation (Liang et al. 2009). Considering that more than 50% of all mammalian mRNAs have been estimated to be under selective pressure to maintain miRNA binding sites (Friedman et al. 2009), a significant fraction of the 160 blood pressure candidate genes investigated in the current study may be expected to be under miRNA regulation. Several algorithms have been developed to computationally predict potential mRNA-miRNA interactions and the generated predictions are available in dedicated databases (John et al. 2004; Krek et al. 2005; Lewis et al. 2005). These predictions provide an excellent tool to form hypotheses and identify potential targets for more focused analyses.

In this study, an investigation was carried out regarding possible miRNA regulation of candidate genes implicated in blood pressure control (**Figure 4**).

First, the question whether any miRNAs may be specifically devoted to the regulation of blood pressure related genes was examined. To this end, a bioinformatic analysis of the predicted miRNA binding sites in the 3'UTRs of the 160 blood pressure candidate genes was carried out. Three target site prediction programs (Targetscan, PicTar and MiRanda) (John et al. 2004; Krek et al. 2005; Lewis et al. 2005) were used. The predicted sites were analysed using binomial tests to detect any possible saturation of binding sites of each miRNA in blood pressure related genes. The number of observed binding sites in blood pressure candidate genes was compared to the number expected by chance alone given the known lengths of the 3'UTRs of candidate genes and the combined lengths of the 3'UTRs of all genes. In the primary analysis only predictions from Targetscan were used due to the strict and statistically solid criteria employed in this algorithm, which minimizes false positive signals.

3.1.2.1. None of the analyzed miRNAs are specifically targeting blood pressure regulating genes

A bioinformatic analysis of the 3'UTRs of the 160 blood pressure candidate genes using the target site prediction tool Targetscan 4.2 revealed that no miRNAs are specifically devoted to regulating blood pressure controlling genes. Each of the 162 miRNA families (groups of miRNAs with identical seed regions) in the Targetscan database were analyzed with binomial tests. The miRNAs with strongest evidence of enrichment in blood pressure candidate genes are given in **Table 5**. None of the tests remained significant after Bonferroni correction ($\alpha = 0.05/162 = 0.0003$). This result is not unexpected given that very few miRNAs have been shown to preferentially target genes in a specific pathway. However, the top miRNA – mir-140 (P=0.0053) has been shown to be upregulated in coronary artery disease patients (Taurino et al. 2010). This finding suggests that the observed over-representation of target sites for mir-140 in BP candidate genes may actually be indicative of its role in cardiovascular functions.

Table 5. Top 10 miRNA families with largest discrepancy between expected and observed number of Targetscan miRNA target site predictions in 3'UTRs of 160 hypertension candidate genes.
P values were obtained with binomial tests. None of the tests reached Bonferroni corrected significance level ($\alpha = 0.05/162 = 3.09\times10^{-4}$).

	No of miRNA target site predictions			
	Observed			
miRNA family	All genes	Candidate genes	Expected	P value
miR-140	180	6	1.555	0.0053
miR-122	144	4	1.244	0.0377
miR-365	168	4	1.451	0.0597
miR-214	402	7	3.473	0.0632
miR-19	770	11	6.652	0.0756
miR-150	183	4	1.581	0.0762
miR-200bc/429	684	10	5.909	0.0778
miR-455	126	3	1.088	0.0973
miR-185	203	4	1.754	0.1014
miR-496	723	10	6.6.246	0.1019

3.1.2.2. Several genes involved in renal water-salt balance regulation are potentially targeted by miRNAs

In the second phase a reverse question was asked: are any BP affecting genes regulated by miRNAs? The analysis focused only on genes involved in renal salt-water balance regulation – the process implicated in all monogenic blood pressure regulation disorders known so far (Lifton et al. 2001). To improve the quality of the binding site predictions, the results of three separate prediction algorithms: Targetscan, PicTar and miRanda were combined and the binding sites that had support from more than one prediction algorithm were considered as stronger candidates. miRNA binding sites were identified in 13, 12 and 27 genes by Targetscan, PicTar and miRanda, respectively. Six of these genes (*ADD1, AGTR2, KCNJ1, NEDD4L, NR3C2* and *SCNN1A*) contained miRNA target site predictions by all three algorithms (**Table 6**). The 3'UTRs of these genes are unusually long, ranging from 1026 bp to 2575 bp. Long 3'UTRs have been considered an indication of miRNA regulation (Stark et al. 2005). Among the studied genes *NR3C2* (the mineralocorticoid receptor gene) appears to be under especially complex miRNA control. Its long (~2.5 kb) 3'UTR was found to contain 23, 64 and 411 predicted miRNA binding sites by Targetscan, PicTar and miRanda, respectively. *NEDD4L* was also found to be a likely candidate for miRNA regulation (7, 28 and 262 predicted sites by Targetscan, PicTar and miRanda, respectively), but as the evidence for miRNA binding in this and other genes was weaker, only the *NR3C2* gene was taken forward to experimental validation.

Table 6. Number of predicted miRNA target sites in blood pressure candidate genes identified by Targetscan, PicTar and MiRanda.

Gene	Targetscan	PicTar	MiRanda	3'UTR length (bp)
ADD1	4	5	34	1891
AGTR2	1	3	58	1195
KCNJ1	1	8	22	1105
NEDD4L	7	28	262	1888
NR3C2	23	64	411	2575
SCNN1A	2	2	131	1026
Average*	1.97	5.39	74.5	722

* – Average target prediction counts and 3'UTR lengths are shown for 35 candidate genes.

3.1.2.3. Regulation of *NR3C2* by miRNAs

Three criteria were used to select the best-supported miRNAs out of several hundred predictions:

1) Overlap between prediction algorithms. Ten miRNA target sites were predicted by all three algorithms.
2) Number of target site predictions for each miRNA. It has been shown that the number of miRNA binding sites in the 3'UTR of the target gene affects the magnitude of target repression (Doench et al. 2003). Three target sites of mir-124 and two target sites of mir-135 were predicted by at least two algorithms.
3) Binding site prediction quality (Targetscan quality score was used). The target site for mir-30 was chosen because it had the best aggregate P_{CT} score for a single site in the *NR3C2* 3'UTR.

According to these criteria the target sites for miRNAs miR-124, miR-135, miR-30, miR-19 and miR-130 were considered to be most likely biologically relevant. Many miRNAs possess several highly similar variants that are transcribed from different genomic loci and are denoted by lowercase letters (e.g. mir-23a, mir-23b, mir-23c). In this study the miRNA variants with strongest complementarity to the target site in *NR3C2* 3'UTR were selected for validation using luciferase assays.

The 3'UTR of *NR3C2* was cloned into a reporter vector (pGL3-Control), capable of producing luciferase protein in eukaryotic cells. This configuration allows the detection of miRNA-mediated repression of the luciferase gene. The miRNA genes were amplified from the same individual and cloned into pQM-Ntag-A vector for expression under the control of CMV promoter.

3.1.2.4. miRNAs mir-124 and mir-135 can downregulate NR3C2 expression

Luciferase assays were conducted in HeLa cells with reporter constructs containing the cloned 3'UTR of the *NR3C2* gene. First, the miRNA expression constructs were tested by qPCR (with miRNA probes from Applied Biosystems) for their capacity to produce mature miRNAs. All constructs were capable of expressing their respective miRNAs (**Figure 8C**). HeLa cells were also found to express mir-30e, mir-19b and mir-130a endogenously. Expression of miR-135a was detected in these cells in very low amounts and miR-124 was undetectable (**Figure 8C**).

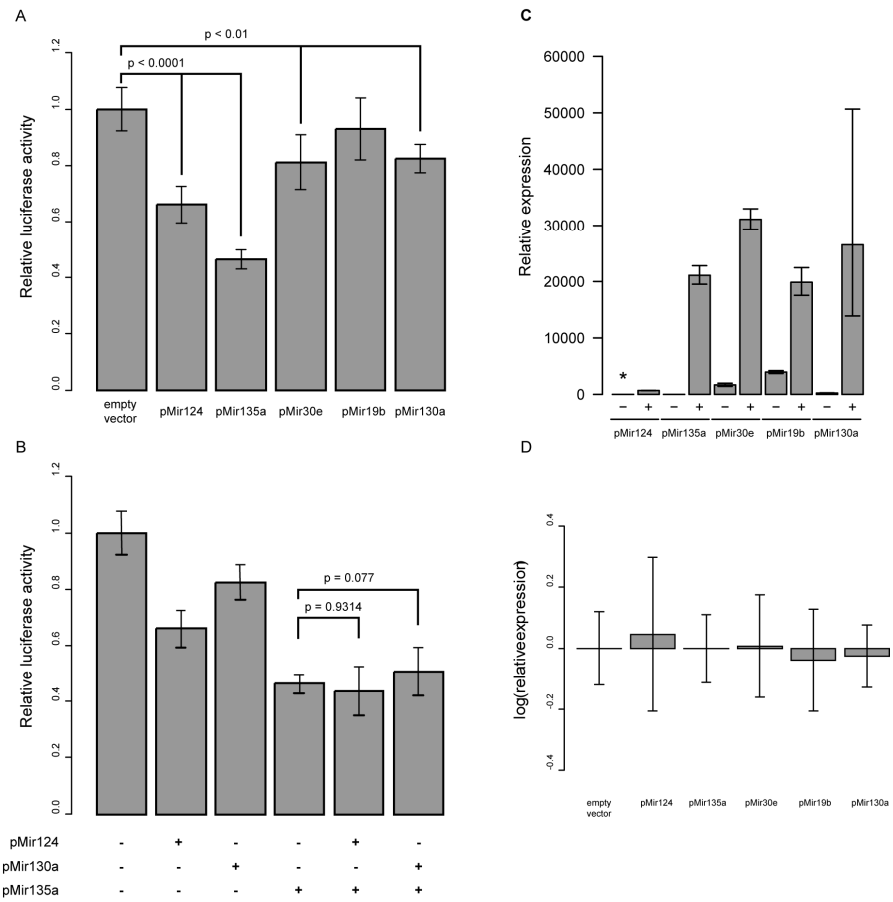

Figure 8. miRNA transfection experiments in HeLa cells. A) Repression of *NR3C2* 3'UTR reporter by single miRNAs. B) Cotransfection of two miRNAs. C) Endogenous and exogenous expression of studied miRNAs in HeLa cells. D) mRNA levels of NR3C2 reporter upon transfection of miRNA expression constructs detected by qPCR. ***** – No detectable expression.

The NR3C2 3'UTR luciferase reporter construct was transfected into HeLa cells along with the miRNA expression plasmids. The luciferase reporter activity was significantly ($P<0.0001$) reduced in cells expressing miR-124 and miR-135a compared to cells transfected with an empty expression vector. The signal was reduced 2.2 fold in cells expressing miR-135a and 1.5 fold in cells expressing miR-124 (**Figure 8A**). In cells expressing miR-19b, miR-130a or miR-30e repression of the signal was not detected.

Co-expression of more than one miRNA did not reveal additive or synergistic effects of miRNAs on *NR3C2* repression. The effects of two miRNAs with the strongest effect (miR-135a and miR-124) and miRNA with strong effect (miR-135a) combined with a miR with weaker effect (miR-130a) were studied. In both cases the addition of a second miRNA did not exert additional repression compared to miR-135a alone (**Figure 8B**).

3.1.2.5. miR-135a and miR-124 affect *NR3C2* translation and not transcription level

It is known that miRNAs are capable of both repressing the translation and reducing the mRNA amounts of the target gene (Bartel 2004; Guo et al. 2010). Quantitative PCR experiments demonstrated that the tested miRNAs reduced neither the mRNA levels of the *NR3C2* reporter construct (**Figure 8D**) nor the endogenous *NR3C2* transcript. Even after accounting for the experimental uncertainty in the qPCR experiments the variation in mRNA levels is not able to explain the previously observed reduction in luciferase activity (**Figure 8A**).

This indicates that the observed repression of the gene takes place at translation level. This study identified mir-124 and mir-135 as potential regulators of the mineralocorticoid receptor, a critical component of the RAAS.

3.1.2.6. Genetic associations with blood pressure traits in NR3C2 and miRNA genes (unpublished data)

To answer the question whether any of the loci identified in the previous analysis may harbor polymorpisms that may affect blood pressure, lookups were performed in the KORA S3 genome-wide association test results in these regions. *NR3C2* gene was included in the previously detailed survey of blood pressure candidate genes and contained a marker rs17024456 that was associated with hypertension ($P=6.64 \times 10^{-3}$).

There are two copies of miR-135a in the human genome. One (miR-135a-1) is transcribed from the opposite strand of the *GLYCTK* 3'UTR on chromosome 3 (p21.1) and the other (miR-135a-2) is located on chromosome 12. The strongest association within 10 kb of the miR-135a-1 is with rs3796343 (diastolic blood pressure; $P=0.028$) located 3.5 kb upstream from the miRNA gene in the first intron of *GLYCTK*. The miR-135a-2 gene region contains an

association with marker rs1349418 (systolic blood pressure; P=0.0096) located ~9kb upstream. Three copies of miR-124 are present in the genome: two on chromosome 8 (miR-124-1 and miR-124-2) and one on chromosome 20 (miR-124-3). No markers within 10 kb from the miR-124 genes are associated with any of the blood pressure traits (P>0.05).

3.2. Part 2 – Genome-wide association studies (Ref III and unpublished data)

3.2.1. Genome-wide association study of blood pressure traits in three European populations

To assess the impact of common genetic polymorphisms on blood pressure, 395,912 SNPs were genotyped using Affymetrix 500k genotyping arrays in 1644 individuals from the KORA S3 cohort from southern Germany (**Table 4**). These samples were genotyped in the framework of the KORA S3 GWAS consortium (led by T. Meitinger, H.-Erich Wichmann and C. Gieger), which aimed to identify the genetic causes of several phenotypes and diseases in the German population. It is considered a typical European population and exhibits low amounts of population stratification (inflation factor $\lambda = 1.02$). Three blood pressure related phenotypes were tested under additive and dominant genetic models: SBP and DBP as quantitative traits and hypertension (HTN) as a binary trait. Recessive model was not considered due to power limitations. Based on the initial scan, 77 SNPs were selected for further testing according to the observed association strength and proximity to plausible candidate genes or previously reported linkage peaks. The second stage of the study was performed on the KORA S4 cohort (**Table 4**), an additional sample from the same southern German population (n = 1566). Five of these SNPs displayed concordant effect directions and magnitudes across the two samples. These SNPs and additional three SNPs from nearby loci were subsequently taken to the third stage of the study. The SNP with the strongest association signal from stage two (but opposite effect direction in stage 1 and stage 2) rs12731181 was also carried over to the third stage (**Table 7**). In total, nine SNPs from six distinct genomic loci were taken to the third stage. The SNPs were genotyped in the HYPEST (n = 1246) cohort (**Table 4**) (an Estonian sample of unrelated subjects) and tested for association as in previous stages. The marker rs11646213 (16q23.3), lying ~20 kb upstream from the *CDH13* gene was associated with HTN in all three populations (**Table 7**). Tests performed for HTN in a British family based case-control sample from the BRIGHT study (n = 4370) (**Table 4**) revealed a non significant trend for association with the rs11646213 marker (additive model $P = 8.22\times10^{-2}$; dominant model P = 0.141) (**Table 7**).

Table 7. Association results with HTN for 9 markers taken to the third stage of the study.

Study stage	Sample collection	Test	Marker (nearest gene)																	
			rs12731181 (*PTGFR*)		**rs6784190 (*SLITRK3*)**		**rs448559 (*LAMB4*)**		**rs1994547 (*ANO3*)**		**rs11646213 (*CDH13*)**		**rs3784990 (CDH13)**		**rs254340 (CDH13)**		**rs9948310 (*KCTD1*)**		**rs506038 (KCTD1)**	
			OR	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value	OR	*P*-value
Stage 1	KORA S3	Add	0.74	1.60×10^{-2}	1.11	3.34×10^{-1}	0.67	1.33×10^{-4}	1.76	2.96×10^{-4}	0.67	1.39×10^{-4}	0.62	2.21×10^{-5}	1.26	4.38×10^{-2}	1.35	3.07×10^{-2}	0.65	7.22×10^{-4}
(GWA 500K)		Dom	0.65	3.23×10^{-3}	1.05	7.24×10^{-1}	0.59	4.17×10^{-4}	1.89	1.19×10^{-4}	0.49	2.34×10^{-6}	0.53	1.12×10^{-5}	1.35	3.76×10^{-2}	1.39	3.97×10^{-2}	0.60	5.50×10^{-4}
Stage 2	KORA S4	Add	1.48	1.63×10^{-4}	1.28	7.48×10^{-3}	0.88	1.38×10^{-1}	1.27	4.94×10^{-2}	0.75	1.00×10^{-3}	1.13	1.84×10^{-1}	0.98	8.15×10^{-1}	1.28	2.71×10^{-2}	0.92	4.26×10^{-1}
		Dom	1.57	2.33×10^{-4}	1.24	6.82×10^{-2}	0.74	1.30×10^{-2}	1.27	7.25×10^{-2}	0.70	2.00×10^{-3}	1.18	1.58×10^{-1}	0.92	5.08×10^{-1}	1.22	1.22×10^{-1}	0.87	2.62×10^{-1}
Stage 3a	HYPEST	Add	0.96	6.51×10^{-1}	0.96	6.93×10^{-1}	1.07	4.79×10^{-1}	1.17	4.21×10^{-1}	0.93	4.38×10^{-1}	1.00	9.68×10^{-1}	0.91	3.65×10^{-1}	0.93	5.10×10^{-1}	1.02	8.70×10^{-1}
		Dom	1.01	9.68×10^{-1}	0.90	3.99×10^{-1}	1.01	9.37×10^{-1}	1.13	5.46×10^{-1}	0.79	4.80×10^{-2}	1.02	9.06×10^{-1}	0.93	5.38×10^{-1}	0.91	4.68×10^{-1}	0.93	5.44×10^{-1}
Meta-analysis: KORA S3+KORA S4+HYPEST		Add	1.05	4.17×10^{-1}	1.11	6.01×10^{-2}	0.87	1.23×10^{-2}	1.38	**1.84×10^{-4}**	0.78	**8.27×10^{-6}**	0.93	1.90×10^{-1}	1.02	7.09×10^{-1}	1.15	3.97×10^{-2}	0.88	4.17×10^{-2}
		Dom	1.06	4.23×10^{-1}	1.06	4.14×10^{-1}	0.78	8.67×10^{-4}	1.40	2.46×10^{-4}	0.67	**5.3×10^{-8}**	0.91	2.00×10^{-1}	1.02	7.74×10^{-1}	1.13	1.21×10^{-1}	0.81	5.00×10^{-3}
Stage 3b	BRIGHT	Add	1.06	3.47×10^{-1}	0.96	4.31×10^{-1}	0.99	8.60×10^{-1}	1.11	1.52×10^{-1}	0.92	8.22×10^{-2}	1.08	1.95×10^{-1}	1	9.94×10^{-1}	0.78	5.70×10^{-4}	0.95	3.33×10^{-1}
		Dom	1.05	4.74×10^{-1}	0.96	5.02×10^{-1}	0.95	4.23×10^{-1}	1.13	1.36×10^{-1}	0.9	1.41×10^{-1}	1.11	1.30×10^{-1}	0.97	6.41×10^{-1}	0.78	1.06×10^{-3}	0.95	4.80×10^{-1}

Age and sex of the individuals were included in the logistic regression models as covariates.

3.2.2. Meta-analysis of genome-wide association study results in three European populations

Meta-analyses allow combining the results of several independently conducted studies as long as the design of the original studies is fairly uniform.

The results for the markers taken to the third stage of the study were combined by a weighted meta-analysis using fixed effects (**Table 7**). Two markers exhibited improved evidence for association after combining the data: rs1994547 in the *ANO3* locus and rs11646213 in the *CDH13* locus. For rs11646213 associations with the HTN phenotype both additive and dominant tests indicate a stronger association signal in the combined analysis than in any of the cohorts separately. The association strength for the dominant model ($P = 5.30\times10^{-8}$) is improved to the level that satisfies the Bonferroni correction threshold ($\alpha = 0.05/395{,}912 = 1.26\times10^{-7}$). The BRIGHT sample is a family based case-control sample, the collection of which relied on somewhat different design principles and the inclusion of this cohort in the meta-analysis did not improve the overall significance of the observed associations despite the detected trend in this cohort (**Table 7**).

3.2.3. KORA S3 results for top associations from published meta-analyses (unpublished data)

The Global BPGen consortium performed a very large meta-analysis of blood pressure traits in many cohorts including tens of thousands of individuals from different European populations (Newton-Cheh et al. 2009). This study began with a discovery sample of 34,433 European individuals and replicated the results in up to 130,000 European samples. The *CDH13* locus was not among the top loci in this study after combining of the discovery and replication cohorts. In total, eight loci were discovered to be significantly ($P < 5\times10^{-7}$) associated with SBP or DBP. As the effect sizes of the identified associations are small – ~ 1 mmHg for SBP and ~0.5 mmHg for DBP and majority of the associations display varying degrees of population specificity this analysis demonstrates the difficulties involved in determining the genetic factors influencing human blood pressure and the magnitude of the efforts required to overcome them.

Table 8. Associations with top SNPs from the CHARGE (Levy et al. 2009) and Global BPGen (Newton-Cheh et al. 2009) meta-analyses in KORA S3 samples.

			Global BPGen				KORA S3		
SNP	**Gene**	**Coded allele**	**Trait**	**MAF**	**Beta**	**P-value**	**MAF**	**Beta**	**P-value**
rs17367504	*MTHFR-NPPB*	G	SBP	0.15	–0.85	2×10^{-13}	0.15	–0.047	0.33
rs16998073	*FGF5*	T	DBP	0.21	0.50	1×10^{-21}	0.23	0.10	0.017
rs1530440	*c10orf107*	T	DBP	0.19	–0.39	1×10^{-9}	0.18	–0.06	0.16
rs11191548	*CYP17A1-NT5C2*	T	SBP	0.09	1.16	7×10^{-24}	0.10	0.02	0.70
rs653178	*ATXN2*	T	DBP	0.47	–0.46	3×10^{-18}	0.47	–0.05	0.12
rs1378942	*CYP1A1-ULK3*	C	DBP	0.36	0.43	1×10^{-23}	0.32	–0.005	0.87
rs12946454	*PLCD3*	T	SBP	0.28	0.57	1×10^{-8}	0.26	0.06	0.14
rs16948048	*ZNF652*	G	DBP	0.39	0.31	5×10^{-9}	0.35	0.11	0.0021
			CHARGE						
rs1004467	*CYP17A1*	A	SBP	0.10	1.05	1.28×10^{-10}	0.18	0.089	0.087
rs381815	*PLEKHA7*	T	SBP	0.26	0.65	1.89×10^{-9}	0.33	0.024	0.55
rs2681492	*ATP2B1*	T	SBP	0.20	0.85	3.76×10^{-11}	0.23	0.017	0.73
rs9815354	*ULK4*	A	DBP	0.17	0.49	2.54×10^{-9}	0.25	0.11	0.014
rs11014166	*CACNB2*	A	DBP	0.34	0.37	1.24×10^{-8}	0.40	0.034	0.33
rs3184504	*SH2B3*	T	DBP	0.48	0.48	2.58×10^{-14}	0.48	0.050	0.14
rs2384550	*TBX3-TBX5*	A	DBP	0.35	–0.35	3.75×10^{-8}	0.39	0.0024	0.95
rs6495122	*CSK-ULK3*	A	DBP	0.42	0.40	1.84×10^{-10}	0.47	0.019	0.58
rs2681472	*ATP2B1*	A	HTN	0.17	0.15	1.75×10^{-11}	0.21	0.14	0.35

SBP – systolic blood pressure; DBP – diastolic blood pressure; MAF – minor allele frequency; HTN – hypertension.

The KORA S3 cohort was included in the Global BPGen study (Newton-Cheh et al. 2009), but with 1644 individuals it constitutes only a small fraction of the overall study population. Concurrently with the Global BPGen study, another large meta-analysis was reported by the CHARGE consortium (Levy et al. 2009). To gain insights about the robustness of the detected associations, it is of interest how the top loci detected in the Global BPGen and CHARGE consortia perform in the smaller constituent cohorts. The KORA S3 association results for the eight significant SNPs from the Global BPGen and 9 top SNPs from the CHARGE studies are given in **table 8**. The effect sizes of all SNPs are significantly smaller in the KORA S3 cohort compared to the overall studies. The effect directions, however, are in good agreement as 7 out of 8 SNPs from Global BPGen and 8 out of 9 SNPs from CHARGE display similar effect directions (sign test P value = 0.0012). Only two of the SNPs: rs1378942 (*CYP1A1* locus) from Global BPGen and rs2384550 (*TBX3-TBX5* locus) from CHARGE failed to recapitulate the effect direction of the whole study. The *FGF5* and *ZNF652* loci from Global BPGen and *ULK4* from CHARGE exhibit the strongest confirmatory signals in the KORA S3 cohort.

DISCUSSION

For a long time any studies involving direct genotyping had to be focused on specific candidate genes due to technological limitations. This changed with the advent of genome-wide genotyping technology. Despite the initial setbacks, GWAS approach has been proven to be effective even in the case as difficult as blood pressure regulation. This raises the question whether candidate gene based studies are any longer practical or should they be phased out in favor of modern array-based or genome-wide sequencing technologies.

In this thesis, both approaches were used. The most thorough investigation of genetic associations in blood pressure candidate genes published to date was carried out. Genetic associations in blood pressure candidate genes were mapped in several populations, the contribution of haplotype information was assessed and the effects of smoking, alcohol consumption and obesity were explored. In addition, the role of miRNA system in blood pressure regulation was interrogated both bioinformatically and experimentally. As an alternative approach the results of several hypothesis free genome-wide association studies were integrated in meta-analyses.

The investigation of genetic associations in blood pressure candidate genes relied on genotyping data from Affymetrix 500k genotyping arrays and suffered from the relatively low number of markers by today's standards (**Figure 5**). The sparse coverage of a significant proportion of the candidate genes (nearly half of the studied genes display <50% coverage of common variation) may contribute to the lack of associations detected in these genes. The newer and denser genotyping arrays are less prone to this problem (**Figure 5**). Overall, the study did not detect any significant associations in the candidate genes. For many genes this is likely to reflect true lack of associations, as these loci may be too highly conserved to harbor common functional variation (Kepp et al. 2007). In other cases poor coverage and small effect sizes of the associations are more likely to result in the lack of significant findings. Power analysis revealed that a 4 mmHg effect on systolic blood pressure would have been necessary for the association to attain conclusive significance in our study. In the context of current knowledge, the existence of common variants with such drastic effects seems unrealistic. The currently published and upcoming large collaborative studies can provide significantly more power to detect weaker associations and it could be rewarding to explore the candidate gene regions in these study results more thoroughly.

As opposed to genetic analyses where large-scale interrogation of large numbers of genotype variants is currently feasible, clear hypotheses about the involved factors are a necessary prerequisite in functional studies. In this thesis a hypothesis that miRNAs may be involved in blood pressure regulation was raised. A bioinformatic survey was used to test whether any miRNAs are preferentially targeting blood pressure candidate genes. Alternatively, the best candidate genes were screened for evidence of miRNA regulation. The study did not reveal any miRNA to be specifically targeting blood pressure regulating

genes, but several candidate genes are shown to possess necessary target sites in their 3'UTRs for miRNA regulation. One of the studied genes, the mineralocorticoid receptor *NR3C2*, is a critical component of the RAAS and mutations in this gene are responsible for two Mendelian forms of blood pressure dysregulation (Geller et al. 1998; Geller et al. 2000). This is in accordance with the finding that transcription factors and other regulatory genes display an increased propensity for miRNA regulation (John et al. 2004). *In vitro* experiments showed that miRNAs miR-124 and miR-135a are capable of downregulating the translation of the *NR3C2* gene while leaving its mRNA level unchanged. Coupled with the fact that *NR3C2* is coexpressed with miR-124 in the brain (de Kloet et al. 2000; Sempere et al. 2004) and with miR-135 in both the kidney and the brain (Hsu et al. 2010) it is plausible that these miRNAs can reduce the signaling in the RAAS by downregulating the mineralocorticoid receptor expression.

No synergistic effect between these miRNAs were observed upon cotransfection, possibly due to the saturation of the repression pathways by the high levels of exogenous miRNAs used in the experiments. The strongest repression was observed by miRNAs with low or undetectable endogenous expression in the HeLa cells (**Figure 8C**) and the possibility that the other tested miRNAs (miR-30e, miR-19b and miR-130a) can also repress *NR3C2* can not be ruled out. On the other hand, the miRNAs with the strongest observed effects had also the best and most numerous predicted target sites in the NR3C2 3'UTR (two target sites for miR-135a and three for miR-124).

As an alternative and complementary approach to the candidate gene based strategies, a genome-wide association study of blood pressure traits was conducted. After the initial scan in the German KORA S3 cohort and replication stages in KORA S4 and Estonian HYPEST populations, one marker, rs11646213 (chr16q23.3) attained genome-wide significance in meta-analysis combining the data from all three stages for the hypertension binary phenotype. A trend towards association was also observed in the British BRIGHT cohort. This marker is located ~20 kb upstream from the *CDH13* gene.

This gene encodes the cadherin-13 preproprotein, a good candidate for involvement in blood pressure regulation. It is expressed in the heart, aorta and the arteries (Ivanov et al. 2001). It has been identified as the adiponectin receptor (Hug et al. 2004) and genome-wide association and linkage studies have implicated this gene in regulating adiponectin levels (Ling et al. 2009; Jee et al. 2010) as well as susceptibility for ADHD (Franke et al. 2009) and alcohol dependence (Treutlein et al. 2009). The *CDH13* locus has also been identified as one of the top associations with coronary artery disease in the WTCCC study (WTCCC 2007). *CDH13* locus was also among the top loci for SBP in the Framingham Heart Study GWAS (Levy et al. 2007) and showed association with SBP and SBP in African Americans (Adeyemo et al. 2009).

Interestingly, in our study the strongest evidence was observed with the binary HTN phenotype while later studies have indicated that quantitative phenotypes (SBP and DBP) are more powerful for detecting associations with

blood pressure. This can possibly be explained by the study design, as in this study the individuals receiving antihypertensive medication were excluded from the analysis of quantitative traits. The current practice of imputing theoretical blood pressure values for treated individuals appears to allow for greater detection power for quantitative blood pressure traits.

The *CDH13* locus has not been successfully identified as one of the top blood pressure associated loci by the large meta-analyses by Global BPGen and CHARGE consortia, possibly due to allelic heterogeneity in the region, population specific differences in LD structure or differences in phenotype definitions. Very few loci have so far been associated with hypertension case-control phenotype. The difficulties of replication are further underlined by the performance of Global BPGen top markers in the KORA S3 cohort. The results indicate that although the directions of effects are in remarkably good agreement between KORA S3 and the whole Global BPGen data, the effects are much reduced in KORA (**Table 8**). This indicates that even the largest meta-analyses published so far are underpowered to estimate true effect sizes of associated markers and the reported associations are likely to suffer from the 'winners curse' of having randomly inflated effect sizes. This lack of power is also likely to be responsible for the small overlap in the results of Global BPGen and Charge consortia. In the light of these considerations it is prudent to consider the possibility that the association of rs11646213 near CDH13 could be a chance finding and not indicative of true association. Alternatively, the association may be caused by a secondary correlation of blood pressure to a different underlying phenotype – coronary artery disease, adiponectin signaling or obesity. On the other hand, it is clear that many true associations are still missed by the largest of meta-analyses and particular associations should not be dismissed solely for not appearing in these studies.

The meta-analysis approach itself, while being used extensively in the modern era of collaborative association studies, has its limitations. They allow taking maximal advantage of the collected data, as the costs associated with sample collection, genotyping and phenotyping of the individuals remain large. Also the problem of population stratification associated with studies involving participants of different ancestries is naturally circumvented. The drawbacks of the method lie in making sure that the combined studies are sufficiently uniform and comparable in their design criteria. Also, these studies are more prone to suffer from genetic heterogeneity as populations involved in the studies may be genetically vastly different and differences in LD or haplotype structure may mask true associations. Newly proposed gene based strategies, as opposed to the traditional marker centric approaches, may alleviate these issues (Peng et al. 2010).

Current genetic methods are only capable of targeting common genetic variation and the statistical power of the current methods drops drastically for rare variation. Also, even the common genetic variants are incompletely covered by the currently employed genotyping technologies. This problem can be somewhat alleviated by imputation methods that allow predicting the genotypes

of unmeasured markers, but these probabilistic methods are inherently error-prone. Consequently, a significant proportion of the total genetic inter-individual variation remains untraceable. This may be the explanation for the apparent shortcomings of present genetic association studies. It is a common theme across almost all genome-wide association studies regardless of the studied phenotype that the discovered associations are only capable of explaining at best a few percent of the total genetic variation. The unexplained proportion of the variation is often colloquially referred to as the "genetic dark matter" or "missing heritablility" and untangling this phenomenon is perhaps one of the major challenges for the future of genetics. As the attention of the researchers turns more towards the discovery of rare variants and whole genome sequencing the potential of approaches utilizing information about candidate genes is becoming more apparent. A rare variant discovered in or near a candidate gene is much more likely to affect blood pressure than a similar variant located in a gene desert. Therefore it is likely that future studies will need to integrate available functional information about candidate genes as well as their regulatory mechanisms like transcription factor binding sites, miRNAs, epigenetics etc. to overcome the reduction in power due to the vast number of variants in the human genome.

Also, the relationship between the already discovered genes and their actual physiological and biochemical roles remain to be determined. These questions will likely not be answered by population genetic studies and much experimental work needs to be carried out in order to explain the discovered associations and put them into the context of actual biochemical pathways.

Genetic heterogeneity is prevalent in blood pressure associated loci and also different loci are likely to be responsible for BP differences is different populations. Even though currently the contribution of blood pressure candidate genes to BP genetics appears to be limited they should not be discounted as important in affecting genetic predispositions to higher or lower blood pressure. Genome-wide association studies have undoubtedly contributed a lot to blood pressure genetics and all confirmed loci detected so far have gained a place in any list of blood pressure regulating candidate genes. Nevertheless, the explained fraction blood pressure genetic variation remains small and as the understanding of rare variation increases the candidate gene based methods are likely to gain importance.

CONCLUSIONS

The results of the conducted analyses point to the fact that the contribution of polymorphisms in previously known blood pressure candidate genes to common genetic variation responsible for inter-individual differences in blood pressure might be limited. This however does not invalidate these candidate genes as important in the blood pressure regulation pathways.

Common polymorphisms were unevenly covered by SNPs on Affymetrix 500k genotyping arrays. Nevertheless, most genes were adequately captured and potential associations with blood pressure were uncovered in *LEPR* and *ADRA2A* genes. Life-style factors: BMI, smoking and alcohol consumption were found to modify these associations. Consideration of haplotype effects did not produce a significant improvement over single marker tests in the detection of associations, but haplotypes correlated with blood pressure traits were found in *LEPR* and *PTGER3* genes.

It is shown in this work that there are no miRNAs that are specifically targeting blood pressure regulating genes, but some blood pressure genes are targeted by miRNAs. The potential role of miRNAs miR-124 and miR-135a as regulators of RAAS is demonstrated.

The meta-analyses conducted in the framework of this study implicated markers near two loci – *CDH13* and *ANO3* in affecting blood pressure in the general population and highlighted the difficulties in replicating discovered associations in different populations.

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SUMMARY IN ESTONIAN

Funktsionaalsetel kandidaatgeenidel põhinevate uuringute roll kaasaegses vererõhu geneetikas

Veri on imetajatel ja paljudel teistel loomadel vajalik hapniku ja toitainete transpordiks organitesse ja kudedesse, samuti ainevahetuse jääkproduktide eemaldamiseks. Nende eesmärkide täitmiseks peab olema tagatud pidev vereringe. Vere voolamise soontes põhjustab vererõhu gradient (rõhk on kõrgem arterites ja madalam veenides), mille tekitab südame töö. Südame kokkutõmbe ajal on rõhk arterites suurem (süstoolne vererõhk) ja südame lõõgastumise ajal madalam (diastoolne vererõhk). Vererõhku mõõdetakse inimestel enamasti sfügmomanomeetriga õlavarrelt ja väljendatakse millimeetrites elavhõbedasammast (mmHg).

Inimese vererõhk võib laias vahemikus kõikuda sõltuvalt füüsilisest koormusest, kellaajast, tervislikust ja vaimsest seisundist. Samuti erinevad vererõhu väärtused inimeste vahel suures ulatuses. Vererõhk on kompleksne tunnus, see tähendab, et iga indiviidi vererõhu väärtuse määravad nii pärilikud kui ka keskkonna parameetrid. Päriliku komponendi osakaalu vererõhu määramisel hinnatakse 30–60 protsendile. Aastakümnete jooksul on kogunenud märkimisväärne hulk andmeid vererõhku määravate regulatsioonimehhanismide kohta. Füsioloogiliste ja molekulaarbioloogiliste uuringute käigus on leitud rida erinevaid geene ja signaalradasid, millede koosmõju tagab vererõhu püsimise optimaalsel tasemel. Häired vererõhu regulatsioonis viivad kõrgvererõhutõve (hüpertensioon) tekkeni. See haigus on väga levinud eeskätt vanemaealiste inimeste hulgas ja on peamiseks riskiteguriks südameinfarktide ja insultide tekkimisel.

Esimesed edusammud vererõhu geneetikas leidsid aset tänu vererõhku tugevalt mõjutavate mutatsioonide leidmisele, mis põhjustavad perekondlikke, Mendeli seaduste järgi päranduvaid kõrget või madalat vererõhku põhjustavaid sündroome. Tänu aheldusuuringutele, mis kaardistavad genoomipiirkondade pärandumist perekondades, on kindlaks tehtud kaheksateist geeni milledes olevad mutatsioonid põhjustavad perekondlikku kõrget või madalat vererõhku. Ühe geeni mutatsioonidest põhjustatud sündroomid on haruldased ja neis leiduvad geneetilised variandid ei seleta vererõhu varieeruvust üldpopulatsioonis. Sellise varieeruvuse geneetiliste põhjuste leidmiseks on kasutusele võetud assotsiatsiooniuuringud, kus võrreldakse geneetiliste variantide esinemissagedusi kõrge ja madala vererõhuga indiviidides. Viimastel aastatel on saanud võimalikuks sadade tuhandete geneetiliste variantide tuvastamine igas uuritavas indiviidis. Niisuguseid uuringuid nimetatakse ülegenoomseteks assotsiatsiooniuuringuteks ja siiani on selle lähenemise abil leitud enam kui kakskümmend üldpopulatsioonis levinud inimese vererõhku mõjutavat geneetilist varianti. Nende uuringute tulemused on osutunud üllatavateks, kuna tuvastatud geenide hulgas on väga vähe varem tuntud vererõhku mõjutavaid kandidaatgeene. Seetõttu tekib küsimus, kas varasemalt tuvastatud geenide roll on inimeste vaheliste vererõhuerinevuste põhjustamises väiksem kui varem arvatud.

Käesolevas töös rakendati nii kandidaatgeenidel põhinevaid kui ka ülegenoomseid analüüsimeetodeid, et selgitada vererõhu geneetilist tausta ja hinnata tuntud kandidaatgeenide regulatsiooni ja osatähtsust inimestevaheliste vererõhuerinevuste kujunemisel. Töö esimene osa keskendub 160 füsioloogilistes uuringutes, aheldusanalüüsil või loommudelites kindlaks tehtud vererõhu kandidaatgeenile. Kandidaatgeenide piirkondades on kindlaks määratud 2411 erinevat geneetilist varianti 1017 Lõuna Saksamaalt pärinevas indiviidis kasutades Affymetrixi ülegenoomseid genotüpiseerimiskiipe. Töö tulemused näitasid, et genotüpiseeritud variandid katavad uuritud geene ebaühtlaselt ja ligi pooltes geenides ei anna genotüpiseeritud variandid informatsiooni enam kui 50% sagedate variantide (sagedus populatsioonis üle 5%) kohta. Rangetele statistiliste olulisuse kriteeriumitele vastavaid vererõhku mõjutavaid geneetilisi variante uuritud kandidaatgeenides ei tuvastatud, kuigi leptiini retseptori (*LEPR*) ja prostaglandiin E retseptor 3 (*PTGER3*) geenides olevad variandid omavad võimalikku seost vererõhuga.

Samuti viitavad töö tulemused inimeste elustiili (suitsetamine, alkoholi tarbimine ja kehamassiindeks) koosmõjule geneetiliste teguritega: kõrgenenud vererõhk avaldus geeni *ADRA2A* uuritava DNA-variandi puhul ainult ülekaalulistel indiviididel ja geeni *LEPR* analüüsitava geenivariandi puhul ainult suitsetajatel. Töö tulemuste kinnitamiseks on tugevaimad leitud seosed kontrollitud ka Eesti ja Inglismaa päritoluga valimites.

Täiendavalt analüüsiti mikroRNAde võimalikku mõju vererõhu kandidaatgeenide avaldumisele. MikroRNAd on väikesed (keskmiselt 22 nukleotiidi pikkused) üheahelalised RNA molekulid, mis seonduvad sihtmärk-geenide mRNAdega ja vähendavad nende geenide avaldumist. Bioinformaatiline analüüs näitas, et ükski mikroRNA ei ole pühendatud spetsiifiliselt vererõhku mõjutavate geenide regulatsioonile. Samas on mitmed vererõhu kandidaatgeenid potentsiaalseteks märklaudadeks mikroRNAdele. Katsed rakukultuuris näitasid, et mikroRNAd miR-124 ja miR-135a on võimelised vähendama valgu sünteesi mineralokortikoidi retseptori (*NR3C2*) geenilt mõjutamata mRNA taset. Mineralokortikoidi retseptor (aldosterooni retseptor) on oluline komponent reniin-angiotensiin-aldosteroon süsteemis, mis on üks põhilisi vererõhu regulatsiooni mehhanisme. Seega võivad uuritud mikroRNAd mõjutada signaali ülekannet reniin-angiotensiin-aldosteroon süsteemis ja seeläbi osaleda vererõhu kontrollis.

Töö teises osas viidi läbi ülegenoomne assotsiatsiooniuuring leidmaks seoseid geneetiliste variantide ja inimese vererõhu vahel. Analüüsiti 395912 geneetilist varianti Lõuna-Saksa populatsioonis. Tulemused kombineeriti andmetega lisaindiviididest samast populatsioonist ning Eestist ja Inglismaalt pärit valimitest. Leiti mitmeid potentsiaalseid vererõhku mõjutavaid variante, milledest tugevaimad asuvad kaderiin 13 (*CDH13*) ja anoktamiin 3 (*ANO3)* geenide ümbruses. Kaderiin 13 on adiponektiini retseptor ja hea funktsionaalne kandidaat osalemaks vererõhu regulatsioonis.

Läbiviidud uuringute tulemused viitavad vererõhu kandidaatgeenides leiduvate sagedaste geneetilise variantide piiratud osalusele vererõhu määramisel.

Sellegi poolest ei ole põhjust kandidaatgeenidel põhinevaid uuringuid alahinnata. Töös kirjeldatud kandidaatgeenidel põhinev mikroRNAde analüüs näitas, et mikroRNAd miR-124 ja miR-135a võivad osaleda inimese vererõhu regulatsioonis. Teostatud ülegenoomne assotsiatsiooniuuring ning meta analüüsid erinevates Euroopa populatsioonides kinnitavad geneetiliste markerite ja vererõhu vaheliste seoste leidmise keerukust. Üksikute leitavate variantide mõju on vererõhule väike ja populatsiooniti erinev, mis takistab tulemuste replitseerimist. Vaatamata raskustele, on ülegenoomsed assotsiatsiooniuuringud oluliselt täiendanud teadmisi vererõhu geneetikast. Tulevikku vaadates võib eeldada, et kandidaatgeenidel põhinevad strateegiad muutuvad järjest olulisemaks, kui uurijate tähelepanu hakkab keskenduma harvade geneetiliste variantide tuvastamisele.

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APPENDIX

Supplementary Table 1. List of studied candidate genes

Genes involved in renal water-salt balance			Association studies		
Gene	**Name**	**Chr**	**Type of evidence****	**Results (+/-)***	**References**
ACE	angiotensin I converting enzyme	17	physiological	14/16	[1–7]
ACSM3	SA hypertension-associated homolog	16	linkage	2/3	[1, 7, 8]
ADD1	adducin 1 (alpha)	4	linkage	5/7	[1, 6, 7, 9, 10]
AGT	angiotensinogen preproprotein	1	physiological	18/12	[1, 4, 6, 7, 11–16]
AGTR1	angiotensin II receptor, type 1	3	physiological	7/9	[1–3, 6, 7, 17]
AGTR2	angiotensin II receptor, type 2	X	physiological	1/0	[18]
ATP1A1	Na+/K+ -ATPase alpha 1 subunit isoform b	1	linkage	1/0	[19]
BSND	barttin	1	monogenic	0/2	[20, 21]
CLCNKA	chloride channel Ka	1	physiological	0/1	[21]
CLCNKB	chloride channel Kb	1	monogenic	0/2	[7, 21]
CYP11B1	cytochrome P450, family 11, subfamily B	8	monogenic	1/1	[7, 22]
CYP11B2	cytochrome P450, subfamily XIB polypeptide 2	8	monogenic	6/9	[1, 7, 22]
CYP17A1	cytochrome P450, family 17	10	monogenic	2/0	[23, 24]
GNB3	guanine nucleotide-binding protein, beta-3	12	physiological	4/7	[1, 6, 7, 25]
HSD11B1	11-beta-hydroxysteroid dehydrogenase 1	1	physiological	0/2	[7, 21]
HSD11B2	hydroxysteroid (11-beta) dehydrogenase 2	16	monogenic	2/3	[1, 7]
KCNJ1	potassium inwardly-rectifying channel J1	11	monogenic	1/0	[21]
KLK1	kallikrein 1 preproprotein	19	physiological	0/2	[7, 26]
NEDD4L	neural precursor cell expressed, developmentally	18	physiological	1/2	[10, 27, 28]
NPPA	natriuretic peptide precursor A	1	physiological	4/6	[1, 6, 7, 29]
NPPB	natriuretic peptide precursor B preproprotein	1	physiological	1/1	[7, 29]
NPPC	natriuretic peptide precursor C	2	physiological	1/0	[30]
NPR1	natriuretic peptide receptor 1	1	physiological	2/2	[1, 7, 31]
NPR2	natriuretic peptide receptor B precursor	9	physiological	1/2	[1]
NR3C2	nuclear receptor subfamily 3, group C, member 2	4	monogenic	1/1	[7, 21]
PNMT	phenylethanolamine N-methyltransferase	17	animal	0/1	[32]
PTGIS	prostaglandin I2 (prostacyclin) synthase	20	physiological	1/0	[33]
REN	renin precursor	1	physiological	2/3	[1, 7]
SCNN1A	sodium channel, nonvoltage-gated 1 alpha	12	monogenic	0/6	[6, 21, 27, 34, 35]
SCNN1B	sodium channel, nonvoltage-gated 1, beta	16	monogenic	1/5	[7, 21, 27, 36–39]
SCNN1G	sodium channel, nonvoltage-gated 1, gamma	16	monogenic	3/2	[21, 27, 40–42]
SGK	serum/glucocorticoid regulated kinase 1	6	physiological	0/1	[27]
SLC12A3	solute carrier family 12, member 3	16	monogenic	0/1	[21]
SLC14A2	solute carrier family 14 (urea transporter)	18	physiological	0/0	
SLC22A2	solute carrier family 22 member 2	6	physiological	0/0	
SLC8A1	solute carrier family 8	2	physiological	0/1	[7]
WNK1	WNK lysine deficient protein kinase 1	12	monogenic	6/2	[7, 10, 43–48]
WNK4	WNK lysine deficient protein kinase 4	17	monogenic	0/3	[7, 48, 49]
Other blood pressure candidate genes					
ABCC8	ATP-binding cassette, sub-family C, member 8	11	diabetes	0/0	
ADD2	adducin 2	2	linkage	0/2	[1, 7]
ADD3	adducin 3 (gamma)	10	linkage	0/1	[7]
ADM/AM	adrenomedullin	11	physiological	0/0	
ADORA1	adenosine A1 receptor	1	physiological	0/0	
ADORA2A	adenosine A2a receptor	22	physiological	0/0	
ADRA1A	alpha-1A-adrenergic receptor	8	physiological	1/1	[7, 50]
ADRA2A	alpha-2A-adrenergic receptor	10	physiological	0/1	[7]
ADRB2	adrenergic, beta-2-, receptor, surface	5	physiological	3/5	[1, 6, 7]
ADRB3	adrenergic receptor, beta 3	8	physiological	3/1	[1]
AKR1B1	aldo-keto reductase family 1, member B1	7	diabetes	0/0	
APOA1	apolipoprotein A-I preproprotein	11	cardiovascular	0/0	
APOA2	apolipoprotein A-II preproprotein	1	cardiovascular	0/0	
APOC2	apolipoprotein C-II precursor	19	cardiovascular	0/0	
APOC3	apolipoprotein C-III precursor	11	cardiovascular	1/1	[1]
APOC4	apolipoprotein C-IV	19	cardiovascular	0/0	
APOE	apolipoprotein E precursor	19	cardiovascular	0/1	[7]
AQP2	aquaporin 2	12	animal	0/0	
AVP	arginine vasopressin-neurophysin II	20	physiological	0/0	
AVPR1A	arginine vasopressin receptor 1A	12	physiological	0/0	
AVPR1B	arginine vasopressin receptor 1B	1	physiological	0/0	
AVPR2	arginine vasopressin receptor 2	X	physiological	0/0	

Genes involved in renal water-salt balance			Association studies		
Gene	**Name**	**Chr**	**Type of evidence****	**Results (+/-)***	**References**
BDKRB2	bradykinin receptor B2	14	physiological	1/0	[51]
BRS3	bombesin-like receptor 3	X	metabolic	0/0	
CACNA1C	calcium channel, voltage-dependent, L type,	12	physiological	0/0	
CALCA	calcitonin isoform CALCA preproprotein	11	physiological	0/0	
CMA1	chymase 1, mast cell preproprotein	14	physiological	0/1	[7]
CYP4A11	cytochrome P450, family 4, subfamily A	1	physiological	0/0	
DBH	dopamine beta-hydroxylase precursor	9	physiological	0/0	
DRD1	dopamine receptor D1	5	physiological	1/0	[7]
DRD2	dopamine receptor D2 isoform short	11	physiological	2/0	[1]
ECE1	endothelin converting enzyme 1	1	physiological	0/1	[7]
EDN1	endothelin 1	6	physiological	4/1	[1, 7, 52]
EDN2	endothelin 2	1	physiological	1/1	[7, 53]
EDN3	endothelin 3	20	physiological	0/1	[7]
EDNRA	endothelin receptor type A	4	physiological	1/0	[49]
EDNRB	endothelin receptor type B	13	physiological	0/1	[7]
F2R	coagulation factor II receptor precursor	5	cardiovascular	0/0	
GAL	galanin preproprotein	11	physiological	0/0	
GALR	galanin receptor 1	18	physiological	0/0	
GCG	glucagon preproprotein	2	physiological	0/0	
GCK	glucokinase	7	diabetes	1/0	[33]
GFPT1	glucosamine-fructose-6-phosphate	2	diabetes	0/0	
GH1	growth hormone 1	17	physiological	0/0	
GH2	growth hormone 2	17	physiological	0/0	
GIPR	gastric inhibitory polypeptide receptor	19	animal	0/0	
GLP1R	glucagon-like peptide 1 receptor	6	physiological	0/0	
GNAI1	guanine nucleotide binding protein (G protein)	7	physiological	0/0	
GYS1	glycogen synthase 1 (muscle)	19	animal	1/1	[1]
GYS2	glycogen synthase 2 (liver)	12	animal	0/0	
HP	haptoglobin	16	physiological	0/0	
IAPP	islet amyloid polypeptide precursor	12	physiological	0/0	
ICAM1	intercellular adhesion molecule 1 precursor	19	physiological	0/2	[6, 7]
ICAM2	intercellular adhesion molecule 2 precursor	17	physiological	0/0	
ICAM3	intercellular adhesion molecule 3 precursor	19	physiological	0/0	
IGF1	insulin-like growth factor 1	12	physiological	0/1	[7]
IGF2	insulin-like growth factor 2	11	physiological	0/0	
INS	proinsulin precursor	11	diabetes	0/1	[7]
INSR	insulin receptor	19	diabetes	3/1	[1, 7]
PDX1	pancreatic and duodenal homeobox 1	13	animal	0/0	
KCNJ11	potassium inwardly-rectifying channel J11	11	physiological	0/0	
KCNJ6	potassium inwardly-rectifying channel J6	21	physiological	0/0	
KCNK2	potassium channel, subfamily K, member 2	1	physiological	0/0	
KNG	kininogen 1	3	physiological	0/0	
LDLR	low density lipoprotein receptor precursor	19	physiological	0/1	[7]
LEP	leptin precursor	7	linkage	0/1	[7]
LEPR	leptin receptor	1	physiological	0/1	[7]
LPL	lipoprotein lipase precursor	8	physiological	1/3	[1, 7]
LRP8	low density lipoprotein receptor-related protein	1	physiological	0/0	
MTHFR	5,10-methylenetetrahydrofolate reductase	1	cardiovascular	1/2	[6, 7, 24]
NOS1	nitric oxide synthase 1 (neuronal)	12	physiological	1/0	[7]
NOS2A	nitric oxide synthase 2A	17	physiological	1/3	[1, 6, 7]
NOS3	nitric oxide synthase 3 (endothelial cell)	7	physiological	6/13	[1, 6, 7, 54, 55]
NPR3	natriuretic peptide receptor C/guanylate cyclase	5	physiological	1/0	[31]
NPY	neuropeptide Y	7	physiological	0/1	[7]
NPY1R	neuropeptide Y receptor Y1	4	physiological	0/0	
NR3C1	nuclear receptor subfamily 3, group C, member 1	5	monogenic	0/0	
PLA2G1B	phospholipase A2, group IB	12	metabolic	0/0	
PPP1CA	protein phosphatase 1, catalytic subunit, alpha	11	cardiovascular	0/0	
PPP1CC	protein phosphatase 1, catalytic subunit, gamma	12	cardiovascular	0/0	
PRCP	prolylcarboxypeptidase	11	physiological	0/1	[7]
PRKCE	protein kinase C, epsilon	2	physiological	0/0	
PRKCQ	protein kinase C, theta	10	physiological	0/0	
PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	14	physiological	0/0	
PTGER3	prostaglandin E receptor 3, subtype EP3	1	physiological	0/0	
PTGIR	prostaglandin I2 (prostacyclin) receptor (IP)	19	physiological	0/0	
PTGS1	prostaglandin-endoperoxide synthase 1	9	physiological	0/0	
PTGS2	prostaglandin-endoperoxide synthase 2 precursor	1	physiological	0/0	
PTHLH	parathyroid hormone-like hormone	12	physiological	0/0	
PTHR1	parathyroid hormone receptor 1 precursor	3	physiological	0/0	

Genes involved in renal water-salt balance			Association studies		
Gene	**Name**	**Chr**	**Type of evidence****	**Results (+/-)***	**References**
RENBP	renin binding protein	X	physiological	0/0	
SCN5A	voltage-gated sodium channel type V alpha	3	physiological	0/0	
SCNN1D	sodium channel, nonvoltage-gated 1, delta	1	physiological	0/1	[7]
SELE	selectin E precursor	1	physiological	0/2	[6, 7]
SERPINA4	serine (or cysteine) proteinase inhibitor, clade	14	physiological	0/0	
SLC12A1	sodium potassium chloride cotransporter 2	15	monogenic	0/1	[21]
SLC12A4	solute carrier family 12, member 4	16	physiological	0/0	
SLC2A1	solute carrier family 2	1	physiological	0/0	
SLC2A2	solute carrier family 2	3	physiological	0/0	
SLC2A3	solute carrier family 2	12	physiological	0/0	
SLC2A4	glucose transporter 4	17	physiological	0/0	
SLC4A1	solute carrier family 4, anion exchanger	17	physiological	0/1	[7]
SLC4A2	solute carrier family 4, anion exchanger, member	7	physiological	0/1	[7]
SLC4A3	solute carrier family 4, anion exchanger	2	physiological	0/0	
SLC4A4	solute carrier family 4, sodium bicarbonate	4	physiological	0/0	
SLC5A2	solute carrier family 5, member 2	16	physiological	0/0	
SLC6A2	solute carrier family 6 member 2	16	physiological	0/1	[7]
SLC8A2	solute carrier family 8 member 2	19	physiological	0/1	[7]
SLC9A1	solute carrier family 9, isoform A1	1	physiological	0/0	
SLC9A2	solute carrier family 9, member 2	2	physiological	0/0	
SLC9A5	solute carrier family 9	16	physiological	0/0	
TBXA2R	thromboxane A2 receptor	19	physiological	0/0	
TBXAS1	thromboxane A synthase 1	7	physiological	0/0	
TGFB1	transforming growth factor, beta 1	19	cardiovascular	0/1	[6]
TH	tyrosine hydroxylase	11	physiological	0/0	
TRH	thyrotropin-releasing hormone	3	physiological	0/0	
TRHR	thyrotropin-releasing hormone receptor	8	physiological	0/0	
TRIP10	thyroid hormone receptor interactor 10	19	physiological	0/0	
UCP3	uncoupling protein 3	11	metabolic	0/0	
VEGFA	vascular endothelial growth factor A	6	physiological	0/0	
VEGFB	vascular endothelial growth factor B	11	physiological	0/0	
VEGFC	vascular endothelial growth factor C	4	physiological	0/0	

***** Studies with no statistically significant results / Studies with statistically significant findings. ****** Evidence for inclusion to the candidate gene list. Physiological – the protein has been shown to affect blood pressure or belongs to a known blood pressure regulating pathway. Monogenic – mutations in the gene have been shown to cause monogenic forms of hyper- or hypotension. Metabolic – involved in metbolic processes or is implicated in the metabolic syndrome. Linkage – is located in or near a linkage peak. Cardiovascular – is involved in cardiovascular pathologies. Diabetes – implicated in increased risk of diabetes. Animal – Identified in knock-out animals.

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PUBLICATIONS

CURRICULUM VITAE

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Education

1995–1998	Hugo Treffner Gymnasium, Tartu
1998–2002	B.Sc. studies in Gene Technology, Department of Microbiology and Virology, Institute of Molecular and Cell Biology, University of Tartu (Supervisor: Reet Kurg, Ph.D.).
2002–2004	M.Sc. studies in Gene Technology, Department of Microbiology and Virology, Institute of Molecular and Cell Biology, University of Tartu. Thesis: "Cloning, expression and purification of Bovine Papillomavirus type 1 capsid proteins". Supervisor: Reet Kurg, Ph.D.
2004–2005	Service in Estonian Defense Forces.
2005–	Ph.D. studies in Molecular and Cell Biology, Institute of Molecular and Cell Biology, University of Tartu

Professional employment

2002	Biodata Ltd., software developer
2010–	University of Tartu, Institute of Molecular and Cell Biology, specialist

Scientific work and activity

Main research topics (1999–2011)

1. Properties of papillomavirus late gene products. Expression and purification of papillomavirus capsid proteins (B.Sc. studies).
2. Late phase of papillomavirus replication, mechanisms of capsid assembly (M.Sc. studies).
3. Marker selection for genotyping studies (Biodata OÜ).
4. The role of noncoding RNA molecules in blood pressure regulation (PhD. studies)
5. Genetics of blood pressure regulation, genetic interactions and epistasis (Ph.D. studies).

List of publications:

1. Kepp K, Org E, **Sõber S**, Kelgo P, Viigimaa M, Veldre G, Tõnisson N, Juhanson P, Putku M, Kindmark A, Kozich V, Laan M (2010) "Hypervariable intronic region in NCX1 is enriched in short insertion-deletion polymorphisms and showed association with cardiovascular traits" BMC Med Genet. 11:15.
2. **Sõber S**, Laan M, Annilo T (2010) "MicroRNAs miR-124 and miR-135a are potential regulators of the mineralocorticoid receptor gene (NR3C2) expression" Biochem Biophys Res Commun. 391(1):727–32
3. **Sõber S**, Org E, Kepp K, Juhanson P, Eyheramendy S, Gieger C, Lichtner P, Klopp N, Veldre G, Viigimaa M, Döring A; Kooperative Gesundheitsforschung in der Region Augsburg Study, Putku M, Kelgo P; HYPertension in ESTonia Study, Shaw-Hawkins S, Howard P, Onipinla A, Dobson RJ, Newhouse SJ, Brown M, Dominiczak A, Connell J, Samani N, Farrall M; MRC British Genetics of Hypertension Study, Caulfield MJ, Munroe PB, Illig T, Wichmann HE, Meitinger T, Laan M (2009) "Targeting 160 candidate genes for blood pressure regulation with a genome-wide genotyping array" PLoS One. 4(6):e6034
4. Org E, Eyheramendy S, Juhanson P, Gieger C, Lichtner P, Klopp N, Veldre G, Döring A, Viigimaa M, **Sõber S**, Tomberg K, Eckstein G; KORA, Kelgo P, Rebane T, Shaw-Hawkins S, Howard P, Onipinla A, Dobson RJ, Newhouse SJ, Brown M, Dominiczak A, Connell J, Samani N, Farrall M; BRIGHT, Caulfield MJ, Munroe PB, Illig T, Wichmann HE, Meitinger T, Laan M. (2009) "Genome-wide scan identifies CDH13 as a novel susceptibility locus contributing to blood pressure determination in two European populations." Hum Mol Genet. 18(12):2288–96

Fellowships:

SA Archimedes Kristjan-Jaak fellowship, 01.04.2008–01.09.2008, to practice in Pontificia Universidad Católica de Chile in Chile.

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Haridus

1995–1998	Hugo Treffneri Gümnaasium, Tartu
1998–2002	B.Sc geenitehnoloogia erialal, Mikrobioloogia ja viroloogia õppetool, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool (juhendaja: Reet Kurg, Ph.D).
2002–2004	M.Sc geenitehnoloogia erialal, Mikrobioloogia ja viroloogia õppetool, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool. M.Sc teema: "Papilloomiviiruse L1 valgul põhinevate viiruslaadsete partiklite moodustamine in vitro" (juhendaja: Reet Kurg, Ph.D).
2004–2005	Ajateenistus eesti kaitseväes.
2005–	Doktoriõpe molekulaar- ja rakubioloogia erialal, Molekulaar ja rakubioloogia instituut, Tartu Ülikool.

Erialane teenistuskäik:

2002	Biodata OÜ, tarkvara arendaja
2010–	Tartu Ülikool, Molekulaar ja rakubioloogia instituut, spetsialist

Teadustegevus:

Peamised uurimisvaldkonnad (1999–2011)

1. Papilloomiviiruste hiliste geenide ja nende poolt kodeeritud valkude omadused. Papilloomiviiruste kapsiidivalkude ekspresseerimine ja puhastamine (B.Sc õpingud).
2. PCR ja APEX praimerite kvaliteedi bioinformaatiline ennustamine (Biodata OÜ).
3. Markerite valik genotüpiseerimisuuringuteks (Biodata OÜ).
4. Papilloomiviiruste replikatsiooni hiline faas, viiruse kapsiidi moodustumise etapid ja tingimused (M.Sc õpingud).

5. Mittekodeerivate RNA molekulide osa inimese vererõhu regulatsioonis osalevate geenide ekspressiooni kontrollis (Ph.D õpingud).
6. Kõrgvererõhutõve geneetika, geneetilised interaktsioonid ja epistaas (Ph.D õpingud).

Publikatsioonid:

1. Kepp K, Org E, **Sõber S**, Kelgo P, Viigimaa M, Veldre G, Tõnisson N, Juhanson P, Putku M, Kindmark A, Kozich V, Laan M (2010) "Hypervariable intronic region in NCX1 is enriched in short insertion-deletion polymorphisms and showed association with cardiovascular traits" BMC Med Genet. 11:15.
2. **Sõber S**, Laan M, Annilo T (2010) "MicroRNAs miR-124 and miR-135a are potential regulators of the mineralocorticoid receptor gene (NR3C2) expression" Biochem Biophys Res Commun. 391(1):727–32
3. **Sõber S**, Org E, Kepp K, Juhanson P, Eyheramendy S, Gieger C, Lichtner P, Klopp N, Veldre G, Viigimaa M, Döring A; Kooperative Gesundheitsforschung in der Region Augsburg Study, Putku M, Kelgo P; HYPertension in ESTonia Study, Shaw-Hawkins S, Howard P, Onipinla A, Dobson RJ, Newhouse SJ, Brown M, Dominiczak A, Connell J, Samani N, Farrall M; MRC British Genetics of Hypertension Study, Caulfield MJ, Munroe PB, Illig T, Wichmann HE, Meitinger T, Laan M (2009) "Targeting 160 candidate genes for blood pressure regulation with a genome-wide genotyping array" PLoS One. 4(6):e6034
4. Org E, Eyheramendy S, Juhanson P, Gieger C, Lichtner P, Klopp N, Veldre G, Döring A, Viigimaa M, **Sõber S**, Tomberg K, Eckstein G; KORA, Kelgo P, Rebane T, Shaw-Hawkins S, Howard P, Onipinla A, Dobson RJ, Newhouse SJ, Brown M, Dominiczak A, Connell J, Samani N, Farrall M; BRIGHT, Caulfield MJ, Munroe PB, Illig T, Wichmann HE, Meitinger T, Laan M. (2009) "Genome-wide scan identifies CDH13 as a novel susceptibility locus contributing to blood pressure determination in two European populations." Hum Mol Genet. 18(12):2288–96

Stipendiumid:

SA Archimedes Kristjan-Jaagu nimeline stipendium, 01.04.2008–01.09.2008, erialane praktika, Pontificia Universidad Católica de Chile, Tšiilis.

DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS

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