Analysis of genetic variation within urotensin-II system in regulation of blood pressure and renal function

Thesis submitted for the degree of

Doctor of Philosophy at the University of Leicester

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1. ABSTRACT

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Finding genetic association with blood pressure and renal function – from candidate gene to large scale genetic association analysis

Elevated blood pressure (BP) and reduced glomerular filtration rate (GFR) are risk factors for cardiovascular disease. BP and GFR are influenced by heritable factors. Only small proportion of this heritability has been explained so far. This project aimed to identify genetic loci contributing to population variation in BP or GFR through application of candidate gene and large scale genotyping approaches.

The candidate gene approach utilised tagging single nucleotide polymorphisms (SNP) in genes of the urotensin-II (U-II) pathway in a sample of white European subjects (3 family collections and 5 unrelated subject studies – altogether 10,748 subjects). This was followed by gene expression studies in 2 collections of human kidneys and phylogenetic analysis of the system to examine its evolutionary conservation from fish to human.

The large scale genotyping project utilised data from 50K IBC genotyping array in a cohort of families (520 pedigrees) from general population of UK.

None of the 28 SNPs in U-II pathway genes was associated with BP or GFR. Gene expression levels of *UTS2* and *UTS2R* were strongly correlated ($r=0.83$, $p<0.0001$) but renal expression was not associated with human hypertension. The phylogenetic analysis showed that strong purifying selection acting on this system in lower vertebrates was lost in primates.

The large scale genotyping approach showed strong signal of association in the 5,10-methylenetetrahydrofolate reductase (NAD(P)H) gene (*MTHFR*) locus with clinic diastolic BP. Each minor copy (G) of rs17037388 was associated with 2.03mmHg reduction in clinic diastolic BP ($p=3.01 \times 10^{-06}$).

Gene candidate and large scale genotyping approaches performed in parallel provide useful information about genetic architecture of complex traits. The data from genetic association in candidate U-II system genes did not provide evidence on its association with BP or GFR. Large scale genotyping experiment led to identification of genuine association signal with clinic diastolic BP.

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3. PUBLICATIONS AND PRESENTATIONS

Publications

Charchar FJ, Bloomer LD, Barnes TA,…Debiec R, et al. Inheritance of coronary artery disease in men: an analysis of the role of the Y chromosome. Lancet;2012;379:915-22.

Tomaszewski M, Debiec R, Braund PS, et al. Genetic architecture of ambulatory blood pressure in the general population: insights from cardiovascular gene-centric array. Hypertension;2010;56:1069-76.

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Tomaszewski M, Charchar FJ, Nelson CP,…Debiec R, et al. Pathway analysis shows association between FGFBP1 and hypertension. J Am Soc Nephrol;2011;22:947-55.

Presentations:

Debiec R, Denniff M, Barnes TA, et al: Urotensin 2 signalling pathway and genetic regulation of blood pressure. British Hypertension Society Annual Meeting – 12-14/09/2011, Queens' College, Cambridge.
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Debiec R, Nelson CP., Braund PS, et al. Genetic control of heart rate in the general population - a large-scale genetic analysis. European Society of Cardiology Congress 28/08-02/09/2010, Stockholm.
Young Investigators Award on Population Sciences.

Abstracts:

R Debiec, M Denniff, TA Barnes, et al. Urotensin 2 signalling pathway and genetic regulation of blood pressure. Journal Hum Hypertens;2011;25:524-525.

Debiec R, Nelson CP., Braund PS, et al. Genetic control of heart rate in the general population - a large-scale genetic analysis. Eur. Heart J.;2010;31;1087.

4. LIST OF ABBREVIATIONS

AA - arachidonic acid

ACTH - adrenocorticotropic hormone

ADMA - asymmetric dimethylarginine

ApoE - apolipoprotein E

BMI - body mass index

bp - base pairs

BP - blood pressure

CAD - coronary artery disease

cDNA - complementary DNA

CKD - chronic kidney disease

CLCN6 - chloride channel 6 gene

CVD - cardiovascular disease

DAG - diacylglycerol

DBP - diastolic blood pressure

DNA - deoxyribonucleic acid

EDHF - endothelium derived hyperpolarizing factor

eGFR - estimated glomerular filtration rate

NOS - nitric oxide synthase

ERK - extracellular regulated kinase

ESRD - end stage renal disease

FBAT - Family Based Association Test

FGF1 - fibroblast growth factor 1

FGFBP1 - fibroblast growth factor binding protein 1

GEE - generalised estimating equations

GFR - glomerular filtration rate

GPR - G protein coupled receptor

GRAPHIC - Genetic Regulation of Arterial Pressure of Humans in the Community

GWAS - genome wide association study

HDL-C - high-density lipoprotein cholesterol

HWE - Hardy-Weinberg equilibrium

ICAM-1 - intracellular adhesion molecule-1

IGF2 - and insulin-like growth factor 2 gene

IP3 - inositol triphosphate

KDOQI - Kidney Disease: Outcomes Quality Initiative

LD - linkage disequilibrium

LDL-C - low-density-lipoprotein cholesterol

MAF - minor allele frequency

MDRD - Modification of Diet in Renal Disease

MLCK - myosin light chain kinase

mRNA - messenger ribonucleic acid

MTHFR - 5,10-methylenetetrahydrofolate reductase (NAD(P)H) gene

NCBI - National Centre for Biotechnology Information

NO - nitric oxide

NPPA - natriuretic peptide precursor A gene

NPPB - natriuretic peptide precursor B gene

PAI - plasminogen activation inhibitor

PCR - polymerase chain reaction

PDE1A - phosphodiesterase 1A gene

PER3 - region of period homolog 3 gene

PGE2 - prostaglandin E2

PIP2 - phosphatidyl inositol bisphosphate

PKC - protein kinase C

PPARG - proliferator-activated receptor gamma gene

QTL - quantitative trait loci

ROS - reactive oxygen species

RT-PCR - real time polymerase chain reaction

SBP - systolic blood pressure

SCS - Silesian Cardiovascular Study

SE - standard error

SHS - Silesian Hypertension Study

SNER - sensory epithelium neuropeptide-like receptor

SNP- single nucleotide polymorphism

SOLAR - Sequential Oligogenic Linkage Analysis Routines

SRTB - Silesian Renal Tissue Bank

TNFα - tumour necrosis factor α

UCE - urotensin-II converting enzyme

U-II - urotensin-II

URP - urotensin-II related peptide

UT - urotensin-II receptor

UTS2 - urotensin-II gene

UTS2D - urotensin-II related peptide gene

UTS2R - urotensin-II receptor gene

VSMs - vascular smooth muscle cells

WPKP - Western Poland Kidney Project

YMCA - Young Men Cardiovascular Association study

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6. INTRODUCTION

Epidemiological data

Epidemiology of high blood pressure and impaired renal function

Cardiovascular disease (CVD) is one of the major causes of mortality and disability worldwide.[1] Of these, coronary artery disease (CAD) and cerebrovascular disease were listed as first and second (respectively), among the 30 leading causes of death worldwide in 1990.[1] For both of these conditions high BP and reduced renal function are strong risk factors.[2,3] Moreover, high BP and reduced renal function worsen clinical outcomes in acute manifestations of CVD.[4,5] Approximately 972 million people were hypertensive in 2000; this accounts for 26.4% (26.6% in males and 26.1% in females) of the total world population.[6] Furthermore, the prevalence of hypertension is predicted to rise to 29.2% by 2025, mainly due to changes in lifestyle and aging of populations in the developing countries.[6] Prevalence of high BP is strongly affected by sex and geographical origin with the highest rates reported in Latin America, the Caribbeans and established Western economies.[6] High BP is more prevalent in women than men from Eastern Europe, India, Middle East and Sub-Saharan Africa but the opposite was observed for Western Europe, USA, Latin America, China and other parts of Asia.[6]

Impaired renal function is diagnosed in 7.2% of adults (≥30 years). The prevalence of decreased GFR increases substantially with age up to 35.8% in those >64 years.[7] A steady rise in the numbers of patients with chronic kidney disease (CKD) has been observed, most likely due to ageing of populations and

increase in the prevalence of type 2 diabetes.[8] Large epidemiological studies showed that the incidence of CKD is higher in males than females independent of other risk factors.[9] Male sex has also been identified as a risk factor of further decline in renal function and cardiovascular mortality in subjects already diagnosed with CKD.[10] Prevalence of CKD is strongly affected by geographical and racial factors with the highest rates reported for subjects from developed Western economies and of African and Afro-Caribbean origin.[8]

High blood pressure and impaired renal function as cardiovascular risk factors

Elevated BP has been identified as a risk factor of several cardiovascular disorders including ischemic heart disease, stroke, heart failure, vascular disease, and CKD. It has been estimated that the cardiovascular mortality related to high BP doubles with each 20/10 mmHg increment in BP.[11] Reduced GFR and markers of renal injury are also increasingly recognised as important cardiovascular risk factors in general population.[12] Reduction in GFR to below the normal range exponentially increases the risk of cardiovascular morbidity and mortality.[13] Significant albuminuria is also associated with more than 2 fold increase in cardiovascular mortality in general population.[14]

Blood pressure and coronary artery disease

Of the cardiovascular complications of high BP, CAD is the most prevalent in the general population. The relationship between the risk of CAD and BP is

linear and present even at BP levels considered as normal.[15] An increase of 20mmHg in systolic BP (SBP) and 10mmHg in diastolic BP (DBP) is associated with 1.85 increase in risk of death from CAD in European populations.[11] The relationship between BP and CAD is apparent in populations from different ethnicities and across all age categories.[16]

Impaired renal function and coronary artery disease

There is a strong association between impaired renal function and CAD.[12] The relationship, first observed in end stage renal disease (ESRD) patients, was confirmed by prospective studies in general population; even a mild reduction in glomerular filtration (GFR <60ml/min*1.73m2) was associated with 1.4-increase in risk of CAD.[17] In a prospective study of the general population CKD was an independent risk factor of CAD (adjusted hazard ratios for CAD were 1.55 for stage 1 CKD; 1.72 for stage 2, 1.39 for stage 3a, 1.0 for stage 3b and 4.29 for stage 4), only slightly weaker than for diabetes or smoking.[18]

Blood pressure and heart failure

Elevated BP is the most prevalent risk factor for heart failure in the general population.[19] Although myocardial infarction is the single strongest risk factor of cardiac failure, high BP, due to high prevalence in the general population, confers greater population-attributable risk.[20] In the longitudinal cohort study of the Framingham population (Massachusetts, USA; over a 20 year period) hypertension increased the risk of heart failure by 2-fold in men and 3-fold in

women (independent of other risk factors).[19] Hypertension increases the risk of heart failure most likely by directly affecting the myocardium (left ventricle hypertrophy) as well as indirectly by increasing the risk of CAD.[21]

Impaired renal function and heart failure

Moderate levels of renal function impairment are also associated with an increased risk of heart failure.[22] Subjects from the lower quartile of the renal function distribution have approximately 2-fold increase in risk of heart failure independent of other risk factors when compared with those from the highest quartile.[23]

Blood pressure and the risk of stroke

High BP is the single strongest, modifiable risk factor of ischaemic stroke.[24] Large scale longitudinal, epidemiological observations suggest that hypertension more than doubles the risk of ischaemic stroke over the lifetime.[25] There is a proportional relationship between the degree of BP elevation and the risk of stroke (SBP being a better predictor of risk than DBP).[21] In subjects aged 40-69 years the risk of mortality related to stroke doubles with each 20mmHg increase of SBP and 10mmHg increase of DBP across a wide range of blood pressure values.[26] It has also been suggested that the long-term time averaged BP, “antecedent BP”, has better predictive value than BP determined on a single occasion.[27]

Impaired renal function and the risk of stroke

Renal function impairment increases the risk of ischaemic stroke in patients with cardiovascular disease.[28] Mild renal impairment (GFR<60ml/min*1.73m2) is associated with 1.54-fold increase in risk of stroke or transient ischaemic attack.[3] However, this association has not been confirmed in the in general population without pre-existing cardiovascular disease, where mild renal impairment does not predict the risk of stroke.[29]

Impaired renal function and high blood pressure

The association between high BP and impaired renal function is bi-directional. Diseases affecting the kidney and associated arteries (reno-vascular) are a common cause of secondary hypertension in adults. GFR declines with advancing age but observational studies showed that this age related decline was markedly accelerated by elevated BP.[21] Furthermore, there is a linear relationship between BP elevation and the rate of renal function decline even for BP values in the “normal” range.[30] Elevated SBP seems to be a better predictor of risk of developing CKD than DBP.[31]

Pathophysiology of high blood pressure and chronic kidney disease

The pathophysiology of human hypertension is not fully understood.[32] Physiologically, BP is a product of cardiac output and vascular resistance.[32] However, complex interactions of short- and long-term adaptive mechanisms

adjust BP to the changes in environment. The interplay between the "sympathetic and parasympathetic nervous systems" hormonal pathways of water and ion handling (i.e. renin angiotensin aldosterone system[33], natriuretic peptides[34]), vasoconstricting and vasodilatating molecules (i.e. endothelin[35], nitric oxide[36]) directly influence cardiac output and/or vascular resistance. Many novel pathways (i.e. U-II pathway[37], endocanabinoids[38], fibroblast growth factors[39]) have also emerged from genetic studies as strong candidates with potential to contribute to BP regulation. Currently, it is not clear how these mechanisms interact together to produce excessive and long lasting BP increase and hypertensive complications.

Over the lifetime, a gradual loss of functioning nephrons in the kidney leads to a reduction in glomerular filtration.[40] Several factors accelerate this process and participate in the development of CKD.[30] The diabetes-hypertension interaction is by far the most common and important factor leading to deterioration of renal function. Others include serum lipids, current smoking or obesity.[21] These factors lead to endothelial dysfunction, microvascular damage and possibly damage of the filtration barrier in the glomerulus.[41] Under "normal" physiological conditions glomerular filtration is maintained by the auto-regulatory mechanisms. This protects the glomerulus from the fluctuations of BP and mechanical overload. In the presence of the renal risk factors microvascular changes lead to a reduction in the overall renal perfusion and disruption of the glomerular autoregulation.[42] The glomerular capillary pressure increases in order to maintain filtration. The increased pressure damages

filtration barrier, leads to mesangial hypertrophy, increased glomerular and interstitial fibrosis and eventually to the loss of the functioning nephrons.[40]

Heritability of blood pressure and eGFR

Both hypertension and renal injury share similar risk factors and are strongly influenced by the environment. The risk factors affecting both of these phenotypes can be divided into non-modifiable factors such as increasing age, male sex or genetic predisposition and modifiable factors such as diabetes or impaired glucose tolerance, serum lipids abnormalities (high low-density lipoprotein cholesterol /LDL-C/, high triglycerides, low high-density lipoprotein-cholesterol /HDL-C/), obesity and excessive alcohol consumption.[43,44] There is substantial evidence on familial clustering of BP.[45] Presence of strong significant correlation of blood pressure between spouses pictures great impact of the environmental factors in shaping this trait.[46] However, the familial aggregation of blood pressure also provided indirect evidence on the existence of heritable component of this phenotype. The first insights into the contribution of heritable factors in blood pressure regulation came from twin studies which showed much higher interclass correlation between BP levels in mono- than di-zygotic twins.[47] The familial studies showed much greater inter- than intra-familial variation in BP and allowed for the mathematical estimation of the heritable component.[32] In a study of 1006 subjects from extended pedigree of Dutch origin van Rijn et al. found that mean arterial pressure showed the highest heritable component estimated at 40% with slightly lower heritability for

DBP (37%) and SBP (34%).[48] In the longitudinal study of American Indians (from 13 communities) The Strong Heart Study (altogether 950 individuals) North et al. estimated heritability of clinic SBP and clinic DBP at 23% and 34%, respectively.[49] Tomaszewski et al. estimated heritability of clinic SBP and DBP at 31% and 32% in a sample of 520 families from the general population of Leicestershire UK.[50]

Also, GFR shows substantial heritable component. This differs significantly depending on the cohort and phenotype used to assess excretory renal function. In a study on 249 healthy siblings from 110 Swedish families heritability for creatinine clearance was estimated at 51% for, 58% for the estimated Cockcroft–Gault creatinine clearance and 40% for the eGFR (measured using the Modification of Diet in Renal Disease /MDRD/ formula).[51] Similar results were reported by Bochud et al. who calculated gender-adjusted heritabilities of 41% for inulin measured clearance and 52% for creatinine clearance in 348 participants from 77 hypertensive families of African descent.[52] These studies clearly show that the majority of intra-population variation in renal function or blood pressure is due to the environmental factors. However, it is also evident that at least part of this variation originates from the action of the environmental factors on specific genetic background.

Strategies to identify genetic association

Presence of genetic diversity in the population contributes to phenotypic variation of its subjects. However, the overall contribution of genetic factors the expression of a phenotypic traits vary. The phenotypic trait may be primarily genetically regulated (i.e. Mendelian traits - presence of genetic variant is almost consistent with its phenotypic manifestation) or be a result of a complex interaction of environment with multiple genetic variants of various effects sizes and penetrance (complex traits i.e. BP).[53] Traditionally mapping of Mendelian traits used linkage analysis. The analysis of linkage is based on a concept of co-segregation (linkage) of genetic markers with a phenotype of interest.[54] This linkage is broken down by events of recombination, rate of which depends on the physical distance from the causative variant (the closer the causative variant the less recombination occurs).[54] Linkage studies have been most successful in identification of highly penetrant mutations responsible for Mendelian forms of diseases.[55] However, due to their familial design they suffer several disadvantages. Linkage studies require availability of deoxyribonucleic acid (DNA) from biologically related subjects (families, siblings) with a given phenotype/disease which makes recruitment of a study cohort more difficult and expensive.[56] Loci identified under a linkage peak usually span several millions of bases of DNA and encompass multiple genes which makes identification of the causative variant difficult.[57] Genetic association is commonly used for tracing of complex genetic traits. Unlike the linkage scans association studies

do not require subjects of the study to be related. Association focuses on the assessment of deviation of genotypes distribution in the population related to trait/phenotype of interest.[54] Genetic association may be identified through application of different research approaches.

Candidate gene approach

Candidate gene studies utilise existing knowledge about biology of a given phenotypic trait (usually obtained through in vivo and in vitro experiments) to point at molecules, systems and pathways which could be involved in the regulation of a trait.[58] Genes of molecules with presumed function in the biological process are then saturated with genetic markers (usually SNPs or micro-satellites) and genotyped in the studied population.[59] Subsequently prevalence of genotypes is compared between carriers and non-carriers of a given trait (in case of binary trait) or the correlation between quantitative value of a phenotype and presence/number of specific genetic variants is assessed. Alleles/genotypes co-segregating with a given phenotype or with higher/lower values of quantitative trait are considered associated with a given trait.[59] Replication in independent cohorts with identical phenotypes is then necessary to obtain confirmation of result obtained in the discovery cohort.[60]

Candidate gene approach is widely applied to finding genetic association but has several pros and cons. One of the main advantages of such design is the flexibility in the choice of genetic markers used to study association. Usually the research can be based on tagging polymorphism, which through linkage

disequilibrium (LD) provide information about neighbouring SNPs, reducing need for direct genotyping.[61] The tagging variants can be enriched by genetic variants with plausible function in order to obtain more comprehensive assessment of association in a given region.[62] Also, investigator may decide to add functional polymorphism specific for a given population (which would not be present on a commercially available platform.[62] Due to the need of genotyping only limited number of genetic variants candidate gene approach are relatively inexpensive and involve less resources than whole genome approaches. Candidate gene studies are usually characterised by relatively good power (related to the number of performed statistical tests and size of the study population) as they do not suffer from the problem of multiple testing correction to the extent of whole genome approaches.[63] By far the most important drawback of candidate gene approaches is the fact that a candidate pathway is chosen based on an already existing knowledge or suspicion about involvement of a biological pathway in regulation of a phenotype.[59] This way the choice of candidate is influenced by the quality and reliability of previous experiments and interpretation of results. Due to genotyping variants in limited number of genes, candidate gene studies have limited usefulness in studying gene-gene interactions.[59]

Candidate pathway approach

Investigation of the whole candidate pathway is an extension of the traditional candidate gene approach. This was possible thanks to the

development of knowledge regarding molecular interactions in a human body. Traditionally pathway approach was a sequel of a discovery of a genuine association. This was followed by the analysis of genetic variants in molecules closely interacting with the primary target.[39,64] The concept of pathway analysis changed with the development of high output genotyping technologies. Currently data from a genome wide association are processed using specific bioinformatics software. These programs use existing databases of biological interactions to search for pathway or biological processes with over-representation of nominally significant association signals.[65]

Whole genome approaches

Whole genome approaches utilise different strategy to find signal of genetic association. Rather than focusing on a limited number of genes densely saturated with genetic markers, they use specific platforms with genetic markers uniformly spread across the genome.[66]

Subsequently presence of association is assessed with each individual genetic markers. Therefore, genome wide approaches are not biased by a priori hypothesis as the distribution of genetic markers is not affected by *a priori* existing knowledge.[59] By far the biggest strength of genome wide approach is the possibility of discovering a novel pathway involved in genetic regulation.[63] Some of the performed research identified strong association signal in regions without any obvious candidate gene or even not known to harbour any protein coding transcripts – so called “gene deserts”.[67] Another pros of whole genome

approach comes from using standardised genotyping platform. This greatly facilitates meta-analysis attempts as the same genetic variants have been genotyped in all cohorts (in case when the same type and version of platforms were used in all studies). However, genome wide approaches also suffer from drawbacks related to their design. One of the biggest disadvantages of such design is large number of executed statistical tests, ranging from several thousands to several millions.[68] This reduces greatly the power of the study and imposes stringent cut off criteria for statistical significant findings.[68] Due to the high price of the commercially available platforms and large populations which have to be used to obtain required statistical power, genome wide approaches are usually expensive and consumes significant resources. Another drawback lies in the design of the platforms. Based on an existing knowledge about the structure of the genome in representative population, commercially available platforms may not be accurate in studies of ethnically different or isolated cohorts.[69] Finally, due to the use of tagging approach, genome wide approaches tend not to identify causative variants but rather their proxys. Therefore initial findings apart from replication usually need fine genotyping of the region and biological studies.

Constituents of the urotensin-II pathway

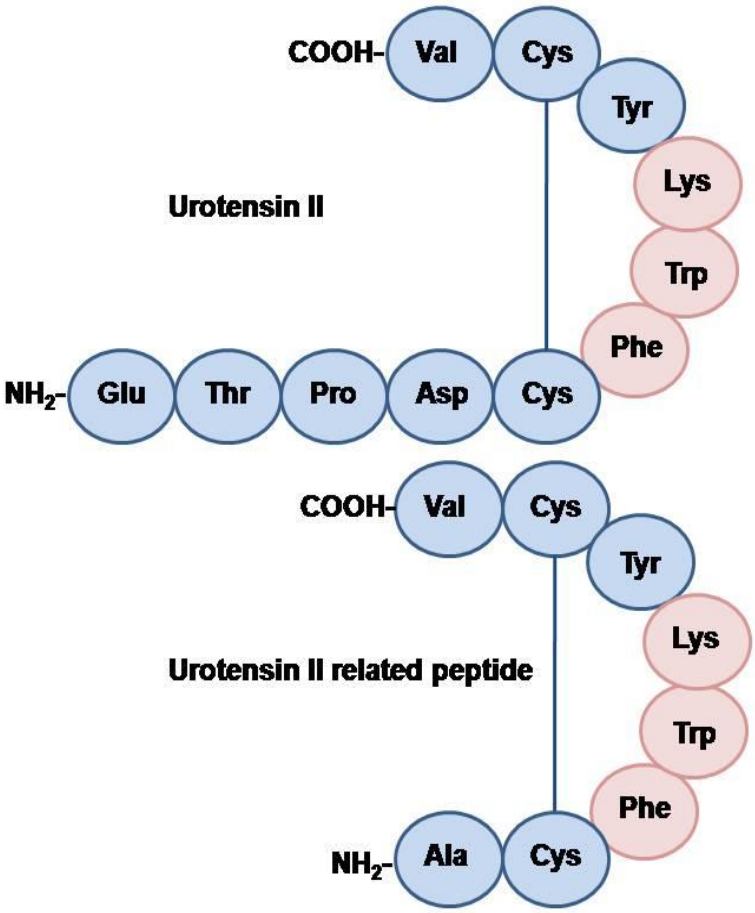
Urotensin-II system overview

Urotensin-II is an undeca- cyclic- peptide first isolated from the urophysis - neurosecretory organ of teleost fish.[70] It has been shown to regulate ion transport in secretory and absorptive tissues and influence osmoregulation in fish.[71] Subsequently, the peptide has been found to regulate blood vessel tone and evoke vasoactive responses in vertebrates. Sequences of U-II precursor genes have been cloned and identified in mouse, rat and human[72,73] and their expression beyond the nervous system has been reported. Comparative analysis of the amino acid sequence of U-II revealed that the active, cyclic C-terminal region of this peptide has been conserved across evolution from fish to human, whereas the N-terminus is highly variable in these species.[72] G protein coupled receptor (GPR) GPR14, was formally identified as the receptor for U-II and called urotensin-II receptor.[74] Subsequently, a novel agonist – 8-amino acid peptide, urotensin-II related peptide (URP), capable of specific urotensin-II receptor (UT) binding and evoking cellular effects, was identified.[75] Physiological and pharmacological studies on U-II system led to the discovery of its potential in the regulation of the cardiovascular system. Serum levels of U-II and altered expression of the peptide and its receptor have been linked to several cardiovascular disorders such as hypertension, type 2 diabetes and CKD, making it a relevant target for new therapies.[76]

Urotensin-II

Human U-II is an undeca- peptide composed of 11 amino acid residues (Figure 1). The cyclic part is produced from the formation of a disulphide bridge between Cys5-Cys10, which creates a conformation necessary for a ligand - receptor interaction.[77] This conserved cyclic element of the peptide is surrounded by a highly flexible N terminal chain, and in humans, by a hydrophobic Val residue at the C terminus.[77] Minimal biologically active sequence of U-II comprises eight amino acid – cyclic region flanked by single amino acids at both N- and C-terminal extremities.[78] Substitution of any of the amino acids within the cyclic sequence suppresses the activity of the peptide, whereas cleavage of the amino acid residues outside the cyclic region reduces the biological potency of the peptide by 1000–fold.[78] U-II shows homology to somatostatin in the cyclic region – both peptides share Phe-Tryp-Lys amino acids (Figure 1).[79] Furthermore, because of this homology U-II as well as URP are capable of weakly interacting with somatostatin type 2 and 5 receptors.[80]

Figure 1. The structures of human urotensin-II and urotensin-II related peptide.



Urotensin-II is an 11-amino acid peptide with a disulphide bridge between Cys5-Cys10. Urotensin-II related peptide is an 8 amino acid peptide with disulphide bridge between Cys5-Cys10. The Phe-Tryp-Lys segment (in pink) is homologous to somatostatin.

Urotensin-II related peptide

URP is an endogenous, functional ligand of UT and shows slightly higher affinity to the receptor in transfected cells but is less potent in tissues than U-II.[75] URP is an 8-amino acid peptide that contains cyclic domain identical to that of U-II (Figure 1). The cyclic domain is flanked by one amino acid on each N- and C-terminal region. Whereas the C-terminal residue is identical to U-II, the N-terminal amino acid differs.[75] Several studies suggest that residues within the cyclic domain of the peptide (especially Phe-Tryp-Lys triplet homologous with somatostatin) are of particular functional importance – an amino acid substitution in this region markedly affects biological function of URP. Only the Ala8 analogue of URP retains its high biological activity, indicating the lack of crucial role of Val8 for biological function.[81] URP, similarly to U-II has been shown to activate somatostatin type 2 and 5 receptors.[80] However, in contrast to U-II, URP has no significant effect on cell proliferation mediated by somatostatin receptor type 5, which might suggest relevance of the N-terminal region of U-II in this process.[80]

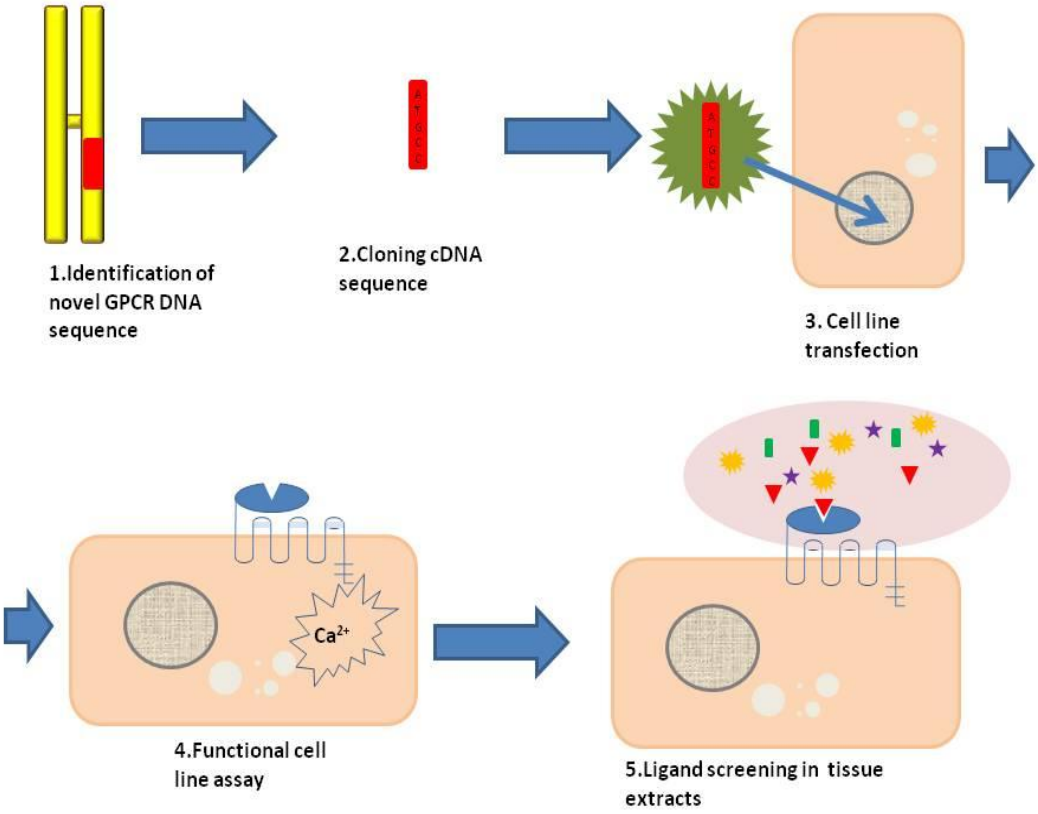
Molecular physiology of the urotensin-II receptor

UT (G protein coupled orphan receptor [GPR14], sensory epithelium neuropeptide-like receptor [SNER]) belongs to class A family of G protein coupled receptors and is activated by U-II and URP. It has been deorphanised (Figure 2) and subsequently shown as homologous (in 27% overall and 41% in the transmembrane domains) with somatostatin receptor (SSTR4) and opioid

receptors.[74,82] Due to this structural similarity the receptor can be activated by somatostatin but at much lower efficacy than that seen for U-II.[83] As with all the GPRs, UT consists of seven transmembrane domains.[84] UT shares many conserved motifs and residues with other receptors of this family, mutations of which lead to loss of function or constitutive activity phenotype.[84] The ligand binding site is located within the extracellular domain and top portions of the transmembrane domains. The II, III, and IV extracellular domains of this receptor are agonist binding sites.[85,86] The intracellular domain is associated with activation of heterotrimeric G protein Gαq/11.[87] There is also evidence for coupling of the receptor to Gαi/o but this may be the result of high receptor expression in transfected cells assays.[84] Other than a role in cell signalling, the cytoplasmatic tail is important for receptor desensitisation, internalisation and turnover. The pivotal role of the four Ser residues in this process, at positions 364-367, has been confirmed by truncation and mutagenesis studies.[87] U-II and URP bind specifically to UT and cause activation of intracellular signalling pathways. Agonist binding to the receptor is characterized by high affinity and very slow dissociation rate, which makes the result of this interaction pseudo-irreversible.[88] Stimulation of the receptor evokes not only cellular signalling, but also regulates functioning of the receptor. Following agonist binding and signal transduction, uncoupling from the trimeric G protein and desensitization occur.[87] The receptor-agonist complex is subsequently internalized to the endosomal compartment via clathrin-coated vesicles.[87,89] Participation of the β-arrestin protein family has been implicated in these processes. However, the exact role

of these proteins in the internalisation of the complex is not fully elucidated.[87,89] The receptor is then quantitatively recycled to the cell membrane. It is thought that rapid internalisation with slightly slower recycling, together with low dissociation rate explain the reduced response to subsequent doses of agonists.[89]

Figure 2. Deorphanisation of G protein coupled receptor.



Process of identification of a new G-protein coupled receptor starts with identification of its DNA sequence. The identification is based on sequence homology to known receptors and assessed *in silico* using specific protocols. Subsequently, a cDNA encoding receptor is cloned and transfected to obtain a cell line with expression of the receptor, enabling functional studies. The expression system forms the basis for screening for active ligand in tissue extracts. Binding of the ligand to the receptor can be detected by analysis of second messenger such as cAMP or Ca2+. Following this step the active ligand is isolated from tissue extract and identified.

Urotensin-II converting enzyme

Both U-II and URP are produced as immature proteins.[75,90] Proteolytic cleavage of these pre-peptides is necessary for them to acquire biological activity.[91] However, a specific protease(s) has not been identified to date. The enzymatic activity of urotensin-II converting enzyme (UCE) has been reported in protein extract of porcine kidney.[92] Following this finding, Russel et al. investigated the activity of UCE in human cells.[93] They examined the presence and localisation of a putative enzyme in cultures of human epicardial mesothelial cells and cardiac fibroblasts.[93,94] The cleavage product – U-II was detected in the supernatant of permeabilised cells whereas only low levels were present in supernatants of intact cells, suggesting intracellular localisation of the enzyme. Furthermore, cell buffer modification and comparison with the activity of recombinant furin (mammalian protease primarily present in trans-Golgie network) on the substrate were suggestive of furin-like activity of UCE. It has also been shown that the sequence between amino acid residues 11-17 corresponds to the consensus sequence of the furin cleavage site. The possible conversion of pre-U-II within the circulation was also reported.[93] This process was sensitive to aprotinin suggesting presence of a trypsin like protease, capable of converting pro-U-II into an active peptide. It is not clear which of these processes are biologically relevant but further investigation of UCE is necessary as proteolytic enzymes have proven to be important pharmacological targets.

Organ and tissue distribution of urotensin-II, urotensin-II related peptide and urotensin-II receptor

U-II was initially considered a nervous system specific peptide. However, subsequent studies revealed reactivity of cells derived from different tissues to exogenous U-II and provided important insight into the expression and systemic distribution of UT and its ligands.

Within the nervous system of vertebrates U-II immunoreactivity was reported predominantly in neurons from the medulla and ventral horns of the spinal cord.[95] U-II positive neurones showed co-expression of acetylcholine which might suggest their involvement in motor functions. The expression of URP precursor protein showed a similar pattern to the expression of U-II but at slightly lower levels in the spinal cord.[75] In a comprehensive study of localisation and function of UT in rat brain, Jégou et al. found U-II binding sites were restricted to discrete brain areas such as the lateral septum, anteroventral thalamus, pedunculopontine reticular area, the pontine nuclei and cerebellar cortex.[96] However, semi-quantitative real time polymerase chain reaction (RT-PCR) analysis revealed low, but widespread, distribution of UT messenger ribonucleic acid (mRNA) in all samples examined.

The presence of U-II pathway constituents was also reported in peripheral organs and tissues. In humans U-II precursor mRNA is expressed in the kidney (mainly in the tubular epithelial cells), heart (atria and ventricle), arteries, pancreas, liver, spleen, small intestine, thymus, prostate, pituitary gland, adrenal gland and ovary.[73,97,98]. The pattern of URP mRNA expression is similar

to that of U-II in peripheral tissues.[75] Similarly, UT mRNA expression is apparent in cardiovascular and renal system. However, there are substantial differences when compared to U-II and URP. The level of UT mRNA expression appears similar in these tissues.[98] Immunohistochemical studies of human tissues revealed diffuse U-II-like immunoreactivity in cardiomyocytes, endothelial cells of human arteries and in atherosclerotic plaques (mainly within lipid laden smooth muscle/macrophage regions).[37,99] Quantitative UT autoradiography indicated moderate levels of UT expression in kidney, heart vasculature and skeletal muscle.[99] Of particular importance were data showing U-II like immunoreactivity in secretory vesicles of human endothelial cells, suggesting the secretion of this peptide is via a constitutive pathway.[99]

Several reports have shown the presence of U-II in plasma, urine as well as correlations of its concentrations with hypertension, CKD, heart failure and diabetes.[100-103] Charles et al. reported the presence of an arterio-venous gradient of this peptide suggestive of cardiac, renal and hepatic production.[104] Also, the cardiopulmonary circulation has been shown to be a source of U-II in patients with congestive heart failure.[91] The role of the kidneys in the systemic production of U-II may, however, be even more complex. Matsushita et al. have reported the presence of significant amounts of this peptide in urine.[98] Furthermore, by comparison of urinary clearance of U-II with glomerular filtration rate, they suggested renal tubular production and excretion of this peptide.[98] Substantial discrepancies in levels of circulating plasma U-II were reported in literature.[105] Comprehensive analysis of commercially available U-II

assays (radioimmunoassay and enzyme-linked immune sorbent assay) and comparison with reverse phase high performance liquid chromatography, proved a lack of specificity of the described assay to mature U-II and URP.[105] Thus, findings from a majority of studies on plasma and urine concentrations of U-II should be interpreted with caution.

Effects of urotensin-II receptor activation

Given that UT is expressed in many different tissues, it is not surprising that its activation may evoke different, and sometimes opposite, outcomes in different organs with the systemic result being the sum of the individual organ effects. Not only do these effects vary at the cell, tissue and system level, but also are species specific.[106,107] In pharmacological studies, route of administration, dose and disease phenotype influence the ultimate molecular response.[108,109]

Molecular mechanisms of vasoconstriction

Perhaps the most significant effect of U-II in vertebrates is its influence on vascular tone regulation.[110] Indeed, U-II has been identified as the most potent mammalian vasoconstrictor with >10 times higher potency than endothelin-1 in rat arteries in vitro.[37,111] U-II-mediated vasoconstriction of vascular smooth muscle cells (VSMs) has been subsequently confirmed in other vertebrates.[112,107] The potency and efficacy of these effects was highly variable

between species, vascular beds and strongly modified by the endothelial function.[113-115,116]

There are at least 3 potential molecular pathways of U-II mediated vasoconstriction. (Figure 3) The primary mechanism of VSMs contraction occurs through regulation of calcium dependent myosin phosphorylation. Binding of U-II to its receptor activates effector enzymes and leads to the production of secondary messengers. Specifically, phospholipase C mediates hydrolysis of phosphatidyl inositol bisphosphate (PIP2) into inositol triphosphate [(IP3) - releases Ca2+ from the sarcoplasmatic reticulum)] and diacylglycerol (DAG) that activates Protein Kinase C [PKC]) while activation of phospholipase A2 results in increase in arachidonic acid (AA).[117,118] The subsequent increase in intracellular calcium is the effect of its influx via voltage gated membrane calcium channels and release of intracellular calcium.[119,117] Ca^{2+} binds to calmodulin and the complex activates myosin light chain kinase (MLCK).[120] Phosphorylation of myosin light chains stimulate the activity of actin-activated ATPase, leading to muscle filament sliding and contraction.[120]

The second mechanism, capable of modification of VSMs contraction in response to U-II, is calcium sensitisation. Secondary messengers such as AA, PKC and Rho-kinase (activated by RhoA) inhibit action of myosin light chain phosphatase.[120] This translates into myosin phosphorylation status in a calcium independent manner.[121,122] As ATPase activity of phosphorylated myosin may be further increased by direct binding of calcium to myosin this mechanism enhances vasoconstriction.[123]

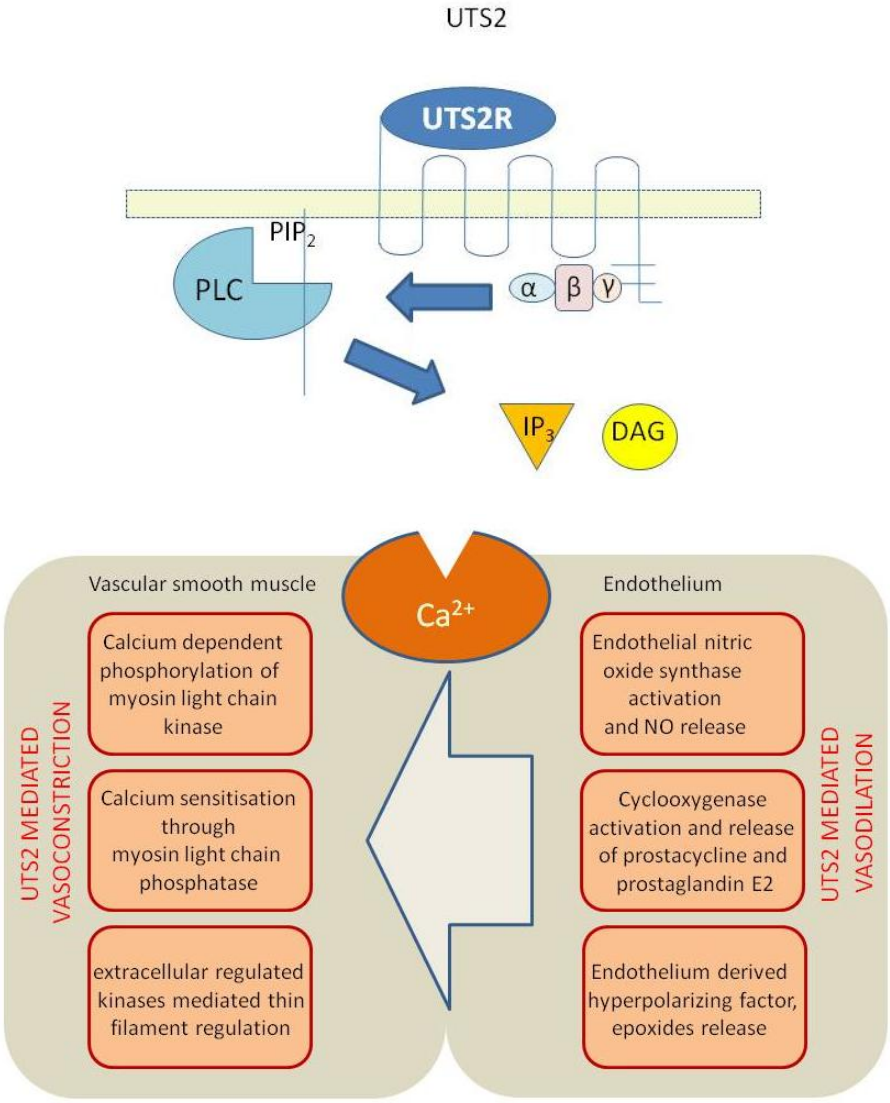
Finally, it has been proposed that U-II influences VSMs contraction through a third mechanism related to thin filament regulation.[121,124] U-II stimulation evokes increases in the activity of extracellular regulated kinases (ERK). These enzymes are thought to act by phosphorylation of calponin (a thin filament protein which, by binding to actin and myosin, inhibits the action of actin-activated ATPase).[124] Phosphorylation of this protein diminishes its affinity to myosin and actin promoting contraction.[124]

Molecular mechanisms of vasodilatation

Apart from its vasoconstrictor activity U-II has been shown to produce hypotensive responses in vivo and vasodilatation in various vascular compartments of different species.[125,126] The mechanism of U-II evoked vasodilatation is mediated by the release of certain vasoactive agents from the endothelium. (Figure 3) Removal of the endothelium attenuates this response and augments vasoconstriction.[110] UT activation in endothelial cells leads to the activation of endothelial nitric oxide synthase (eNOS) and cyclooxygenase with subsequent release of nitric oxide (NO) and arachidonic acid metabolites such as prostacyclin and prostaglandin E2 (PGE2).[114,127,128] Other less well documented mechanisms postulated to contribute to U-II-mediated vasodilatation in rats include endothelium derived hyperpolarizing factor (EDHF) and epoxide related products.[125] The mechanisms of U-II evoked vasodilation may differ between the vascular beds in the same subject and are age dependent.[129] Vasodilation is mediated by NO and PGE2 in young rats but

not in aged animals where it is mediated by AA metabolites only.[114] It is proposed that the ultimate vascular response to U-II stimulation is not only dependent on the density and localisation of UT but is also a reactive response of VSMs and endothelium.

Figure 3. Urotensin-II and its action on blood vessels.



Urotensin-II binding to urotensin-II receptor leads to activation of heterotrimeric G protein Gαq/11. This, in turn, activates membrane bound enzyme phospholipase C, which by degrading phosphatidyl inositol (3, 4, 5) triphosphate produces second messengers: diacylglycerol (DAG) and inositol triphosphate (IP3). IP3 releases intracellular calcium which mediates effects in endothelial and smooth muscle cells.

Cellular proliferation and tissue remodelling

Proliferation and remodelling of tissues within blood vessels and the heart plays an important role in almost all pathological processes within the cardiovascular system.[130] Remodelling includes a wide variety of molecular processes such as endothelial dysfunction, protease-mediated rearrangement of intercellular matrix, fibrosis and stiffening related to increased collagen production, matrix deposition as well as cellular proliferation and hypertrophy.[131] The concept of involvement of the U-II pathway in vascular remodelling is supported by evidence from in vivo and in vitro experiments. Serum levels of U-II are raised in diseases such as hypertension, chronic heart failure or atherosclerosis where vascular remodelling seems to play a key pathophysiological role.[100,101,132] Constituents of U-II system also show significant over-expression in remodelled tissues.[35,133] In vitro experiments have shown stimulatory effects of U-II on mechanisms engaged in remodelling. Specifically, human umbilical vein endothelial cells stimulated with U-II showed increased DNA synthesis and proliferation and were less prone to tumour necrosis factor-α (TNFα)-mediated apoptosis.[134] Upon activation by U-II, endothelial cells also showed a significant increase in collagen 1 expression and a decrease in matrix metalloproteinase 1 gene expression.[135] U-II also leads proliferation and altered plasminogen activation inhibitor (PAI) gene expression in VSMs from the systemic and pulmonary circulation.[136,137] This proliferation effect is intensified by factors of known importance in atherosclerosis such as oxidised LDL-C, serotonin or reactive oxygen species

(ROS).[138,139] Adventitial fibroblasts are also stimulated by the U-II pathway in vascular remodelling. Upon U-II stimulation, adventitial fibroblasts show an increase in DNA production as well as a dose-dependent increase in the production and secretion of collagen. Furthermore, U-II might act as a chemotactic factor for adventitial fibroblasts leading to their migration and profound changes in the structure of blood vessels.[140,141] U-II also regulates cardiac remodelling. Isolated cardiac fibroblasts show proliferation response to U-II which is mediated by the intracellular production of ROS.[142] Cardiomyocytes from neonatal rats, transfected with UT and stimulated with U-II, showed hypertrophic responses including increase in cell size, protein production, myofibril reorganisation and induction of other morphologic changes.[143] A number of in vitro findings have been confirmed in vivo. Chronic U-II infusion in rats decreased myocardial contractility and increased collagen I/III ratio – a marker of myocardial fibrosis, whereas UT blockade with SB-611812 reduced cardiac hypertrophy in rats with coronary artery ligation.[144,145] UT blockade has also been shown to reduce re-stenosis following balloon angioplasty of the common carotid artery in rats.[146] The exact molecular mechanisms underlying U-II effects on vascular remodelling have not been fully elucidated. Phosphorylation and activation of ERK – a member of the mitogen activated kinase family, is likely to be a key element in this process. This is thought to be mediated by Ca^{2+}/calmodulin - RhoA/Rho kinase pathways.[147,148] Alternative pathways include the participation of Gi/o protein, trans-activation of epidermal growth factor receptor or induction of ROS production.[136,142,147]

Finally, participation of cell to cell interaction and the involvement of adhesion mediated integrin signalling in this process has also been postulated.[137] The accumulated evidence for the participation of the U-II pathway in cardiovascular remodelling is strong but a majority of data is from in vitro experiments and animal models rather than humans. Further clinical studies in patients with different cardiovascular disorders along with details of the underlying molecular mechanisms are necessary to confirm the clinical significance of these findings.

Inotropic effects of urotensin-II

U-II system was shown to influence contractile function of the myocardium. Indeed, positive inotropic effects were observed in rat cardiomyocytes stimulated by human U-II in vitro.[149] The increase in contractility was also reported in human heart. Russel et al. showed that contractility of human right atrial trabecule and right ventricle muscle samples increased following U-II stimulation in vitro.[150] Furthermore, the effect was concentration dependent but complicated by pro-arrhythmogenic activity.[150] Interestingly, the effects of U-II on myocardium depends on 'health' status of cardiomyocytes; U-II increased contractility of non-failing myocardium but had a negative inotropic effect on failing cardiomyocytes.[151] Furthermore, UT antagonist administration in the absence of exogenous U-II was capable of improving contractility only in failing myocardium.[151] Using specific inhibitors Russel et al. examined possible signalling pathways that could mediate the effects of the peptide on the myocardium.[152] In human myocardial samples they

showed that U-II dependent increase in contractile force were accompanied by a gain in phosphorylation of myosin light chains.[152] The inotropic effect was mediated by PKC, but this was not the mechanism responsible for increased myosin light chain phosphorylation - RhoA-Rho kinase did not seem to play a significant role in this process.[152] Paradoxically, in vivo experiments in primates gave results contrary to in vitro findings. Specifically, intravenous administration of high doses of human U-II led to fatal cardiovascular collapse in cynomolgus monkeys (macaque).[153] Careful examination of the haemodynamic mechanisms led to the conclusion that the overall response was the result of negative chrono- and inotropic effects combined with a reduction in total peripheral resistance.[153] Overall haemodynamic response to UT stimulation is even more complex. Administration of U-II into the central nervous system increases heart rate, contractility and cardiac output.[154] This effect is mediated by increased sympathetic activity and can be blocked by administration of beta adrenergic receptor blockers.[154,155]

Urotensin-II as a marker of cardiovascular disease

Several studies linked U-II system to clinical phenotypes such as hypertension, CKD, heart failure, diabetes mellitus, atherosclerosis, cardiomyopathy, or portal hypertension.[101,156-159]

Urotensin-II pathway and hypertension

U-II is involved in haemodynamic regulation at various levels: central – via an influence on the autonomic nervous system, vascular – via direct actions on blood vessels, cardiac – through an influence on myocardial contractility and finally renal – possibly via an influence on glomerular filtration and sodium excretion.[37,71,150,154] High potency of vasoconstriction shown in various species has made the U-II pathway a plausible contributor to the pathophysiology of essential hypertension.[107] There is a synergistic action of U-II and angiotensin II in producing vasoconstriction.[160] Several studies showed correlation between serum U-II and SBP in patients with essential hypertension.[98,100] Indeed, an elevation in post-exercise serum U-II levels was shown in hypertensive but not normotensive patients.[161] Serum levels of U-II are significantly reduced by antihypertensive therapy.[162] Sondermeijer, demonstrated a direct vascular effect of U-II on human blood vessels in vivo by iontophoretic administration of the peptide.[109] Importantly, peripheral vasoconstriction was produced in patients with hypertension, but not in healthy controls (in whom it caused vasodilatation).[109] However, substantial discrepancies exist in the results of

many experiments. No difference in serum and cerebrospinal fluid levels of U-II-like immunoreactivity was found between 10 hypertensive and 10 normotensive subjects.[163] No vascular effects were recorded upon direct infusion of human U-II or urantide (UT antagonist) into the brachial artery in either healthy controls or patients with cardiovascular disease.[164] A randomised clinical trial performed in a group of hypertensive patients (54 subjects in treatment group and 54 controls) with diabetic nephropathy did not show any clinical benefit of addition of palosuran (U-II antagonist) to standard antihypertensive treatment.[165] Most importantly, neither urinary albumin excretion nor BP were affected by the treatment.[165] Although the vasoactive properties of U-II are well established, its role in the pathophysiology of human hypertension needs further evaluation. It is possible that the observed discrepancies are due to different experimental protocols. The trials with U-II antagonists have, in turn, been criticised for low power, inadequate dosing of the active agent and confounded affects from subjects already receiving multi-drug effective antihypertensive treatment.[166]

Urotensin-II pathway and chronic kidney disease

There is a general lack of consistency in data from studies on renal effects of the U-II system. U-II was shown to regulate fluid and mineral homeostasis in fish and these actions were subsequently confirmed in other vertebrates.[167] Experiments in animal models suggest that U-II exerts its renal actions through regulation of kidney blood flow, glomerular filtration and direct effects on tubular electrolyte reabsorbtion.[71,168,169] While most studies showed a negative effect

on glomerular filtration and natriuresis, Zhang et al. reported increased renal blood and sodium excretion following administration of U-II into the renal artery in rats.[71,170,128] The observed discrepancies were hypothesised to originate from differences in experimental conditions and U-II administration protocols. The animal studies were supported by some clinical observations. Plasma U-II immunoreactivity was positively correlated with impairment of renal function when assessed in a group of 14 patients with renal dysfunction, 12 patients on heaemodialysis (highest plasma U-II immunoreactivity) and in 24 healthy controls.[102] Totsune et al. reported increased U-II-like immunoreactivity in plasma and urine of patients with diabetic nephropathy and, based on those findings, proposed that increased U-II renal production and tubular excretion of U-II could play a role in renal complications of diabetes.[171] Indeed, increased U-II and UT expressions (45- and 2000- fold respectively) were found in kidney biopsy samples from patients with diabetic nephropathy.[172] In preparation for clinical studies the nephroprotective effect of palosuran (UT antagonist) was shown in diabetic rats with nephropathy.[173] Sidharta et al. showed that chronic administration of palosuran in macroalbuminuric diabetic patients decreased urinary albumin excretion.[174] However, a small number of subjects in the study (19 individuals) and the low palosuran dose make the results of this study unconvincing.[166] A randomised clinical trial performed in a representative group of 54 subjects with diabetic nephropathy did not show any difference in glomerular filtration or albuminuria following administration of palosuran (at 4 and 8 weeks) when compared to placebo.[166] However, again a low dose of

palosuran was used in this study. Ravani et al. showed that low serum U-II levels were predictors of cardiovascular complications in patients with CKD.[175] Zoccali et al. observed a direct correlation between elevated plasma levels of U-II and left ventricular function and geometry in patients with cardiomyopathy and ESRD.[176] Further carefully designed trials in homogenous groups of patients are necessary to confirm these findings.

Urotensin-II pathway and diabetes mellitus

In addition to a postulated primary role in vascular regulation, the U-II system may be involved in regulation of metabolic processes. Presence of U-II and its receptor transcripts and protein products have been confirmed in pancreatic and liver tissues of various species.[104,177,178] In perfused rat pancreas native and non-native U-II have been shown to inhibit insulin secretion in response to glucose.[179,180] This effect was mediated by UT and could be attenuated by blocking the UT receptor.[177,181] Furthermore, palosuran and urantide blocked the effect of endogenous U-II and potentiated insulin release.[181] Wang et al. showed over-expression and insulinostatic effect of U-II in skeletal muscles of diabetic mice.[182] Clozel et al. showed that chronic palosuran treatment improved survival in diabetic rats.[173] The beneficial effects of UT blockade are not only limited to improved metabolic control of glycaemia but also positively influence serum lipid profile and slowed the progression of renal complications (i.e. decrease in albuminuria, increase in renal plasma flow and glomerular filtration rate, decreased occurrence and severity of renal

lesions in histopathological examination).[173] Clinical observations also revealed a link between U-II system and diabetes. Serum levels of the peptide have been reported as elevated in patients with diabetes and metabolic syndrome.[103,183] Plasma U-II levels correlate with severity of retinopathy and carotid atherosclerosis as well as nephropathy in diabetic patients.[156,171] Iontophoretic administration of U-II produced vasoconstriction in the peripheral vasculature of diabetic patients but not in healthy controls, indicating its role in predisposition to vascular complications of diabetes.[184] These data stimulated studies on U-II antagonists in the treatment of diabetes. In a pilot study of U-II antagonist palosuran in 18 subjects with diabetic nephropathy a reduction in urinary albuminuria was observed. [174] The effect of palosuran treatment was assessed in a double-blind, placebo-controlled, randomized, two-way crossover study of 20 patients with diet controlled diabetes. Palosuran treatment was not associated with a change in insulin response during the hyperglycaemic glucose clamp and did not affect insulin or blood glucose response during a meal tolerance test.[185] This indicated a lack of clinical usefulness of palosuran in the treatment of type 2 diabetes but left the question on the significant discrepancies between human studies and animal models unanswered.

Urotensin-II pathway and atherosclerosis

U-II has been shown to influence cellular proliferation and vascular remodelling and has also been linked to impaired endothelial function. Heringlake et al. reported a correlation between the number of coronary arteries

affected by atherosclerosis and increasing serum levels of U-II in patients with ischaemic myocardial diastolic dysfunction.[186] In two independent groups of patients with essential hypertension and type 2 diabetes Suguro et al. showed that carotid artery intima-media thickness (an early marker of atherosclerosis) and plaque score (an indicator of the severity of carotid atherosclerosis) were directly correlated with circulating levels of U-II.[156,187] However, a study in patients with ESRD and high cardiovascular risk showed no positive correlation between serum levels of U-II and traditional risk factors of atherosclerosis.[157] In fact, serum U-II was inversely correlated with fibrinogen, intracellular adhesion molecule-1 (ICAM-1), and plasma asymmetric dimethylarginine [ADMA (inhibitor of NO synthesis)].[157] Expression studies revealed that atherosclerotic plaques in coronary arteries showed stronger expression of U-II than arteries obtained from normal hearts. Furthermore, levels of expression were dependent on the severity of the underlying process with the strongest expression in endothelial cells underlined by accumulation of inflammatory cells.[188] Using samples of carotid and aortic atherosclerotic plaques, Bousette et al. demonstrated U-II immunoreactivity in endothelial, inflammatory and intimal smooth muscle cells.[133] The expression of UT was also significantly increased in plaque tissue. However, in contrast to U-II (which was most abundantly found in endothelial cells covering the plaque) levels of UT expression were the highest in monocytes and macrophages suggesting the role of U-II in paracrine signalling within a plaque.[133] The role of U-II in pathogenesis of atherosclerosis was also confirmed in mice model. Shiraishi et

al. showed that infusion of exogenous U-II promotes foam cell formation and atherosclerosis in apolipoprotein E (ApoE) knockout mice on a high fat diet.[189] UT antagonist (4-aminoquinoline) reversed the effect of U-II administration and decreased atherosclerosis in ApoE knockout mice on a high fat diet. Interestingly, chronic administration of U-II in ApoE knockout mice that were not on a high fat diet did not influence the severity of atherosclerosis. Several in vitro studies have suggested mechanisms by which U-II might accelerate atherogenesis. Segain et al. have analysed the distribution and role of U-II and UT in peripheral blood white cells.[190] UT was mainly expressed in monocytes and natural killer (NK) cells and this expression was further increased by stimulation with pro-inflammatory agents such as lipopolysaccharide, interleukin-10 or TNF.[190] UT was mainly expressed in CD14+ and CD16+ cells characterised by a higher potential of trans-endothelial trafficking. Furthermore, stimulation of UT is a strong chemotactic signal for peripheral blood monocytes and significantly increases expression and activity of acyl-coenzyme A: cholesterol acyltransferase.[190,191] The accumulated data strongly suggest contribution of U-II to atherogenesis. Released by endothelial and inflammatory cells during early plaque formation, U-II is likely to attract certain populations of monocytes and alter their phenotype promoting foam cell formation.

Genetic Studies on urotensin-II system

Linkage analyses were the first to provide insights into the possible involvement of U-II pathway in genetic regulation of BP and renal function.

A study of 3665 American Indians from the Strong Heart Family Study (multipoint variance component linkage analysis) showed presence of a suggestive linkage peak for eGFR on 1p.36 (LOD=2.3), encompassing *UTS2*.[192] In the same cohort Franceschini et al. used multipoint variance component linkage analysis to discover suggestive SBP linkage signal (LOD=2.4) mapping to 17q.25 locus (encompassing *UTS2R*).[193]

In silico analysis of rodent genomes provides further support for the role of U-II system in genetic regulation of BP and renal function. Indeed, rat chromosomes 5, 10 and 11 (harbouring *UTS2*, *URP*, and *UT*, respectively) contain quantitative trait loci (QTLs) for renal function and BP.[194] Specifically, rat *UTS2* locus harbours BP QTLs 147, 292, 139, 103, 131, 210; rat *UTS2D* locus: BP QTL cluster 10, renal function QTL 19 and urinary albumin excretion QTL 10; rat *UTS2R* locus (chr 10): BP QTLs 9, 186 and 250.[194] This indicates that segments of chromosomes containing genes belonging to U-II pathway are involved in genetic regulation of blood pressure and renal function in rodents.

Several studies on genetic association of U-II system genes with human cardiovascular phenotypes have been performed over the last decade. Fine mapping of the 17q25 SBP linkage peak, identified by Franceschini in the population of American Indians, revealed association of exonic, synonymous

SNP rs11654140 in UTS2R with blood pressure.[195] Ong et al. examined associations of SNPs (rs7367534, rs228647 rs228648, rs2890565) in *UTS2* and *UTS2R* (rs7502620 and rs7211435) with hypertension and metabolic traits (glucose metabolism, insulin resistance) as well as serum U-II in 224 hypertensive and 306 normotensive Japanese subjects.[196] No association was found between any of the SNPs and hypertension but there was significant association of both UTS2R SNPs (rs7502620 and rs7211435) as well as haplotype in *UTS2* gene (rs228647, rs228648 and rs2890565) with insulin resistance and glucose metabolism.[196] In contrast to findings reported by Ong et al. missense *UTS2* SNP rs2890565 was associated with hypertension in two Chinese population (Han population - 198 hypertensive subjects and 131 controls and Dongxiang population - 120 hypertensive patients and 102 controls).[197] The rs2890565 was not associated with pre-eclampsia in 144 subjects of Turkish origin in a study by Dikensoy.[198]

These studies did not provide conclusive evidence on the role of genetic variation in U-II system in predisposition to hypertension and impaired renal function. A majority of these studies were conducted in populations of small size (up to a few hundred individuals) and positive findings were reported mainly in Asian populations and not replicated in other ethnicities.

7. HYPOTHESIS

Common genetic variant(s) within U-II pathway control(s) its expression in the human kidney and thus contribute(s) to regulation of BP and/or renal function.

8. AIMS

The overall goal of this project was to look for common genetic variants associated with BP and eGFR. The specific aims included:

1) To calculate heritability of BP and eGFR.

2) To examine associations of common genetic variants within *UTS2*, *UTS2D* and *UTS2R* with BP and eGFR.

3) To examine differences in renal expression of U-II system genes between normotensive and hypertensive subjects.

4) To analyse evolutionary forces acting on U-II system across various levels of phylogeny.

5) To look for common genetic variants associated with eGFR and clinic BP phenotypes using data from large scale genotyping (50K IBC array) in the GRAPHIC Study.

9. MATERIALS AND METHODS

Study groups

Genetic Regulation of Arterial Pressure of Humans in the Community (GRAPHIC) Study

GRAPHIC Study consists of 2037 subjects (all of white British origin) from 520 nuclear families recruited from the general population in Leicestershire (UK).[50,58] Index subjects (mothers aged 40-60 years) were randomly selected through general practitioners registry. Families were recruited if both parents (aged 40 to 60 years) and 2 offspring (≥18 years) agreed to participate. Detailed medical history was taken from all participants using coded questionnaires. All subjects underwent anthropometric measurements (height and weight). Clinic BP were recorded using Omron HEM-705CP digital BP monitors with an appropriate size cuff after a minimum of 10 minutes rest and with an interval of at least 3 minutes between readings (the average was calculated from the 2nd and 3rd readings). Ambulatory BP was measured (at 30-minute intervals between 08:00 and 21:59 [day time] and at 1-hour intervals between 22:00 and 07:59 [night time]) using a Spacelabs 90207 monitor (Spacelabs, Wokingham, UK) for approximately 26 hours. The first 2 hours of each recording were excluded from further analysis. Fasting samples of venous blood and 24-hour urine were collected from each individual. These were subsequently used for biochemical measurements (including 24-hour urinary sodium and potassium excretion) and DNA extraction. The eGFR was calculated using MDRD formula

[GFR = 186.3* serum creatinine-1.154 * age-0.203 * 1.212 (if patient is black) * 0.742 (if female)].[199]

Silesian Cardiovascular Study (SCS)

SCS families consist of 213 Polish pedigrees (703 subjects) recruited through index subjects with high cardiovascular risk.[39,200] High cardiovascular risk was defined as co-existence of CAD and hypertension or Any cardiovascular disease (CAD or hypertension) accompanied by at least 2 cardiovascular risk factors (smoking, waist circumference >102 cm men or >88 cm women, clinically documented history of hyperglycaemia, hyperlipidaemia or lipid-lowering medication, parental history of CAD or hypertension) or clustering of at least 3 cardiovascular risk factors (as above).[200]All subjects underwent detailed phenotyping including clinical history (recorded using anonymous coded questionnaires), physical examination, anthropometric measurements and biochemical laboratory tests according to protocols described previously.[200] Clinic BP was measured in triplicate using an oscillometric method and averaged to calculate final clinic SBP and DBP.[201] Weight and height were used to calculate body mass index (BMI) according to the formula: weight (kg)/height^2 (m^2). A fasting sample of whole blood was obtained by venepuncture for further biochemical analysis and DNA isolation. The eGFR (calculated from serum creatinine using MDRD formula)[199] and urinary albumin: creatinine ratio (measured using turbidimetric method in a spot urine sample) were used as indicators of renal function and structural damage, respectively.

435 unrelated Polish individuals were collected together with the SCS families in the same medical centres, using the same entry criteria and the same phenotyping protocol for the purpose of replication analysis – (SCS extension).[200]

Silesian Hypertension Study (SHS)

SHS consists of 207 families (629 subjects) recruited in Silesia (south of Poland) through the identification of a hypertensive index offspring.[39,64,202,203] Each subject underwent detailed phenotyping including medical history (standardised coded questionnaires), physical examination, anthropometric measurements (weight and height) and BP measurements. Clinic BP was measured (in triplicate and averaged) in a sitting position (after a minimum of 10 minutes rest) using manual sphygmomanometer. Weight and height of the subjects were used to calculate BMI. Hypertension status was defined according to the International Society of Hypertension criteria (SBP and/or DBP >140/90 mmHg on 3 separate occasions and/or remaining on antihypertensive treatment).[204] A sample of blood was obtained by venepuncture for further biochemical analysis and DNA isolation. Serum creatinine level was used to calculate eGFR using MDRD formula.[199]

Young Men Cardiovascular Association (YMCA) study

YMCA study consists of 1157 apparently healthy male subjects recruited from secondary schools in Silesia (south of Poland).[205] Included were subjects

who gave informed consent (>18 years) [or consent was given by subjects parents if an individual was younger <18].[200] Detailed clinical history was recorded using standardised coded questionnaires and subjects underwent anthropometric measurements. BP was measured in triplicate using a mercury sphygmomanometer with a cuff size adjusted to the arm, after 20 minutes rest. Weight and height of the subjects were used to calculate BMI. A fasting blood sample of whole blood was secured for DNA isolation and biochemical analyses. In all subjects aged≥18 years MDRD formula was used to calculate eGFR.[199]

An additional 597 young, healthy males were later recruited in the south of Poland later and included in the YMCA extension. The recruitment strategy, phenotyping protocol and further biochemical analyses were conducted using the protocol as those used in the YMCA study.[206]

Polish End Stage Renal Disease (PESRD) cohort

The study consists of 109 nuclear families (both parents and one offspring) identified through the index patient (offspring) diagnosed with CKD (stages 3-5 according to Kidney Disease: Outcomes Quality Initiative [KDOQI]) due to non-diabetic nephropathy.[39,207] In 30 subjects CKD was caused by chronic primary glomerulonephritis, tubulointerstitial nephritis was the cause of CKD in the remaining subjects.[207] Individuals with renal disease due to heritable conditions for example: polycystic kidney disease, Alport`s syndrome and of unknown aetiologies were excluded from this study. A detailed medical history

was collected from all participants.[207] Blood samples were obtained from each individual for DNA extraction.

Silesian Renal Tissue Bank (SRTB)

The SRTB was designed to explore renal gene expression profiles in hypertensive and normotensive subjects.[39,64] A total of 62 Polish subjects, who underwent elective, unilateral nephrectomy for non-invasive renal cancer, were included in the analysis. All subjects were of white European background and were recruited in Silesia (Poland). BP was measured using mercury sphygmomanometer with a cuff size adjusted to the arm, after at least 10 minutes of rest. An average of 3 readings was used in calculation of final clinic SBP and DBP values. Each subject was phenotyped and classified as hypertensive or normotensive using the International Society of Hypertension criteria.[204] Renal tissue samples from the healthy (unaffected by cancer) pole of the kidney were excised and transferred to the laboratory in containers with RNAlater and preserved at –70°C before mRNA expression analysis.[64]

Western Poland Kidney Project (WPKW)

WPKP is an ongoing collection of biological material (renal tissue, serum and urine) from patients undergoing unilateral nephrectomy for non-invasive renal cancer. Patients scheduled for elective nephrectomy, who gave informed consent, underwent detailed phenotyping including past medical history, medication and anthropometric measures (weight, height, waist and hip

circumferences). Blood pressure was measured using automatic, digital BP monitor after 10 min of rest, using cuff size adjusted to the arm. Hypertension was defined based on SBP and/or DBP >140/90 mmHg on 3 separate occasions and/or remaining on antihypertensive treatment. Similar to the protocol in SRTB, samples of renal tissue are excised from unaffected (by tumour) kidney pole and preserved in RNAlater before mRNA expression analysis.

CoLaus Study

To further strengthen results of association study, replication of the top SNP was requested in an external cohort - the CoLaus Study.[208] CoLaus cohort consists of 6188 subjects from general population of Lausanne, Switzerland. Subjects of the study were randomly selected from the list of all inhabitants of Lausanne (n=56694) in year 2003, who were between 35 and 75 year of age, as per population register. To be eligible for the study subjects had to give informed consent for participation and acquisition of biological samples, be of white Caucasian origin and provide information about parents and grandparents (ancestors had to originate from restricted list of European countries). Subsequently, all subjects had to fill in questionnaires regarding demographic, socio-economical, marital status as well as provide information about lifestyle factors i.e. alcohol consumption and level of physical activity. This was followed by an interview aiming at obtaining of familial and medical history. All subjects of the CoLaus study underwent detailed phenotyping including anthropometric

measurements i.e. body weight, height. BP and heart rate were measured in triplicate, in a sitting position after a minimum of 10 minutes of rest using Omron HEM-907 automated oscillometric sphygmomanometer, with cuff adjusted to arm size. Fasting venous blood samples were used to obtain serum measurements and isolate DNA. Genotyping was performed using the Affymetrix 500k SNP chip following manufacturers instruction.

Bioethical approval

All studies were approved by appropriate local bioethical committees. All subjects gave informed, written consent for participation.

Phenotypes

Clinical phenotypes

In all cohorts clinic SBP, clinic DBP and eGFR (calculated using MDRD formula)[199] were used as main phenotypes. Additionally, in the GRAPHIC Study ambulatory blood pressure measurements (mean 24 hour SBP and mean 24 hour DBP) and additional renal phenotypes (24-hour urinary sodium and potassium excretions, obtained from 24-hour urine collection) were available. Urinary albumin: creatinine ratio – a marker of glomerular injury was available in the SCS and SCS extension.

Traditionally, clinic blood pressure is measured on the brachial artery using mercury sphygmomanometer. For the reliability and reproducibility of findings the measurement should be performed in a patient after an adequate rest (usually 10 minutes) and the cuff should be placed at the level of the heart.[209,210] An elastic cuff (covering 2/3 of patient arm) is placed over patient's arm and inflated until compression of the brachial artery stops the blood flow to the forearm.[209,210] Using a stethoscope, placed over the brachial artery distally to the cuff, examiner auscultates the artery to identify Korotkoff sounds while gradually lowering pressure in the cuff. First Korotkoff sound appears due to turbulent blood flow, when the blood start flowing in the artery (systolic pressure in the artery is higher than the pressure in the cuff) and denotes SBP. Lowering of pressure in the cuff is continued until no sound can be heard in the stethoscope, which denotes DBP.[209,210]

Similarly to auscultatory-, oscilloscopic method uses inflatable elastic cuff placed on the arm over the brachial artery. However, electronic sensor measures the oscillations in the cuff pressure (which are related to blood flow in the brachial artery) to determine SBP and DBP.[211]

Although, widely used for the diagnostic and management purposes, clinic blood pressure has several disadvantages. As a single time-point measurements it is likely to suffer from interferences and therefore be biased in the approximation of the real underlying phenotype.[212] Blood pressure shows significant short pattern variability and circadian pattern which adds to the uncertainty of the obtained result.[213] Finally it is affected by the sole fact of measurement, especially in the clinical setting – the so called "white coat hypertension".[214]

Ambulatory blood pressure measurement is performed using automatic recorders with integrated inflatable cuff, in regular time intervals, over given time period (usually 24 hours).[215] Due to repeated measurements performed in the environment usual for the patient, ambulatory blood pressure is less likely to be influenced by random interferences.[215] Also, by providing information in long time periods ambulatory blood pressure is informative not only about mean BP but also about diurnal patterns and overall variation of the phenotype. Ambulatory blood pressure has been shown to be superior over clinic blood pressure in predicting complications and organ damage related to blood pressure.[216-219]

Unlike in the case of blood pressure, no direct way exists to assess excretory renal function. Excretory renal function is usually expressed as glomerular filtration rate and defined as the volume of plasma that is filtered by the kidney (and cleared of an hypothetical ideal substance) within an unit of time.[220]

An ideal substance that could be used for assessment of renal function should be characterised by stable plasma concentration, free glomerular filtration, not be reabsorbed or excreted by the kidney or eliminated by any other organ of the body.[221] None of the endogenous substances behave like the ideal substance that could be used to assess renal function.[221]

Historically renal function was assessed using serum creatinine levels. Creatinine is an endogenous metabolite of skeletal muscle and only small amounts of serum creatinine come from ingested food.[220] It is filtrated in the glomerulus and not reabsorbed but undergoes active excretion in the renal tubules.[220] Of all of the body metabolites it is the closest to an ideal substance which would be used for the estimation of glomerular filtration.[220]

Given the simplicity of creatinine measurements its serum concentration is being used to estimate renal function. This can be done by measurement of the creatinine clearance or prediction of the glomerular filtration rate using mathematical equations, taking into account other variables such as age sex and ethnicity.[222]

Creatinine clearance can be calculated using measured concentrations of creatinine in the serum and urine. However, owing to tubular excretion

creatinine clearance is a poor estimate of the glomerular filtration. Also, there is a marked diurnal variation in creatinine clearance (up to 25%) which, makes its interpretation difficult.[220,223]

Prediction of glomerular filtration rate using mathematical formulas directly from serum creatinine is nowadays the most widely used method of estimating renal excretory function. Several mathematical formulas exist. The most recognised are Cockcroft-Gault equation (uses serum creatinine, age, sex and weight to predict glomerular filtration) and MDRD formula (based on serum creatinine, patients age, sex and ethnicity).[222] The MDRD 4 formula [GFR = 186.3* serum creatinine$^{-1.154}$ * age$^{-0.203}$ * 1.212 (if patient is black) * 0.742 (if female)][199] has been validated in numerous studies and is extensively used in clinical and epidemiological research.[224]

Urinary albumin creatinine ratio is calculated from the concentrations of albumin and creatinine in the first voided, morning, midstream urine specimen.[225] Under “normal” physiological conditions only small amounts of albumin is excreted with urine.[226] Excessive excretion of albumin is related to increase vascular permeability related to haemodynamic, structural and functional alternation in the glomeruli.[226] Increased leakage of albumin to the urine is therefore a marker of widespread vascular damage, endothelial dysfunction and chronic inflammation.[225] Increased albumin: creatinine ratio suggestive of microalbuminuria has been shown to correlate with overall mortality, risk of coronary artery disease, left ventricular hypertrophy and cerebrovascular disease.[227]

24-urinary sodium and potassium excretions are calculated from the 24 hour urine collection. They are the product of glomerular filtration and tightly regulated reabsorption/secretion of the minerals in further parts of the nephron and collecting ducts of the kidney aiming at maintaining the minerals homeostasis in the body fluids.[228] Urinary electrolyte excretions are linked to ingestions of these electrolytes with food, and as so have been used in the large epidemiological studies.[229] Apart from being only marker of dietary intake minerals excretion correlate with cardiovascular risk.[229] The association between mortality and urinary sodium excretion is J-shaped with excess mortality due to congestive heart failure on the low excretion end of the spectrum and excess cardiovascular mortality on the high excretion end of the spectrum.[230] Higher urinary potassium excretion is in turn inversely related to the risk of stroke.[230]

Phenotypes – adjustment analysis

Prior to genetic analysis, distributions of all quantitative traits (eGFR, clinic SBP, clinic DBP, 24-hour urinary sodium excretion, 24-hour urinary potassium excretion, mean 24-hour SBP, mean 24-hour DBP, urinary albumin: creatinine ratio) were assessed for normality by visual inspection of histograms and by Shapiro-Wilk statistical test. Phenotypes showing the biggest deviation from theoretical distribution (24-hour urinary sodium excretion, 24-hour urinary potassium excretion and urinary albumin: creatinine ratio) were log10-transformed prior to analysis.

In the cohorts of unrelated subjects stepwise forward multiple regression was used to identify significant predictors of each examined phenotype.

In family cohorts (the GRAPHIC Study, SHS, SCS families) generalised estimating equations (GEE) was used to identify significant predictors of the phenotype.[231] In brief GEE is a statistical technique developed to deal with correlated data. GEE performs calculation of the correlation of observations within a cluster (in our case family) and takes them into account by using relevant weights. Subsequently a matrix representing identified correlations is fitted into the regression equation and the coefficients of the equation are recalculated.[231]

Prior to association analysis all phenotypes were adjusted for age and sex. In family studies also age^2 was tested for significant contribution to multiple regression (due to the two generational structure of family cohorts).

The effect of antihypertensive treatment on BP was corrected for using a semi-parametric algorithm as suggested by Tobin.[232] In subjects on anti-hypertensive treatment the value of so called “underlying BP” reading (BP which would be recorded, if the subject was on no anti-hypertensive medications) is masked by the effect of therapy. This is usually addressed by ignoring the effect of treatment, by exclusion of the treated group from the analysis or adjusting for the treatment effect by using it as a binary covariate in the regression model. However, all these approaches have been shown to be invalid. Tobin et al. used 3 sets of different simulation data and data from real Speedwel cohort study, where BP reading were available on subjects before

and after starting anti-hypertensive treatment. He showed that adding arbitrary constant value (10mmHg to SBP and 5-10mmHg to DBP) to BP of individuals on treatment (as previously proposed by Cui et al.) or constant obtained from non-parametric analysis is the most robust method to correct for the effect of anti- hypertensive treatment.[232,233] According to this algorithm an adjustment value is calculated by subtracting the mean of the regression based residuals of treated individuals from the mean residuals of non-treated subjects (matched for BP readings). In individuals on treatment, treatment adjusted BP value is then a sum of the measured BP and adjustment value.[232]

Urotensin-II system genes

Urotensin-II gene

UTS2 is located on the reverse strand of the short arm of chromosome 1 (1p.36). Its coding sequence (10,430 base pairs [bp]) is organised into 5 exons. The primary transcript of this gene is processed to form alternative mRNA products, translated into 4 prepro-urotensin-II peptide chains ranging from 124 to 139 amino acids. These peptides undergo cleavage processing to finally form mature U-II.[90]

Urotensin-II related peptide gene

UTS2D is located on the reverse strand of the long arm of chromosome 3 (3q.28). The genes (63,382 bp) coding sequence is organised into 9 exons. The primary transcript is processed into a 2394 bp mRNA and is translated into its precursor peptide of 119 amino acids. Unlike the prepro-urotensin-II that lacks typical cleavage sites, URP can be processed from the precursor by cleaving Lys-Arg site, adjacent to the N-terminal end of the mature peptide.[75]

Urotensin-II receptor gene

UTS2R is located on the plus strand of the long arm of chromosome 17 (17q.25.3). *UTS2R* (1310 bp) contains no intronic fragments. The mRNA product of this gene consists of 1170 bp and is translated into a 389 amino acid chain.

Genotyping

For the purpose of association analysis of U-II pathway genes with BP and renal phenotypes in the GRAPHIC Study genotypes were obtained from 50K IBC genotyping array and (if unavailable) from wet-lab genotyping using TaqMan assay (Applied Biosystems).

50k Illumina Human CVD BeadChip genotyping

The 50K IBC array was designed to provide information on approximately 45000 genetic markers in ≥2000 loci previously implicated in cardiovascular physiology/pathophysiology.[234] The loci were assigned differing levels of importance (based on supporting evidence for their involvement in cardiovascular regulation/disease) and the density of tagging was adjusted accordingly (increased tagging in higher levels of importance).[234] Loci of Tier 1 genes had a high likelihood of functional significance and therefore tagging SNPs for these loci were chosen to capture variation with minor allele frequency (MAF)>0.02 and an r^2≥0.8 (according to HapMap populations and Seattle SNPs database). Tier 2 loci were tagged for MAF>0.05 with an r^2≥ 0.5 (according to HapMap populations and Seattle SNPs database). Tier 3 loci comprised mainly of larger genes (>100 kb) which were of lower interest a priori. Only non-synonymous SNPs and known functional variants of MAF>0.01 were captured in these loci.[234] Genotyping on the HumanCVD BeadChip array was performed by an external company as specified in manufacturer's protocol.

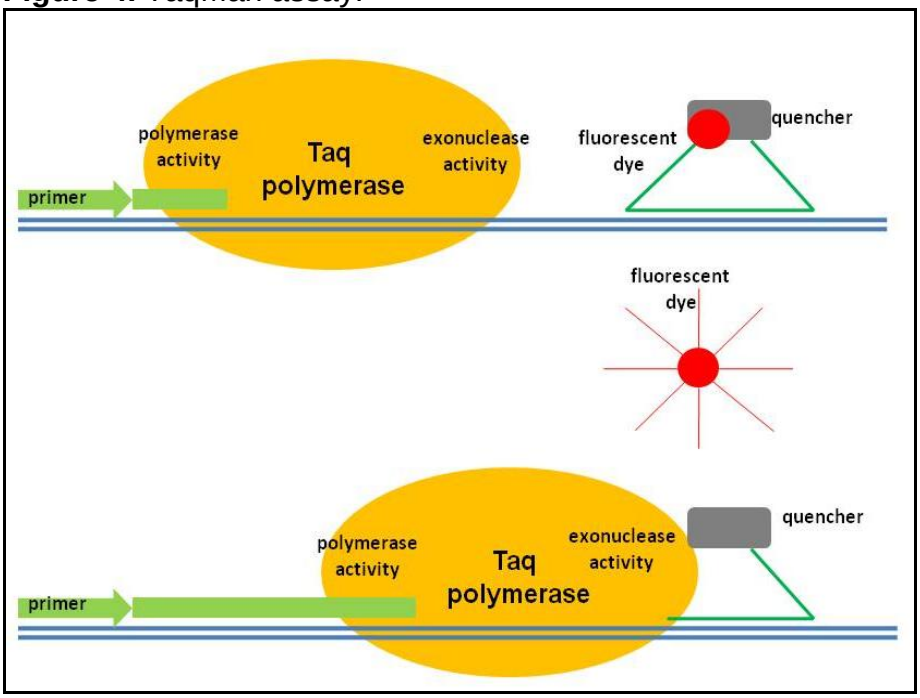
200ng of DNA from each subject was hybridized to the assay. Allelic discrimination was performed using the Illumina BeadStudio (v3) Genotyping Module (based on GenCall Software algorithms for clustering, calling, and scoring genotypes). Two experienced investigators independently assessed the scatter plots to ensure appropriate quality of allele calling.[50]

TaqMan genotyping

The TaqMan assay (Applied Biosystems) is a polymerase chain reaction (PCR) based method of allelic discrimination using 2 different probes for each of the alleles. The probes are oligonucleotides containing a covalently joined fluorophore at the 5'-end and quencher at the 3'-end. The close proximity of the fluorophore to the quencher inhibits fluorescence of the probe after laser excitation. (Figure 4) The allele specific probe hybridizes to the native DNA during the annealing step of the PCR reaction. In the elongation step the polymerase elongates the primer and uses its 5' to 3' exonuclease activity to degrade the probe which leads to the separation of fluorophore from the quencher. Laser excitation of the PCR products produces fluorescence of the allele specific fluorophore (which is now separate from the quencher due to degradation of the probe). Discrimination of genotypes in a set of samples is thus based on the intensity of emission of specific light waves. Each PCR reaction mix consisted of 10-15 ng of sample DNA, 3.125 µL of Applied Biosystems Genotyping Master Mix, 2.291 µL of ultrapure water (Milli-Q water purification system, Millipore) and 0.031 µL SNP specific TaqMan probe

(Applied Biosystems). Subsequently the samples were run in GeneAmp 977 PCR System (Applied Biosystems) with 40 repeats of a cycle consisting of 15s denaturation (92^{o}C) and 1 min annealing/elongation (60^{o}C). Allelic discrimination was conducted using ABIPrism 7900 Sequence Detection System (Applied Biosystems).

Figure 4. TaqMan assay.



During elongation the polymerase complex (yellow) propagates and digests the TaqMan probe. This leads to the separation of the fluorescent probe from the quencher and allows for detection of fluorescence in the supernatant upon laser excitation. Different fluorescent dyes are used for allele specific probes which allows for the discrimination between alleles.

Urotensin-II system - SNPs investigated in the discovery cohort – the GRAPHIC Study

In the GRAPHIC Study I took advantage of the availability of *UTS2* and *UTS2D* genotypes from a previous large scale, gene-centric genotyping project completed using the 50k Illumina HumanCVD BeadChip array.[50,234] A total of 16 SNPs were genotyped in the GRAPHIC Study in *UTS2*. 11 SNPs were genotyped using 50k Illumina HumanCVD BeadChip and another 5 SNPs were chosen based on HapMap derived tagging algorithm. The following criteria were used: MAF>5% and r^2>0.8. The additional 5 SNPs were genotyped using TaqMan® SNP Genotyping Assay (Applied Biosystems). (Table 1)

Only 1 SNP from *UTS2D* was genotyped using the 50k Illumina HumanCVD BeadChip array. Therefore, 6 additional SNPs were selected from HapMap (CEU) using tagging algorithm under the following criteria (MAF>5% and r^2>0.8). The additional 6 SNPs were genotyped using TaqMan® SNP Genotyping Assay (Applied Biosystems). (Table 1)

Of the 5 *UTS2R* SNPs genotyped, 2 (rs11077991, rs6502104) were chosen from the HapMap (CEU) using a tagging algorithm under the following criteria: MAF>5% and r^2>0.8. The additional SNPs in this gene (rs7502620, rs7211435, rs11650469) were chosen based on their putative biological function (as assessed by Brainarray SNP Function Annotation Portal (http://brainarray.mbni.med.umich.edu/Brainarray/Database/SearchSNP/snpfunc.asp). All SNPs in *UTS2R* were genotyped using TaqMan® SNP Genotyping Assay (Applied Biosystems). (Table 1)

A total of 28 SNPs in the U-II pathway were selected for genotyping in the GRAPHIC Study.

Table 1. SNPs in the urotensin-II system genes - GRAPHIC Study.

SNP	Gene	Chr	Position (bp)	MAF	MA	Location	Genotyping format
rs707472	UTS2	1	7906008	0.167	T	3'	50K IBC
rs228652	UTS2	1	7908888	0.264	T	intron	50K IBC
rs2890565	UTS2	1	7909737	0.295	A	exon	50K IBC
rs170629	UTS2	1	7910391	0.306	T	intron	TaqMan
rs228651	UTS2	1	7911099	0.278	A	intron	TaqMan
rs34305100	UTS2	1	7913029	0.181	C	exon	TaqMan
rs228648	UTS2	1	7913430	0.403	C	exon	TaqMan
rs13306061	UTS2	1	7913445	0.167	A	exon	TaqMan
rs4908486	UTS2	1	7914835	0.3	T	5'	50K IBC
rs707476	UTS2	1	7918106	0.264	T	5'	50K IBC
rs228638	UTS2	1	7918598	0.486	A	5'	50K IBC
rs2859389	UTS2	1	7920423	0.278	A	5’	50K IBC
rs531485	UTS2	1	7921952	0.181	C	5’	50K IBC
rs12566535	UTS2	1	7924094	0.222	A	5'	50K IBC
rs500508	UTS2	1	7927456	0.292	A	5'	50K IBC
rs579992	UTS2	1	7927981	0.083	G	5'	50K IBC
rs6772358	UTS2D	3	190986740	0.362	A	intron	TaqMan
rs17465071	UTS2D	3	190987067	0.264	G	intron	TaqMan
rs9809263	UTS2D	3	190989466	0.333	T	intron	TaqMan
rs2886062	UTS2D	3	190990163	0.153	G	intron	TaqMan
rs4399929	UTS2D	3	190994143	0.306	G	intron	TaqMan
rs6444532	UTS2D	3	190999760	0.324	T	intron	TaqMan
rs6788319	UTS2D	3	19099997	0.333	C	exon	50K IBC
rs7502620	UTS2R	17	80320561	0.347	A	5'	TaqMan
rs7211435	UTS2R	17	80323686	0.342	T	5'	TaqMan
rs11650469	UTS2R	17	80330676	0.264	G	5'	TaqMan
rs11077991	UTS2R	17	80330774	0.194	G	5'	TaqMan
rs6502104	UTS2R	17	80331778	0.342	C	5'	TaqMan

SNP – single nucleotide polymorphism, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, Chr – chromosome, Position – genetic position on chromosome according to NCBI (CEU), MAF – minor allele frequency, MA – minor allele, 3’ – 3’ untranslated region, 5’ – 5’ untranslated region, TaqMan – genotyping using TaqMan probes (Applied Biosystems), 50K IBC – genotyped using 50k Illumina HumanCVD BeadChip.

Urotensin-II system - SNPs analysed in the replication cohorts

One SNP located in the 5' region of *UTS2* (rs531485) was genotyped in the remaining cohorts (SHS, SCS families, SCS extension, YMCA study and YMCA extension) in a replication experiment after the association analysis in discovery cohort (the GRAPHIC Study). The genotyping was performed using TaqMan® SNP Genotyping Assay (Applied Biosystems) on the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Urotensin-II system - SNPs analysed in the SCS families, SCS extension, PESRD and SRTB

19 SNPs were selected for genotyping in the U-II signalling pathway to examine its association with markers of structural renal damage (albumin: creatinine ratio) and ESRD, alongside renal expression of *UTS2*, *UTS2D* and *UTS2R*. These SNPs were chosen based on a HapMap derived tagging algorithm and criteria described above (MAF>5% and r^2>0.8). All SNPs were genotyped using TaqMan® SNP Genotyping Assay (Applied Biosystems) on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

A total of 8 *UTS2* SNPs (rs228652, rs170629, rs228651, rs34305100, rs228648, rs13306061, rs2859389, rs531485), 6 *UTS2D* SNPs (rs6772358, rs17465071, rs9809263, rs2886062, rs4399929, rs6444532) and 5 *UTS2R* SNPs (rs7502620, rs7211435, rs11650469, rs11077991, rs6502104) were genotyped in SCS families, SCS extension, ESRD, and SRTB.

Quality control filters

Quality control filters were applied after 50K IBC array and TaqMan-based genotyping. Distributions of the scatter plots were manually inspected and if the clusters were poorly determined the genotypes were removed from further analysis. In family cohorts presence of Mendelian inconsistencies was assessed and if present genotypes of family members were set as missing. Due to the uncertainty regarding performance of the genotyping assay SNPs with >10% missingness rate, these violating Hardy Weinberg Equilibrium (HWE) [$\chi 2$ P-value<0.0001] were removed from further analysis.[235] SNPs with MAF< 5% were excluded from the analysis due to possibility of calling algorithms error and issues with statistical power.[235]

Population stratification in the GRAPHIC Study

To assess for the presence of population stratification in the GRAPHIC Study, for the purpose of large scale genetic association analysis I used a subset of 11534 SNPs. After the standard genotyping quality criteria have been applied SNPs have been assessed for the presence of LD using multiple regression as implemented in PLINK. SNPs with multiple correlation coefficient with other markers of >0.2 were excluded from further analysis. Subsequently the remaining subset of pruned SNPs was used to create matrix of identity by state distances of all individuals in the study. This matrix was further transformed using multidimensional scaling technique to principal components. First and second components from this analysis were used as coordinates to

map individuals on a two dimensional graph and visually assess for the presence of outliers. The same subset of SNPs was also obtained from HapMap resource for each of the HapMap model populations and individuals from the GRAPHIC Study were visualised against the HapMap populations to show that they form separate group most closely genetically related to the CEU HapMap population.

Additionally, presence of population stratification was assessed by calculation of genomic control and creation of quantile-quantile plots. In brief genomic control compares distribution of the statistics obtained from association testing to the theoretical distribution. If no stratification exists the statistic should follow χ^2 distribution with one degree of freedom.[236] In the presence of stratification calculated genomic control informs about the inflation of test statistic.[236]

Genotype imputation in the CoLaus study

For the purpose of replication in the CoLaus study the rs531485 genotypes were imputed using IMPUTE v0.3 software. http://mathgen.stats.ox.ac.uk/impute/impute_v0.5.html using HapMap genotypes as reference. In brief, imputation is based on an available reference resource with full information on correlations between genetic markers. These correlations are then extrapolated on data from genotyping array used in a given experiment.[237] Subsequently the genotype of the missing marker (not available on the array used in the experiment) is inferred from the observed

nearby markers.[237] This relies on the assumptions that the correlations observed between markers in the reference resource are true for population used in the experiment.

Data analysis

Heritability analysis

Heritability analysis of clinic SBP, DBP and eGFR in the GRAPHIC Study, was performed using variance component analysis algorithm implemented in SOLAR (Sequential Oligogenic Linkage Analysis Routines) v2.0 software.[238] After the effect of covariates had been accounted for, the residual variance was partitioned into components due to genetic (both polygenic additive and dominant effects) and non-genetic variance. The genetic component was further divided into polygenic additive effect - narrow sense heritability (explained by the regression slope) and the non-additive effect of alleles.[239] Estimates were then modelled to best fit the observed data using a maximum likelihood approach and genetic component tested against the null hypothesis (that heritable component equals to 0).

Calculation of statistical power – PBAT

Calculation of statistical power for family based association testing in the GRAPHIC Study was performed using PBAT Tools for Family-Based Association Studies software.[240] The software uses a flexible approach which allows the user to model underlying genetic parameters. In the GRAPHIC Study, calculations of statistical power were computed under an additive model of inheritance for alleles with MAF ranging from 5-90%. Heritability estimates of 5 phenotypes used in the model were previously calculated in MERLIN and a

P=0.0007 threshold (calculated as described in the section regarding multiple testing correction) was used to assign statistical significance. The calculations were performed on the assumption of “no ascertainment condition” (subjects of the study were to represent random sample of a population) for the variant with genetic attributable fraction of the phenotype variation at 0.05. The PBAT showed adequate statistical power (>90%) to detect true association of SNPs with MAF≥0.05 with eGFR, 24-hour urinary sodium and potassium excretion, clinic SBP and clinic DBP at the level of statistical significance calculated after correction for multiple testing.

Genetic association analysis in family cohorts

For the purpose of candidate gene project the analysis of genetic association in families was performed using Family Based Association Test (FBAT).[241] The principle of this statistical approach is similar to the original transmission disequilibrium test.[242] The FBAT uses a conditional score test to contrast between the transmission of alleles among the offspring, who have high and low values of the quantitative trait.[243] Under the null hypothesis (no linkage and no association between genetic marker and a trait) the probability of transmission of a given allele from parents to offspring is calculated according to Mendel’s law of independent assortment and is conditioned on the parental genotypes only.[244] The alternative hypothesis (assuming presence of linkage and association between the trait and the marker allele) assumes increased or decreased transmission of risk alleles which arises due to

ascertainment of the families through index affected child. The main advantage of the FBAT over classic transmission disequilibrium test is its ability to use data from different pedigrees (not only parent-child trios) and to obtain the genotype distribution under the null hypothesis for incomplete parental data conditioned on the available parental and offspring genotypes.[245] By using this conditional distribution instead of population data, the test statistic is robust against population stratification and admixture.[244]

For all analysed traits an additive model of inheritance (assuming linear relation between number of minor alleles and a phenotype) was used. All traits were adjusted for relevant covariates using GEE. A linear regression model including relevant covariates was constructed (with GEE to correct for familial correlation) in STATA and the residuals were used as a final adjusted phenotype in FBAT software.

Genetic association analysis in the large experiment using 50K IBC array data in the GRAPHIC Study.

GEE was used in the large scale genetic association analysis using 50K IBC array data with clinic SBP, clinic DBP and eGFR in the GRAPHIC study. As previously described on page 75, GEE is a statistical technique used to take into account and correct for correlation within the data.[231] Unlike FBAT GEE is not immune to population stratification, which has to be assessed and if present corrected for. For each of the tested genetic variants an additive genetic model

with phenotype as an outcome and age, age^2, sex and number of minor copies of a given genetic variant was utilised.

Genetic association analysis in unrelated subjects

The analysis of association in cohorts of biologically unrelated subjects was performed using PLINK.[246] For quantitative traits, PLINK uses a linear regression with the trait of interest (the model is conditional on the number of copies of minor allele [0, 1 or 2]), under the assumption of an additive genetic effect. All analyses were adjusted for appropriate covariates. The β regression coefficient represents the size and direction of the effect of each extra minor allele on the phenotype and the P-value statistical significance of the finding.

Multiple testing correction in the urotensin-II pathway genes association experiment

To calculate a correction for multiple testing in the discovery cohort a spectral decomposition of linkage disequilibrium statistics, proposed by Nyholt was used.[247] Genetic markers with close physical distances show high levels of correlation arising from their origin on a common ancestral haplotype.[248] The same is true for closely correlated phenotypes. Thus, use of Bonferroni correction, assuming independence of all tested SNPs and phenotypes, would be overly conservative.[249] The spectral decomposition identifies the number of truly independent statistical tests in the dataset after taking into account correlation between the tested variables.[247] Nyholt's correction calculates

eigenvalues from a pair-wise correlation matrix of examined variables. The variance of the eigenvalues represents the overall correlation in the dataset (ranging from 0 [when all variables are independent] to n [n - total number of variables]). A ratio of observed to maximal eigenvalues variance represents a proportional reduction in the number of variables. This allows for the identification of the true number of independent variables in the data. The statistical significance threshold is then generated based on the number of independent statistical tests.

Spectral decomposition of SNPs genotyped in the discovery cohort revealed; 13.89, 5.84 and 3.39 fully independent genetic markers in *UTS2*, *UTS2D* and *UTS2R*, respectively. (Table 2)

Table 2. Spectral decomposition of SNPs in the urotensin-II pathway genes in the GRAPHIC Study.

Gene	Number of genotyped SNPs	Effective number of markers
UTS2	16	13.89
UTS2D	7	5.84
UTS2R	5	3.39
Total	28	23.12

Effective number of markers – the total number of fully independent SNPs per locus.

Of the 28 SNPs genotyped in U-II system genes the number of fully independent genetic markers was calculated at 23.12.

Spectral decomposition of the correlation matrix for 7 phenotypes examined in GRAPHIC Study (eGFR, clinic SBP, clinic DBP, 24-hour urinary sodium excretion, 24-hour urinary potassium excretion, mean 24-hour SBP and mean 24-hour DBP) revealed 6.08 independent traits. (Table 3)

Table 3. Correlation matrix of phenotypes analysed in the GRAPHIC Study.

Trait	eGFR	clinic SBP	clinic DBP	24-hour urinary sodium excretion	24-hour urinary potassium excretion	mean 24-hour SBP	mean 24-hour DBP
eGFR	-						
clinic SBP	-0.1566*	-					
clinic DBP	-0.2372*	0.7653*	-				
24-hour urinary sodium excretion	-0.0073*	0.6775	0.5295*	-			
24-hour urinary potassium excretion	-0.2094*	0.5759	0.6493	0.6186*	-		
mean 24-hour SBP	0.2190	-0.0190*	-0.0772*	0.1049*	0.0787*	-	
mean 24-hour DBP	0.0580*	0.0064*	-0.0001*	-0.0309*	0.0210	0.7398*	-

Data are pair-wise Pearson's correlation coefficients, * denotes significant correlations at $p<0.05$.

The total number of independent statistical tests was calculated at 140.57 in the discovery cohort. Based on the total number of performed statistical tests threshold of statistical significance in the primary association analysis was set up at p=0.0007 to keep type 1 error rate below 10%.

Multiple testing correction using false discovery rate in the large scale genetic association experiment using 50K IBC genotype data in the GRAPHIC Study

To correct for multiple testing in the large scale genotyping experiment for association of 50K Illumina HumanCVD BeadChip markers with eGFR, clinic SBP and DBP I calculated false discovery rate (FDR), using QVALUE software (http://genomine.org/qvalue/). In general the traditional Bonferroni correction for multiple testing is considered overly conservative as it assumes total independence of genetic markers. Rather than focusing on a probability of type I statistical error for a given test FDR sets quantity q, which specifies the proportion of expected false positive findings among all significant findings.[250]

Regional association plots

In order to facilitate interpretation of the results the graphical presentations of association were plotted using SNAP – web based graphic tool.[251] SNAP uses the user submitted SNP identifiers with corresponding P-values from genetic association analyses to generate the plot. Apart from plotting physical positions of the SNPs (on the X axis) and P-values (on the Y

axis) SNAP provides information about statistical correlation of the analysed SNPs (r^2 based on HapMap estimates) depicted as the intensity of the SNP marker colours. The presence of recombination hot spots is marked on the graphs by means of vertical blue lines to help clarify, whether given association signal occurs within one haploblock or extends beyond.

Meta–analysis

rs531485 taken forward for replication were meta-analysed in SCS families, SCS extension, SHS, YMCA study and YMCA extension and CoLaus study. In family cohorts variance component model, implemented in MERLIN, was used to estimate effect sizes and confidence intervals (CI) prior to the meta-analysis.[252] The implemented model takes advantage of the known family structure to correct for intra-familial correlation between subjects and calculates parametric estimates of effect sizes. In cohorts of unrelated subjects the estimates were obtained from linear regression analysis. Meta-analysis was conducted using a fixed effect, inverse variance model implemented in Metan script.[253] Prior to the analysis heterogeneity between studies was assessed by χ^2 test.[254] If no significant heterogeneity was observed, fixed effect model was used (assuming that all of the contributing studies were drawn from one population – white Europeans).[254] Effect sizes for each study were weighted and the assigned weights were inversely proportional to the variance of a given estimate. The final β-coefficient, standard error (SE) and P-value reflect

estimated magnitude of effect size and its statistical significance in subjects from all replication cohorts.

Gene expression analysis

mRNA expression levels of *UTS2*, *UTS2D* and *UTS2R* were quantified in human kidneys from SRTB and WPKP.

RNA extraction was performed using the RNeasy mini kit (Qiagen). Samples of kidney tissue (30 mg) stored in RNAlater (Ambion Inc.) were suspended in lysis buffer, disrupted and homogenised using a rotor-stator homogeniser. The lysate was subsequently centrifuged and supernatant used in subsequent steps. After mixing with 70% ethanol, the remaining supernatant was further cleaned using an RNeasy mini absorption column. The nucleic acid absorbed on the column was purified of DNA using DNase buffer. Subsequently RNA was eluted from the column and suspended in an RNAase free buffer.

The assessment of concentration and quality of RNA was performed using NanoDrop (Labtech) spectrophotometer using 260/280 nm ratio. This approach is based on the Beer-Lambert law which predicts a linear change in absorbance of light with increasing concentration of the substance. The 260nm wave is absorbed by RNA whereas 280nm absorbance is a measure of sample contamination. Following absorbance measurement at 260nm and 280nm a 260/280 ratio was calculated (2.1 is the ratio for pure RNA) and quality of the solution assessed.

Extracted RNA was used to create a cDNA library. cDNA was synthesised using a total of 200 ng RNA and a reverse transcription kit (Applied Biosystems) containing a murine leukaemia reverse transcriptase and a combination of oligo dT16 and random hexamer primers (Applied Biosystems).

The mRNA expression was subsequently quantified using designed TaqMan gene expression assays (Applied Biosystems) and normalized to the expression of a housekeeping control gene (β-microglobulin) on the 7900HT Sequence Detection System (Applied Biosystems). Real time PCR was carried out in a 20µL volume using a reaction buffer containing 10µL TaqMan Genotyping PCR Master Mix, 1µL TaqMan probe/primer mix (both Applied Biosystems), 8µL RNase-free water (Milli-Q system, Millipore Ltd) and 1µL cDNA template from the reverse transcription reaction. Conditions of the reactions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Reactions were performed in duplicates.

Data were expressed as cycle threshold (Ct) and used to determine dCT values. The SRTB and WPKP individuals were split into groups based on phenotypes and the fold difference in mean expression was calculated according to the following formula: fold difference $=2^{-\text{difference in dCt.}}$

Pearson's linear correlation was used to assess relationship between expressions of pairs of U-II system genes.

Statistical analysis of gene expression in hypertensive and normotensive subjects was performed using a linear regression model. dCT values were

conditioned on the hypertensive and normotensive status and adjusted for relevant covariates (age, sex and BMI).

A linear regression model (adjusted for relevant covariates) was used to assess whether any of the tagging SNPs within UTS2, UTS2D and UTS2R affected the expression of relevant genes in SRTB.

Evolutionary analysis

The analysis of evolutionary pressure acting on protein coding sequences can be estimated using ratios of non-synonymous to synonymous genetic substitutions.[255] Synonymous substitutions do not lead to changes in the amino acid composition of a protein and are generally evolutionary neutral. Non-synonymous substitutions lead to changes in the amino acid composition of a protein and therefore provide an evolutionary disadvantage or advantage for the carrier.[256] The analysis of the ratio between these substitutions in related sequences allows the detection of evolutionary forces acting on a protein.[257]

DNA and peptide sequences for *UTS2*, *UTS2D* and *UST2R* are all available in vertebrate species and were obtained from ENSEMBL database http://www.ensembl.org/index.html. Where more than one splice variant was available, the longest was used as the reference sequence.

Multiple peptide sequence alignments were performed using Teecoffee@igs web server (http://igs-server.cnrs-mrs.fr/Tcoffee/_tcoffee_cgi/index.cgi). In brief, for each pair of sequences the software calculates best global, and 10 best local alignments and stores them in a library. Subsequently,

libraries for all pairs of sequences are used to assemble global alignment, to maximise the overall level of consistency.[258] Following automatic processing, visual inspection was performed. Sequences missing the biologically important C-terminal fragments of U-II or URP or sequences substantially shorter than the average (introducing large gaps into the alignment) were removed from further analysis (Details in the supplementary materials). The ultimate peptide alignments were used to guide complementary DNA (cDNA) alignment using RevTrans 1.4 web server (http://www.cbs.dtu.dk/services/RevTrans/). This software takes advantage of the higher signal to noise ratio in protein alignments. The peptide alignment undergoes “reverse translation” producing a template for cDNA alignment.[259]

The cDNA alignments were used in evolutionary analysis conducted with the use of Adaptive Evolution Server http://datamonkey.org. HKY85 nucleotide substitution model was chosen using a genetic algorithm available on http://datamonkey.org. The algorithm assumes different rates of substitutions between codon pairs and divides them into classes based on the estimation from the alignment. Other parameters of the model (substitution rates, codon frequencies and distances) were estimated using maximum likelihood procedures.[260]

Phylogenetic trees were created using a neighbour-joining algorithm. A distance matrix was calculated for combinations of all sequence pairs. The pair showing the smallest distance is bound by a node of the tree and distances from the node to all other sequences are re-calculated.

Phylogenetic line specific evolutionary models were fitted using GABranch algorithm available on http://datamonkey.org server.[261] The algorithm uses a previously specified codon substitution model and phylogenetic tree to calculate a tree wide estimate of non-synonymous/synonymous substitution ratio. Alternative models with different classes of branches (each class differs by non-synonymous/ synonymous substitutions ratio) were created. Fitness of each model to the data was assessed and the best model was chosen.

10. RESULTS

Genetic association analysis of common genetic variants in urotensin-II pathway with renal function and blood pressure

Characteristics of study cohorts

GRAPHIC Study

The basic clinical characteristics of subjects from the GRAPHIC Study are shown in Table 4. The age of participants ranged from 18 to 61 years (mean age of subjects from parental generation was 52.8 years, whereas mean age of offspring was 25.4 years). The overall prevalence of hypertension among participants of the GRAPHIC Study was 28.5% (43.6% in parental generation and 13% in offspring). 6.67% of all participants were on antihypertensive treatment (12.8% in parental generation and 0.4% of offspring). The mean eGFR among subjects of the GRAPHIC Study was 83.9 mL/min*1.73m^2 (76.2 mL/min*1.73m^2 in the parental generation and 91.8 mL/min*1.73m^2 among offspring). Altogether 68.7% (1392 subjects) had eGFR<90 mL/min*1.73m2 – consistent with presence of CKD.

The distributions (histograms) of phenotypes (eGFR, clinic SBP, clinic DBP, mean 24-hour SBP, mean 24-hour DBP, 24-hour urinary sodium excretion and 24-hour urinary potassium excretion) in subjects from the parental generation of the GRAPHIC Study are presented on Figure 5. 24-hour urinary

sodium and potassium excretions had been log10 transformed prior to the analysis. (Figure 6)

The GEE analysis (exchangeable correlation structure) with basic demographic parameters as predictors identified age, sex and BMI as independent determinants of all analysed phenotypes in the GRAPHIC Study. (Table 5) Additionally, age^2 was included as a covariate to account for the non-linear relation with age, originating from the 2-generational design of the GRAPHIC Study.

Age was a negative predictor of renal function phenotypes. eGFR and log10 transformed 24-hour urinary sodium excretion were lower by 0.97 mL/min*1.73m^2 and 0.01 with each year of life, respectively. (Table 5) Advancing age was also associated with higher values of both systolic and diastolic BP. Clinic and mean 24-hour SBP were higher by 0.68 mmHg and 0.61mmHg with each year of life, respectively; age had slightly weaker influence on clinic and mean 24-hour DBP, which were higher by 0.45 mmHg and 0.46 mmHg with each year of life, respectively. (Table 5)

Sex had also significant influence on all of the analysed phenotypes. Females in the GRAPHIC Study had eGFR lower by on average 7.1 mL/min*1.73m^2 than males. (Table 5) Similarly, the log10 transformed 24-hour urinary excretions of sodium and potassium in females were lower by on average 0.09 and 0.05. (Table 5) As expected, all BP phenotypes were lower amongst women than men from the GRAPHIC Study (clinic SBP and mean 24-

hour SBP by 10.6 mmHg and 7.1 mmHg, respectively; clinic DBP and mean 24-hour DBP by 3.6 mmHg and 3.7 mmHg, respectively). (Table 5)

BMI was also amongst the independent predictors of renal function and BP phenotypes. Although eGFR was slightly reduced with increasing BMI (by 0.17 mL/min*1.73m^2 per unit of BMI) the log10 transformed 24-hour urinary sodium and potassium excretions were slightly increased with increasing BMI. (Table 5) Consistently with expectation BP phenotypes were positively correlated with BMI. BMI had bigger effect on clinic SBP and mean 24-hour SBP (increase by 0.93 mmHg and 0.69 mmHg per unit of BMI, respectively) than DBP phenotypes (clinic DBP and mean 24-hour DBP increased by 0.59 mmHg and 0.19 mmHg per unit of BMI, respectively). (Table 5)

Of all analysed phenotypes age, age^2, sex and BMI explained the biggest proportion of variation in mean 24-hour SBP accounting for 21.4% variance of this trait in the parental generation of the GRAPHIC Study. Similarly, age age^2, sex and BMI explained high proportions of variance of other BP phenotypes in the parental generation of the GRAPHIC Study: 17.2% of clinic SBP, 16.5% of mean 24-hour DBP and 13.7% for clinic DBP. Age, age^2, sex and BMI explained also significant but smaller proportion of eGFR, log10 transformed urinary sodium and potassium excretions accounting for 10.4%, 14.3% and 6.5% of the overall variance of this phenotypes in the parental generation of the GRAPHIC Study.

Table 4. Clinical characteristics of subjects from the GRAPHIC Study.

Variable	Mothers (n=516)	Fathers (n=516)	Sons (n=512)	Daughters (n=493)
Age (years)	51.9±4.4	53.8±4.3	25.0±5.0	25.9±5.4
BMI (kg/m^2)	27.1±4.6	27.8±4.0	24.9±4.1	24.5±4.9
eGFR (mL/min*1.73m^2)	73.6±10.2	78.7±11.7	96.3±13.1	87.1±12.9
Clinic SBP (mmHg)	130.3±19.9	140.2±20.1	127.8±13.1	113.9±11.9
Clinic DBP (mmHg)	81.8±10.9	87.3±11.5	76.0±9.7	73.6±8.7
Mean 24-hour SBP (mmHg)	118.1±12.4	125.9±12.5	120.8±8.2	112.8±7.2
Mean 24-hour DBP (mmHg)	72.5±7.9	78.7±7.8	69.3±6.6	67.9±5.2
Hypertension (%, N)	35.6 (184)	51.8 (267)	21.0 (108)	4.7 (23)
Antihypertensive treatment (%, N)	10.4 (54)	15.2 (78)	0.6 (3)	0.2 (1)
24-hour urinary sodium excretion (mmol/24h)	68.3±30.8	92.2±38.8	109.3±50.4	96.9±45.2
24-hour urinary potassium excretion (mmol/24h)	41.7±15.8	50.2±20.0	51.0±22.6	48.3±22.9

Data are means and standard deviations, percentages and counts; BMI – body mass index, eGFR – estimated glomerular filtration rate, SBP – systolic blood pressure, DBP – diastolic blood pressure.

Table 5. Generalised estimating equations analysis of variables influencing blood pressure and renal function phenotypes. Data come from model with phenotype as an outcome and age, age^2, sex and body mass index as predictors.

Phenotype	Age (years)		Age^2 (years^2)		Sex		BMI (kg/m^2)	
	β±SE	p	β±SE	p	β±SE	p	β±SE	p
eGFR (mL/min*1.73m^2)	-0.97±0.15	$2.3x10^{-11}$	0.005±0.002	0.0042	-7.14±0.48	$5.7x10^{-51}$	-0.17±0.06	0.0049
Clinic SBP (mmHg)	0.68±0.19	0.0006	0.014±0.003	$4.4x10^{-8}$	-10.58±0.71	$6.7x10^{-50}$	0.93±0.09	$3.7x10^{-24}$
Clinic DBP (mmHg)	0.45±0.13	0.0003	0.002±0.001	0.2399	-3.63±0.43	$2.1x10^{-17}$	0.59±0.05	$3.5x10^{-28}$
Mean 24-hour SBP (mmHg)	0.61±0.13	$3.4x10^{-6}$	0.009±0.002	$3.9x10^{-8}$	-7.09±0.46	$5.6x10^{-54}$	0.69±0.60	$5.4x10^{-31}$
Mean 24-hour DBP (mmHg)	0.46±0.09	$6.5x10^{-8}$	0.002±0.001	0.0096	-3.73±0.31	$1.2x10^{-33}$	0.19±0.04	$1.3x10^{-6}$
Log10 24-hour urinary sodium excretion	-0.01±0.002	$9.4x10^{-5}$	0.0008±0.0003	0.0227	-0.09±0.008	$2.3x10^{-25}$	0.009±0.001	7.4x10-19
Log10 24-hour urinary potassium excretion	-0.003±0.002	0.29236	0.0002±0.0001	0.4971	-0.05±0.01	$8.3x10^{-9}$	0.004±0.001	$6.1x10^{-5}$

BMI – body mass index, eGFR – estimated glomerular filtration rate, SBP – systolic blood pressure, DBP – diastolic blood pressure, β – estimated effect size; SE – standard error of the estimate; p – level of the statistical significance in the model.

Figure 5. Distributions of the analysed phenotypes in the parental generation of the GRAPHIC Study.

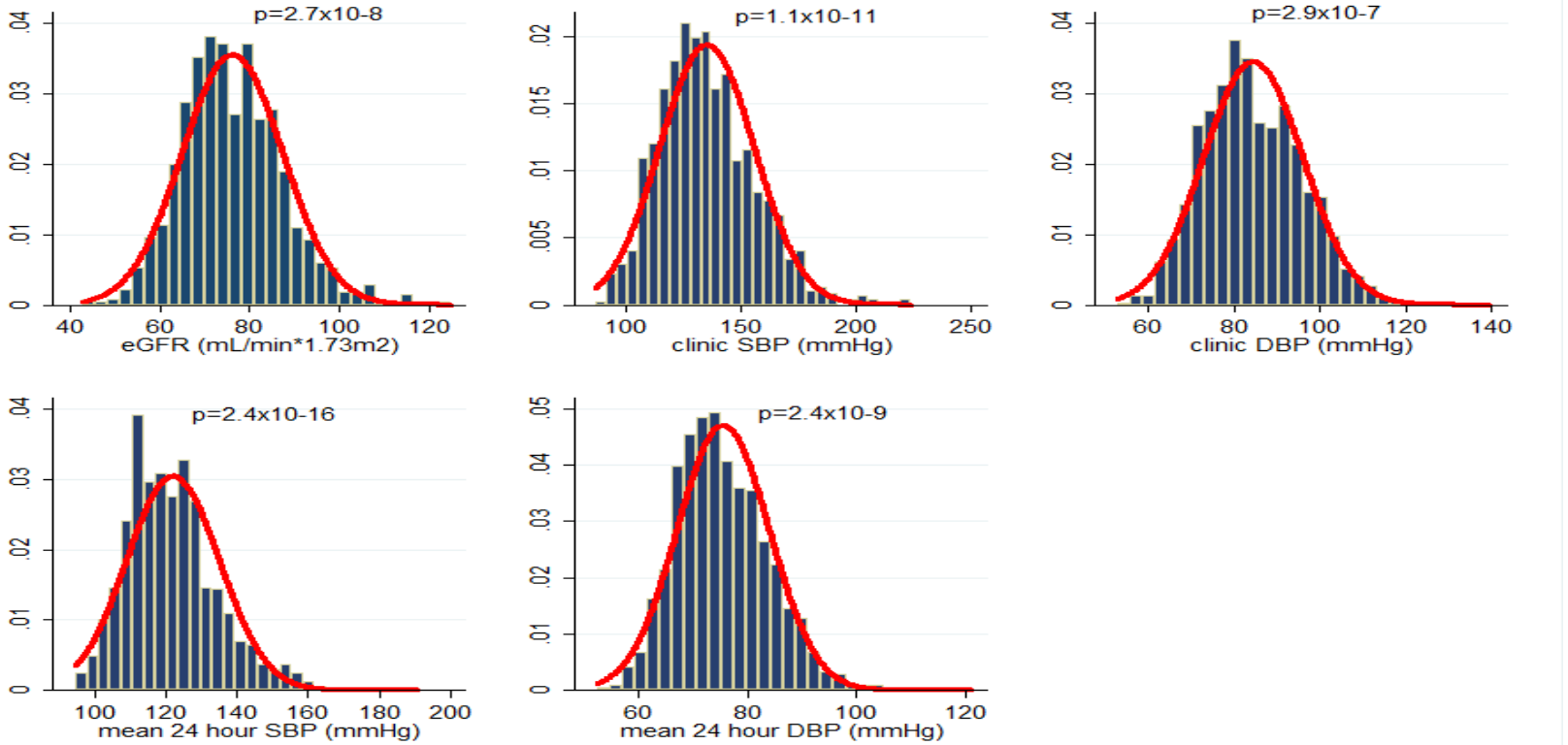


Figure presents distributions of phenotypes in the parental generation of the GRAPHIC Study in form of histograms. Red line depicts theoretical distribution; eGFR – estimated glomerular filtration rate; SBP – systolic blood pressure, DBP – diastolic blood pressure; p – statistical significance of the Shapiro-Wilk test with the null hypothesis that the sample is normally distributed.

Figure 6. Distribution of the analysed phenotypes in the parental generation of the GRAPHIC Study.

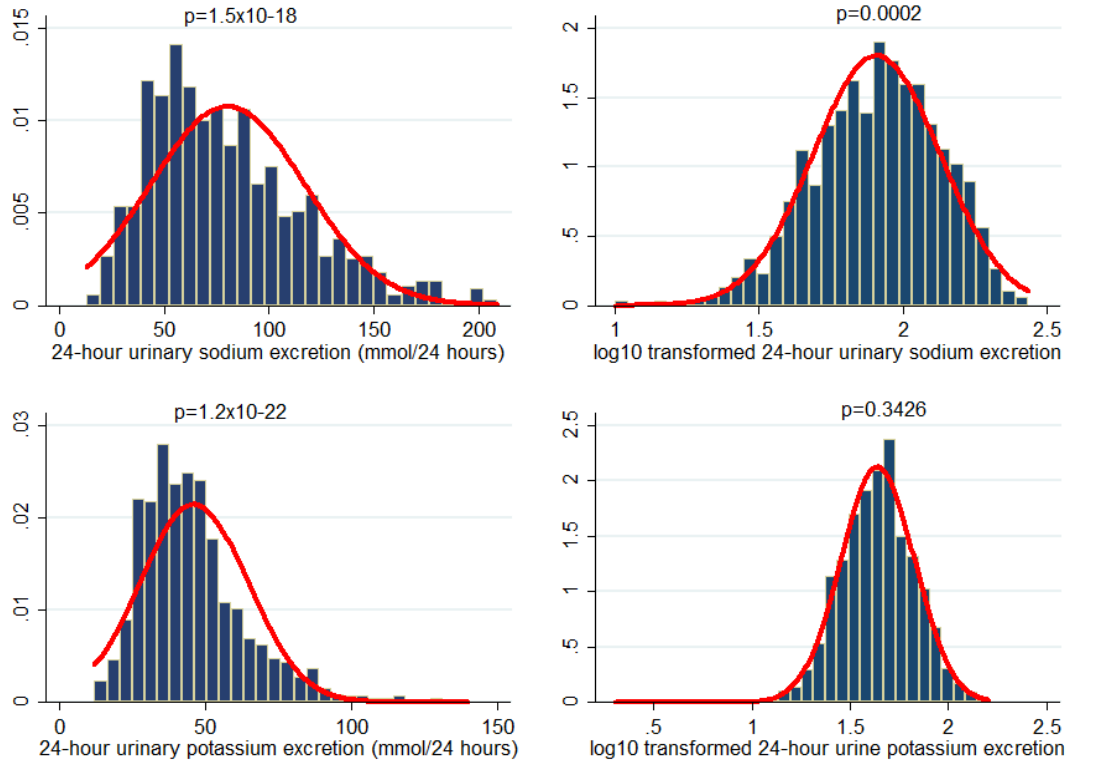


Figure presents distributions of phenotypes in the parental generation of the GRAPHIC Study in form of histograms. Red line depicts theoretical distribution; p – statistical significance of the Shapiro-Wilk test with the null hypothesis that the sample is normally distributed.

Silesian Cardiovascular Study – families

The basic clinical characteristics of subjects from SCS families are presented in Table 6. The mean age of subjects from SCS families was 43.4 years (55.3 years for parents and 30.9 for offspring). Altogether, 51.7% of subjects were hypertensive, of which 35.8% were on active antihypertensive treatment. 6.6% of subjects were diabetic. Mean eGFR in the SCS families was 82.7 mL/min*1.73m^2 (76.5 mL/min*1.73m^2 in the parental generation and 89.9 mL/min*1.73m^2 among offspring). 63% (N=449) of subjects from SCS families had eGFR<90 mL/min*1.73m^2, consistent with the presence of CKD.

Histograms showing distributions of analysed phenotypes (eGFR, clinic SBP, clinic DBP and urinary albumin: creatinine ratio) in the parental generation of SCS families are presented on Figure 7. Urinary albumin: creatinine ratio had been log10 transformed prior to analysis. (Figure 7)

Results of a GEE analysis (exchangeable correlation structure) with basic demographic parameters as predictors of all analysed phenotypes are presented in Table 7.

In the parental generation of SCS families age and sex were important determinants of eGFR. eGFR was lower in women than in men by on average 8.5 mL/min*1.73m^2 and was negatively correlated with age, dropping by on average 0.55 mL/min*1.73m^2 per year of life. (Table 7)

Among subjects from SCS families clinic SBP and clinic DBP were significantly affected by BMI. Clinic SBP was on average higher by 1.24 mmHg and clinic DBP by 0.41mmHg, respectively with increase of each unit of BMI.

Additionally, clinic DBP showed strong significant positive correlation with age, increasing by on average 0.79 mmHg per each year of life. (Table 7)

As expected log transformed urinary albumin to creatinine ratio was significantly higher in women.

In the parental generation of SCS families age, age^2 sex and BMI were important predictors of clinic SBP and eGFR accounting for 13.8% and 13.2% of the total variance of this traits. However, age, age^2, sex and BMI explained only 5.9% of variance of the log10 transformed urinary albumin/creatinine ration and 3.7% of the total variance in clinic DBP among parents form SCS families.

Table 6. Clinical characteristics of subjects from SCS families.

Variable	Mothers (n=194)	Fathers (n=166)	Sons (n=164)	Daughters (n=179)
Age (years)	53.0±9.9	55.0±8.6	28.0±10.3	30.0±11.7
BMI (kg/m^2)	27.7±3.6	27.1±4.9	25.1±4.1	22.7±5.0
eGFR (mL/min*1.73m^2)	73.7±16.1	79.9±17.5	96.5±18.3	83.7±18.5
Clinic SBP(mmHg)	137.5±18.3	130.0±17.6	128.0±14.4	121.5±17.5
Clinic DBP (mmHg)	77.5±13.7	77.0±10.8	71.5±9.4	70.5±12.1
Albumin: creatinine ratio (mg/mmol)	0.83	0.49	0.40	0.56
Diabetes mellitus (%, N)	10.0 (19)	10.8 (18)	1.84 (3)	3.37 (6)
Hypertension (%, N)	67.5 (131)	77.7 (129)	31.7 (52)	27.4 (49)
Antihypertensive treatment (%, N)	49.9 (97)	63.9 (106)	12.2 (20)	16.8 (30)

Data are means and standard deviations (geometric mean for albumin: creatinine ratio), percentages and counts; BMI – body mass index, eGFR – estimated glomerular filtration rate, SBP – systolic blood pressure, DBP – diastolic blood pressure.

Table 7. GEE analysis of variables influencing blood pressure and renal function phenotypes in the Silesian Cardiovascular Study families. Data come from generalised estimating equations model with phenotype as an outcome and age, age^2, sex and body mass index as predictors.

Phenotype	Age (years)		Age^2 (years^2)		Sex		BMI (kg/m^2)	
	β±SE	p	β±SE	p	β±SE	p	β±SE	p
eGFR (mL/min*1.73m^2)	-0.55±0.22	0.0137	-0.0001±0.002	0.9639	-8.56±1.38	6.1x10-10	0.03±0.15	0.8326
Clinic SBP (mmHg)	-0.21±0.21	0.3168	0.007±0.002	0.0023	-1.19±1.21	0.3263	1.24±0.18	$7.3x10^{-12}$
Clinic DBP (mmHg)	0.79±0.13	$3.1x10^{-9}$	-0.005±0.002	0.0003	-0.42±0.79	0.5944	0.41±0.11	0.0004
Log10 urine albumin: creatinine ratio	-0.0006±0.007	0.9335	0.0005±0.0007	0.4645	0.19±0.3	$9.9x10^{-9}$	-0.002±0.004	0.6639

BMI – body mass index, eGFR – estimated glomerular filtration rate, SBP – systolic blood pressure, DBP – diastolic blood pressure, β – estimated effect size; SE – standard error of the estimate; p – level of the statistical significance in the model.

Figure 7. Distribution of the analysed phenotypes in the parental generation of the Silesian Cardiovascular Study families.

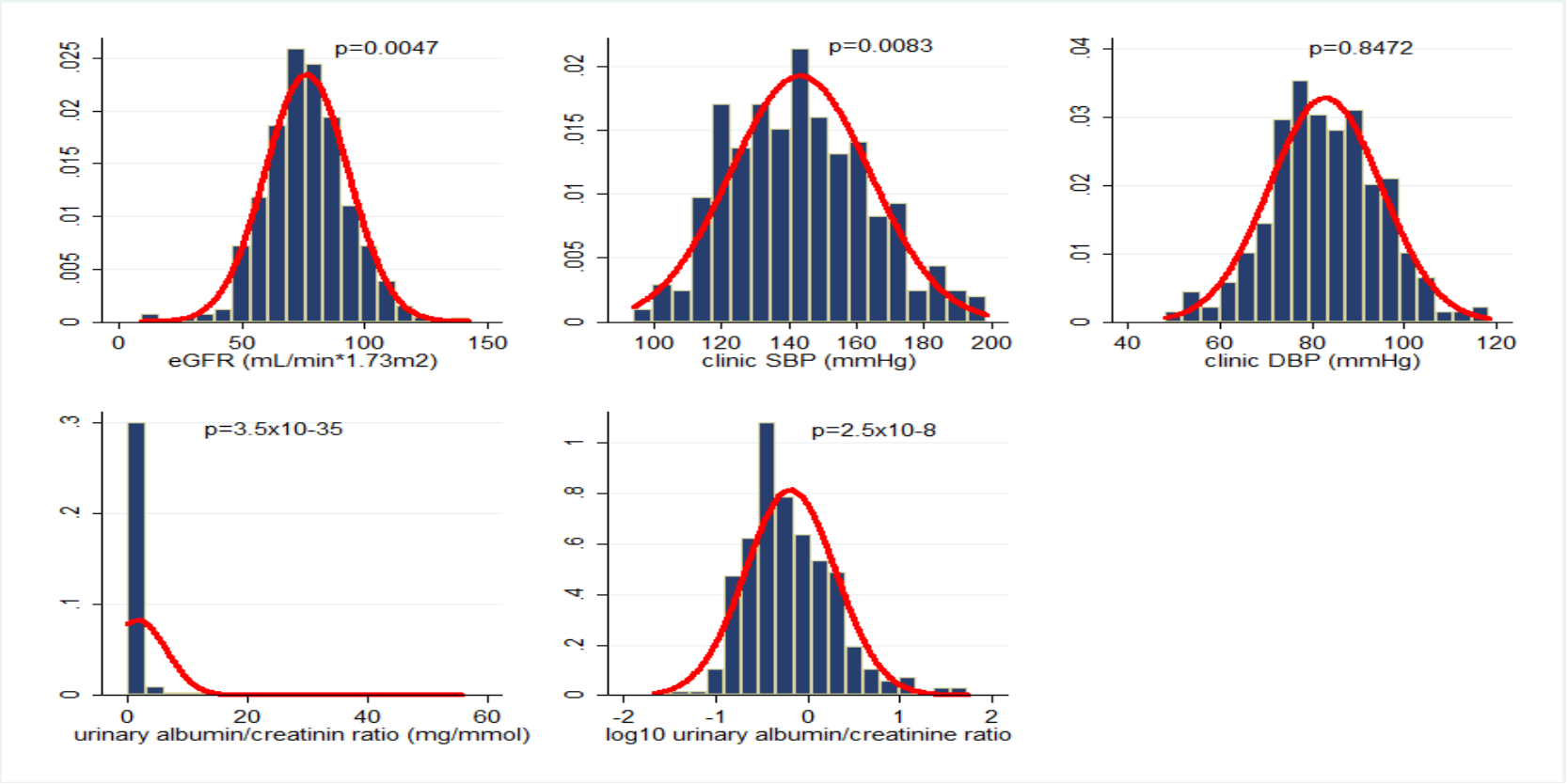


Figure presents distribution of given phenotypes in the parental generation of the SCS families in form of histograms. Red line depicts theoretical distribution; eGFR – estimated glomerular filtration rate; SBP – systolic blood pressure, DBP – diastolic blood pressure; log10 urinary albumin/creatinine ratio – urinary albumin: creatinine ratio following log10 transformation; p – statistical significance of the Shapiro-Wilk test with the null hypothesis that the sample is normally distributed.

Silesian Hypertension Study

The mean age of participants from SHS was 45.8 years (54.9 years in parents and 38.8 years in offspring). The overall prevalence of hypertension was 58.6% - 52.5% among parents and 63.4% among children. 42.7% of participants were on active antihypertensive treatment. The detailed characteristics of SHS subjects are presented in Table 8. The mean eGFR in subjects of the SHS was 68.9 ml/min*1.73m^2 (65.8 ml/min*1.73m^2 in the parental generation and 71.7 ml/min*1.73m^2 among the offspring). Altogether, almost 85% of subjects in the SHS had CKD. (Table 8)

Histograms of the distribution of eGFR, clinic SBP and clinic DBP in the parental generation of the SHS are presented on Figure 8. Due to binominal distribution clinic SBP and clinic DBP were exclude from further analyses.

The GEE model showed that among the subjects of SHS age and sex were important predictors of eGFR. eGFR was lower in women by on average 10.8 mL/min*1.73m^2 and was dropping by on average 0.52 mL/min*1.73m^2 with each year of life. (Table 9)

In the parental generation of the SHS age, age^2 sex and BMI account for high proportion of the overall variance in the eGFR - 31.9%.

Table 8. Clinical characteristics of subjects from the Silesian Hypertension Study.

Variable	Mothers (N=144)	Fathers (N=130)	Sons (N=183)	Daughters (N=172)
Age (years)	54.2±10.8	55.8±10.1	35.1±14.9	42.8±15.4
BMI (kg/m^2)	27.5±5.1	27.7±4.1	26.4±4.0	26.0±5.2
eGFR (mL/min*1.73m^2)	60.4±11.6	71.6±11.85	78.4±13.4	65.0±12.3
Clinic SBP(mmHg)	139.4±23.3	138.6±19.8	138.3±15.6	137.6±21.2
Clinic DBP (mmHg)	88. 7±13.6	87.2±11.6	88.4±10.6	87.86±12.3
Hypertension (%, N)	58.5 (84)	46.6 (61)	65.0 (119)	61.6 (106)
Antihypertensive treatment (%, N)	40.2 (58)	32.3 (42)	45.9 (84)	49.4 (85)

Data are means and standard deviations or percentages and counts, BMI – body mass index, SBP – systolic blood pressure, DBP – diastolic blood pressure, eGFR – estimated glomerular filtration rate.

Table 9. Generalised estimating equations analysis of variables influencing estimated glomerular filtration rate in the Silesian Hypertension Study. Data come from model with phenotype as an outcome and age, age^2, sex and body mass index as predictors.

Phenotype	Age (years)		Age^2 (years^2)		Sex		BMI (kg/m^2)	
	β±SE	p	β±SE	p	β±SE	p	β±SE	p
eGFR (mL/min*1.73m^2)	-0.52±0.14	0.0001	0.0007±0.001	0.6336	-10.81±0.8	$2.7x10^{-41}$	-0.18±0.10	0.0718

BMI – body mass index, eGFR – estimated glomerular filtration rate, β – estimated effect size; SE – standard error of the estimate; p – level of the statistical significance in the GEE model.

Figure 8. Distribution of the analysed phenotypes in the parental generation of the Silesian Hypertension Study.

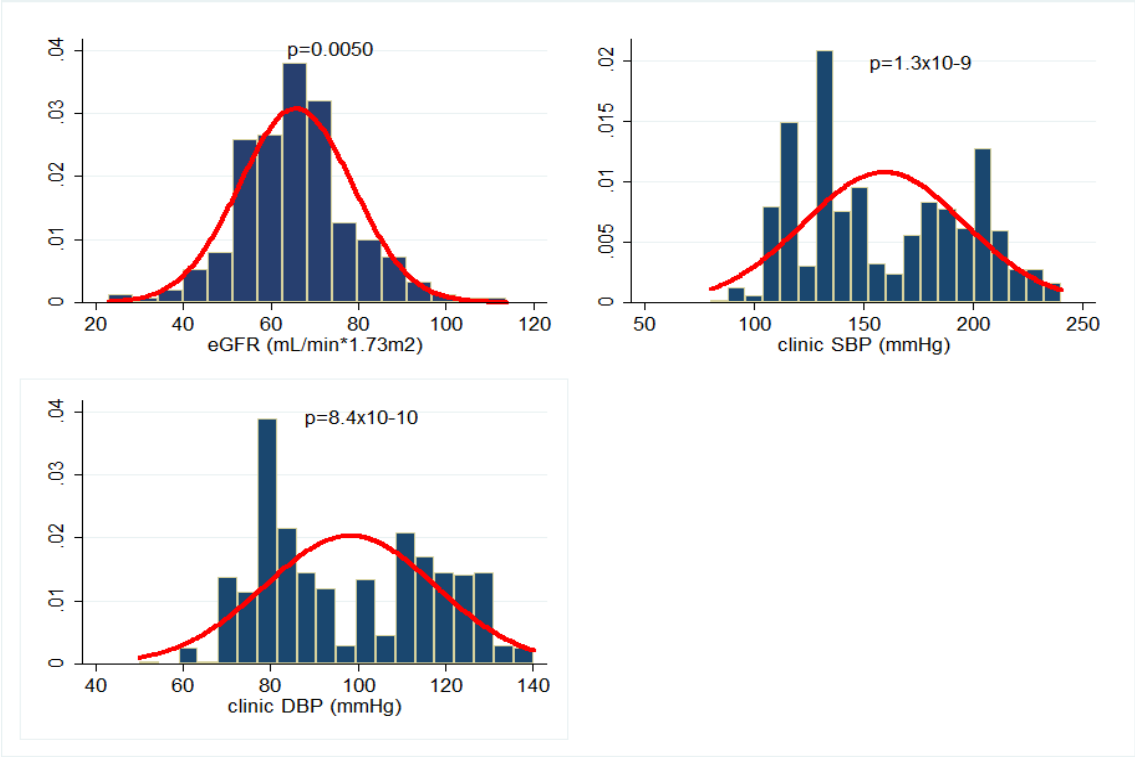


Figure presents distributions of given phenotypes in the parental generation of the Silesian Hypertension Study in form of histograms. Red line depicts theoretical distribution; eGFR – estimated glomerular filtration rate; SBP – systolic blood pressure, DBP – diastolic blood pressure; p – statistical significance of the Shapiro-Wilk test with the null hypothesis that the sample is normally distributed.

Silesian Cardiovascular Study extension

The basic clinical characteristics of subjects from SCS extension are presented in Table 10. Generally, subjects from SCS extension were older (mean age 55.4 years) than subjects from SCS families (mean age 43.4 years). Similarly, the prevalence of hypertension (72.9% of which 62.4% on treatment) and diabetes (14.2%) were higher than in SCS families. The mean eGFR among subjects of the SCS extension was 75.5 mL/min*1.73m^2. 80% of subjects in the SCS extension had eGFR<90 mL/min*1.73m^2.

Histograms showing distributions of the main phenotypes (eGFR, clinic SBP, clinic DBP and albumin: creatinine ratio) in the SCS extension are presented on Figure 9. Urinary albumin: creatinine ratio had been log10 transformed prior to analysis. (Figure 9)

Multivariate regression analysis showed that age and sex were independent predictors of eGFR in SCS extension. eGFR decreased on average by 0.6 mL/min*1.73m^2 per each year of life and was lower in women (by almost 6 mL/min*1.73m^2) than in men. (Table 11)

Age, sex and BMI were influencing clinic SBP and clinic DBP amongst individuals recruited into SCS extension. Whereas clinic SBP increased on average by approximately 0.5 mmHg per each year of life, the increase in clinic DBP was estimated at 0.14 mmHg. (Table 11) Clinic SBP was on average 3.9 mmHg higher in men than women in the SCS extension. Increasing BMI was related to higher values of BP (by on average 0.9 mmHg and 0.5mmHg per unit of BMI for clinic SBP and clinic DBP, respectively). (Table 11)

Overall the multiple regression model including age, sex and BMI explained 18% of the total variance in eGFR. Age sex and BMI explained smaller proportions of the overall variance in clinic SBP and clinic DBP accounting for almost 8% and 3.5% of the total variance, respectively.

Table 10. Clinical characteristics of subjects from the Silesian Cardiovascular Study extension.

Variable	Males (N=294)	Females (N=141)
Age (years)	56.8±11.4	52.5±15.8
BMI (kg/m^2)	27.6±3.6	26.6±4.6
eGFR (mL/min*1.73m^2)	76.7±18.5	73.2±15.27
Clinic SBP(mmHg)	128.0±19.1	130.7±20.0
Clinic DBP (mmHg)	51.5±11.3	52.2±13.8
Albumin: creatinine ratio (mg/mmol)	0.53	0.72
Diabetes mellitus (%, N)	15.8 (46)	10.9 (15)
Hypertension (%, N)	78.5 (231)	61.4 (87)
Antihypertensive treatment (%, N)	68.3 (201)	55.6 (78)

Data are means and standard deviations or geometric means (albumin: creatinine ratio) and percentages and counts; BMI – body mass index, eGFR – estimated glomerular filtration rate, SBP – systolic blood pressure, DBP – diastolic blood pressure.

Table 11. Multiple regression analysis of variables influencing blood pressure and renal function phenotypes in the Silesian Cardiovascular Study extension. Data come from multiple regression model with phenotype as an outcome and age, sex and body mass index as predictors.

Phenotype	Age (years)		Sex		BMI (kg/m^2)	
	β±SE	p	β±SE	p	β±SE	p
eGFR (mL/min*1.73m^2)	-0.56±0.06	$4.4x10^{-19}$	-5.97±1.66	0.0004	-0.10±0.19	0.6089
Clinic SBP (mmHg)	0.51±0.06	$1.8x10^{-16}$	-3.93±1.48	0.0082	0.94±0.23	$3.6x10^{-23}$
Clinic DBP (mmHg)	0.14±0.04	0.0002	-0.48±0.89	0.5824	0.53±0.13	0.0001
Log10 albumin: creatinine ratio	0.007±0.001	$1.0x10^{-6}$	0.20±0.03	$1.4x10^{-9}$	-0.003±0.004	0.4555

BMI – body mass index, eGFR – estimated glomerular filtration rate, SBP – systolic blood pressure, DBP – diastolic blood pressure, β – estimated effect size; SE – standard error of the estimate; p – level of the statistical significance in the multiple regression model.

Figure 9. Distribution of the analysed phenotypes in subjects from the Silesian Cardiovascular Study extension.

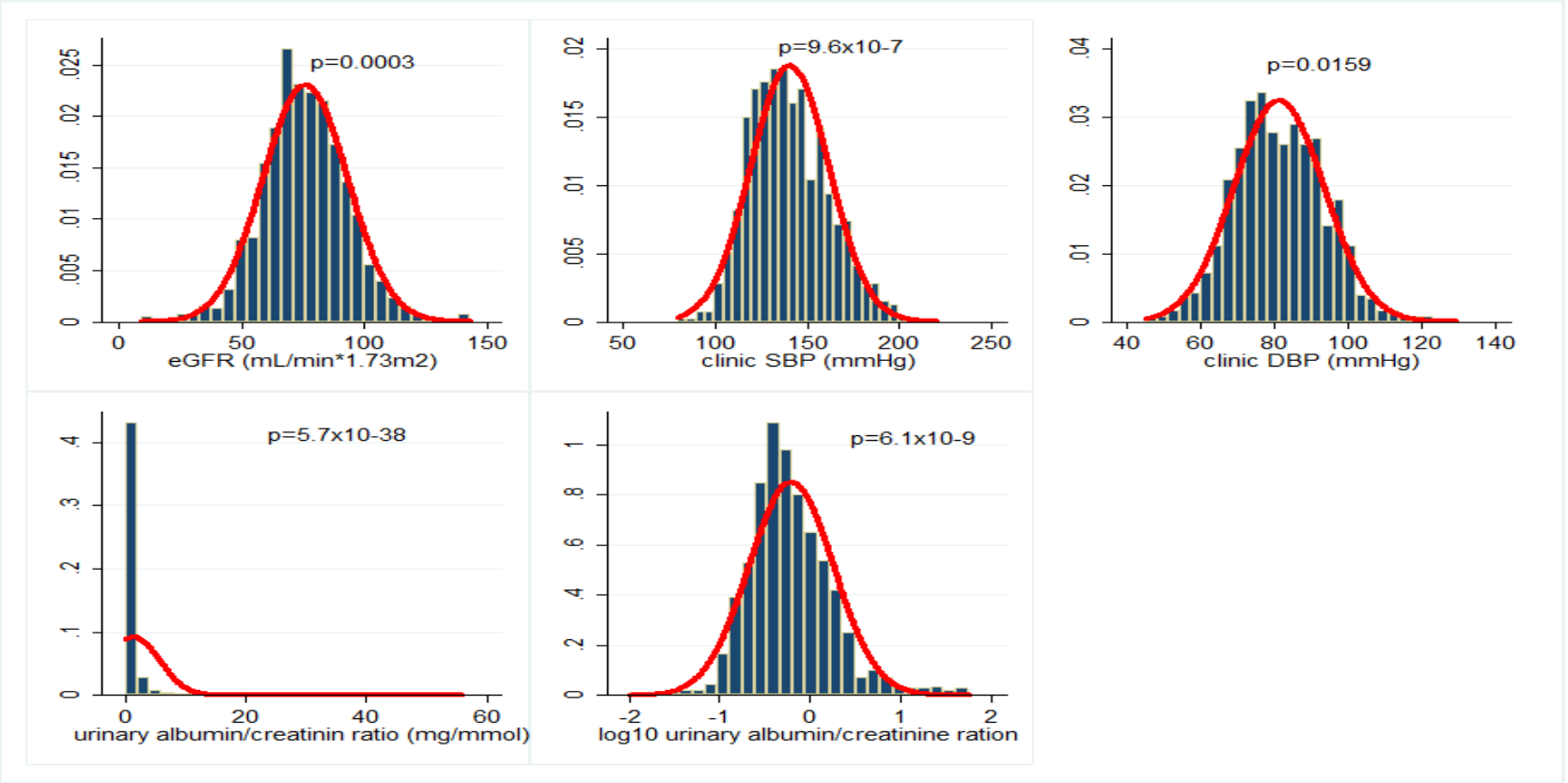


Figure presents distribution of given phenotypes in the parental generation of the SCS families in form of histograms. Red line depicts theoretical distribution; eGFR – estimated glomerular filtration rate; SBP – systolic blood pressure, DBP – diastolic blood pressure; log10 urinary albumin/creatinine ratio – urinary albumin/creatinine ratio following log10 transformation; p – statistical significance of the Shapiro-Wilk test with the null hypothesis that the sample is normally distributed.

Young Men Cardiovascular Association (YMCA) study and Young Men Cardiovascular Association study extension

The clinical characteristics of subjects from YMCA study and YMCA extension are presented in Table 12.

Histograms of distributions of the main phenotypes (eGFR, clinic SBP and clinic DBP) in the YMCA and YMCA extension are presented on Figure 10 and Figure 11, respectively.

The regression analysis confirmed that age and BMI were significant independent predictors of eGFR - there was on an average a decrease of 2 mL/min*1.73m^2 per year of life in the YMCA study and 2.1 mL/min*1.73m^2 in YMCA extension. (Table 13) Contrary to expectations, BMI was positively correlated with eGFR in the YMCA but not in the YMCA extension. (Table 13) Clinic SBP and clinic DBP increased by on average 0.28 and 0.19 mmHg per year of life in the YMCA study whereas, in YMCA extension the increase per year of life for clinic SBP and clinic DBP were 0.21 mmHg and 0.8 mmHg, respectively. (Table 13) Both clinic SBP and clinic DBP showed strong positive correlation with BMI in both cohorts. (Table 13)

In the YMCA age and BMI explained 30% of the overall variance in eGFR, 12% of variance in clinic SBP and 7% of variance in clinic DBP. In the YMCA extension age and BMI accounted for 19% of variance in eGFR, 5.5% of variance in clinic SBP and 3.8% of variance in clinic DBP.

Table 12. Clinical characteristics of subjects from Young Men Cardiovascular Association study and Young Men Cardiovascular Association study extension.

Variable	YMCA study (N=1157)	YMCA extension (N=597)
Age (years)	19.1±3.6	18.9±3.5
BMI (kg/m^2)	22.8 ±3.0	22.6±3.0
eGFR (mL/min*1.73m^2)	131.7±25.2	124.5±19.1
Clinic SBP(mmHg)	117.9±13.2	119.0±13.5
Clinic DBP (mmHg)	74.2±7.9	74.4±8.2
Hypertension (%, N)	10.4 (120)	8.4 (50)
Antihypertensive treatment (%, N)	1.6 (19)	0.9 (5)

Data are means and standard deviations and percentages and counts; BMI – body mass index, eGFR – estimated glomerular filtration rate, SBP – systolic blood pressure, DBP – diastolic blood pressure.

Table 13. Multiple regression analysis of variables influencing blood pressure and renal function phenotypes in the Young Men Cardiovascular Association study and Young Men Cardiovascular Association study extension. Data come from multiple regression model with phenotype as an outcome and age and body mass index as predictors.

Phenotype	YMCA				YMCA extension			
	Age (years)		BMI (kg/m^2)		Age (years)		BMI (kg/m^2)	
	β±SE	p	β±SE	p	β±SE	p	β±SE	p
eGFR (mL/min*1.73m^2)	-2.01±0.18	1.8×10^{-27}	5.08±0.23	6.8×10^{-22}	-2.09±0.23	1.0×10^{-18}	-0.67±0.27	0.0133
Clinic SBP (mmHg)	0.28±0.11	0.0089	1.41±0.12	1.2×10^{-27}	0.21±0.17	0.2149	0.96±0.20	2.1×10^{-6}
Clinic DBP (mmHg)	0.19±0.07	0.0042	0.59±0.07	2.1×10^{-14}	0.80±0.10	0.4192	0.50±0.12	5.3×10^{-5}

BMI – body mass index, eGFR – estimated glomerular filtration rate, SBP – systolic blood pressure, DBP – diastolic blood pressure, β – estimated effect size; SE – standard error of the estimate; p – level of the statistical significance in the multiple regression model.

Figure 10. Distribution of the analysed phenotypes in subjects from the Young Men Cardiovascular Association study.

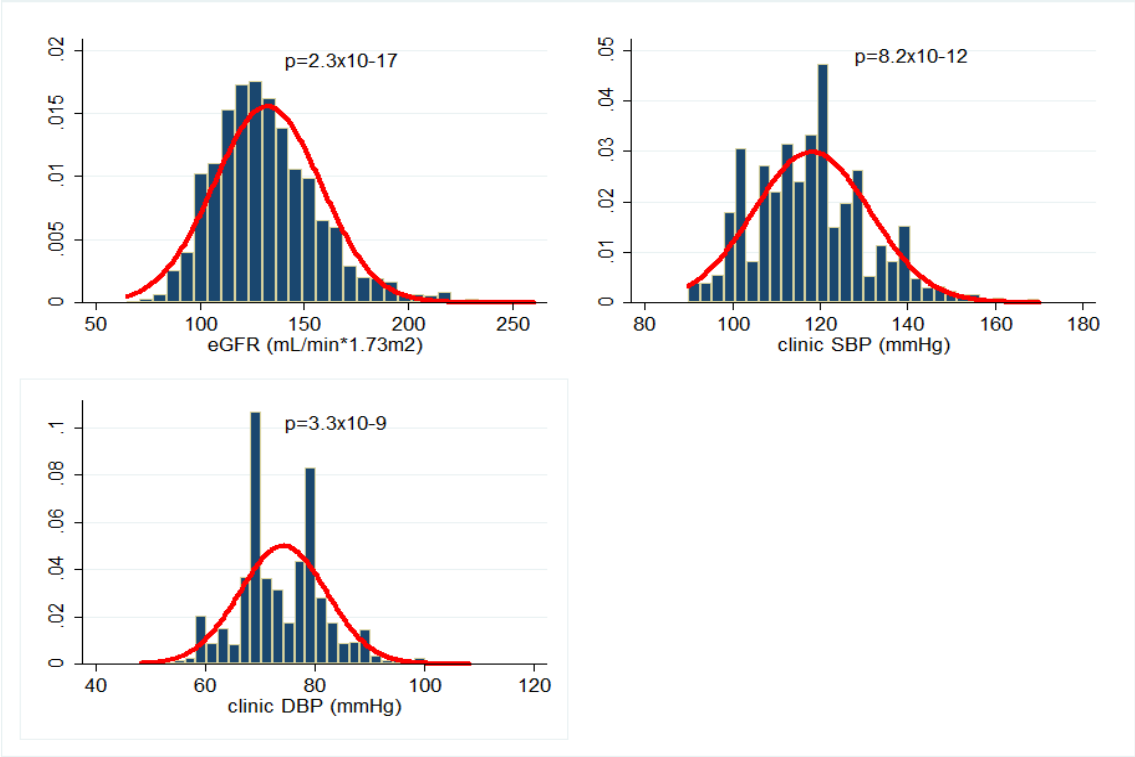


Figure presents distribution of given phenotypes in the Young Men Cardiovascular Association study in form of histograms. Red line depicts theoretical distribution; eGFR – estimated glomerular filtration rate; SBP – systolic blood pressure, DBP – diastolic blood pressure; p – statistical significance of the Shapiro-Wilk test with the null hypothesis that the sample is normally distributed

Figure 11. Distribution of the analysed phenotypes in the subjects from the Young Men Cardiovascular Association study extension.

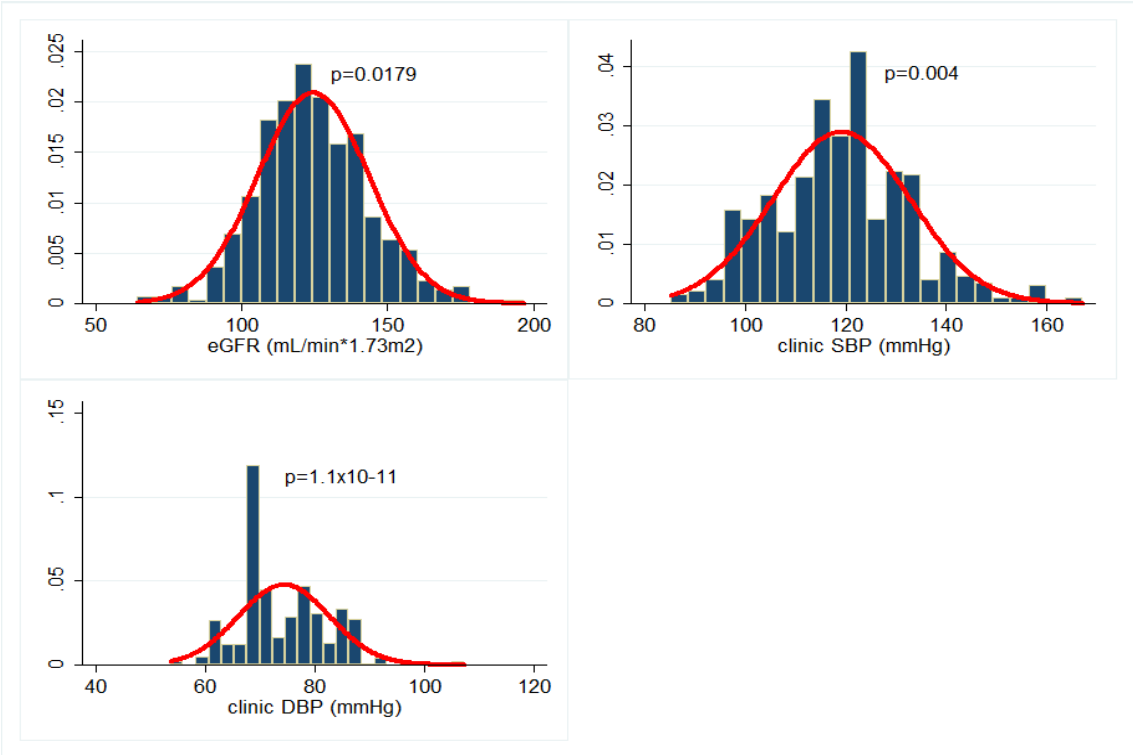


Figure presents distribution of given phenotypes in the Young Men Cardiovascular Association study extension in form of histograms. Red line depicts theoretical distribution; eGFR – estimated glomerular filtration rate; SBP – systolic blood pressure, DBP – diastolic blood pressure; p – statistical significance of the Shapiro-Wilk test with the null hypothesis that the sample is normally distributed.

Analysis of heritability

Taking advantage of the family structure of the GRAPHIC Study (520 pedigrees) I estimated heritability of the main phenotypes: eGFR, clinic SBP and clinic DBP. The age, age^2 and sex adjusted eGFR showed heritability of 38.4% ($p=5.5x10^{-25}$). (Table 14) Both clinic SBP and clinic DBP showed also significant heritable component; medication, age, age^2 and sex adjusted clinic SBP showed heritability of 33.1%, while 31% was calculated for adjusted clinic DBP. Narrow sense heritability estimates of clinic SBP were slightly higher than that of clinic DBP. Conversely, analysis of mean 24-hour BP phenotypes showed that adjusted mean 24-hour DBP had higher heritable component than adjusted mean 24-hour SBP. (Table 14) Whereas, mean 24-hour SBP showed heritability of 33% the mean 24-hour DBP showed the highest heritability of all analysed phenotypes - 41%.

The 2 remaining renal phenotypes log10 transformed age, age^2 and sex adjusted 24-hour urinary sodium and potassium excretions showed lower heritabilities estimated at 11.3% and 17.4%, respectively. (Table 14)

Table 14. Heritability of blood pressure and renal function phenotypes in the GRAPHIC Study.

Phenotype	No of informative families	h^2	SE	p
eGFR	520	0.384	0.038	$5.5x10^{-25}$
Clinic SBP	520	0.305	0.043	$2.5x10^{-13}$
Clinic DBP	520	0.317	0.041	$2.2x10^{-15}$
Mean 24-hour SBP	520	0.332	0.052	$8.4x10^{-14}$
Mean 24-hour DBP	520	0.411	0.054	$3.7x10^{-20}$
Log10 transformed 24-hour urinary sodium excretion	520	0.113	0.131	$6.9x10^{-10}$
Log10 transformed 24-hour urinary potassium excretion	520	0.174	0.098	$5.6x10^{-8}$

h^2 – estimate of narrow sense heritability, SE – standard error, p – level of statistical significance, eGFR – estimated glomerular filtration rate; SBP – systolic blood pressure, DBP – diastolic blood pressure.

Genes of U-II system, blood pressure and renal function – family based association analysis in the GRAPHIC Study

Quality control of genotyping

Prior to statistical analysis, genotyping quality of all SNPs was thoroughly checked. Based on visual inspection of scatter plots SNPs with poor quality genotyping were re-genotyped (rs9809263, rs11077991). The familial structure of the GRAPHIC Study allowed Mendelian inconsistencies to be identified. All SNPs genotyped in *UTS2*, *UTS2D* and *UTS2R* had MAF similar to those reported in National Centre for Biotechnology Information (NCBI) database for Caucasian population. All SNPs were common (MAF between 10.8% and 45.9%) except rs2890565 (MAF – 3.2%). The quality of genotyping was generally good and the proportion of missing genotypes was small. No SNPs violated the threshold of Hardy Weinberg equilibrium ($p<0.0001$).[67] The details of quality control results for each SNP are presented in Table 15.

Table 15. SNPs in the U-II system genes – quality control filters (GRAPHIC Study).

SNP	Gene	Chr	Position	MAF	Missingness (%)	HWE P-value
rs707472	UTS2	1	7906008	0.179	0.8	0.7493
rs228652	UTS2	1	7908888	0.252	0.7	0.9340
rs2890565	UTS2	1	7909737	0.032	0.7	0.6205
rs170629	UTS2	1	7910391	0.369	1.4	0.1385
rs228651	UTS2	1	7911099	0.374	1.3	0.1604
rs34305100	UTS2	1	7913029	0.197	3.3	0.2372
rs228648	UTS2	1	7913430	0.433	2.1	0.4409
rs13306061	UTS2	1	7913445	0.200	0.7	0.0241
rs4908486	UTS2	1	7914835	0.315	2.3	0.1255
rs707476	UTS2	1	7918106	0.311	0.7	0.1257
rs228638	UTS2	1	7918598	0.459	0.7	0.1862
rs2859389	UTS2	1	7920423	0.296	0.7	0.0611
rs531485	UTS2	1	7921952	0.220	0.7	0.2741
rs12566535	UTS2	1	7924094	0.116	0.7	0.2216
rs500508	UTS2	1	7927456	0.312	0.8	0.0412
rs579992	UTS2	1	7927981	0.108	0.7	0.1416
rs6772358	UTS2D	3	190986740	0.384	3.3	0.1597
rs17465071	UTS2D	3	190987067	0.257	2.1	0.3229
rs9809263	UTS2D	3	190989466	0.431	2.7	0.0820
rs2886062	UTS2D	3	190990163	0.324	2.5	0.1145
rs4399929	UTS2D	3	190994143	0.267	2.6	0.6876
rs6444532	UTS2D	3	190999760	0.353	3.3	0.0068
rs6788319	UTS2D	3	190999917	0.394	0.7	0.0307
rs7502620	UTS2R	17	80320561	0.293	2.7	0.7036
rs7211435	UTS2R	17	80323686	0.296	2.4	1.0000
rs11650469	UTS2R	17	80330676	0.322	2.1	0.4707
rs11077991	UTS2R	17	80330774	0.272	2.8	0.8114
rs6502104	UTS2R	17	80331778	0.325	1.7	0.4311

SNP – single nucleotide polymorphism, "rs" – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, Chr – chromosome, position – location of the polymorphism on chromosome, MAF – frequency of the minor allele in the parental generation, HWE P-value – the level of statistical significance in Hardy-Weinberg equilibrium test.

Genes of urotensin-II system and eGFR in the GRAPHIC Study – family based association analysis

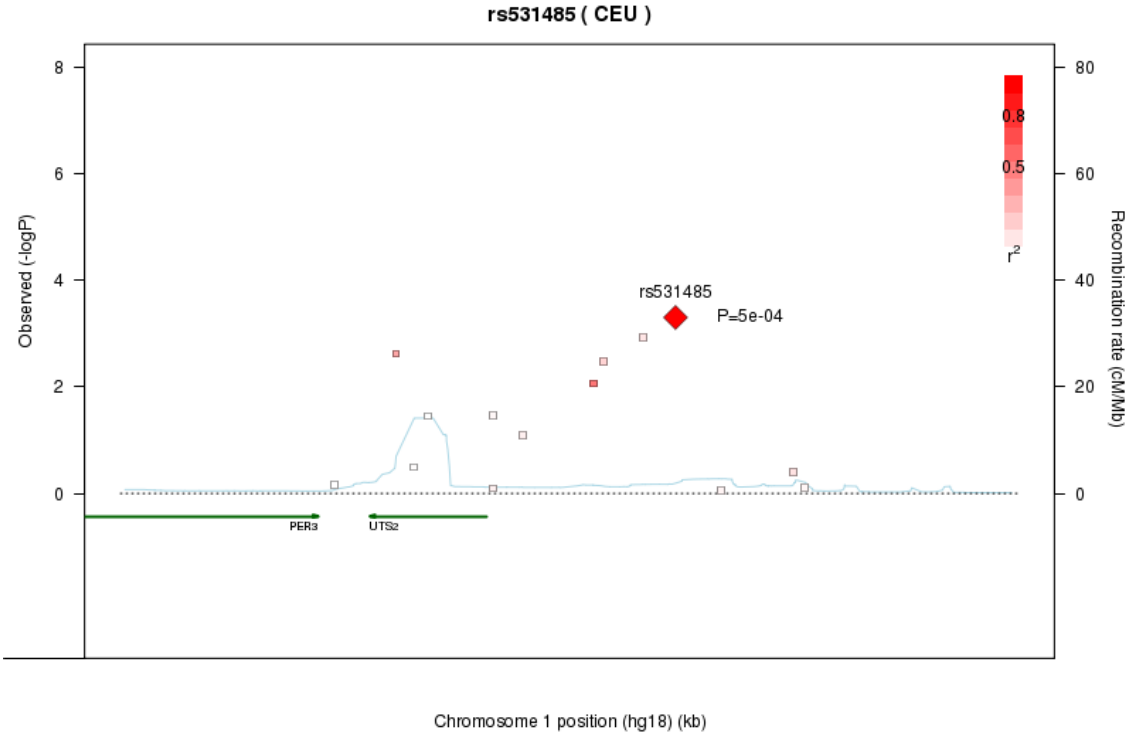
Family based association testing revealed that only one of the 28 SNPs (rs531485 in the 5' region of *UTS2*) showed significant association with adjusted eGFR (p=0.0005). (Table 16) Specifically, minor allele (C) of rs531485 was associated with significantly higher values of eGFR. The association remained significant in sensitivity analysis (additionally adjusted for BMI and clinic SBP) (p=0.0005). Two other SNPs in this locus (rs2859389 in the 5' region of *UTS2* and rs228652 located in the intronic region of *UTS2*) showed trends towards suggestive association (p=0.0012 and p=0.0024 respectively) but the signal was not strong enough to survive the multiple testing correction. (Figure 12) Both of these SNPs were in strong LD with the leading SNP rs531485 – $r^2_{rs531485xrs2859389}$=0.97; $r^2_{rs531485xrs228652}$=0.61. (Figure 13)

Table 16. Association between U-II system genes and estimated glomerular filtration rate in the GRAPHIC Study.

SNP	Gene	Alleles	MA	MAF	Informative families	Z	P-value
rs707472	UTS2	C/A	A	0.179	250	-0.39	0.7002
rs228652	UTS2	C/T	T	0.252	308	3.04	0.0024
rs2890565	UTS2	A/G	A	0.032	63	1.00	0.3190
rs170629	UTS2	C/T	T	0.369	368	-2.11	0.0353
rs228651	UTS2	A/G	A	0.374	367	-2.24	0.0251
rs3435100	UTS2	C/T	C	0.197	281	-0.58	0.5650
rs228648	UTS2	C/T	C	0.433	376	2.12	0.0345
rs13306061	UTS2	A/G	A	0.200	291	-0.25	0.8005
rs4908486	UTS2	G/A	A	0.315	347	1.73	0.0828
rs707476	UTS2	G/A	A	0.311	345	2.62	0.0087
rs228638	UTS2	G/T	T	0.459	389	2.94	0.0033
rs2859389	UTS2	A/G	A	0.296	341	-3.24	0.0012
rs531485	UTS2	A/C	C	0.220	291	3.48	0.0005
rs12566535	UTS2	T/C	T	0.116	184	0.17	0.8667
rs500508	UTS2	A/C	A	0.312	356	-0.85	0.3972
rs579992	UTS2	A/G	G	0.108	181	-0.28	0.7787
rs6772358	UTS2D	A/T	T	0.384	348	-0.04	0.9648
rs17465071	UTS2D	A/G	G	0.257	300	1.23	0.2179
rs9809263	UTS2D	C/T	T	0.431	353	0.06	0.9553
rs2886062	UTS2D	G/C	G	0.324	328	0.76	0.4482
rs4399929	UTS2D	A/G	G	0.267	311	-0.01	0.9905
rs6444532	UTS2D	G/T	T	0.353	310	2.16	0.0308
rs6788319	UTS2D	T/G	G	0.394	344	2.46	0.0139
rs7502620	UTS2R	A/G	A	0.293	334	-1.01	0.3151
rs7211435	UTS2R	C/T	C	0.296	328	-0.62	0.5338
rs11650469	UTS2R	A/G	G	0.322	345	-0.48	0.6337
rs11077991	UTS2R	C/G	G	0.272	321	-0.25	0.8060
rs6502104	UTS2R	A/C	C	0.325	343	-0.28	0.7767

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele in the parental generation, Z – Z score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance.

Figure 12. SNPs in urotensin II gene and estimated glomerular filtration rate in the GRAPHIC Study – regional association plot.



Y axis - –log10 transformed p-values from the family based association tests; X axis is the chromosomal position of the relevant SNPs; green line represent relevant genes; light blue line on the graph depicts recombination rate giving information about recombination hot-spots, breaking down the structure of LD. The top SNP rs531485 is marked as a diamond; colours of the remaining markers represents strength of the LD with the top marker; UTS2 – urotensin-II gene, PER3 – period homolog 3 gene.

Figure 13. Linkage disequilibrium structure of the urotensin-II locus.

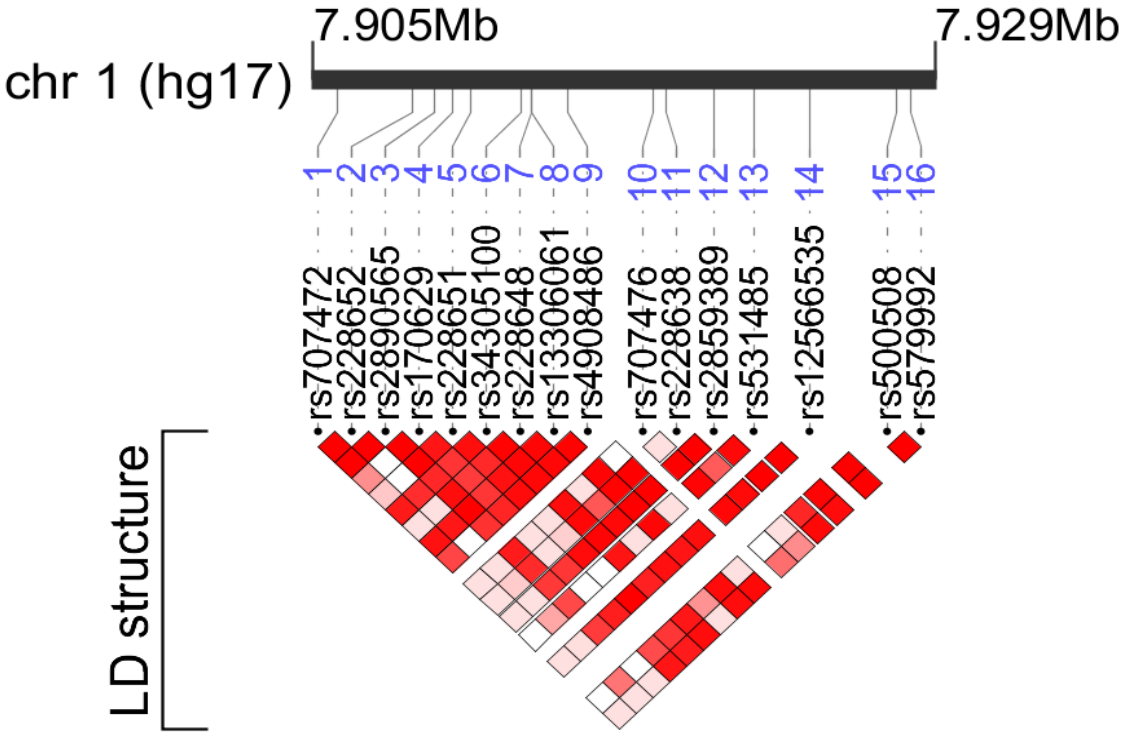


Figure presents LD structure of the UTS2 locus with the strength of LD coded with intensity of colour. Dark red corresponds to LD r^2=1 – maximal LD, white to r^2=0 – no LD.

Genes of U-II system and clinic systolic blood pressure in the GRAPHIC Study – family based association analysis

Only two SNPs in *UTS2* (rs707476, rs579992) were associated with clinic SBP in the GRAPHIC Study at the nominal level of statistical significance p=0.0303 and p=0.0402, respectively. (Table 17) Both SNPs mapped to the 5' region of *UTS2*. The magnitude of these associations was weak and neither survived the correction for multiple testing. None of *UTS2D* and *UTS2R* SNPs showed association with clinic SBP (Table 17). The SNP previously associated with eGFR (rs531485) showed no evidence of association with clinic SBP (p=0.4158); (in a model additionally adjusted for BMI and eGFR p=0.3906).

Table 17. Association between U-II system genes and clinic systolic blood pressure in the GRAPHIC Study.

SNP	Gene	Alleles	MA	MAF in parents	Informative families	Z	P-value
rs707472	UTS2	C/A	A	0.179	250	-1.27	0.2026
rs228652	UTS2	C/T	T	0.252	309	-0.62	0.5324
rs2890565	UTS2	A/G	A	0.032	63	-0.87	0.3842
rs170629	UTS2	C/T	T	0.369	369	1.07	0.2857
rs228651	UTS2	A/G	A	0.374	368	1.09	0.2746
rs3435100	UTS2	C/T	C	0.197	282	0.34	0.7343
rs228648	UTS2	C/T	C	0.433	377	-0.94	0.3471
rs13306061	UTS2	A/G	A	0.200	292	0.28	0.7775
rs4908486	UTS2	G/A	A	0.315	348	-0.13	0.8998
rs707476	UTS2	G/A	A	0.311	346	-2.17	0.0303
rs228638	UTS2	G/T	T	0.459	390	-0.50	0.6208
rs2859389	UTS2	A/G	A	0.296	342	1.28	0.2018
rs531485	UTS2	A/C	C	0.220	291	-0.81	0.4158
rs12566535	UTS2	T/C	T	0.116	184	0.50	0.6208
rs500508	UTS2	A/C	A	0.312	357	-1.01	0.3129
rs579992	UTS2	A/G	G	0.108	182	-2.05	0.0402
rs6772358	UTS2D	A/T	T	0.384	348	-1.00	0.3173
rs17465071	UTS2D	A/G	G	0.257	353	-0.88	0.3776
rs9809263	UTS2D	C/T	T	0.431	328	-1.24	0.2145
rs2886062	UTS2D	G/C	G	0.324	312	1.34	0.1817
rs4399929	UTS2D	A/G	G	0.267	310	-1.47	0.1406
rs6444532	UTS2D	G/T	T	0.353	301	-0.40	0.6866
rs6788319	UTS2D	T/G	G	0.394	345	-1.64	0.1006
rs7502620	UTS2R	A/G	A	0.293	334	-1.36	0.1739
rs7211435	UTS2R	C/T	C	0.296	328	-0.92	0.3591
rs11650469	UTS2R	A/G	G	0.322	346	1.17	0.2424
rs11077991	UTS2R	C/G	G	0.272	322	0.10	0.9247
rs6502104	UTS2R	A/C	C	0.325	344	1.13	0.2598

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele in the parental generation, Z – Z score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance.

Genes of the urotensin-II system and clinic diastolic blood pressure – family based association analysis

There was no association between clinic DBP (adjusted for age, age^2 sex and medication) and *UTS2*, *UTS2D*, *UTS2R* SNPs in family based association analysis. (Table 18) rs531485, which showed association with eGFR in the GRAPHIC Study was weakly associated with clinic DBP (p=0.0405), but this association did not retain its statistical significance after the correction for multiple testing. In the sensitivity analysis (additionally adjusted for BMI and eGFR) the association was slightly stronger p=0.03828.

Table 18. Association between urotensin-II system genes and clinic diastolic blood pressure in the GRAPHIC Study.

SNP	Gene	Alleles	MA	MAF	Informative families	Z	P-value
rs707472	UTS2	C/A	A	0.179	250	-0.31	0.7575
rs228652	UTS2	C/T	T	0.252	309	0.42	0.6714
rs2890565	UTS2	A/G	A	0.032	63	0.77	0.4419
rs170629	UTS2	C/T	T	0.369	369	-1.01	0.3131
rs228651	UTS2	A/G	A	0.374	368	-0.83	0.4055
rs3435100	UTS2	C/T	C	0.197	282	0.76	0.4463
rs228648	UTS2	C/T	C	0.433	377	0.32	0.7509
rs13306061	UTS2	A/G	A	0.200	292	0.59	0.5585
rs4908486	UTS2	G/A	A	0.315	348	0.97	0.3301
rs707476	UTS2	G/A	A	0.311	346	0.45	0.6563
rs228638	UTS2	G/T	T	0.459	390	1.91	0.0566
rs2859389	UTS2	A/G	A	0.296	342	-1.95	0.0515
rs531485	UTS2	A/C	C	0.220	291	2.05	0.0405
rs12566535	UTS2	T/C	T	0.116	184	1.10	0.2718
rs500508	UTS2	A/C	A	0.312	357	-0.30	0.7674
rs579992	UTS2	A/G	G	0.108	182	-1.26	0.2091
rs6772358	UTS2D	A/T	T	0.384	348	-1.04	0.2982
rs17465071	UTS2D	A/G	G	0.257	301	-0.22	0.8245
rs9809263	UTS2D	C/T	T	0.431	353	-0.24	0.8109
rs2886062	UTS2D	G/C	G	0.324	328	-0.99	0.3227
rs4399929	UTS2D	A/G	G	0.267	312	1.69	0.0908
rs6444532	UTS2D	G/T	T	0.353	310	-2.00	0.0461
rs6788319	UTS2D	T/G	G	0.394	345	-1.68	0.0933
rs7502620	UTS2R	A/G	A	0.293	334	-0.27	0.7906
rs7211435	UTS2R	C/T	C	0.296	328	-0.23	0.8198
rs11650469	UTS2R	A/G	G	0.322	346	1.41	0.1599
rs11077991	UTS2R	C/G	G	0.272	322	0.51	0.6139
rs6502104	UTS2R	A/C	C	0.325	344	1.32	0.1868

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele in the parental generation, Z – Z score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance.

Genes of U-II system and 24-hour urinary sodium excretion – family based association analysis

None of the *UTS2*, *UTS2D*, *UTS2R* SNPs was associated with log10 transformed 24-hour urinary sodium excretion (adjusted for age, age^2 and sex) in the GRAPHIC Study. (Table 19) Three SNPs in *UTS2* (rs170629, rs228651, rs2859389) showed nominal association with adjusted log10 transformed 24-hour urinary sodium excretion (p-values 0.0067, 0.0094 and 0.0407, respectively) though none survived correction for multiple testing. The rs531485 polymorphism which showed association with eGFR was not associated with log10 24-hour urinary sodium excretion (p=0.8695). In the sensitivity analysis (additionally adjusted for BMI) rs531485 did not show statistically significant association with log10 24-hour urinary sodium excretion (p=0.8708).

Table 19. Association between U-II system genes and log10 transformed 24-hour urinary sodium excretion in the GRAPHIC Study.

SNP	Gene	Alleles	MA	MAF	Informative families	Z	P-value
rs707472	UTS2	C/A	A	0.179	248	0.1	0.9206
rs228652	UTS2	C/T	T	0.252	304	0.80	0.4214
rs2890565	UTS2	A/G	A	0.032	63	-1.36	0.1723
rs170629	UTS2	C/T	T	0.369	366	-2.71	0.0067
rs228651	UTS2	A/G	A	0.374	364	2.59	0.0094
rs3435100	UTS2	C/T	C	0.197	276	-0.04	0.9645
rs228648	UTS2	C/T	C	0.433	373	-1.33	0.1832
rs13306061	UTS2	A/G	A	0.200	287	-0.02	0.9858
rs4908486	UTS2	G/A	A	0.315	343	-0.86	0.3875
rs707476	UTS2	G/A	A	0.311	341	-0.44	0.6584
rs228638	UTS2	G/T	T	0.459	385	0.74	0.4587
rs2859389	UTS2	A/G	A	0.296	338	2.05	0.0407
rs531485	UTS2	A/C	C	0.220	287	-0.16	0.8695
rs12566535	UTS2	T/C	T	0.116	183	1.11	0.2652
rs500508	UTS2	A/C	A	0.312	352	-0.71	0.4757
rs579992	UTS2	A/G	G	0.108	179	0.94	0.3449
rs6772358	UTS2D	A/T	T	0.384	344	0.76	0.4457
rs17465071	UTS2D	A/G	G	0.257	296	-0.04	0.9686
rs9809263	UTS2D	C/T	T	0.431	349	1.14	0.2541
rs2886062	UTS2D	G/C	G	0.324	326	1.85	0.0648
rs4399929	UTS2D	A/G	G	0.267	308	1.09	0.2743
rs6444532	UTS2D	G/T	T	0.353	306	-0.38	0.7007
rs6788319	UTS2D	T/G	G	0.394	340	-0.11	0.9145
rs7502620	UTS2R	A/G	A	0.293	331	0.04	0.9661
rs7211435	UTS2R	C/T	C	0.296	325	0.55	0.5851
rs11650469	UTS2R	A/G	G	0.322	340	-0.51	0.6096
rs11077991	UTS2R	C/G	G	0.272	318	-0.39	0.6998
rs6502104	UTS2R	A/C	C	0.325	338	-0.30	0.7616

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele in the parental generation, Z – Z score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance.

Genes of U-II system and 24-hour urinary potassium excretion – family based association

Family based association analysis showed that 2 SNPs in *UTS2* (rs170629, p=0.0258; rs2859389, p=0.0182) and 1 in *UTS2D* (rs9809263, p=0.0446) were nominally associated with log10 transformed 24-hour urinary potassium excretion. (Table 20) However, none of these SNPs retained their associations with the phenotype after applying correction for multiple testing. The SNPs in *UTS2R* were not associated with mean 24-hour urinary potassium excretion in the family based association analysis. (Table 20) rs531485 previously associated with eGFR, showed no association with log10 transformed 24-hour urinary potassium excretion (p=0.2088); this was consistent in the sensitivity analysis (additionally adjusted for BMI) p=0.2058.

Table 20. Association between U-II system genes and log10 transformed 24-hour urinary potassium excretion in the GRAPHIC Study.

SNP	Gene	Alleles	MA	MAF	Informative families	Z	P-value
rs707472	UTS2	C/A	A	0.179	248	1.36	0.1735
rs228652	UTS2	C/T	T	0.252	304	1.52	0.1276
rs2890565	UTS2	A/G	A	0.032	63	-1.22	0.2212
rs170629	UTS2	C/T	T	0.369	366	-2.23	0.0258
rs228651	UTS2	A/G	A	0.374	364	1.79	0.0742
rs3435100	UTS2	C/T	C	0.197	276	0.37	0.7123
rs228648	UTS2	C/T	C	0.433	373	-1.39	0.1623
rs13306061	UTS2	A/G	A	0.200	287	0.25	0.8002
rs4908486	UTS2	G/A	A	0.315	343	-0.68	0.4997
rs707476	UTS2	G/A	A	0.311	341	-1.38	0.1682
rs228638	UTS2	G/T	T	0.459	385	1.65	0.0989
rs2859389	UTS2	A/G	A	0.296	338	2.36	0.0182
rs531485	UTS2	A/C	C	0.220	287	1.26	0.2088
rs12566535	UTS2	T/C	T	0.116	183	0.52	0.6042
rs500508	UTS2	A/C	A	0.312	352	0.18	0.8545
rs579992	UTS2	A/G	G	0.108	179	-0.09	0.9214
rs6772358	UTS2D	A/T	T	0.384	344	1.15	0.2488
rs17465071	UTS2D	A/G	G	0.257	296	0.02	0.9828
rs9809263	UTS2D	C/T	T	0.431	349	2.01	0.0446
rs2886062	UTS2D	G/C	G	0.324	326	1.94	0.0530
rs4399929	UTS2D	A/G	G	0.267	308	1.25	0.2122
rs6444532	UTS2D	G/T	T	0.353	306	-1.65	0.0988
rs6788319	UTS2D	T/G	G	0.394	340	1.17	0.2422
rs7502620	UTS2R	A/G	A	0.293	331	-1.38	0.1671
rs7211435	UTS2R	C/T	C	0.296	325	-0.90	0.3680
rs11650469	UTS2R	A/G	G	0.322	340	0.58	0.5617
rs11077991	UTS2R	C/G	G	0.272	318	0.42	0.6766
rs6502104	UTS2R	A/C	C	0.325	338	0.49	0.6226

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele in the parental generation, Z – Z score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance.

Genes of the U-II system and mean 24-hour systolic blood pressure – family based association analysis

Taking advantage of the availability of 24-hour ambulatory BP monitoring in the GRAPHIC Study, I examined if the *UTS2*, *UTS2D* and *UTS2R* SNPs are associated with mean 24-hour SBP. The family based association analysis revealed that only one common polymorphism (rs707476), located in the 5' region of *UTS2*, showed nominal association with mean 24-hour SBP (p=0.0046). (Table 21) However, after correction for multiple testing this association was no longer statistically significant. None of the SNPs in *UTS2D* or *UTS2R* showed association with mean 24-hour SBP. (Table 21) The polymorphism previously associated with eGFR (rs531485), showed no association with mean 24-hour SBP (p=0.0660).The association signal of rs531485 with mean 24-hour SBP became nominally significant after additional adjustment for BMI and eGFR (p=0.0332).

Table 21. Association between urotensin-II system genes and mean 24-hour systolic blood pressure in the GRAPHIC Study.

SNP	Gene	Alleles	MA	MAF	Informative families	Z	P-value
rs707472	UTS2	C/A	A	0.179	250	-1.05	0.2943
rs228652	UTS2	C/T	T	0.252	309	-1.22	0.2216
rs2890565	UTS2	A/G	A	0.032	63	0.76	0.4464
rs170629	UTS2	C/T	T	0.369	369	0.64	0.5213
rs228651	UTS2	A/G	A	0.374	368	0.31	0.7564
rs3435100	UTS2	C/T	C	0.197	282	1.44	0.1493
rs228648	UTS2	C/T	C	0.433	377	-0.45	0.6565
rs13306061	UTS2	A/G	A	0.200	292	1.28	0.1999
rs4908486	UTS2	G/A	A	0.315	348	0.40	0.6911
rs707476	UTS2	G/A	A	0.311	346	-2.83	0.0046
rs228638	UTS2	G/T	T	0.459	390	-0.10	0.9216
rs2859389	UTS2	A/G	A	0.296	342	0.92	0.3596
rs531485	UTS2	A/C	C	0.220	291	-1.84	0.0660
rs12566535	UTS2	T/C	T	0.116	184	0.36	0.7206
rs500508	UTS2	A/C	A	0.312	357	0.51	0.6084
rs579992	UTS2	A/G	G	0.108	182	-1.33	0.1842
rs6772358	UTS2D	A/T	T	0.384	348	0.11	0.9093
rs17465071	UTS2D	A/G	G	0.257	301	-0.95	0.3441
rs9809263	UTS2D	C/T	T	0.431	353	1.12	0.2611
rs2886062	UTS2D	G/C	G	0.324	328	0.19	0.8477
rs4399929	UTS2D	A/G	G	0.267	312	0.33	0.7437
rs6444532	UTS2D	G/T	T	0.353	310	-0.06	0.9533
rs6788319	UTS2D	T/G	G	0.394	345	0.06	0.9505
rs7502620	UTS2R	A/G	A	0.293	328	0.24	0.8095
rs7211435	UTS2R	C/T	C	0.296	322	0.69	0.4909
rs11650469	UTS2R	A/G	G	0.322	338	0.67	0.5008
rs11077991	UTS2R	C/G	G	0.272	314	0.88	0.3810
rs6502104	UTS2R	A/C	C	0.325	336	1.04	0.2986

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele in the parental generation, Z – Z score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance.

Genes of the U-II system and mean 24-hour diastolic blood pressure – family based association

I also examined associations between *UTS2*, *UTS2D* and *UTS2R* and mean 24-hour DBP. No association was identified between SNPs of *UTS2* and *UTS2R* and mean 24-hour DBP in the GRAPHIC Study. (Table 22) An intronic polymorphism in *UTS2D* (rs9809263) showed statistically significant, nominal association with mean 24-hour DBP (p=0.0170) but this did not survive multiple testing correction. (Table 22) The polymorphism associated with eGFR (rs531485) in the GRPAHIC Study showed no association with mean 24-hour DBP (p= 0.6464), this remained unchanged even after additional adjustment for BMI and eGFR (sensitivity analysis p=0.7149).

Table 22. Association between U-II system genes and mean 24-hour diastolic blood pressure in the GRAPHIC Study.

SNP	Gene	Alleles	MA	MAF	Informative families	Z	P-value
rs707472	UTS2	C/A	A	0.179	250	-0.21	0.8369
rs228652	UTS2	C/T	T	0.252	309	0.57	0.5683
rs2890565	UTS2	A/G	A	0.032	63	1.72	0.0861
rs170629	UTS2	C/T	T	0.369	369	-0.70	0.4854
rs228651	UTS2	A/G	A	0.374	368	-0.64	0.5199
rs3435100	UTS2	C/T	C	0.197	282	-0.43	0.6672
rs228648	UTS2	C/T	C	0.433	377	-0.57	0.5686
rs13306061	UTS2	A/G	A	0.200	292	-0.32	0.7527
rs4908486	UTS2	G/A	A	0.315	348	-0.40	0.6915
rs707476	UTS2	G/A	A	0.311	346	-0.42	0.6730
rs228638	UTS2	G/T	T	0.459	390	0.34	0.7345
rs2859389	UTS2	A/G	A	0.296	342	-0.78	0.4332
rs531485	UTS2	A/C	C	0.220	291	0.46	0.6464
rs12566535	UTS2	T/C	T	0.116	184	1.16	0.2468
rs500508	UTS2	A/C	A	0.312	357	-0.93	0.3512
rs579992	UTS2	A/G	G	0.108	182	-0.68	0.4939
rs6772358	UTS2D	A/T	T	0.384	348	1.25	0.2125
rs17465071	UTS2D	A/G	G	0.257	301	-1.80	0.0713
rs9809263	UTS2D	C/T	T	0.431	353	2.39	0.0170
rs2886062	UTS2D	G/C	G	0.324	328	0.81	0.4164
rs4399929	UTS2D	A/G	G	0.267	312	0.86	0.3875
rs6444532	UTS2D	G/T	T	0.353	310	-1.42	0.1567
rs6788319	UTS2D	T/G	G	0.394	345	-1.36	0.1729
rs7502620	UTS2R	A/G	A	0.293	334	0.28	0.7807
rs7211435	UTS2R	C/T	C	0.296	328	0.25	0.8043
rs11650469	UTS2R	A/G	G	0.322	346	0.15	0.8830
rs11077991	UTS2R	C/G	G	0.272	322	0.20	0.8393
rs6502104	UTS2R	A/C	C	0.325	344	0.19	0.8481

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele in the parental generation, Z – Z score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance.

Effect size of the identified genetic associations in the GRAPHIC Study

To quantify the effect size of rs531485 on eGFR in the GRAPHIC Study I used MERLIN variance component method (that provides β-coefficients and standard errors). In this MERLIN-based analysis each minor allele copy of rs531485 was associated with 1.04 mL/min*1.73m^2 increase in eGFR (SE=0.47, p=0.0281).

Summary of the findings from the genetic association analysis in the discovery cohort (GRAPHIC Study)

The detailed family-based analysis of *UTS2*, *UTS2D* and *UTS2R* in the GRPAHIC Study showed that only 1 common SNP (rs531485) in *UTS2* showed significant association with eGFR. This association survived multiple testing correction and thus was unlikely to be a result of type 1 statistical error. Importantly, two SNPs in strong LD with rs531485 also showed association with eGFR providing additional evidence that the observed association signal was not a result of genotyping error. None of the nominal associations with the other examined phenotypes remained significant after the correction for multiple testing. To check the robustness of the association between *UTS2* and eGFR, the significant SNP (rs531485) was taken forward for replication in additional cohorts with available information on renal function (eGFR).

Urotensin-II and renal function – replication analysis

Association of rs531485 with eGFR in replication cohorts

Following studies in the discovery cohort (GRAPHIC Study) rs531485 was genotyped in a total of 3521 individuals from 5 other studies (SHS, SCS families, SCS extension, YMCA study and YMCA extension). The collected replication resource consisted of 2 family-based studies (SCS families and SHS) with a total of 420 pedigrees. In addition, 2189 unrelated individuals were available from 3 studies (SCS extension, YMCA study and YMCA extension). After genotyping and prior to statistical analysis in each individual study genotyping scatter plots were manually assed and quality filters (10% missingness rate, violation of HWE or MAF< 5%) applied. In addition, the presence of Mendelian errors was assessed in the SHS and SCS families, pedigrees with errors were removed from further analysis.

The overall quality of genotyping of rs531485 in the replication cohorts was good. (Table 23)

The family-based analysis revealed that rs531485 was not associated with eGFR in either of the two family studies (SCS families or SHS) even at a nominal level of statistical significance. (Table 24) This was also confirmed in MERLIN-based analysis.

In cohorts of unrelated subjects rs531485 showed association with eGFR only in YMCA extension, where each copy of its minor allele (C) was associated

with 3.29 mL/min*1.73m2 decrease in eGFR. (Table 25) However, the direction of this association was opposite to the one observed in the GRAPHIC Study.

Table 23. Quality control of rs531485 genotyping in Silesian Cardiovascular Study families, Silesian Hypertension Study, Silesian Cardiovascular Study extension, Young Men Cardiovascular Association study and Young Men Cardiovascular Association study extension.

Study	MAF	Missingness (%)	HWE P-value
SCS families	0.249	2.7	0.4780
SHS	0.273	5.2	0.0822
SCS extension	0.242	1.9	0.8451
YMCA study	0.249	3.3	0.2984
YMCA extension	0.228	2.9	0.3915

SCS families – families from Silesian Cardiovascular Study, SHS - Silesian Hypertension Study, SCS extension – unrelated subjects from Silesian Cardiovascular Study, YMCA study– Young Men Cardiovascular Association study, YMCA extension – extension of Young Men Cardiovascular Association study, MAF – frequency of the minor allele (calculated in parental generation for SCS families and SHS), HWE P-value – the level of statistical significance in Hardy-Weinberg equilibrium test.

Table 24. Association of rs531485 and estimated glomerular filtration rate in the Silesian Cardiovascular Study families and Silesian Hypertension Study.

Study	Minor allele	No of informative families	Z-score	P-value	β	SE	P-value*
		Family-based association test			MERLIN		
SCS families	C	101	0.42	0.6779	1.34	1.11	0.2288
SHS	C	83	0.39	0.6984	-0.54	0.73	0.4624

SCS families – families from Silesian Cardiovascular Study, SHS – Silesian Hypertension Study; Z-score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance, β – effect size per each copy of minor allele calculated using MERLIN, SE – standard error, P-value* – significance of the MERLIN estimate.

Table 25. Association of rs531485 and estimated glomerular filtration rate in the Silesian Cardiovascular Study extension, Young Men Cardiovascular Association study and Young Men Cardiovascular Association study extension.

Study	Minor allele	No of informative subjects	β	SE	P-value
SCS extension	C	430	-0.42	0.95	0.6564
YMCA study	C	1119	-0.06	1.21	0.9612
YMCA extension	C	539	-3.29	1.32	0.0130

SCS extension – unrelated subjects from Silesian Cardiovascular Study, YMCA study – Young Men Cardiovascular Association study, YMCA extension – Young Men Cardiovascular Association study extension, β – effect size per each copy of minor allele, SE – standard error, P-value – level of statistical significance.

Association of rs531485 and eGFR in the CoLaus study – in silico replication

To further strengthen replication of the findings from the discovery cohort I seek replication in a large cohort of white Caucasian origin with available data on eGFR - the CoLaus study (6188 subjects). The genotyping data in CoLaus study come from Affymetrix 500K SNP chip, which does not contain rs531485. Therefore, the genotypes of rs531485 have been imputed based on the available nearby markers using Impute 0.3 software. The accuracy of imputation was good – the coefficient of accuracy 0.94. Subsequently, the imputed genotypes were used in the linear regression model (adjusted for age and sex) to test for association between number of copies of minor allele or rs531485 and eGFR. rs531485 did not show significant association with adjusted eGFR (β=0.04±0.35; p=0.9019). Also, there was no association between number of copies of minor allele of rs531485 and adjusted clinic SBP and clinic DBP (β=0.14±0.42; p=0.7339) and (β=0.28±0.27; p=0.2900), respectively.

rs531485 polymorphism and eGFR in replication cohorts – inverse variance meta-analysis

The results of individual associations between rs531485 and eGFR in all replication studies (SHS, SCS families, SCS extension, YMCA study, YMCA extension and CoLaus study – altogether 8724 subjects) were meta-analysed using inverse variance weighted fixed effect size model. This meta-analysis showed that rs531485 was not associated with eGFR (p=0.5834). (Table 26) There was no significant heterogeneity between individual replication studies (p=0.1190).

Table 26. rs531485 polymorphism and eGFR in the replication resource – meta-analysis.

Study	Informative subjects	β±SE	P-value	P-value*
SCS families	684	1.34±1.11	0.2288	
SHS	596	-0.54±0.73	0.4624	
SCS extension	430	-0.42±0.95	0.6564	
YMCA study	1119	-0.06±1.21	0.9612	0.1494
YMCA extension	539	-3.29±1.32	0.0130	
CoLaus study	5356	0.04±0.35	0.9019	
Combined	**8724**	**-0.15±0.28**	**0.5834**	

SCS families – families from Silesian Cardiovascular Study SHS –- Silesian Hypertension Study, SCS extension – Silesian Cardiovascular Study extension, YMCA study –- Young Men Cardiovascular Association study, YMCA extension – Young Men Cardiovascular Association study extension, CoLaus study – results of *in silico* replication in the CoLaus cohort, β – effect size, SE – standard error, P-value – level of statistical significance, P-value* - level of the statistical significance in the $\chi 2$ test of heterogeneity.

Summary of association findings - rs531485 and eGFR in the replication resource

The extensive replication experiment (based on direct genotyping in 3521 individuals from 5 studies and *in silico* replication in 5356 unrelated subjects from the general population (altogether 8724 subjects) did not confirm the association between the *UTS2* polymorphism (rs5314850) and eGFR. This suggests that this polymorphic variant is unlikely to contribute to population variation in eGFR.

Genes of urotensin-II system, structural renal damage and end stage renal disease

Genes of U-II system and urinary excretion of albumin (urinary albumin: creatinine ratio)

To examine if genetic variation in the U-II system is associated with urinary albumin: creatinine ratio; a total of 19 tagging SNPs in the candidate pathway [*UTS2* (n=8), *UTS2D* (n=6) and *UTS2R* (n=5)] were selected for genotyping. These SNPs captured >80% of the common genetic variation in *UTS2*, *UTS2D* and *UTS2R* with r^2=0.8 and MAF>0.1. The SNPs were genotyped in both SCS families and in SCS extension (where information on this phenotype was available) and then meta-analysed. Prior to statistical analysis the usual quality control checks were applied (Table 27) and one polymorphism (rs9809263) was excluded from further analysis because of excessive missing genotypes (12.9%). None of the SNPs that passed quality control filters showed association with log-transformed urinary albumin: creatinine ratio in family based analysis. (Table 28) In the SCS extension only 1 SNP (rs11077991 in *UTS2R*) showed nominal association with urinary albumin: creatinine ratio (p=0.036) - each copy of the minor allele of this SNP was associated with 0.05 increase in log-transformed urinary albumin: creatinine ratio. (Table 28) However, rs11077991 did not show association with urinary excretion of albumin in the meta-analysis of SCS families and SCS extension.

(Table 28) Likewise, no other SNP in *UTS2* or *UTS2D* showed association with this phenotype in meta-analysis of SCS families and SCS extension. (Table 28)

Table 27. SNPs in urotensin-II system genes – quality control filters in Silesian Cardiovascular Study families and Silesian Cardiovascular Study extension.

SNP	Gene	Chr	Position	SCS families			SCS extension		
				MAF	Missingness (%)	HWE P-value	MAF	Missingness (%)	HWE P-value
rs228652	UTS2	1	7908888	0.275	6.3	0.8909	0.268	5.5	0.7106
rs170629	UTS2	1	7910391	0.446	2.8	0.3866	0.427	1.9	0.1886
rs228651	UTS2	1	7911099	0.433	2.9	1	0.415	2.1	0.5070
rs34305100	UTS2	1	7913029	0.157	6.1	0.8365	0.172	4.3	0.3711
rs228648	UTS2	1	7913430	0.415	4.4	0.6597	0.422	3.1	0.6052
rs13306061	UTS2	1	7913445	0.161	6.5	0.5517	0.174	4.7	0.4486
rs2859389	UTS2	1	7920423	0.354	3.6	1	0.339	3.4	0.5199
rs531485	UTS2	1	7921952	0.249	2.7	0.4780	0.242	1.9	0.8451
rs6772358	UTS2D	3	190986740	0.354	7.7	0.4770	0.348	4.7	0.6308
rs17465071	UTS2D	3	190987067	0.288	5.8	0.6915	0.288	5.4	1
rs9809263	UTS2D	3	190989466	0.447	12.9	0.7886	0.431	3.6	0.526
rs2886062	UTS2D	3	190990163	0.258	5.3	0.3953	0.269	4.8	0.8536
rs4399929	UTS2D	3	190994143	0.248	4.4	0.5663	0.242	1.9	0.4947
rs6444532	UTS2D	3	190999760	0.363	5.3	0.1280	0.345	3.3	0.3385
rs7502620	UTS2R	17	80320561	0.294	5.1	0.6941	0.288	4.7	0.2157
rs7211435	UTS2R	17	80323686	0.279	6.9	0.6886	0.275	4.4	1
rs11650469	UTS2R	17	80330676	0.295	4.6	0.5181	0.297	4.8	0.0362

SNP	Gene	Chr	Position	SCS families			SCS extension		
				MAF	Missingness (%)	HWE P-value	MAF	Missingness (%)	HWE P-value
rs11077991	UTS2R	17	80330774	0.239	3.8	0.0540	0.256	3.3	0.3933
rs6502104	UTS2R	17	80331778	0.286	5.4	0.2850	0.297	4.4	0.1925

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, Chr – chromosome, position – location of the polymorphism on chromosome, MAF – frequency of the minor allele in the parental generation, HWE P-value – the level of statistical significance in Hardy-Weinberg equilibrium test.

Table 28. Association between urotensin-II system genes and urinary albumin: creatinine ratio in Silesian Cardiovascular Study families and Silesian Cardiovascular Study extension.

SNP	Gene	SCS families FBAT			SCS families MERLIN			SCS extension			Meta-analysis		
		Informative families (n)	Z	P-value	β	SE	P-value	β	SE	P-value	β	SE	P-value
rs228652	UTS2	100	0.75	0.4513	-0.007	0.031	0.8137	-0.033	0.026	0.202	-0.022	0.019	0.2637
rs170629	UTS2	127	1.35	0.1756	-0.024	0.029	0.4206	0.003	0.023	0.906	-0.007	0.018	0.6803
rs228651	UTS2	126	1.45	0.1460	-0.014	0.029	0.6235	0.010	0.024	0.667	0.0002	0.018	0.9895
rs34305100	UTS2	71	-0.15	0.8783	0.061	0.040	0.1255	-0.056	0.030	0.066	-0.014	0.024	0.5631
rs228648	UTS2	121	-1.19	0.2323	-0.007	0.029	0.8187	-0.032	0.024	0.181	-0.022	0.018	0.2376
rs13306061	UTS2	75	-0.43	0.6658	0.050	0.039	0.1997	-0.026	0.030	0.388	0.002	0.024	0.9245
rs2859389	UTS2	100	0.75	0.4513	0.013	0.030	0.6585	-0.002	0.025	0.946	0.004	0.019	0.8292
rs531485	UTS2	127	1.35	0.1756	-0.029	0.032	0.3725	0.014	0.027	0.596	-0.004	0.021	0.8507
rs6772358	UTS2D	99	-0.31	0.7551	-0.009	0.030	0.7554	0.012	0.025	0.620	0.003	0.019	0.8597
rs17465071	UTS2D	101	1.66	0.0966	-0.024	0.030	0.4407	0.022	0.026	0.394	0.003	0.020	0.8801
rs2886062	UTS2D	89	-0.89	0.3742	-0.011	0.030	0.7616	-0.014	0.026	0.593	-0.013	0.021	0.5382
rs4399929	UTS2D	101	-0.82	0.4121	-0.015	0.030	0.6830	0.049	0.026	0.063	0.027	0.021	0.1991
rs6444532	UTS2D	115	-1.03	0.3056	-0.009	0.030	0.7832	0.045	0.024	0.064	0.025	0.189	0.1919
rs7502620	UTS2R	95	-0.21	0.8362	-0.006	0.032	0.8592	-0.021	0.025	0.402	-0.015	0.019	0.4639
rs7211435	UTS2R	97	-0.14	0.8906	-0.018	0.032	0.5730	-0.030	0.026	0.243	-0.025	0.020	0.2112
rs11650469	UTS2R	107	1.03	0.3036	-0.024	0.031	0.4463	0.026	0.024	0.283	0.007	0.019	0.7019

SNP	Gene	SCS families FBAT			SCS families MERLIN			SCS extension			Meta-analysis		
		Informative families (n)	Z	P-value	β	SE	P-value	β	SE	P-value	β	SE	P-value
rs11077991	UTS2R	107	1.06	0.2893	-0.041	0.034	0.2277	0.054	0.026	0.036	0.019	0.020	0.3589
rs6502104	UTS2R	105	0.13	0.8949	-0.027	0.032	0.4004	0.036	0.025	0.1450	0.012	0.019	0.5383

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, FBAT – family-based association test, Z – Z-score – statistic calculated by the family-based association test showing the direction of association, , SCS families MERLIN – statistics obtained using MERLIN, β – effect size per each copy of minor allele, SE – standard error, P-value – level of statistical significance.

Genes of U-II system and end stage renal disease in the Polish End Stage Renal Disease Study (PERSD) – family based association analysis

The same set of 19 tagging SNPs in *UTS2* (n=8), *UTS2D* (n=6) and *UTS2R* (n=5) was genotyped in PESRD study to examine their association with ESRD. This cohort consists of 218 parents (mean age of mothers: 42.2±7.8 years; mean age of fathers: 45.1±7.8 years), 59 sons (mean age: 14.9±6.6 years) and 50 daughters (mean age: 16.1±6.1 years).

Following genotyping standard quality checks were applied. (Table 29)

Family based association analysis revealed that alleles of two SNPs (rs7502620, rs7211435 in the 5' region of *UTS2R*) were under-transmitted from parents to offspring with ESRD (p=0.0243 and p=0.0416, respectively). (Table 30) However, neither of these associations survived the correction for multiple testing.

Table 29. SNPs in U-II system genes – quality control filters in Polish End Stage Renal Disease Study.

SNP	Gene	Chr	Position	MAF	MA	Missingness (%)	HWE P-value
rs228652	UTS2	1	7908888	0.245	T	7.3	0.1809
rs170629	UTS2	1	7910391	0.415	T	2.7	0.1184
rs228651	UTS2	1	7911099	0.407	A	3.3	0.5676
rs34305100	UTS2	1	7913029	0.223	C	3.0	0.4268
rs228648	UTS2	1	7913430	0.455	C	4.2	0.6792
rs13306061	UTS2	1	7913445	0.228	A	3.9	0.5586
rs2859389	UTS2	1	7920423	0.312	A	3.3	0.2612
rs531485	UTS2	1	7921952	0.246	C	8.9	1
rs6772358	UTS2D	3	190986740	0.385	T	4.2	0.6619
rs17465071	UTS2D	3	190987067	0.278	G	2.1	0.2359
rs9809263	UTS2D	3	190989466	0.441	T	1.8	0.7811
rs2886062	UTS2D	3	190990163	0.290	G	3.6	1
rs4399929	UTS2D	3	190994143	0.222	G	3.6	1
rs6444532	UTS2D	3	190999760	0.341	T	3.6	0.6481
rs7502620	UTS2R	17	80320561	0.299	A	3.0	0.4128
rs7211435	UTS2R	17	80323686	0.297	C	3.3	0.4151
rs11650469	UTS2R	17	80330676	0.303	G	3.0	0.4160
rs11077991	UTS2R	17	80330774	0.250	G	2.4	0.7175
rs6502104	UTS2R	17	80331778	0.292	C	2.1	0.6195

SNP – single nucleotide polymorphism, "rs" – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, Chr – chromosome, position – location of the polymorphism on chromosome, MAF – frequency of the minor allele in the parental generation, HWE P-value – the level of statistical significance in Hardy-Weinberg equilibrium test.

Table 30. Association between U-II system genes and end stage renal disease in the Polish End Stage Renal Disease Study.

SNP	Gene	Alleles	MA	MAF	Informative families	Z	P-value
rs228652	UTS2	C/T	T	0.252	46	-0.927	0.3538
rs170629	UTS2	C/T	T	0.369	69	-0.64	0.5224
rs228651	UTS2	A/G	A	0.374	71	-0.834	0.4042
rs3435100	UTS2	C/T	C	0.197	54	0.465	0.6419
rs228648	UTS2	C/T	C	0.433	68	1.32	0.1869
rs13306061	UTS2	A/G	A	0.200	52	0.471	0.6374
rs2859389	UTS2	A/G	A	0.296	60	-0.697	0.4855
rs531485	UTS2	A/C	C	0.220	55	0.611	0.5413
rs6772358	UTS2D	A/T	T	0.384	69	-0.726	0.4679
rs17465071	UTS2D	A/G	G	0.257	57	-0.798	0.4250
rs9809263	UTS2D	C/T	T	0.431	75	-0.196	0.8445
rs2886062	UTS2D	G/C	G	0.324	62	-1.835	0.0665
rs4399929	UTS2D	A/G	G	0.267	51	-0.372	0.7098
rs6444532	UTS2D	G/T	T	0.353	69	-0.536	0.5910
rs7502620	UTS2R	A/G	A	0.293	67	-2.251	0.0243
rs7211435	UTS2R	C/T	C	0.296	67	-2.037	0.0416
rs11650469	UTS2R	A/G	G	0.322	65	-0.863	0.3883
rs11077991	UTS2R	C/G	G	0.272	66	-1	0.3173
rs6502104	UTS2R	A/C	C	0.325	65	-1.193	0.2328

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele in the parental generation, Z – Z score – statistic calculated by the family-based association test showing the direction of association, P-value – level of statistical significance.

Summary of findings from genetic analysis of urotensin-II pathway genes and albumin: creatinine ratio as well as end stage renal disease

The genetic analysis of U-II signalling cascade showed no association between its common allelic variants and urinary albumin: creatinine ratio or ESRD. These data suggest that the genetic variation within the U-II pathway is unlikely to play a role in human renal disease.

Hypertension and renal expression of urotensin-II system genes

To further examine the role of U-II system genes in hypertension I analysed the expression of *UTS2*, *UTS2*D and *UTS2R* at the mRNA level using human kidney tissue from SRTB and WPKP.

The brief clinical characteristics of subjects from SRTB and WPKP are presented in Table 31.

The overall expression of *UTS2*, *UTS2D* and *UTS2R* in renal tissue samples from both cohorts was low (the average dCT for *UTS2*, *UTS2D* and *UTS2R* were 34.6, 38.0 and 34.8 in SRTB and 32.2, 36.4, 33.4 in WPKP, respectively).

The renal expression of *UTS2* and its receptor were correlated in the kidney from both SRTB ($r=0.84$, P-value<0.00001) and WPKP ($r=0.83$, $P<0.0001$). (Figure 14) There was a moderate linear correlation between *UTS2D* and *UTS2R* in SRTB ($r=0.46$, $p=0.0004$) but not WPKP ($r=-0.2$, $p=0.88$). (Figure 14) No significant linear correlation in renal expression of *UTS2* and *UTS2D* was found in either SRTB ($r=0.36$, $p=0.08$) or WPKP ($r=0.05$, $p=0.79$). (Figure 14)

The comparative analysis of U-II system genes expression between kidneys from normotensive and hypertensive subjects from SRTB revealed no statistically significant differences. (Figure 15) Similarly, there were no significant differences in renal expression of *UTS2*, *UTS2D* or *UTS2R* between hypertensive and normotensive subjects from WPKP. (Figure 15)

I also investigated whether common variants of *UTS2* (n=8), *UTS2D* (n=6) and *UTS2R* (n=5) were associated with levels of their renal expression. These SNPs

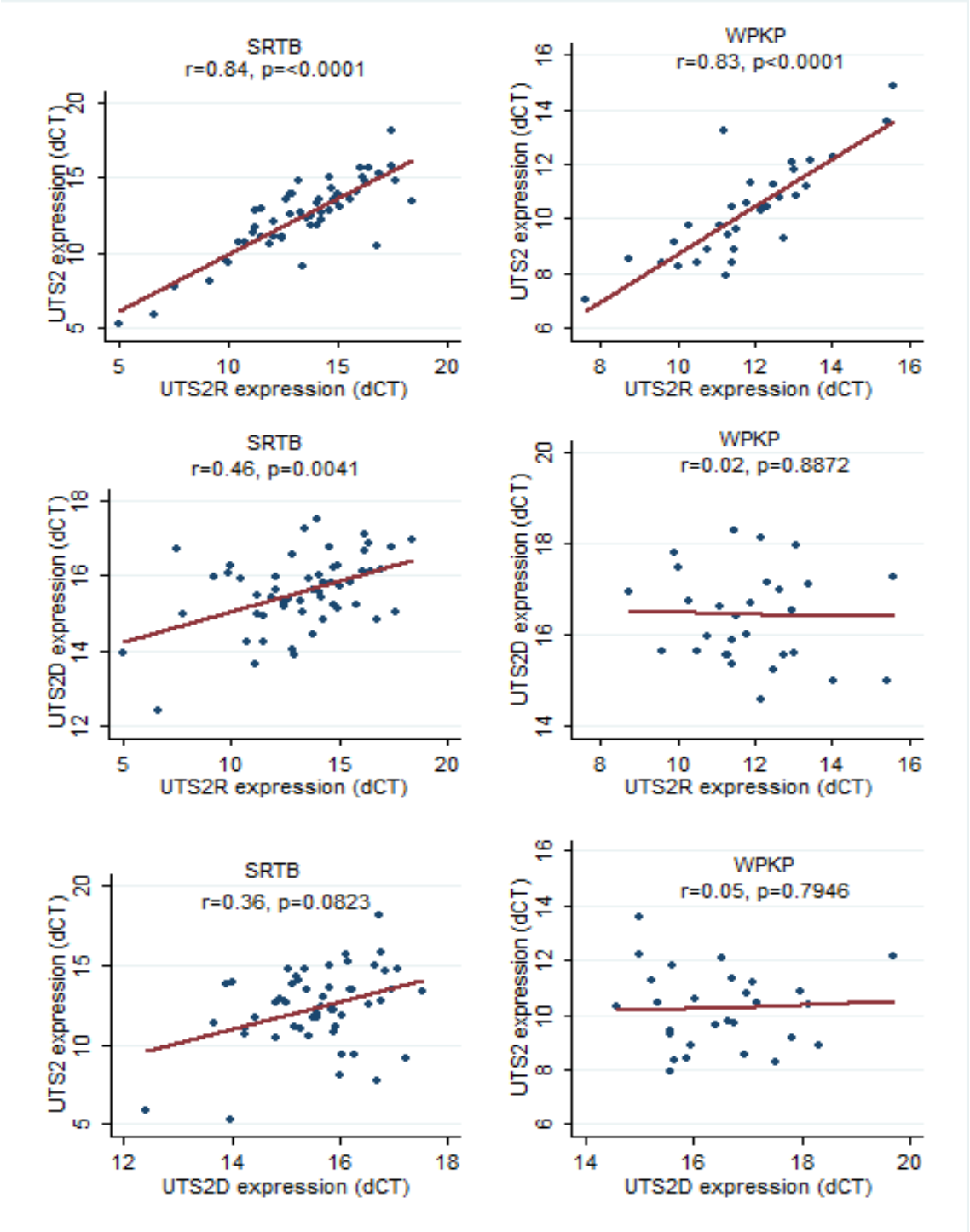
were chosen based on a HapMap derived tagging algorithm using the following criteria MAF>5% and r^2>0.8. None of the tagging SNPs in *UTS2* was associated with its renal expression in SRTB. (Table 32) Likewise, none of the SNPs in *UTS2R* showed association with its expression in kidneys from SRTB. (Table 32) An intronic SNP in *UTS2D* (rs4399929) showed positive association (β=0.64±0.26, p=0.0176) with renal expression of *UTS2D* in SRTB. (Table 32) However, this association did not survive correction for multiple testing.

Table 31. Clinical characteristics of subjects from Silesian Renal Tissue Bank and Western Poland Kidney Project.

Variable	SRTB		WPKP	
	Males (N=34)	**Females (N=28)**	**Males (N=19)**	**Females (N=16)**
Age (years)	57.9±10.6	59.5±12.6	62.0±10.6	65.4±9.5
BMI (kg/m^2)	27.5±3.7	27.2±4.5	26.8±3.6	25.4±7.7
Hypertension (%; N)	65 (22)	57 (16)	73 (14)	75 (12)

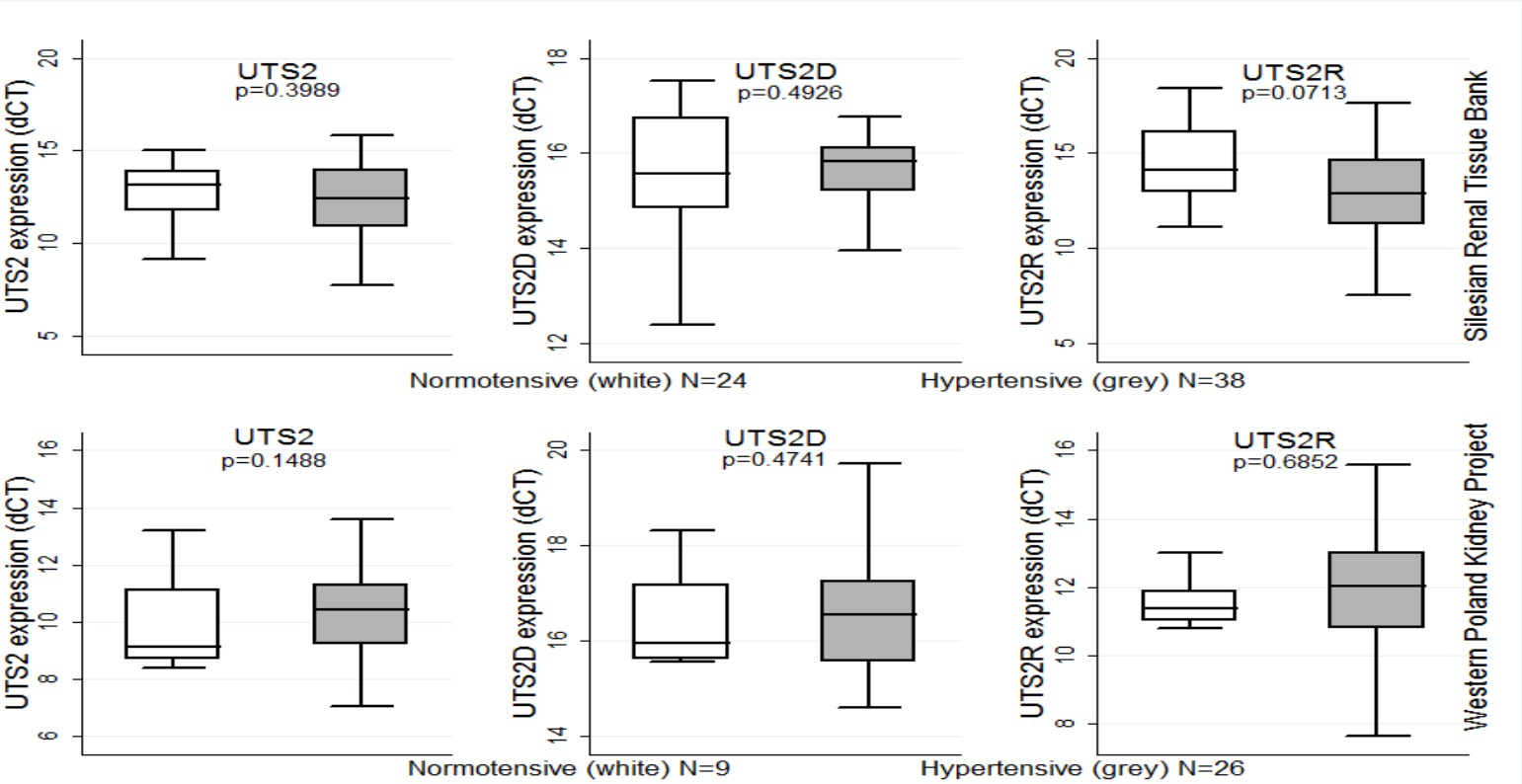
Data are means and standard deviations or percentages and counts; SRTB – Silesian Renal Tissue Bank, WPKP – Western Poland Kidney Project, BMI – body mass index.

Figure 14. Renal expression of U-II pathway genes – linear correlations in SRTB and WPKP.



The r coefficients were calculated using Pearson's linear correlation (P – level of statistical significance). Left side of the figure is SRTB, right side WPKP.

Figure 15. Renal expression of *UTS2*, *UTS2D* and *UTS2R* in normotensive and hypertensive subjects from SRTB (upper panel) and WPKP (lower panel).



Fold difference in mRNA expression - ratio calculated according to the formula: fold difference $=2^{-\text{difference in dCt}}$, P-values (obtained from multiple regression analysis) adjusted for the effect of age, sex and BMI, dCT – difference in cycle threshold between tested gene and control (housekeeping) gene(mean±SD).

Table 32. SNPs in U-II pathway genes and renal expression of *UTS2* in kidneys from SRTB.

SNP	Gene	MA	MAF	β	SE	P-value
rs228652	UTS2	T	0.283	-0.92	0.59	0.1231
rs170629	UTS2	T	0.368	0.10	0.59	0.8592
rs228651	UTS2	A	0.385	0.35	0.58	0.5447
rs3435100	UTS2	C	0.183	-0.27	0.71	0.6994
rs228648	UTS2	C	0.418	-0.48	0.53	0.3655
rs13306061	UTS2	A	0.172	-0.21	0.71	0.7656
rs2859389	UTS2	A	0.305	0.71	0.59	0.2343
rs531485	UTS2	C	0.283	-0.46	0.54	0.3899
rs6772358	UTS2D	T	0.344	-0.14	0.22	0.5312
rs17465071	UTS2D	G	0.361	0.23	0.22	0.2943
rs9809263	UTS2D	T	0.385	-0.05	0.22	0.7965
rs2886062	UTS2D	G	0.295	-0.05	0.25	0.8282
rs4399929	UTS2D	G	0.238	0.64	0.26	0.0176
rs6444532	UTS2D	T	0.336	-0.18	0.21	0.3658
rs7502620	UTS2R	A	0.245	0.82	0.74	0.2761
rs7211435	UTS2R	C	0.250	0.78	0.75	0.3016
rs11650469	UTS2R	G	0.328	0.39	0.64	0.5507
rs11077991	UTS2R	G	0.287	0.03	0.64	0.9629
rs6502104	UTS2R	C	0.328	0.39	0.64	0.5504

SNP – single nucleotide polymorphism, “rs” – SNP identifier in the NCBI, UTS2 – urotensin-II gene, UTS2D – urotensin-II related peptide gene, UTS2R – urotensin-II receptor gene, MA – minor allele, MAF – frequency of the minor allele, β – effect size per each copy of minor allele, SE – standard error, P-value – level of statistical significance (adjusted for age and sex).

Summary of findings from studies on urotensin-II system expression in human kidney

The mRNA experiments showed low expression of U-II system in the human kidney (previously reported as the tissue with high levels of U-II expression).[98] There was a strong positive correlation between renal expression of *UTS2* and *UTS2R* in both SRTB and WPKP. There were no differences in renal expression of *UTS2*, *UTS2D* or *UTS2R* between normotensive and hypertensive subjects from SRTB and WPKP. None of the tagging SNPs in *UTS2*, *UTS2D* or *UTS2R* was associated with altered renal expression of U-II system elements in SRTB.

Evolutionary analysis

Evolutionary analyses of UTS2, UTS2D and UTS2R coding sequences

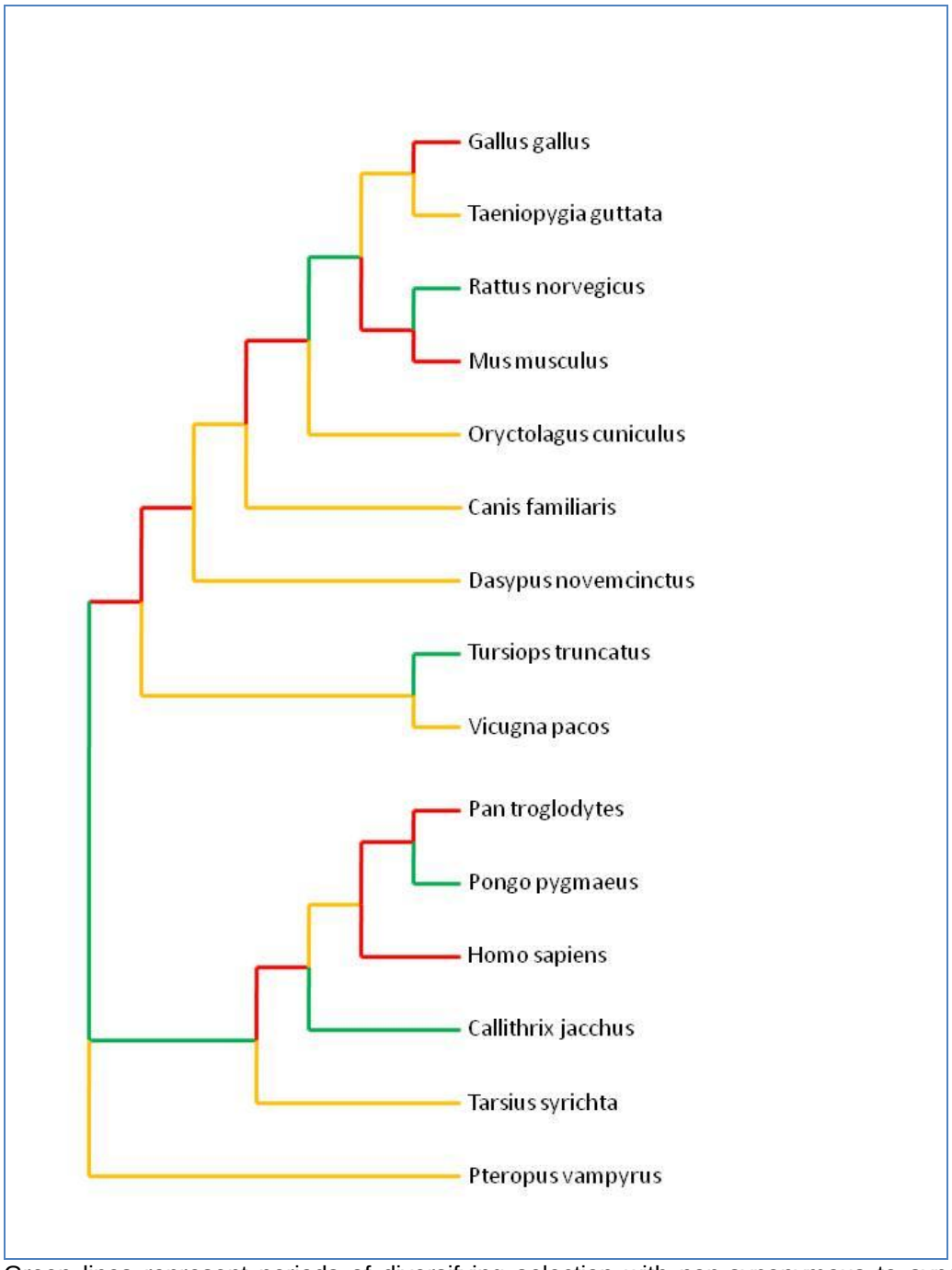
Line specific evolutionary analysis of pre-pro urotensin-II sequences showed that the speed of evolution of this peptide was not the same along all phylogenetic lineages. (Figure 16) There were substantial differences in the phylogeny with non-synonymous to synonymous substitution ratios ranging from 0.27 (strong conservation) to 1.6 (accumulation of mutations - lack of conservation). A division into 2 main groups of phylogenetically older animals and mainly primates was apparent; whereas no trend favouring either purifying or diversifying selection could be observed within each of the groups, they were separated by long period of diversifying selection (represented by green line – Figure 16). These data show that during the phylogenetical history, the *UTS2* sequence underwent diversifying selection (accumulating mutations) since the divergence of primates.

Analysis of evolutionary forces acting on precursor protein of URP showed that variable purifying selection acted on URP precursor across the phylogeny (Figure 17). Whereas the strength of this process varied (as represented by dN/dS ratios between 0.03-0.6) no periods of diversifying selection were discovered.

Similar to URP, the analysis of evolutionary forces acting on UT showed that the purifying selection was acting on the receptor across the phylogeny (Figure 18). The strength of this process varied as represented by dN/dS ratios

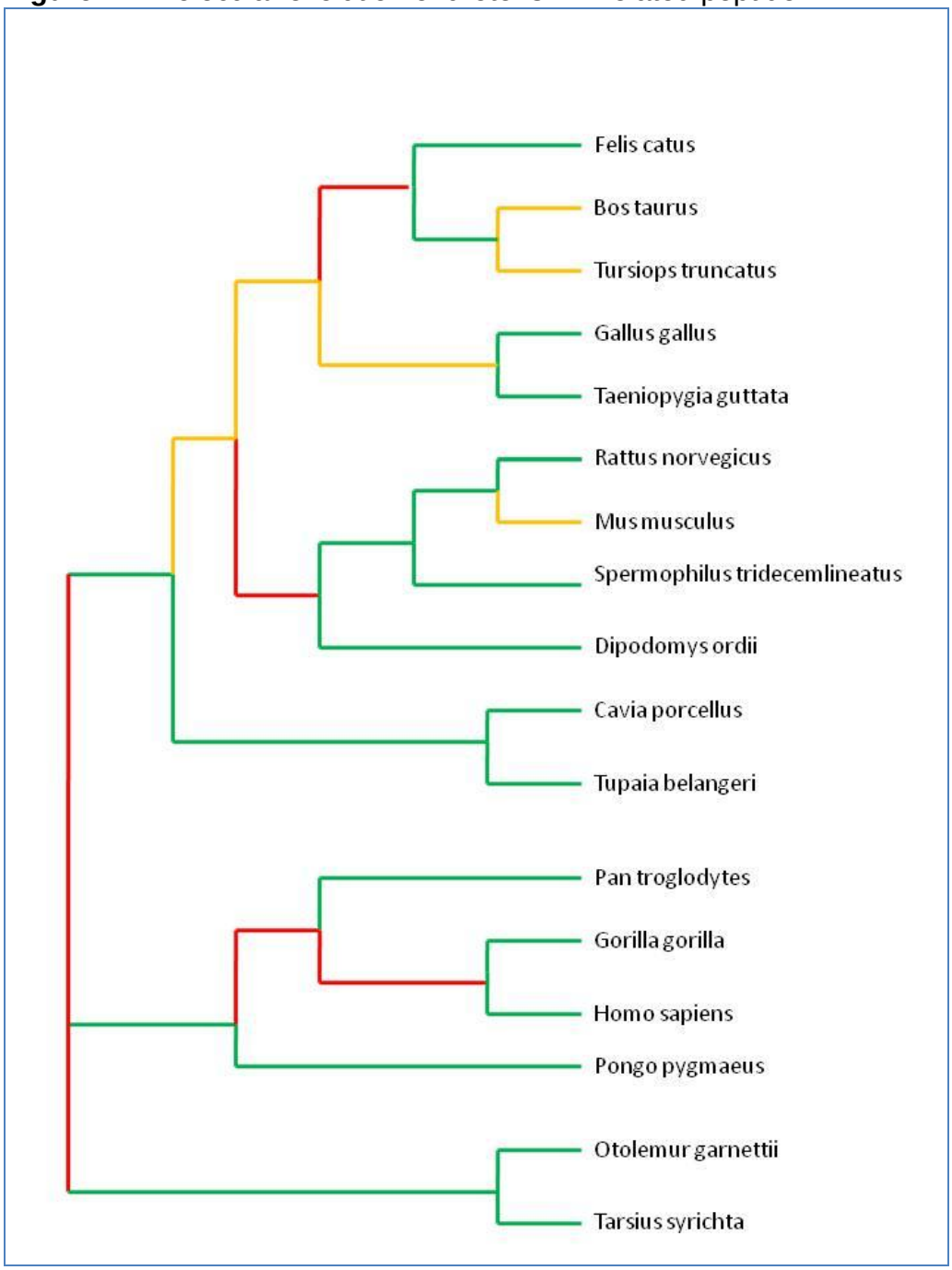
ranging from 0.04 (very strong purifying selection – depicted as red lines – Figure 18) to 0.6 (moderate purifying selection – green lines). However, enrichment for green colour in primates may suggest that this was the part of the phylogeny with relatively weakest purifying selection.

Figure 16. Molecular evolution of urotensin-II.



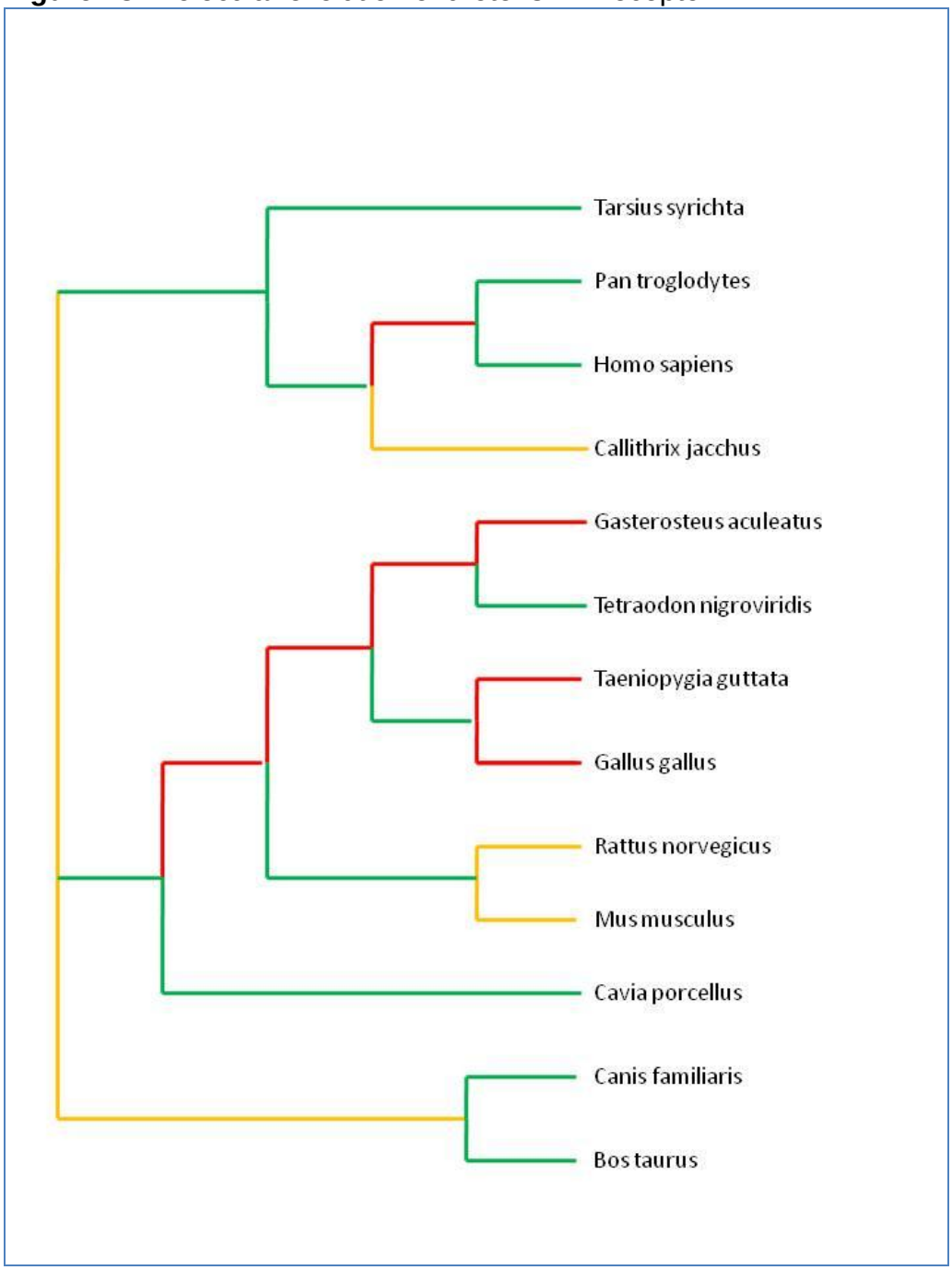
Green lines represent periods of diversifying selection with non-synonymous to synonymous substitution ratio of 1.6 (accumulation of non-synonymous mutations leading to changes in the protein sequence), orange lines - moderate purifying selection with non-synonymous to synonymous substitution ratio of 0.6; red lines – periods of strong purifying selection with non-synonymous to synonymous substitution ratio of 0.27.

Figure 17. Molecular evolution of urotensin-II related peptide.



Green lines represent periods of weak purifying selection with non-synonymous to synonymous substitution ratio of 0.6, orange lines represent strong purifying selection with non-synonymous to synonymous substitution ratio of 0.2; red lines represent periods of very strong purifying selection with non-synonymous to synonymous substitution ratio of 0.03.

Figure 18. Molecular evolution of urotensin-II receptor.



Green lines represent periods of moderately strong purifying selection with non-synonymous to synonymous substitution ratio of 0.25, orange lines represent moderate purifying selection with non-synonymous to synonymous substitution ratio of 0.18; red lines represent periods of very strong purifying selection with non-synonymous to synonymous substitution ratio of 0.04

Summary of the evolutionary analysis

The analysis of evolutionary forces acting on all elements of the system suggests the presence of selection acting on the coding sequences of *UTS2*, *UTS2D* and *UTS2R*. It appears that *UTS2* was under diversifying selection during divergence of primates. Throughout the phylogeny, coding sequence of *UTS2*R has been subject to purifying selection.

Genetic association analysis using data from large scale genotyping with 50K IBC array in the GRAPHIC Study

Quality control

Prior to the analysis of genetic association appropriate quality filters were applied to the data from 50K IBC array genotyping in the GRAPHIC Study.[50] Out of the initial 49094 markers genotyped on the array a total of 15517 markers were excluded from further analysis. Of those, 1775 were the ancestry and population admixture informative markers, 106 were removed as duplicates or copy number variants and 638 excluded SNPs were located on the sex chromosomes. The remaining 12998 SNPs were removed due to failure to meet quality control criteria - 12443 had MAF <0.01, 424 had poor genotyping quality (successful genotyping in <90% subjects of the GRAPHIC Study), 20 SNPs showed Mendelian inconsistencies; 107 SNPs violated the Hardy Weinberg equilibrium (χ^2 test cut off p<0.0001). Finally, 4 markers were excluded due to unambiguous rs identification.

Further quality control checks and exclusion of population stratification

Genomic control (λ) was calculated for each of the analysed phenotypes. The λ for eGFR, clinic SBP and clinic DBP were calculated at 0.98, 0.97 and 0.98 (respectively) and did not show significant inflation of type I statistical errors. This was visualised using the q-q plots for each of the examined phenotypes. The observed distributions did not show visible deviation from the expected distribution. (Figure 19)

To detect population stratification (which could lead to spurious associations) a set of non-correlated SNPs was used to create genetic dissimilarity matrix. This was further transformed using multidimensional scaling and visualised. (Figure 20) No subjects in the parental generation of the GRAPHIC Study showed significant genetic dissimilarity from the rest of the population. (Figure 20) In the combined analysis of the GRAPHIC population with the model HapMap populations (CEU, Yoruba and JPT+CHB) the GRAPHIC Study showed evidence of European ancestry. (Figure 20)

Genetic association

A total of 33577 markers were tested for association with each phenotype (eGFR, clinic SBP, clinic DBP). Only 3 SNPs showed experiment-wide significant association (q-value<0.05) with adjusted clinic DBP in the GRAPHIC Study. (Table 33) There were no statistically significant associations with eGFR or clinic SBP. The distribution of the –log10 transformed p-values of all examined SNPs across the autosomes for each of the phenotypes is presented in Figure 21.

All SNPs showing significant association with adjusted clinic DBP were common - their MAF were between 0.165 (rs17367504) and 0.169 (rs17037390). (Table 33) The MAF of these SNPs in the GRAPHIC population was not different to the reference values for the CEU population, as listed in the National Centre for Biotechnology Information (NCBI) database http://www.ncbi.nlm.nih.gov/snp.

The top 3 SNP showing significant association with clinic DBP mapped to the intronic region of the 5,10-methylenetetrahydrofolate reductase (NAD(P)H) gene (*MTHFR*) and were in strong LD with each other: r^2 for rs17037388 x rs17037390 and rs17037388 x rs17367504 were calculated at 0.98 and 0.96, respectively. (Figure 22)

Further 2 SNPs rs13306561 and rs17037425 showed suggestive associations with clinic DBP. Both were located in the intronic region of *MTHFR* and intronic region of the nearby chloride channel 6 gene (*CLCN6*),

respectively. (Table 34) Both rs13306561 and rs17037425 were common in the GRAPHIC Study (MAF: 0.169 and 0.155, respectively).

None of 7 functional SNPs in the *MTHFR/CLCN6/NPPA/NPPB* locus present on the 50K IBC array showed significant or suggestive association with adjusted clinic DBP. (Table 35) Of those, the missense *MTHFR* SNP rs1801131 (showing moderately strong LD with top association signal from clinic DBP - r^2=0.45) showed the most significant signal of association with clinic DBP (p=0.0007). (Table 35)

All SNPs showing significant and suggestive associations with clinic DBP were located within a large haploblock of strong LD spanning across both *MTHFR* and *CLCN6*. (Figure 23) However, the LD seems to be extending even further covering two nearby genes – natriuretic peptide precursor A gene (*NPPA*) and natriuretic peptide precursor B gene (*NPPB*). (Figure 23)

In the quantitative association analysis each minor copy (G) of the top SNP (rs17037388) was associated with average 2.03 mmHg decrease in clinic DBP. The other 2 significant SNPs (rs17037390 and rs17367504) showed the same direction of association with clinic DBP. (Table 33) The association of rs17037388 with clinic DBP remained significant in the sensitivity analysis, after additional adjustment for BMI and eGFR ($p=2.51x10^{-6}$). Further analysis, where rs17037390 and rs17367504 were included as covariates into the model showed that their association with clinic DBP was not independent and driven by the strong LD with the top SNP - rs17037388.

The 3 SNPS significantly associated with clinic DBP (rs17037388, rs17037390 and rs17367504) were associated at the nominal level of statistical significance with clinic SBP. (Table 36) However, the magnitude of their association with clinic SBP was not sufficient to survive correction for multiple testing. (Table 36) Neither of these SNPs showed association with age, age^2 and sex adjusted eGFR. (Table 36)

A total of 4 SNPs showed suggestive associations with at least one of three examined phenotypes (q-value<0.25). Of these 2 SNPS showed suggestive association with eGFR, one with clinic SBP and one with clinic DBP. (Table 34)

Two SNPs showing suggestive association with eGFR mapped to 2 separate loci - region of period homolog 3 (Drosophila) gene (*PER3*) on chromosome 1 and insulin-like growth factor 2 gene (*IGF2*) on chromosome 1. Each copy of minor allele (T) of the intronic SNP in *PER3* (rs12035969) was associated with average 2.03 mL/min*1.73m^2 decrease in eGFR ($p=1.32x10^{-05}$, q=0.2437). (Table 34) The *IGF2* SNP (rs1004446) showed also a suggestive association with eGFR ($p=1.47x10^{-05}$, q=0.2437). (Table 34)

A rare SNP rs2623410 - MAF=0.01, located in the intronic region of phosphodiesterase 1A gene (*PDE1A*) showed suggestive association with adjusted clinic SBP ($p=4.09x10^{-06}$, q=0.1351). (Table 34) No other SNPs in the *PDE1A* locus showed significant, suggestive or even nominal (p<0.05) association with adjusted clinic SBP. (Table 34)

The minor allele of an intronic SNP (rs1152002) within peroxisome proliferator-activated receptor gamma gene (*PPARG*) was associated with an average 1.39 mmHg increase in adjusted clinic DBP (p=7.41x10-6, q=0.0519). (Table 34) No other SNPs in this locus showed association with clinic DBP.

Table 33. Significant findings from association analysis of 50k Illumina HumanCVD BeadChip in the GRAPHIC Study.

Phenotype	Chr	BP	Locus	SNP	MA/ MAF	β±SE	p/q	Genetic location	Predicted function
cDBP	1	11780623	MTHFR	rs17037388	G/0.168	-2.03±0.43	$3.01x10^{-06}$/ 0.0395	Intron, MTHFR	Possible transcription factor binding site
cDBP	1	11783430	MTHFR	rs17037390	A/0.169	-2.03±0.44	$3.06x10^{-06}$/ 0.0395	Intron, MTHFR	Possible transcription factor binding sit
cDBP	1	11785365	MTHFR	rs17367504	G/0.165	-2.03±0.44	$3.6x10^{-06}$/ 0.0395	Intron, MTHFR	Possible transcription factor binding sit

cDBP – clinic diastolic blood pressure; MTHFR - 5,10- methylenetetrahydrofolate reductase (NAD(P)H) gene; Chr – chromosome; BP – base pair; SNP – single nucleotide polymorphism; MA – minor allele; MAF – frequency of the minor allele; β-coefficient – estimated quantitative effect of each copy of minor allele of a given SNP on clinic DBP (adjusted for age, age^2, sex and anti-hypertensive medication); SE – standard error; p – statistical significance of association; q – false positive discovery rate.

Table 34. Suggestive findings from the association analysis of 50k Illumina HumanCVD BeadChip in the GRAPHIC Study.

Phenotype	Chr	BP	Locus	SNP	MA/ MAF	β±SE	p/q	Genetic location	Predicted function
eGFR	1	7815433	PER3	rs12035969	T/0.217	-2.03±0.47	$1.32x10^{-05}$/ 0.2437	Intron, PER3	Possible transcription factor binding site
eGFR	11	2126719	IGF2	rs1004446	A/0.367	-1.73±0.39	$1.47x10^{-05}$/ 0.2437	Intron, IGF2	Possible transcription factor binding sit
cSBP	2	182880609	PDE1A	rs2623410	T/0.012	-8.02±1.74	$4.09x10^{-06}$/ 0.1351	Intron, PDE1A	Possible transcription factor binding sit
cDBP	3	12446871	PPARG	rs1152002	T/0.483	1.39±0.31	$7.41x^{10-6}$/ 0.0519	Intron, PPRG	Possible transcription factor binding site
cDBP	1	11788391	MTHFR	rs13306561	C/0.169	-1.94±0.43	$7.88x^{10-6}$/ 0.0519	Intron , MTHFR	Possible transcription factor binding sit
cDBP	1	11792970	CLCN6	rs17037425	A/0.155	-1.96±0.45	$1.27x^{10-5}$/ 0.0695	Intron CLCN6	Possible transcription factor binding site

eGFR – estimated glomerular filtration rate; cSBP – clinic systolic blood pressure; cDBP – clinic diastolic blood pressure; PER3 - period homolog 3; IGF2 - insulin-like growth factor 2 gene; PDE1A - phosphodiesterase 1A gene; PPARG peroxisome proliferator-activated receptor gamma gene; MTHFR - 5,10- methylenetetrahydrofolate reductase (NAD(P)H) gene; CLCN6 - chloride channel, voltage-sensitive 6 gene; Chr – chromosome; BP – base pair; SNP – single nucleotide polymorphism; MA – minor allele: MAF – frequency of the minor allele; β-coefficient – estimated quantitative effect of each copy of minor allele on a given phenotype; SE – standard error; p – statistical significance of association; q-value – false positive discovery rate.

Table 35. Known functional genetic variants in the MTHFR/CLCN6/NPPA/NPPB locus and clinic DBP – association analysis in the GRAPHIC Study.

Chr	BP	Gene	SNP	MA/MAF	r^2	molecular function	β±SE	p/q
1	11773514	MTHFR	rs2274976	T/0.053	0.278	missense	-0.88±0.81	0.2778/0.9415
1	11777063	MTHFR	rs1801131	G/0.312	0.445	missense	-1.19±0.35	0.0007/0.4808
1	11778965	MTHFR	rs1801133	A/0.331	0.100	missense	0.64±0.36	0.0784/0.9088
1	11785644	MTHFR	rs2066470	A/0.112	0.603	synonymous substitution	-1.48±0.52	0.0047/0.6922
1	11828655	NPPA	rs5065	G/0.146	0.057	stop/loss of function	-0.76±0.45	0.0963/0.9133
1	11830235	NPPA	rs5063	T/0.054	0.257	missense	-0.89±0.78	0.2536/0.9393
1	11841106	NPPB	rs5229	T/0.010	0.002	missense	-1.91±1.22	0.1189/0.9145

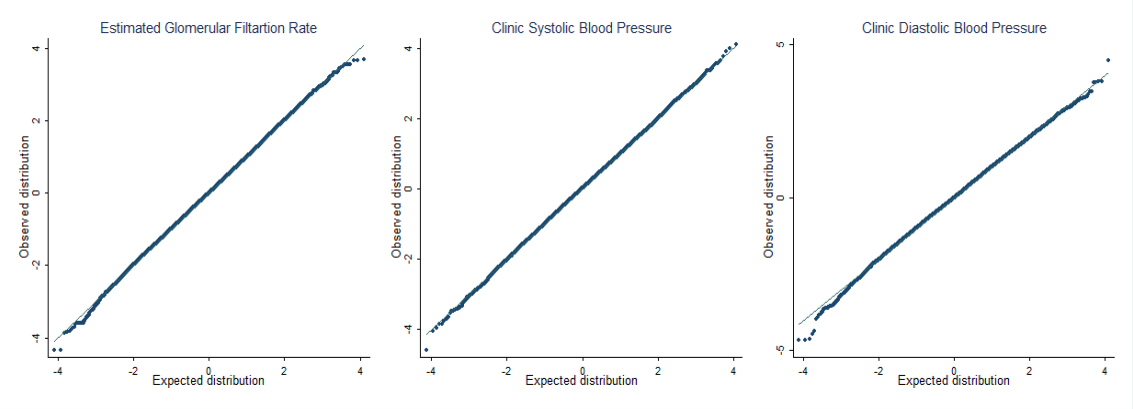
MTHFR - methylenetetrahydrofolate reductase (NAD(P)H); NPPA - natriuretic peptide precursor A, NPPB natriuretic peptide precursor B, Chr – chromosome; BP – base pair; SNP – single nucleotide polymorphism; MA – minor allele: MAF – frequency of the minor allele; r^2 – r^2 coefficient of LD between given SNP and rs17037388 (top association signal with cDBP), β-coefficient – estimated quantitative effect of each copy of minor allele of a given SNP; SE – standard error; p – statistical significance of association; q-value – false positive discovery rate.

Table 36. Association of the top SNPs from the clinic DBP analysis with other phenotypes

Chr	BP	Locus	SNP	MA/MAF	eGFR		cSBP	
					β±SE	p/q	β±SE	p/q
1	11780623	MTHFR	rs17037388	G/0.168	0.74±0.50	0.1409/0.9391	-2.11±0.70	0.0026/0.6804
1	11783430	MTHFR	rs17037390	A/0.169	0.67±0.49	0.1764/0.9505	-2.11±0.71	0.0028/0.6805
1	11785365	MTHFR	rs17367504	G/0.165	0.68±0.50	0.1739/0.9505	-2.24±0.72	0.0018/0.6805

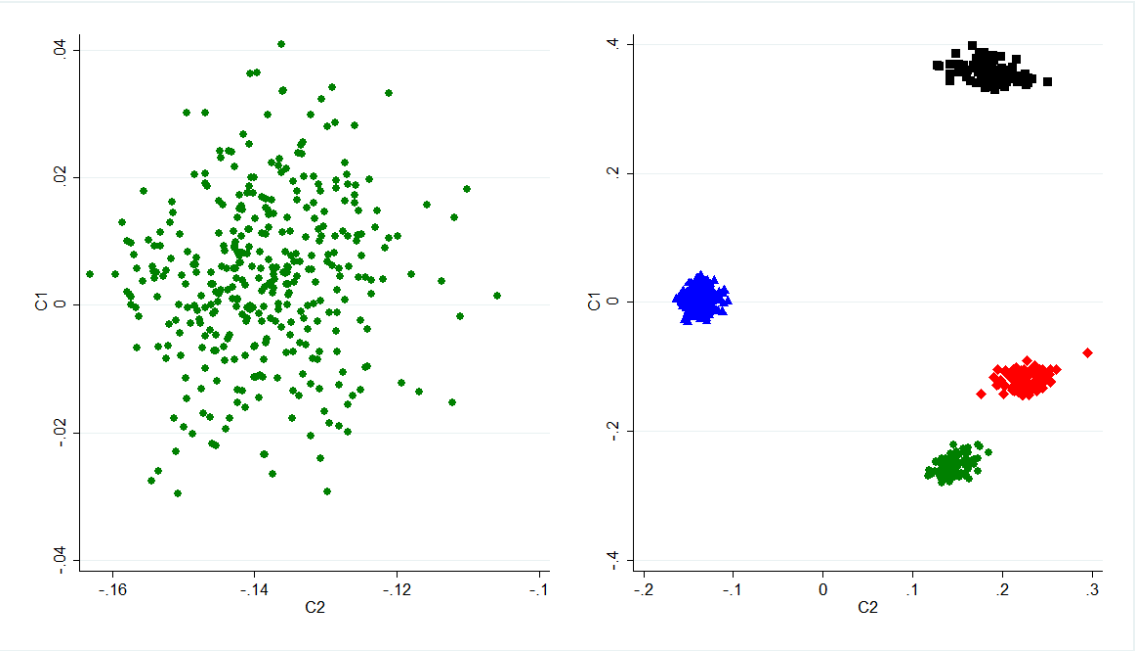
eGFR - estimated glomerular filtration rate; cSBP – clinic systolic blood pressure; MTHFR - methylenetetrahydrofolate reductase (NAD(P)H); Chr – chromosome; BP – base pair; SNP – single nucleotide polymorphism; MA – minor allele: MAF – frequency of the minor allele; β-coefficient – estimated quantitative effect of each copy of minor allele of a given SNP; SE – standard error; p – statistical significance of association; q-value – false positive discovery rate.

Figure 19. Quantile-quantile plots for the analysis of association between adjusted eGFR, clinic SBP, clinic DBP and SNPs from 50K IBC array in the GRAPHIC Study.



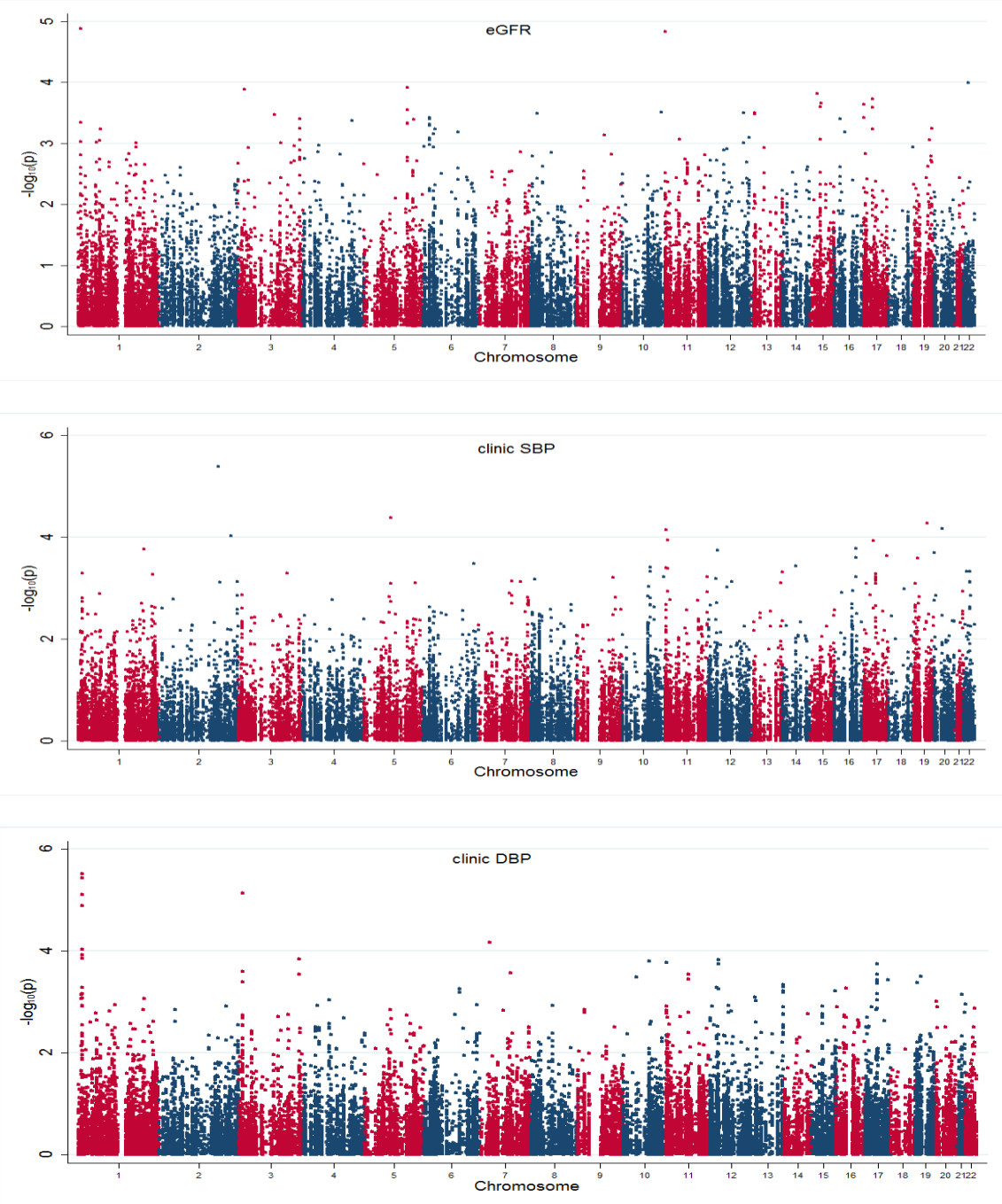
Plots present distribution of observed versus expected –log10 p-values from association analysis using GEE model (under additive genetic model, adjusted for age, age^2, sex and anti-hypertensive treatment, where appropriate.

Figure 20. Analysis of population stratification of the GRAPHIC Study.



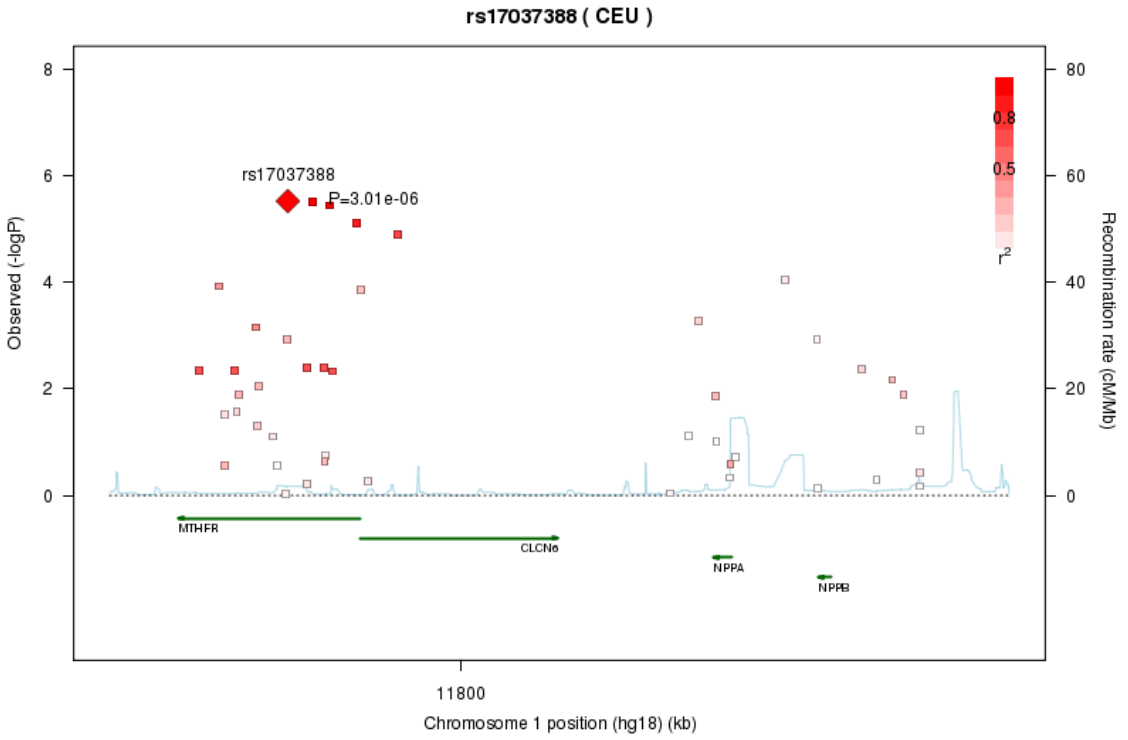
The data were obtained from multidimensional scaling of the genetic dissimilarity matrix of subjects from the parental generation of the GRAPHIC Study. The X and Y axes represent the first two dimensions from multidimensional scaling (MDS) analysis. Panel on the left presents scatter plot of individuals from parental generation of the GRAPHIC Study. Right panel shows genetic similarity between subjects from the parental generation of the GARPHIC Study (green circles) and HapMap CEU population (red diamonds); HapMap YRI and JPT+CHB populations are shown in black and blue, respectively.

Figure 21. Distribution of the –log10 p-values from the 50K Illumina HumanCVD BeadChip association analysis in the GRAPHIC Study.



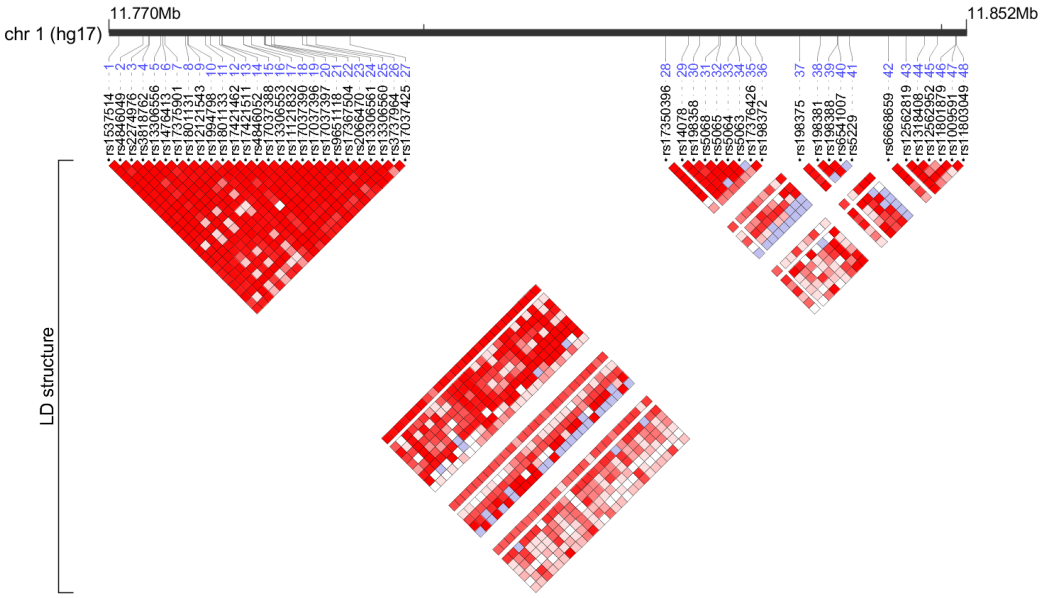
Upper panel - eGFR; middle panel - cSBP, lower panel - cDBP; Y axis –log10 p-values; colour coded on the x axis are the 22 autosomal chromosomes; each point represents single statistical test of association with a given phenotype.

Figure 22. SNPs of the MTHFR/CLCN6/NPPA/NPPB locus and their association with adjusted clinic DBP in the GRAPHIC Study.



Y axis - –log10 transformed p-values from the association tests; X axis is the chromosomal position of the relevant SNPS; green lines represent relevant genes in the area whereas light blue line on the graph depicts recombination rate giving information about recombination hot-spots, breaking down the structure of LD. The top SNP rs17037388 is marked as a diamond, . the colour of the remaining markers represents strength of the LD with the top marker, MTHFR - 5,10-methylenetetrahydrofolate reductase (NAD(P)H) gene; CLCN6 - chloride channel, voltage-sensitive 6 gene; NPPB - natriuretic peptide precursor B gene; NPPA - natriuretic peptide precursor A gene.

Figure 23. Structure of the linkage disequilibrium within the MTHFR/CLCN6/NPPA/NPPB locus.



Dark red corresponds to LD r^2=1 – maximal LD, white to r^2=0 – no LD; red triangle on the left represents area of strong LD comprising MTHFR and CLCN6; area on the right represents location of the NPPA and NPPB; diamond in the middle shows structure of LD between these loci.

11. DISCUSSION

Genetic architecture of quantitative traits like BP or renal function is complex and shaped by genetic variants with different frequencies, penetrance and effect sizes on the ultimate phenotype.[262] Each individual strategy used currently to investigate genetics of complex polygenic traits has its strengths and weaknesses.[62] Therefore, it is common to apply different research techniques and approaches in parallel to increase chances for identification of genuine genetic associations.[59,263] This project focused on genetic analysis of BP and eGFR using a candidate pathway association approach, gene expression studies, evolutionary analysis and large scale screening across genes and pathways relevant to cardiovascular regulation (50 IBC array).

The candidate gene study was designed to systematically assess U-II pathway in relation to renal function and BP at DNA, mRNA and phylogenetic level. As the most potent mammalian vasoconstrictor with evolutionary links to water and electrolyte homeostasis, U-II system has been investigated as a potentially important cardiovascular regulator and pharmacological target.[37,112,167] Data on the physiological functions of the system come mainly from *in vitro* and animal experiments. Many of these studies provided inconsistent messages, at least partly because of the use of different protocols, models and techniques.[71,128,168] Therefore, the results of these studies may not be directly extrapolated to human or clarify the role of the U-II cascade in the cardiovascular and renal regulation.[71,112,167,264]

So far the data on the involvement of U-II pathway in human cardiovascular disease are inconclusive. Several studies reported associations between circulating levels of U-II and hypertension, diabetes or renal impairment.[100,102,175,183,265] These

studies were based on different methods of biochemical analysis (radioimmunoassay and enzyme-linked immune sorbent assay) and showed substantial discrepancies in the reported levels of circulating U-II (differences ranging by three orders of magnitude).[105] The experiments with administration of U-II in humans showed also conflicting reports. Whereas, Sondermeijer demonstrated a direct vascular effect of U-II on human blood vessels in vivo by iontophoretic administration, no haemodynamic effect was reported following infusion of U-II or urantid (U-II antagonist) into the brachial artery by Cheriyan et al.[109,164] Finally, randomised controlled trials with palosuran (U-II antagonist) failed to provide evidence for clinical importance of this system in regulation of BP or renal function. Use of palosuran (U-II antagonist) in patients with diabetic nephropathy did not show any effect on blood pressure or albumin excretion.[165] Lack of consistency between reported results and failure of the clinical trials made it necessary to review the existing evidence.

My genetic association study of U-II pathway genes was designed in two stages. The primary stage used family based association testing (as this is immune to bias of population stratification) in a sample of well phenotyped families from general population with available BP and eGFR. The most significant association signal from the primary analysis was further examined in several external cohorts of families and unrelated subjects and results of the replication meta-analysed. Gene expression experiments were performed in two independent, unique collections of human kidney tissues. These experiments provided information about the level of expression of U-II pathway components and correlations between individual transcripts in the human kidney (previously published reports were based on very

limited sample sizes, which did not allow for such analyses).[98] Availability of the hypertension phenotype allowed for the assessment of differences in renal expression of U-II pathway molecules between normotensive and hypertensive human kidney. Finally using genotypes and expression data from the same cohort I looked for SNPs associated with level of U-II pathway gene expression. The expression analysis was followed by analysis of the evolution of the system in 16 vertebrate species. The opinion about strong evolutionary conservation of U-II system came from evolutionary analysis of the 11 amino-acid terminal sequence of mature U-II but the evolutionary conservation has never been assessed in the context of the whole system including URP and UT.[72]

One of the most important factors nurturing interest in genetics of complex traits is their heritability. Presence of significant heritable component in the population suggests existence of variation in the genotypes of the population affecting variance of the phenotype.[239] Both renal function and BP clearly contain heritable component but quantitative estimates of their heritability vary across populations.[50-52,195,266-268] The available data from family-based studies estimate renal function heritability in the range from 40% to 82%.[51,52,268] BP is reported to show slightly lower heritability with estimates ranging from 24% for pulse pressure to 37% for DBP.[48]

The data from my analysis shows that both renal and BP phenotypes have significant heritable component in the general white Caucasian population of UK. Out of the tested phenotypes mean 24-hour DBP showed the highest heritable component estimated at 41%. Interestingly, both ambulatory BP phenotypes mean 24-hour SBP (h^2=33%) and mean 24-hour DBP (h^2=41%) showed higher heritability

than corresponding clinic values (clinic SBP h^2=31% and clinic DBP h^2=32%). The presence of substantial differences in heritability between clinic and mean 24-hour values are consistent with literature findings. Bochud et al. gave estimates for clinic SBP and DBP at 20% and 5% respectively but mean 24-hour SBP and mean 24-hour DBP showed heritability at 37% and 24%, respectively.[266] Similar findings were described by Fava et al. who reported heritability for mean 24-hour SBP at 30% and mean 24-hour DBP at 29% but did not find a heritable component for office systolic or diastolic BP measurements in 260 siblings from 118 Swedish families (not on antihypertensive treatment).[267] Indeed, it is believed that ambulatory BP measurements show higher reproducibility and are less influenced by environmental interferences.[269] This study showed that the same applies to genetic studies of BP. Given higher heritability and less inter-study variation, ambulatory BP phenotypes may have an advantage over office BP phenotypes in genetic analyses.

Renal phenotypes in the GRAPHIC Study showed also heritable components. Whereas the heritability of eGFR was moderately high h^2=38%, mean 24-hour urinary sodium and potassium excretions showed low heritabilities estimated at 11% and 17%, respectively. The estimate of eGFR heritability calculated in the GRAPHIC Study is in agreement with previously reported values 40-82%.[51,52,268] One of the factors for such a wide spectrum of heritability estimates from these studies is the difference in definition of the phenotype. Indeed, several measures of renal function (inulin clearance, creatinine clearance, eGFR) were used in these studies and it appears that lack of the same reproducible phenotype may underlie, at least to certain extent, the variation in renal function heritability estimates.[51,52,268,270] The

differences in the design of the study (extended families versus twin studies) is also a well-recognised factor that contributes to differences in measurement of heritability estimates across studies (twin studies tend to over-estimate heritability).[271] Classical mono- and di-zygotic twin designs allow for estimation of only three components of variance (covariance of mono-zygotic twins, covariance of di-zygotic twins and total variance of the sample) ignoring non-genetic components of covariance, which may lead to bias in estimation of heritability.[239] Also, twin design are particularly prone to biases related to difficulties in quantification of gene-environment interactions, which (if show over-additive effect) tend to erroneously increase estimations of heritability.[53]

Little published information exists on the heritability of 24-hour urinary excretion of sodium and potassium. Hunt et al. analysed intra-familial correlations between overnight urinary sodium excretion.[272] They found that overnight urinary sodium excretion was moderately correlated between spouses (Pearson's correlation coefficient r=0.29) and siblings (Pearson's correlation coefficient r=0.38).[272] In parent and offspring there was also a moderate correlation for offspring <20 years old (Pearson's correlation coefficient r=0.29), but weak if offspring were >20 years old (Pearson's correlation coefficient r=0.13) suggesting substantial influence of a common environment.[272] In a study of twins (539 monozygotic and 1208 dizygotic twin pairs) Hunter et al. reported heritabilities of 40% and 43% for 24-hour urinary sodium and potassium excretion, respectively.[270] In the GRAPHIC Study 24-hour urinary sodium and potassium excretion showed weak heritability, estimated at 11% and 17%, respectively. Heritability of these phenotypes was lower than reported by

Hunter et al.[270] The most likely explanation for the difference in this quantitative estimation of heritability between the GRAPHIC Study and published data lies in the difference in study designs. Whereas, Hunter et al. based their estimations on interclass correlation coefficients in mono- and dizygotic twins (not accounting for the influence of common environment), estimations in GRAPHIC Study (families) were performed using variance components analysis.[53] The data from GRAPHIC Study suggest that 24-hour urinary sodium and potassium excretions are primarily regulated by the influence of the environment among Caucasian population of UK.

Having confirmed presence of heritable component of the tested phenotypes I conducted analysis of their associations with common genetic variation within *UTS2*, *UTS2D* and *UTS2R*.

The family based association analysis of the discovery cohort showed that only one SNP (rs531485) in 5'-flanking region of *UTS2* showed significant association with eGFR. Specifically, each copy of minor allele was associated with 1.04 ml/min*1.73m^2 increase in eGFR in this population. As expected, SNPs in LD with rs531485 (rs2859389, rs228638) also showed similar strength of association with eGFR (p=0.0012 and p=0.0033, respectively). The association between rs531485 and eGFR was not replicated in other family cohorts (SHS and SCS families). In cohorts of biologically unrelated subjects rs531485 showed association with eGFR only in YMCA extension but not in SCS extension or YMCA study. In subjects from YMCA extension each copy of minor allele of rs531485 was associated with 3.29 ml/min*1.73m^2 decrease in eGFR. The direction of this association was opposite to the effect observed in GRAPHIC (where presence of minor allele was associated

with increased renal function). Also, *in silico* replication in the CoLaus study did not show association between number of minor copies of rs531485 and eGFR (p=0.9019). In fact, in the meta-analysis of 8724 individuals (SHS, SCS families, SCS extension, YMCA study, YMCA extension, CoLaus study) rs531485 polymorphism showed no association with eGFR (p=0.5834). rs531485 did not show any association with related phenotypes such as 24-hour urinary excretion of sodium and potassium or clinic and mean 24-hour ambulatory BP in the GRPAHIC Study.

The analysis of tagging SNPs within *UTS2*, *UTS2D* and *UTS2R* and additional renal phenotypes (urinary albumin: creatinine ratio and ESRD) showed no association.

Taken together these data did not show that common genetic variants in U-II system genes are associated with BP and measures of renal function and kidney injury.

One may argue that this lack of association in the replication resources could be due to differences in ascertainment of the replication populations. Indeed, the GRAPHIC Study represents general population whereas subjects from SCS and SHS were enriched for cardiovascular disease at recruitment. In theory, ascertainment through high cardiovascular risk subject should lead to increase in prevalence of genetic variants increasing cardiovascular risk, if these are present in a given population.[273] This should theoretically increase statistical power of such designs and make them desirable replication resources. More importantly, no replication was observed in other cohorts originating from general population YMCA study, YMCA extension and CoLaus. Whereas YMCA and YMCA extension,

although recruited from the general population, differ significantly from the GRAPHIC Study (young healthy males) the CoLaus study is in its design almost identical to the GRAPHIC Study (apart from family design). Lack of replication in these population samples or their meta-analysis increases the probability that the initial result from the GRAPHIC Study was a false positive finding.

It could be argued that difference in the results of association between GRAPHIC Study and SHS or SCS could be attributed to the difference in geographical origins of these cohorts. Whereas, the GRAPHIC Study was recruited in the UK, SHS, SCS and YMCA study were collected in Poland and CoLaus study consists of individuals from the general population of Switzerland. As a tagging SNP, rs531485 captures information about other common genetic variants within the whole haploblock (all statistically similar SNPs).[69] The possibility that the structure of the haploblocks is different between the studies used for replication cannot be fully excluded.[274] However, it should be noted that rs531485 is a common polymorphism in all three cohorts and there were no major differences in its MAF between these populations. This suggests that neither major evolutionary (selection) nor demographic (population bottleneck, population admixture) events were likely to disrupt the haploblock structure.[69]

Lack of statistical power could be one of the reasons for the overall negative result of my association study. However, use of spectral decomposition procedure in calculating for multiple testing correction increased the accuracy of estimate of cut-off value for statistically significant findings. Furthermore, multiple cohorts used in the analysis provided a sample size much larger than those previously used in the

analysis of association of genetic variation within U-II system genes with cardiovascular phenotypes ensuring reliability of observed results.

So far, the U-II system gene polymorphisms were analysed by several investigators and the results of these reports are inconsistent. Former studies focused mainly on SNPs with suspected molecular function (missense or those mapping to the gene promoter). Unlike tagging approach, this strategy did not provide any information about the overall genetic variance in the candidate locus. In addition, the studies examined associations between common genetic variants of U-II system genes in very small samples.[196-198] Despite small sizes of these populations, none of the studies reported the statistical power of the analysis *a priori*. Therefore, negative association results reported, could not be fully interpreted as the conclusive lack of association between blood pressure and genetic variation in U-II system genes. Finally, only Yi et al. authors attempted to replicate results of the association between *UTS2* SNP and hypertension in an external population (which is considered as a gold standard of genetic association study nowadays).[197]

Previous large scale genotyping experiments published after this projected started did not provide any evidence for the association between U-II pathway genes and BP or renal phenotypes. Indeed, the recent meta-analysis of renal function GWAS (67093 individuals of European ancestry) did not report significant associations between common genetic variants within *UTS2*, *UTS2D* and *UTS2R* and eGFR, serum cystatin C or CKD phenotype.[275] Also no association of variants within U-II pathway genes was reported in a meta-analysis of over 200,000 subjects of European origin with BP phenotypes.[276]

Taken together, these data suggest that urotensin-II system genes do not contribute to regulation of human BP and kidney function.

U-II system components are widely expressed in human cardiovascular and endocrine tissues.[37,172,188,277] Kidneys, in particular have been hypothesised to act as a source of systemic production of U-II.[104,97] U-II is excreted to urine.[98] This excretion of U-II exceeds glomerular filtration suggesting regional production and role in the regulation of functioning of the nephron.[98] Expression of *UTS2* mRNA and presence of mature U-II have been shown in human kidney.[97,98,172] However, the experiments were performed on small number of kidneys[97] or samples from pathologically changed organs.[172] In my study I took advantage of the availability of renal tissue samples from two independent tissue banks. These resources are the largest so far, collections of human kidneys.

The quantitative real-time PCR in SRTB and WPKP (altogether 97 human kidney samples) confirmed that all three molecules of U-II pathway were expressed in human kidney. There was a strong, positive correlation between the expression of *UTS2* and *UTS2R* in human kidneys from both SRTB ($r=0.84$, p-value<0.00001) and WPKP ($r=0.83$, $P<0.0001$). This is the first replicated observation showing a strong correlation in expression of *UTS2R* and its ligand. Indeed, the strong statistically significant correlation in renal expression of *UTS2* and its receptor showed the same direction in both SRTB and WPKP – higher expression of *UTS2* were associated with higher abundance of *UTS2R*. These data might suggest functional co-regulation of the U-II system in human kidney. A study by Matsushita et al. previously reported expression of *UTS2* and *UTS2R* in human renal tissue.[98] However, due to small

sample size (5 samples) and lack of direct analysis of correlation the co-regulation between both transcripts could not be formally confirmed.[98] Interestingly, correlations were not consistently observed for *UTS2D* and *UTS2R*. Whereas a moderate positive correlation between renal expression of *UTS2D* and *UTS2R* was observed in SRTB (r=0.46, p=0.0004) this was absent in kidneys from WPKP (r=-0.02, p=0.8872). Also, no statistically significant correlation was found between renal expression of *UTS2* and *UTS2D* neither in SRTB nor in WPKP (r=0.36, p=0.0823; r=0.05, p=0.7946, respectively). Lack of correlation in renal expression of *UTS2* and *UTS2D* may suggest lack of biologically meaningful interactions between these molecules. This was surprising as both U-II and URP act through binding to the same receptor, and could be expected to show either synergy (positive correlation) or competition (negative correlation) towards U-II receptor.

There were no differences in renal expression of U-II system constituents between hypertensive and normotensive individuals in neither SRTB nor WPKP. The results remained negative even after adjustment for possible covariates including age, sex and BMI. This finding was somewhat surprising given the previous report by Cheung et al. who showed raised plasma U-II levels in hypertensive patients.[100] However, the value of measuring serum levels of U-II by currently available radioimmunoassay has been questioned.[105] In contrast, the gene expression analysis is based on direct measurement of U-II system abundance in the kidney and to the best of my knowledge is the first direct comparative analysis of *UTS2*, *UTS2D* and *UTS2R* expression in human tissue from normotensive and hypertensive subjects.

The resources used in this project were validated in previous studies of gene expression profiles of hypertensive subjects showing adequate statistical power.[64] Indeed, SRTB showed significant up-regulation of fibroblast growth factor 1 gene (*FGF1*) expression and its chaperone - fibroblast growth factor binding protein 1 (*FGFBP1*) in the hypertensive kidney.[64,39] The experiments performed on kidney samples from SRTB showed differential expression of 46 genes (including renin) between hypertensive and normotensive kidney as well as identified micro RNAs regulating renin expression.[278]

I also checked whether common genetic variants within *UTS2*, *UTS2D* and *UTS2R* were associated with renal expression of their mRNAs. Previous analysis by Ong et al. showed that haplotype constructed from three SNPs in *UTS2* (rs228647, rs228648, rs2890565) showed association with serum levels of U-II in Chinese population.[196] Indeed, one of the possible mechanisms through which, this haplotype might influence U-II serum levels, is altered gene expression. Out of the three SNPs forming the haplotype only rs228648 was directly genotyped in samples from SRTB. However, rs228648 did not show association with renal expression of *UTS2* in SRTB. None of the tagging SNPs of genes of U-II system was associated with altered expression of *UTS2*, *UTS2* or *UTS2R* in kidneys collected in SRTB.

The transcript-based data from SRTB and WPKP suggest that mechanisms underlying altered renal expression of U-II system are unlikely to play a major role in human hypertension. Renal over-expression of the U-II system was linked to hypertension and kidney failure in rat.[279,280] Over-expression of *UTS2* and *UTS2R* was reported in kidneys of patients with diabetic nephropathy.[172] Furthermore,

previous research showed elevated serum concentrations of U-II in hypertensive patients as well as reduction of serum U-II levels following antihypertensive treatment.[100,162]

The lack of consistency of our data with the results obtained in rat models can be explained by distant evolutionary origin of both species. Some of the previous studies showed higher serum U-II levels in hypertensive subjects.[100] Unfortunately, this could not be verified directly in our experiments. Firstly, serum levels of U-II in subjects from SRTB or WPKP were not measured. Secondly, uncertainty remains regarding the accuracy of assays measuring U-II.[105] Finally, higher serum levels of U-II in hypertensive subjects may not originate from the kidney but other organs. Taken together, my data do not support the hypothesis of primary up-regulation of the U-II system in the kidney as a mechanism of genetic predisposition to essential hypertension.

Having found no evidence of association between U-II system genes and BP or kidney function in genetic association and gene expression studies I examined the evolution of this system in available vertebrate species. Indeed, one of the factors nurturing ongoing interest in U-II system is its high evolutionary conservation.[72] Direct comparison of pre-pro *UTS2* amino acid sequences from different vertebrate species showed that whilst the overall conservation of the pre-pro-peptide was only moderate, the C-terminal cyclic domain (necessary for the interaction with the receptor) was absolutely conserved.[72] These data were interpreted as an evidence of the importance of U-II in physiology. However, the evolutionary conservation has never been assessed in the context of the whole system including URP and UT.

U-II shows substantial structural similarity to somatostatin.[79] In fact, it is believed that *UTS2* and *UTS2D* genes belong to the somatostatin superfamily and arose through two events of segmental/chromosomal duplication from the common ancestral somatostatin/urotensin gene.[281,282] Furthermore, *UTS2R* shows high sequence identity with somatostatin receptor and this gene is physically close to somatostatin receptor *SST3* gene.[281] There is also an analogous U-II gene and promoter regions organisation, across different species.[283] This would imply that apart from conservation of the structure, the functionality of the system may be also preserved across different species. However, there are substantial differences between functioning of the system in different species.[107] It has been shown that rodent and primate *UTS2R* act differently upon stimulation with an artificial ligand. [284] Similar observation come from the use of native and non-native U-II on tissues, suggesting that also the residues outside the conserved cyclic part may play role in ligand - receptor interaction.[285]

My evolutionary analysis was conducted on coding sequences of *UTS2*, *UTS2D* and *UTS2R* from publicly available databases. The principle of this analysis was based on comparing ratios of non-synonymous DNA changes (dN - changes in DNA introducing changes in amino acid structure of the protein) to synonymous substitutions (dS - DNA changes without effect on protein function – generally considered not important from evolutionary point of view).[257] The analysis uses data about DNA changes and linked to these peptide sequence alternations, therefore the dN/dS ratios can only be calculated for protein coding DNA sequences. Ratios <1 suggest purifying selection aiming at preservation of the structure in unchanged

state. Ratios >1 may indicate both: loss of function (accumulation of random mutations) or directional selection (evolution towards changes increasing fitness of the species).[257] The analysis of the whole pre-pro peptide sequence of *UTS2* showed very different signatures of evolution among different species – non-synonymous to synonymous substitution ratios ranged from 0.14 (purifying selection) to 1.15 (accumulation of mutations). This implies existence of substantial divergence in evolutionary forces across the phylogenetic tree of *UTS2*. Less diversity in evolutionary forces was identified in *UTS2D* and *UTS2R*. Indeed, both molecules are subject to purifying selection with dN/dS ratios ranging from 0.03-0.57 for *UTS2D* and 0.04-0.2 for *UTS2R*. These data suggest stronger conservation of *UTS2R* than *UTS2* and are consistent with the fundamental role of *UTS2R* in signalling of the pathway. It appears that *UTS2* underwent a period of strong diversifying selection following divergence of primates. In this period *UTS2* sequence was rapidly changing by accumulation of non-synonymous mutations. Similar trends are apparent in the analysis of *UTS2R*. Although, the receptor showed overall higher conservation than *UTS2*, the purifying selection in primates was much weaker than in the phylogenetically older vertebrates. Collectively these data suggest that similar evolutionary forces (tendency for weaker conservation) acted at least in part, on the *UTS2* and *UTS2R* since the divergence of primates. Direct extrapolation of results obtained from lower vertebrates to primates should be made with caution.

Collectively, these suggests that U-II system is unlikely to be associated with BP and renal function in European population. This is supported by other findings.[286] No major hemodynamic changes were seen in *UTS2R* knockout mice, apart from

attenuated vascular response to administration of exogenous *UTS2*.[264] Systemic infusion of *UTS2* in human volunteers did not cause any hemodynamic changes either in central or peripheral circulation.[287] The pharmacological regulation of the system is also unclear. Agonists bind to UT with very high affinity, which makes this binding pseudo-irreversible.[288] This results in all binding sites saturated by physiological levels of agonists, leaving *UTS2R* expression and recycling as the most likely way of regulating the system. Higher levels of U-II have been shown as protective in patients with ESRD and acute myocardial infarction casting doubt on previously suggested deleterious action of U-II pathway.[157,265] Therefore, the evolutionary analysis could provide some explanation for these discrepancies. The U-II system may be important in phylogenetically older animals but may have slowly evolved towards loss of function since the divergence of primates. It would be fair to acknowledge that some data do not fit entirely into this theory. For example, infusion of U-II caused circulatory collapse and death in anaesthesized cynomolgus monkeys (primates).[153] This would not be possible if the system was inactive in primates.

Having found no convincing association signal between common genetic variants in U-II pathway genes and BP or eGFR I used the available genotype data from 50K IBC chip genotyping in the GRAPHIC Study to perform large scale genetic association. The overall quality of genotype data in this genetic association analysis was good. Following genotyping, stringent quality control filters were applied to ensure appropriate reliability of results. This was confirmed by the visual inspection of quantile-quantile plots and calculations of genomic control for each of the

phenotypes. The quantile-quantile plots did not show deviation of the statistic from the expected distributions. This was confirmed by the calculation of genomic control. Also, there were no evidence of population stratification in the GRAPHIC Study. All subjects in the parental generation showed random genetic distribution, when spread according to genetic dissimilarity. (Figure 20) As expected, the GRAPHIC Study showed genetic similarity with the HapMap population of white European ancestry (CEU).

Out of the total of 33577 SNPs analysed for association with eGFR, clinic SBP and clinic DBP only 3 markers showed statistically significant association with age, age^2, sex and anti-hypertensive treatment adjusted clinic DBP. All three SNPs had similar MAF, location in the intronic region of MTHFR and had no obvious biological significance. Conditional analysis showed that the signal of association obtained for these 3 markers was not independent, but most likely originated from the strong LD between these SNPs. Furthermore, analysis of the structure of the LD in the region indicated that the haploblock comprising the top findings from association analysis may extend even further and cover two additional candidate genes *NPPA* and *NPPB*.

None of the functional variants within MTHFR/CLCN6/NPPA/NPPB, present on the 50K IBC array, showed significant or even suggestive associations with clinic DBP. The strongest signal came from a missense *MTHFR* SNP rs1801131. This functional variant is in moderate LD (r^2=0.445) with the top signal from association with clinic DBP - rs17037388. The rs1801131 was directly genotyped on the 50K IBC but the strength of the association signal was weaker than for rs17037388.

Therefore, it is likely that the association of clinic DBP and rs1801131 was, at least to some extent, driven by its LD with rs17037388.

The MTHFR/CLCN6/NPPA/NPPB locus comprises 4 genes in a close physical proximity to each other:

MTHFR encodes an enzyme catalysing the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a substrate necessary for the metabolism of homocysteine to methionine. *MTHFR* is widely expressed in the human body especially in the bone marrow, nervous system, heart lung, kidney pancreas and liver (http://www.genecards.org, http://www.ebi.ac.uk).

CLCN6 - encodes a member of the voltage-dependent chloride channel protein family, which functions as a chloride/proton antiporter in the cells. Similarly to *MTHFR*, *CLCN6* is widely expressed in the human body including bone marrow, brain, heart, lung kidneys and liver (http://www.genecards.org, http://www.ebi.ac.uk).

NPPA and *NPPB* encode proteins of the natriuretic peptide family. Natriuretic peptides are involved in the control of extracellular fluid volume and electrolyte homeostasis by influencing diuresis, natriuresis and vasodilatation.[289] Natriuretic peptides are mainly expressed in the brain, heart, kidneys and liver. Given their biological function, all 4 genes in this locus all plausible biological candidates for regulation of blood pressure.

MTHFR/CLCN6/NPPA/NPPB locus has been previously implicated in the regulation of BP in animal models. In rat, *MTHFR* maps to the BP QTLs 7, 103, 139, 147, 155, 210 and 292.[194] The MTHFR/CLCN6/NPPA/NPPB locus shows identical

organisation in rodents as in humans and also comprises 4 genes *MTHFR*, *CLCN6*, *NPPA* and *NPPB*(http://www.ensembl.org).

Genetic associations between BP and MTHFR/CLCN6/NPPA/NPPB locus have been reported before. In the analysis of genome wide association data from the Global BPgen consortium (34,433 subjects of European ancestry) an intronic *MTHFR* SNP rs17367504 (showing significant association with clinic DBP in the GRAPHIC Study) was the second most significant association signal with clinic SBP.[290] Due to the location in an intronic region it was speculated, that this association signal could be driven by the proximity of the functional *MTHFR* SNP - rs1801133 (missense variant previously associated with plasma homocysteine concentration[291] and hypertension[292]) or 3' untranslated *NPPA* SNP rs5068 (previously associated with BP[293]). Both SNPs (rs1801133 and rs5068) have been directly genotyped in the GRAPHIC Study. However, rs1801133 (MAF=0.33) showed association with neither clinic SBP (p=0.4708) nor with clinic DBP (p=0.0784). The rs5068 (MAF=0.06) showed nominal association only with adjusted clinic DBP (p=0.0134). However, due to weak LD with the lead SNP rs17037388 from the association analysis with clinic DBP ($r^2_{rs17037388xrs1801133}$=0.10; $r^2_{rs17037388xrs5068}$=0.26) these functional variants were unlikely to explain the observed association signal in this analysis.

The previous large scale genetic association analysis in the GRAPHIC Study showed association of genetic variant rs13306560 in MTHFR/CLCN6/NPPA/NPPB locus with mean 24-hour DBP.[50] It was speculated that due to its location in the CpG rich 5' region of *MTHFR*, rs13306560 might influence *MTHFR* promoter activity

through its influence on the DNA methylation status.[50] However, no data on the differential expression of *MTHFR* conditioned on rs13306560 genotype were available for the GRAPHIC Study. The rs13306560 SNP (MAF=0.05) showed nominally significant association with adjusted clinic SBP and even stronger with clinic DBP (p=0.0039; p=0.0001, respectively). However, due to moderately weak LD between top clinic DBP SNP rs17037388 and rs13306560 ($r^2_{rs17037388x\ rs13306560}$=0.28) the rs13306560 was unlikely to account for the association signal observed for clinic DBP.

An intronic MTHFR SNP rs17367504 (previously associated with clinic SBP in the study by Newton-Cheh[293]) and associated with adjusted clinic DBP in the GRAPHIC Study) showed negative association with clinic SBP, clinic DBP and hypertension in the meta-analysis of blood pressure GWAS in >200,000 individuals.[276] This SNP showed also association with circulating plasma levels of atrial natriuretic peptide and brain natriuretic peptide in a subset of subjects of the study (Framingham Heart Study offspring cohort).[276]

In the recent meta-analysis of genetic association of blood pressure phenotypes (using 50K IBC array) the most significant signal within MTHFR/CLCN6/NPPA/NPPB locus was located in the 3' region of the *MTHFR* - rs4846049.[294] However, the multiple regression model with rs4846049, and rs5068 and rs17367504 suggested presence of 2 independent association signals.[294] The gene expression analysis showed strong association of rs4846049 with *MTHFR* and *CLCN6* expression in whole blood and monocytes (*MTHFR* expression only).[294] Further *in silico* analysis revealed that rs4846049 lies within a region for binding by

the signal transducer and activator of transcription 1 (*STAT1*).[294] In the GRAPHIC study rs4846049 (MAF=0.321) did not show association with adjusted clinic SBP (p=0.0657) but showed association with clinic DBP at the nominal level (p=0.0001).

Several studies have reported presence of significant signal of BP phenotypes association with markers spread across the MTHFR/CLCN6/NPPA/NPPB locus. Depending on the study the most significant markers were located in 3', 5' or intronic regions of the *MTHFR*.[50,276,290,294,295] Also, in several studies more than one marker in this locus was strongly associated with analysed traits.[50,276,290,294,295] Collectively, the data suggest the presence of functional genetic variant(s) within MTHFR/CLCN6/NPPA/NPPB locus. The difference in the variant showing the strongest association between the studies are likely to originate from the differences in the LD structure of the locus between the populations.[296]

Whereas the exact mechanisms genetic variants in the MTHFR/CLCN6/NPPA/NPPB influence blood pressure in human remains to be elucidated the consistently strong significant association with BP phenotypes in cohorts of different ethnical origins has major practical implications. *MTHFR* together with nutritional folic acid are crucial for lowering plasma homocysteine – a vasculotoxic amino-acid known to cause endothelial dysfunction.[297] The abundance of folic acid and appropriate activity of *MTHFR* warrant efficient blood vessel regulation through nitric oxide synthesis.[298] Although the links between nutritional folic acid intake, plasma homocysteine and cardiovascular risk was long known, the consistency and strength of this association shed new light on the existing evidence on the role of folate metabolism in the pathogenesis of high blood pressure.[298] A

prospective cohort study of more than 150 thousands American women (Nurses' Health Study I and Nurses' Health Study II) showed that consumption of >1mg of folic acid a day was related to reduced risk of developing hypertension (RR=0.54, p<0.001).[299] Similar conclusion came from 20 years prospective observation of 4400 young (18-30 years) African Americans and white Caucasians, where being in the highest quintile of dietary folate intake was related to reduced risk of developing hypertension (RR=0.48, p<0.01).[300] However, among the spectrum of current anti-hypertensive therapies none are aimed at direct influencing the MTHFR/folate pathway. These data strongly suggest that when looking for blood pressure lowering therapies among the novel pathways we may have overlooked already existing evidence on the role of folate metabolism in this condition.

Apart from the SNPs in the MTHFR/CLCN6/NPPA/NPPB locus all other suggestive association signals in the association of clinic SBP, clinic DBP and eGFR in the GRAPHIC Study were driven by single SNPs.

PPARG locus, where rs1152002, common, intronic SNP showed suggestive association signal with adjusted clinic DBP did not find confirmation in any of the previous large scale genetic association studies.[50,276,290,295]

The only suggestive association signal with adjusted clinic SBP was driven by a single SNP rs2623410 within *PDE1A*. This SNP was rare amongst the subjects of the GRAPHIC Study (MAF=0.012); only just above the cut off threshold for quality control filters. Furthermore, no other SNPs in *PDE1A* showed even nominal association with clinic SBP. Apart from one SNP showing suggestive association

with mean 24-hour SBP in the genetic association study in the GRAPHIC cohort[50], no other large scale genetic association study showed any association signals in the *PDE1A* locus.[50,276,290,295]

The solitary SNPs (rs12035969 - in *PER3* and rs1004446 in *IGF2*) showed association with eGFR in the GRAPHIC Study. These 2 loci showed no association with renal function traits in the large scale genetic association studies.[275,301-303] Although the results of association of *PER3* with eGFR in the GRAPHIC Study are essentially negative the locus is an important candidate on the map of genetic factors contributing to increased cardiovascular risk. There is an evidence on the association of genetic variants within *PER3* with sleep and circadian rhythm disturbances, both of which are controlled by the autonomic nervous system.[304] Disturbed autonomic control, manifesting as disruption of the normal circadian pattern of BP or heart rate variability, has been linked to increased cardiovascular mortality.[200,304]

In summary, despite small sample size the results of association in the GRAPHIC Study identified a genuine association signal with adjusted clinic DBP in the MTHFR/CLCN6/NPPA/NPPB locus. The identified association signal has confirmation in all recent meta-analyses of GWAS with BP phenotypes.[276,290,294]

The use of large scale gene centric 50K IBC array for the purpose of our analysis had several advantages. Firstly, the loci included on the array were not random but chosen based on the *a priori* knowledge about their possible association with cardiovascular phenotypes.[234] The density of markers in these loci was therefore higher than on the commercially available genome wide arrays, giving

better tagging resolution. Unlike the genome wide arrays (usually designed using a tagging approach specific for a given population) the markers on the 50K IBC array were selected to ensure consistent coverage across multiple ancestries.[234] To allow for the analysis of functional SNPs, the 50K IBC array was enriched for the known functional genetic variants, often characterised by low MAF and therefore absent on the standard genotyping arrays.[234] Finally, lower than normally number of genotyped markers reduced the cut-off threshold required to correct for multiple testing.

However, the approach chosen here has also several disadvantages. Relatively small sample size of the GRAPHIC cohort may explain low number of association signals detected. The limited power of the study did not allow for the analysis of rare (often functional) genetic variant (MAF<1), which have very good representation on the 50K IBC array. Clustering of genetic markers within candidate loci meant that signal in other potential regions (genes with no clear link to cardiovascular phenotypes and regions without protein coding sequences- so-called “gene deserts”) would be missed in the analysis.[305]

The experiments performed for the purpose of this thesis used two distinct approaches to address the same research problem – identify genetic variants contributing to variation in blood pressure and renal function. The candidate gene approach was based on the existing evidence suggesting pathophysiological role of U-II pathway in predisposition to high blood pressure and impaired renal function. Conversely, genetic association of the 50K IBC array genotyping data with BP and renal function involved analysis of over 2000 loci, each of which had the potential to be associated with phenotypes of interest. However, while examining the same

genetic contribution to BP and renal function, these two experiments aimed at answering different research questions. For the U-II system genes results of association analysis, whether positive or negative were of interest. Positive association result would strengthen the current opinion on the importance of U-II pathway for regulation of blood pressure or renal function, whereas negative results would weaken this belief. Eventually, lack of the association observed in the presented experiment could not be interpreted as a definite evidence against involvement of U-II pathway in pathogenesis of cardiovascular conditions due to several limitations of the study. At the same time lack of signal in genetic association combined with no evidence of altered gene expression between normotensive and hypertensive subjects and uncertainty regarding complete conservation of the U-II system puts its role in the pathophysiology of human high blood pressure and impaired renal function at doubt.

The results of the large scale genetic association experiment using 50K IBC data provided very different information. While, not burdened by *a priori* hypothesis (apart from the assumptions made during design of the array) the positive association can lead to discovery of locus, which could not be predicted based on the current knowledge. Although 50K IBC array was designed to capture common genetic variation within genes with possible involvement in cardiovascular pathology, the genes of U-II pathway (*UTS2D* and *UTS2R*) were not adequately saturated with genetic markers. Finally, due to the number of performed statistical tests lack of significant association in this experiment could only be interpreted in light of inadequate power of the study.

In summary, both approaches used for the same purpose of dissecting genetic architecture of renal function and BP provided valuable information about genetic contribution to population variation in these phenotypes. Due to the difference in their designs, the results obtained from our candidate gene and large scale genetic association analysis are an example of parallel use of different designs in searching for genuine genetic association.

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