UNIVERSITA' DEGLI STUDI DI PADOVA

Sede Amministrativa: Università degli Studi di Padova

Dipartimento di Medicina Clinica e Sperimentale

DOTTORATO DI RICERCA
IPERTENSIONE ARTERIOSA E BIOLOGIA VASCOLARE
CICLO XX

BLOOD PRESSURE AND METABOLIC PHENOTYPES IN RELATION TO SAH GENE VARIANTS AND *ADRB1 Arg389Gly* AND *ADRAB2 I/D* POLYMORPHISMS IN WHITE FAMILY-BASED POPULATION SAMPLES

Coordinatore: CH.MO PROF. ACHILLE C. PESSINA
Supervisore: PROF. EDOARDO CASIGLIA

Dottoranda : VALERIE TIKHONOFF

DATA CONSEGNA TESI

15 marzo 2008

Table of Contents

Summary

Objectives Aim of the present doctoral dissertation is independent confirmation in family-based population samples, using strict appropriate statistical approach, of the associations, if any, between blood pressure (BP) and related metabolic phenotypes, analysed as continuous traits, and variations in candidate genes arising from experimental animal models [Spontaneously hypertensive rat-clone A-Hypertension-associated (SAH) gene], and from physiological cascades of the adrenergic system [α_{2B}- (ADRAB2) and β_1- (ADRB1) adrenergic receptors].

Methods and Results The SAH gene variants were evaluated in the frame of the European Project On Genes in Hypertension. In details, 2603 relatives from 560 families and 31 unrelated subjects (mean age 38.8±15.7 years; 52.1% women) were randomly recruited from six European populations. Systolic/diastolic BP, body mass index, triceps skinfold, waist-to-hip ratio, serum total and HDL cholesterol, serum triglycerides and blood glucose were measured. All subjects were genotyped for the *G-1606A* and *-962 del/ins* polymorphisms and the allele frequencies were 11.8% and 29.5% for *-1606A* and *-962del,* respectively. Lewontin's D' was 0.97 (p<0.0001). Haplotype frequencies were 58.8% for *-1606G* plus *-962ins*, 29.5% for *-1606G* plus *-962del*, and 11.7% for *-1606A* plus *-962ins*. Both before and after adjustment for covariates, none of the phenotype-genotype associations approached statistical significance. Family-based analyses did not reveal any population stratification (P≥0.67) as a possible explanation of those negative results.

The association studies between *ADRB1 Arg389Gly* and *ADRA2B I/D* polymorphisms of the β_1- and α_{2B}-adrenergic receptors with BP and metabolic phenotypes, were conducted in a subsample of the EPOGH cohort. 1802 relatives from 175 families and 79 unrelated subjects (mean age 45.5±15.7 years; 51.1% women) were randomly recruited from a Caucasian population living in Northern Belgium. Systolic/diastolic BP, body mass index, waist-to-hip ratio, serum total and HDL cholesterol were measured. All subjects were genotyped for the *ADRA2B I/D* and *ADRB1 Arg389Gly* polymorphisms. The *ADRA2B* genotypes (*II* 45.7%, *ID* 41.7%, and *DD* 12.5%; *P*=0.05) and the *ADRB1* genotypes (*ArgArg* 56.2%, *ArgGly* 36.9%,

and *GlyGly* 6.9%; *P*=0.66) did not deviate from Hardy–Weinberg proportions. *ADRB1 ArgArg* homozygotes, compared with *Gly* allele carriers, had higher diastolic BP (79.4 vs 78.4 mmHg; *P*=0.012), and higher serum HDL cholesterol (1.33 vs 1.29 mmol/l; *P*=0.020). None of the other cardiovascular or metabolic phenotypes in relation to the two polymorphisms reached significance. The family-based analyses did not reveal population stratification (P≥0.23).

Conclusions The present study gives evidence in favour of association of diastolic BP and HDL cholesterol with the *ADRB1 Arg389Gly* polymorphism in the absence of population stratification. However, the evidences supporting association of hypertension or hypertension−related phenotypes with the SAH gene remain equivocal in human studies.

Riassunto

Obiettivi Scopo della presente tesi è la conferma mediante rigoroso approccio statistico condotto a livello di popolazione a gruppi famigliari, dell'esistenza di possibili associazioni fra fenotipi analizzati come variabili continue (quali pressione arteriosa e fattori metabolici) e polimorfismi di geni candidati originati sia da studi sperimentali su modelli animali [Spontaneously hypertensive rat-clone A-Hypertension-associated (SAH) gene], sia da ben noti processi fisiologici quale il sistema adrenergico [recettori adrenergici α_{2B}- (ADRAB2) e β_1- (ADRB1)].

Metodi e Risultati Le varianti geniche de gene SAH sono state analizzate nell'ambito dell' European Project On Genes in Hypertension. Si sono reclutati in modo casuale 2603 soggetti membri di 560 famiglie e 31 soggetti indipendenti (età media 38.8±15.7 anni; 52.1% femmine) provenienti da 6 popolazioni Europee. Si sono misurati la pressione arteriosa sistolica e diastolica, l'indice di massa corporea, la plica tricipitale, il rapporto vita/fianchi e i livelli sierici di colesterolo totale e HDL, di trigliceridi e di glucosio. In tutti i soggetti si sono determinati i polimorfismi *G-1606A* e *-962 del/ins* del gene SAH. Le frequenze alleliche erano 11.8% e 29.5% rispettivamente per *-1606A* e *-962del*. Il parametro Lewontin's D' era 0.97 (p<0.0001). Le frequenze aplotipiche erano 58.8% per *-1606G* associato a *-962ins*, 29.5% per *-1606G* associato a *-962del*, e 11.7% per *-1606A* associato a *-962ins*. Sia con che senza aggiustamenti per fattori confondenti, nessuna associazione genotipo-fenotipo risultava statisticamente significativa. L'analisi a gruppi famigliari ha escluso la presenza di una stratificazione di popolazione come possibile spiegazione dei presenti risultati negativi (P≥0.67).

Gli studi di associazione tra i polimorfismi *ADRB1 Arg389Gly* e *ADRA2B I/D* dei recettori adrenergici β_1 e α_{2B} con i fenotipi pressione arteriosa e fattori metabolici sono stati condotti in un sottogruppo dello studio EPOGH. Si sono reclutati in modo casuale 1802 soggetti membri di 175 famiglie e 79 soggetti indipendenti (età media 45.5±15.7 anni; 51.1% femmine) nell'ambito di una popolazione Caucasica del Nord del Belgio. Si sono misurati pressione arteriosa sistolica e diastolica, indice di massa corporea, rapporto vita fianchi, livelli sierici di

colesterolo totale e HDL. In tutti i soggetti si è eseguita l'analisi genetica per I polimorfismi *ADRA2B I/D* e *ADRB1 Arg389Gly*. La frequenza dei genotipi *ADRA2B* (*II* 45.7%, *ID* 41.7%, e *DD* 12.5%; *P*=0.05) e *ADRB1* (*ArgArg* 56.2%, *ArgGly* 36.9%, e *GlyGly* 6.9%; *P*=0.66) non devia dall'equilibrio di Hardy–Weinberg. Gli omozigoti *ADRB1 ArgArg*, rispetto ai portatori dell'allele Gly, avevano valori di diastolica più alta (79.4 vs 78.4 mmHg; *P*=0.012), ed elevati livelli sierici di colesterolo HDL (1.33 vs 1.29 mmol/l; *P*=0.020). Nessun altro fra i fattori cardiovascolari o metabolici risultava associato in modo significativo a questi due polimorfismi. L'analisi a gruppi famigliari ha escluso la presenza di una stratificazione di popolazione (P≥0.23).

Conclusioni Nella presente tesi si dimostra come esista una associazione fra pressione diastolica e livelli sierici di colesterolo HDL, ed il polimorfismo *ADRB1 Arg389Gly* in assenza di stratificazione di popolazione. Tuttavia, l'associazione fra pressione arteriosa e fenotipi relati all'ipertensione, e varianti del gene SAH rimangono equivoci negli studi umani.

Introduction

The multifactorial basis of hypertension is well established and 30 to 50% of the inter-individual variation in blood pressure is attributed to genetic factors [1,2]. Studies in twins document greater concordance of blood pressures of monozygotic than dizygotic twins [3], and population studies demonstrate greater similarity of blood pressure within than between families [4]. This familial aggregation of blood pressure is not simply attributable to shared environmental effects, since adoption studies show greater concordance of blood pressure among biological siblings than adoptive siblings living in the same household [5]. However, the genes responsible for blood pressure variation are mostly unknown.

There are two different strategies to identify trait susceptibility genes: the genome-wide linkage analysis and the candidate gene approach. The former makes use of genetic markers covering the whole genome, which are subsequently investigated for linkage with a trait, in families with multiple affected relatives. Markers that segregate with the trait in relatives more often than expected are used to narrow down the location of the specific gene. Therefore, searches for causative variants in chromosome regions identified by linkage analysis have been successful for rare monogenic forms of hypertension only. Technological advances in genomics, proteomics and molecular medicine led to the discovery of 17 human genes causing Mendelian forms of blood pressure dysregulation [6]. However, monogenic forms of hypertension are extremely rare. By contrast, linkage studies have been much less successful in locating genetic variants that affect common complex disorders, such as essential arterial hypertension, because each variant individually contributes only modestly to risk of disease. Until recently, 25 whole-genome scans with focus on blood pressure or hypertension have been performed in sib-pairs or families of African, Asian, or Caucasian origin [7-9]. These studies identified more than 30 loci distributed over almost all the chromosomes, which showed linkage with human hypertension. Until now, none of these genome-wide scans have led to the discovery of common genetic variants, which may have a large impact on blood pressure in the population at large. This is mainly due to lack of power for genome-wide significance for

multiple testing and to the fact that none of the loci found so far have been confirmed by more than one study.

Another approach to identify genetic causes of disease is to search for association with specific candidate genes, selected from systems physiologically implicated in blood pressure regulation, e.g. the renin-angiotensin system or the adrenergic system [10]. More than 150 candidate genes have been studied in relation to hypertension and other cardiovascular phenotypes. Often, strong associations are reported that are not confirmed in subsequent studies. However, a negative finding or a minor genetic effect in a general population may become a major gene effect in a subgroup of people with the appropriate genetic and environmental background. The lack of consistent reproducibility of these studies highlights the difficulties in genetic investigation of multifactorial traits. It seems apparent that firm conclusions will require consistent replication of results in large and rigorously characterized populations that are well suited for detecting alleles imparting small effects. Such populations would include cohorts of unrelated individuals as well as family-based studies that would allow to use tests of transmission disequilibrium. These latter tests minimize the chance of false-positive associations arising from population admixture of individuals of different genetic background [11].

In addition to human studies, investigation of spontaneous and engineered animal models of hypertension hold promise for understanding the long-term regulation of blood pressure. A number of inbred animal models of hypertension has had blood pressure quantitative trait loci mapped [12]. Some authors have presented a comparative genomic map for candidate hypertension loci in humans based on translating these loci between rat and human, predicting 26 chromosomal regions in the human genome that are very likely to arbour hypertension genes [8,12]. To date, however, only few of these have been identified at the molecular level [13]. More instructive thus far have been genetically engineered mice in which gene activities have been specifically increased or decreased [14].

In the present doctoral dissertation the question to what extent blood pressure, analysed as continuous traits in a large family-based population sample, is associated with variations in candidate genes arising from animal models (Spontaneously hypertensive rat-

8

clone A-Hypertension-associated gene), or from available physiological cascades on the adrenergic system (β1 and α2B adrenergic receptors) is addressed. We analysed for each candidate gene the possible associations with metabolic phenotypes as well. Indeed, in aggregate with hypertension, measures of obesity, insulin resistance, and dyslipidemia, represent major causes of morbidity and mortality in industrialized countries. Studies of familial aggregation, of adoption, with twins, and segregation analysis studies indicate that metabolic risk factors are highly heritable with substantial genetic effects, interacting with environmental factors, leading to development of cardiovascular disease [15]. Although the genes for uncommon monogenic forms of obesity, insulin resistance, and dyslipidemia have been identified, these gene variants do not account for most of the heritability of these conditions in the general population. Furthermore linkages to various chromosomal regions have been reported for these disorders in the general population [15-17]. However, genetic heterogeneity and/or allelic variation may contribute to the difficulty in identifying causal genes.

Part 1

SAH gene variants revisited in the
European Project On Genes in Hypertension

Introduction

The expression of SAH (*Spontaneously hypertensive rat-clone A-H*ypertension-associated) gene is markedly higher in the kidneys of spontaneously hypertensive rats compared with normotensive Wistar-Kyoto controls [18]. In rats, SAH is expressed mainly in proximal renal tubules and in hepatocytes [19,20]. The human SAH gene is located on chromosome 16p13.11, spans about 33 kb, consists of 14 exons and 13 introns, and is expressed in the kidney [21]. Transfected cells expressed the SAH protein in the mitochondria [22]. Cell experiments suggest that it has medium chain fatty acid Coenzyme A ligase activity and that it might be involved in lipid metabolism [22,23].

The Suita population study [22,24] and subsequent case-control studies [25-27] reported significant associations of hypertension and related phenotypes, such as obesity, hypercholesterolemia and hypertriglyceridemia, with genetic variation in the SAH gene. More recently, Telgmann et al. [25] identified five polymorphisms in the SAH promoter region (*C-1808T, G-1606A, -962I/D, G-451A, T-67C*), two polymorphisms in introns 5 and 7 (*C+9/In5T, A+20/In7T*), and one missense variant (*K359N*). In the Project d'Etude des Gènes de l'Hypertension Artérielle Sévère à Modérée Essentielle (PEGASE) case-control study, Telgmann and colleagues [25] reported association between obesity-related hypertension and the SAH G-1606A and -962ins/del polymorphisms. Using continuous phenotypes, we sought independent confirmation of Telgmann's findings in the randomly recruited participants of the family-based European Project On Genes in Hypertension (EPOGH).

Methods

General outline of the study

The primary goal of the EPOGH study was to investigate the complex relationship between blood pressure, analyzed as a continuous or binary phenotype, and various candidate genes [28]. The epidemiological methods used in EPOGH have been previously validated [29]. The EPOGH project was conducted according to the principals outlined in the Helsinki declaration for investigations in human subjects [30]. The Ethics Committee of each institution approved the protocol. Participants gave written informed consent.

Study population

The investigators randomly enrolled nuclear and extended families of White ethnicity from the populations of Bucharest (Romania; n=295), Cracow (Poland; n=326), Hechtel-Eksel (Belgium; n=1276), Mirano (Italy; n=355), Novosibirsk (Russian Federation; n=324), and Pilsen and Prague (Czech Republic; n=399). Nuclear families had to include at least one parent and two siblings. The minimum age for participation was 10 years. Family members had to live within a distance of no more than approximately 10 km to make repeated home visits feasible. The overall response rate was 63.7%. Of these 2975 subjects, we excluded 341 from analysis, because DNA could not be extracted or could not be genotyped or failed to amplify (n=308), or because of inconsistency in Mendelian segregation (n=33). Thus, the number of subjects statistically analyzed totalled 2634.

Phenotypes

The blood pressure phenotype was the average of five consecutive readings at one home visit. After the subjects had rested in the sitting position for 10 min or longer, trained observers measured blood pressure with a mercury sphygmomanometer according to the guidelines of the British Hypertension Society [31]. Standard cuffs had a 12-24 cm inflatable bladder, but, if upper arm circumference exceeded 31 cm, larger cuffs with 15-35 cm bladder were used. Quality control of the blood pressure phenotype in this population study is described in detail and refers to the procedures set up at all centres to ensure high quality blood pressure measurements throughout the project [28]. Hypertension was a blood pressure of at least 140 mm Hg systolic or 90 mm Hg diastolic or the use of antihypertensive

drugs. The observers also measured weight and height with the subjects wearing light indoor clothing without shoes. Body mass index was weight in kilograms divided by the square of height in meters. The waist-to-hip ratio, determined by means of a measuring tape, was the ratio of the smallest circumference at the waist to the largest circumference at the hip. The triceps skinfold was measured at the midportion of the muscle by means of a Harpenden Skinfold Caliper (Bedfordshire, UK), providing a constant pressure of 0.01 kg/mm^2 (0.098 N/mm^2) ± 10% at all openings of the 90 mm^2 anvils.

We administered a validated questionnaire [32] to collect information on each subject's medical history, use of medications, and smoking and drinking habits. From the type and number of alcoholic beverages used each day, we calculated alcohol consumption in grams per day. To exclude occasional drinkers, we defined current alcohol intake as a consumption of at least 5 g of ethanol per day. Venous blood samples, collected in the late afternoon (1056 Belgian participants) or after overnight fasting (181 Belgian and 1397 non-Belgian participants), were analysed for serum total and high-density lipoprotein (HDL) cholesterol, serum triglycerides and blood glucose by automated enzymatic methods.

Determination of genotypes

We extracted genomic DNA from white blood cells, using standard kits (Qiagen, Hilden, Germany). For genotyping, we used restriction fragment length polymorphism analysis (RFLP) of the respective PCR products at the frequent polymorphic loci *G-1606A* (restriction enzyme *KspA*I) and *-962 ins/del* (*Xap*I); as C-1808T was completely associated with *G-1606A*, we did not genotype for this variant.

Using the published sequence of human SAH gene (accession number AC004381.1; Figure 1), primers were designed to amplify both fragments covering the SAH polymorphisms *G-1606A* and *-962ins/del*. The PCR amplification was carried out in a 20-µL volume, containing 5 ng genomic DNA, 10 and 25 pmol/L of each primer (for both fragments, respectively), 2.5 mmol/L MgCl$_2$, 200 µmol/L of each dNTPs, 5 mmol/L betaine and 1U AmpliTaq polymerase (Rapidozym, Berlin, Germany). The PCR was performed initially for 3 min at 94°C and followed by 35 cycles, each with 45 sec at 94°C, 45 sec at 54° and 56°C (depending on primer sequence), 60 sec at 72°C and final elongation of 10 min at 72°C.

12

In the presence of the -*1606A* allele, the PCR product (221 bp) was digested by *KspA*I into 2 fragments of 191 and 30 bp in lengths (−*1606G* undigested). In the presence of the -*962del* allele, the PCR product (339 bp) was digested by *Xap*I into 3 fragments of 180, 93 and 66 bp in lengths. For -*962ins*, the PCR product (344 bp) was digested into 4 fragments of 180, 82, 66 and 16 bp in lengths visualized on ethidium bromide-stained 3% agarose gels. Specific amplification protocols for each primer pair and genotyping conditions for all polymorphisms are reported at the web site http://genecanvas.idf.inserm.fr.

Figure 1 Schematic representation of the human SAH gene (accession number AC004381.1) and positions of the analyzed SNPs. The SAH gene is located on chromosome 16p13.11, spans approximately 33 kb and consists of 14 exons (open boxes) and 13 introns. Black arrows indicate the localization of the variants in the 5'-flanking region (*C-1808T, G-1606A, -962ins/del* TTTAA). *G-1606A* is in complete association with *C-1808T*. The bent arrow indicates the transcription starting site. The asterisk marks the starting ATG sequence.
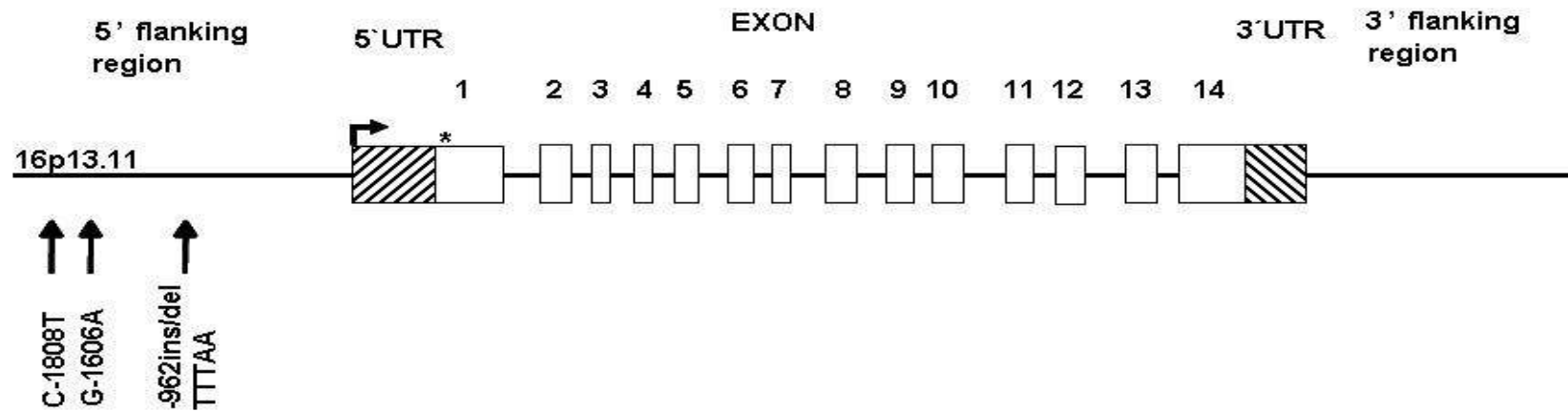
Statistical methods

For database management and statistical analysis, SAS version 9.1.3 (SAS Institute Inc., Cary, NC) was used. Population means and proportions were compared by Tukey's test for multiple comparisons test and the χ^2-statistic with Bonferroni's correction, respectively. Because Shapiro-Wilk's test showed significant departure from normality, we logarithmically transformed triceps skinfold thickness as well as blood glucose and serum triglyceride concentrations. We searched for possible covariates of the metabolic phenotypes, using stepwise multiple regression analysis with the *P* value for independent variables to enter and stay in the models set at 0.10. Depending on the phenotypes, the variables considered for entry into the models were sex, age, age squared, body mass index, use of β-blockers, diuretics or angiotensin-converting enzyme inhibitors, intake of lipid lowering drugs, smoking (0,1), and alcohol consumption (0,1). We adjusted all models for centre and those for blood glucose and serum lipids also for fasting status (0,1).

We tested linkage disequilibrium between the two polymorphisms and we reconstructed haplotypes using the PROC HAPLOTYPE procedure available in the genetics module of the SAS software. In population-based analyses, we applied a generalization of the standard linear model as implemented in the PROC MIXED procedure of the SAS package to test the association between phenotypes and single polymorphisms or haplotypes, while adjusting for covariates as well as for the non-independence of observations within families. In the mixed model, we also tested for heterogeneity across centres, using appropriate interaction terms. To remain consistent with previous studies [25], we compared SAH *-1606GG* homozygotes with carriers of the less frequent *-1606A* allele and SAH *-962ins* homozygotes with carriers of the less frequent *-962del* allele. The present association study had 80% power to detect on two-sided tests at an α-level of 0.05, for SAH G-1606A effect sizes of 1.8/1.3 mm Hg for systolic/diastolic blood pressure, 0.52 kg/m^2 for body mass index, 0.01 units for

the waist-to-hip ratio, 0.96 mm for the triceps skinfold, 0.13 mmol/L and 0.05 mmol/L for total and HDL cholesterol, 0.18 mmol/L for serum triglycerides, and 0.11 mmol/L for blood glucose; for the SAH -962del/ins polymorphism, the corresponding effect sizes were 1.6 /1.2 mm Hg for systolic/diastolic blood pressure, 0.48 kg/m^2 for body mass index, 0.01 units for the waist-to-hip ratio, 0.87 mm for the triceps skinfold, 0.12 mmol/L and 0.04 mmol/L for total and HDL cholesterol, 0.17 mmol/L for serum triglycerides, and 0.10 mmol/L for blood glucose.

To take advantage of the family structures of the data, family-based analyses were performed. We evaluated the within- and between-family components of phenotypic variability using the orthogonal model proposed by Abecasis and colleagues in the quantitative transmission disequilibrium test (QTDT) software, version 2.3 (http://www.sph.umich.edu/csg/abecasis/QTDT) [33]. We implemented the QTDT in SAS, using a mixed model with similar adjustments as in the population-based analyses.

Results

Characteristics of the participants

The study population consisted of 2603 relatives from 560 families and 31 unrelated individuals. Mean ± SD age was 38.8±15.7 years (range, 9.5-80.4). The prevalence of hypertension was 25.3% in the whole study sample and 48.8% among 737 founders. Table 1 provides the characteristics of the participants by centre. There were significant between-centre differences in age, body mass index, waist-to-hip ratio, triceps skinfold, blood pressure, smoking and drinking habits, use of medications, serum total and HDL cholesterol, serum triglycerides and blood glucose, but not in the sex distribution. Among women, 1002 were pre-menopausal (222 on oral contraceptives) and 370 were post-menopausal (none on hormonal replacement therapy)

16

TABLE 1.   Characteristics of the study participants by centres

Characteristics	Belgium (n=1237)	Czech Republic (n=310)	Italy (n=298)	Poland (n=297)	Romania (n=216)	Russian Federation (n=276)
Clinical features						
Female, n (%)	627 (50.7)	149 (48.2)	158 (53.0)	158 (53.2)	121 (56.2)	156 (56.4)
Age (yr)	40.2±17.0	37.3±13.7	40.9±14.2	35.0±13.9	37.0±15.1	37.2±13.7
Body weight (kg)	70.3±14.0	76.7±15.1	70.5±13.6	72.6±14.2	69.2±15.5	70.9±13.7
Body mass index (kg/m^2)*	24.7±4.2	25.6±4.4	25.2±4.1	25.2±4.6	24.4±4.9	24.9±4.7
Waist-to-hip ratio[†]	0.83±0.09	0.83±0.09	0.84±0.08	0.84±0.08	0.82±0.09	0.81±0.07
Triceps skinfolds (cm)	1.78±0.85	1.62±0.87	1.62±0.68	1.80±0.86	1.62±0.44	1.49±0.62
Systolic pressure (mm Hg)[§]	121.5±14.2	121.3±14.1	124.2±14.8	125.4±15.7	121.1±17.9	123.7±17.3
Diastolic pressure (mm Hg)[§]	74.5±10.8	76.5±10.9	79.7±9.6	78.5±11.3	78.1±12.5	79.7±11.6
Heart rate (bpm)	67.8±9.4	71.5±9.3	73.3±9.7	73.7±10.0	76.0±8.2	74.1±8.5
Lifestyle						
Tobacco use, n (%)	317 (25.6)	65 (21.1)	71 (23.7)	78 (26.4)	54 (24.8)	85 (30.7)
Alcohol intake ≥5 g/day, n (%)	276 (22.3)	124 (39.9)	124 (41.6)	52 (17.4)	52 (24.3)	129 (46.6)
Arterial hypertension (%)	273 (22.1)	86 (27.7)	89 (29.9)	89 (30.0)	55 (25.5)	74 (26.8)
Use of drugs						
Antihypertensive agents, n (%)	147 (11.9)	51 (16.5)	41 (13.9)	53 (17.7)	29 (13.3)	32 (11.7)
Diuretics, n (%)	58 (4.7)	19 (6.1)	24 (8.1)	20 (6.7)	11 (5.2)	17 (6.1)
β-blockers, n (%)	93 (7.5)	34 (11.0)	10 (3.4)	28 (9.4)	9 (4.3)	5 (1.8)
ACE inhibitors, n (%)						
Lipid lowering agents, n (%)	36 (2.9)	9 (2.9)	7 (2.4)	3 (1.0)	1 (0.5)	0 (0.0)
Antidiabetic agents, n (%)	12 (0.9)	8 (2.6)	8 (2.7)	7 (2.3)	5 (2.4)	2 (0.7)
Biochemical measurements						
Serum total cholesterol (mmol/liter)	5.25±1.34	5.19±1.12	5.19±1.13	5.00±1.13	4.81±1.24	4.95±1.25
Serum HDL-cholesterol (mmol/liter)	1.40±0.38	1.45±0.33	1.21±0.26	1.58±0.37	0.98±0.24	0.99±0.30
Serum triglycerides (mmol/liter)	2.58 (2.48–2.57)	1.51 (1.36–1.65)	1.08 (1.01–1.16)	1.28 (1.18–1.38)	1.66 (1.48–1.84)	1.50 (1.38–1.62)
Blood glucose (mmol/liter)	4.90 (4.84–4.96)	5.27 (5.18–5.35)	5.00 (4.90–5.10)	4.54 (4.43–4.65)	4.96 (4.84–5.09)	4.77 (4.71–4.84)

Values are arithmetic means ± SD or number of subjects (%). Alternatively, in cases of skewed distributions, values are geometric means (95% confidence interval). Differences between countries were significant ($P \leq 0.05$) except for female sex. To convert values for total and high-density lipoprotein (HDL) cholesterol to milligrams per deciliter, divide by 0.02586. To convert values for triglycerides to milligrams per deciliter, divide by 0.01129. To convert values for glucose to milligrams per deciliter, divide by 0.05551. *The body mass index is weight in kilograms divided by the square of height in meters. †The waist-to-hip ratio is the smallest circumference at the waist divided by the largest circumference at the hip level. § Average of 5 blood pressure readings obtained at one home visit.

Genotype and haplotype frequencies

Across centres (Table 2), allele frequencies for *-1606A* ranged from 10.8% to 13.5% with no significant between-centre differences ($P \geq 0.06$). For the *-962del* allele, frequencies ranged from 27.0% to 32.6% with the highest value in Bucarest ($P < 0.05$). The within-country frequencies of the SAH *G-1606A* and *-962ins/del* genotypes complied with Hardy-Weinberg proportions ($0.12 < P < 0.89$ and $0.22 < P < 0.98$, respectively), except for SAH *G-1606A* polymorphism in Cracow ($P = 0.03$). The two polymorphisms were in significant linkage disequilibrium; Lewontin's disequilibrium coefficient D' was 0.97 ($P < 0.0001$). The haplotype frequencies were 1549 (58.8 %) for the combination of *-1606G* and *-962ins* (H1-G/ins), 777 (29.5 %) for the combination of *-1606G* and *-962del* (H2-G/del), and 308 (11.7 %) for the combination of *-1606A* and *-962ins* (H3-A/ins).

Phenotypes in relation to covariates

Based on results of stepwise multiple regression, we adjusted all models for centre, sex, age, current smoking and alcohol intake. We additionally adjusted systolic and diastolic blood pressures for the quadratic term of age, body mass index and the use of antihypertensive drugs. We additionally adjusted indexes of obesity, serum lipids and blood glucose for the use of lipid lowering drugs, and serum triglycerides, and blood glucose also for fasting status. With these adjustments applied, we did not detect any heterogeneity between centres in the relation of the phenotypes under study with the *G-1606A* ($0.12 > P > 0.99$) or *-962ins/del* polymorphisms ($0.22 > P > 0.96$) of the SAH gene. There was also no heterogeneity between women and men ($0.32 > P > 0.90$ and $0.18 > P > 0.89$, respectively). In all analyses, we therefore pooled all subjects.

TABLE 2. SAH genotypes and allele frequencies by centres

Population	SAH *G-1606A* Genotypes			SAH *G-1606A* Alleles		SAH *-962ins/del* Genotypes			SAH *-962ins/del* Alleles	
	GG	GA	AA	G	A	DD	DI	II	D	I
Belgium (n =1237)	1008 (79.1)	259 (20.3)	8 (0.6)	2275 (89.2)	275 (10.8)	124 (10.0)	522 (42.1)	593 (47.9)	770 (31.1)	1708 (68.9)[†]
Czechia (n=310)	238 (74.4)	78 (24.4)	4 (1.2)	554 (86.6)	86 (13.4)	31 (10.0)	125 (40.3)	154 (49.7)	187 (30.2)	433 (69.8) [†]
Italy (n=298)	227 (74.7)	72 (23.7)	5 (1.6)	526 (86.5)	82 (13.5)	17 (5.7)	127 (42.6)	154 (51.7)	161 (27.0)	435 (73.0)
Poland (n=297)	233 (76.1)	73 (23.9)	0 (0.0)	466 (86.5)	73 (13.5)	17 (5.7)	103 (34.7)	177 (59.6)	137 (23.1)	457 (76.9)
Romania (n=216)	179 (78.5)	45 (19.7)	4 (1.8)	403 (88.4)	53 (11.6)	23 (10.6)	95 (44.0)	98 (45.4)	141 (32.6)	291 (67.4) [†]
Russia (n=276)	218 (76.2)	63 (22.0)	5 (1.8)	499 (87.2)	73 (12.8)	27 (9.8)	105 (38.0)	144 (52.2)	159 (28.8)	393 (71.2) [†]

The within-centre frequencies of genotypes complied with Hardy-Weinberg proportions ($0.12 < P < 0.89$ for G-*1606A* and $0.22 < P < 0.98$ for SAH *-962ins/del*) except for the *G-1606A* polymorphism in Poland (p=0.03). † p<0.05 vs Poland.

Population-based association study

Table 3 shows that for the *G-1606A* polymorphism, both before and after adjustment for covariates, none of the phenotype-genotype associations approached statistical significance. We obtained similarly negative findings when we analyzed the *-962ins/del* polymorphism or carriers vs non-carriers of specific haplotypes. A sensitivity analysis restricted to fasting subjects confirmed that all aforementioned associations were consistently non-significant. Furthermore, in 737 founders (mean age, 53.0±6.7 years; range, 34.7-80.4), we did not find any association between the phenotypes under study, in particular blood pressure, and the *G-1606A* (0.12>*P*>0.62) or *-962ins/del* polymorphisms (0.10>*P*>0.76) of the SAH gene. Finally, when we dichotomised the study population according the presence or absence of hypertension, we could also not demonstrate any association with the SAH genotypes or haplotypes in the whole study population (*P*>0.31), as well as in the founders (*P*>0.62).

Family-based association study

The family-based analyses consisted of 560 pedigrees, of which 103 spanned more than two generations. We adjusted the QTDT analyses as described above. For *G−1606A* and *-962ins/del*, the orthogonal model did not reveal significant population stratification for any phenotype under study (*P*≥0.21 and *P*≥0.28, respectively). Transmission of single alleles or haplotypes from informative parents to offspring was not associated with any difference in the phenotypes under study. Table 4 illustrates these findings for the transmission of the most informative haplotype (H2-G/del) to the offspring.

20

TABLE 3. Characteristics of phenotypes by genotypes of _G-1606A_ polymorphism

	A allele carriers			Homozygotes		
	All (n=596)	**AA** (n=25)	**GA** (n=571)	**GG** (n=2038)	**P trend**	**P_{A-G}**
Clinical features unadjusted						
Systolic blood pressure (mm Hg)*	122.6±0.7	121.8±3.0	122.6±0.7	122.9±0.4	0.85	0.61
Diastolic blood pressure (mm Hg)*	77.1±0.5	76.7±2.2	77.2±0.5	77.5±0.3	0.77	0.49
Body mass index (kg/m^2)§	25.1±0.2	25.6±0.9	25.1±0.2	25.0±0.1	0.79	0.69
Waist-to-hip ratio†	0.83±0.01	0.82±0.02	0.83±0.01	0.83±0.01	0.75	0.61
Triceps skinfold (cm)	1.68 (1.61-1.75)	1.81 (1.49-2.12)	1.68 (1.61-1.75)	1.68 (1.64-1.72)	0.73	0.88
Clinical features adjusted						
Systolic blood pressure (mm Hg)*	125.4±0.6	124.8±2.5	125.4±0.7	126.0±0.5	0.63	0.36
Diastolic blood pressure (mm Hg)*	78.8±0.5	78.4±1.8	78.8±0.5	79.4±0.4	0.17	0.39
Body mass index (kg/m^2)§	25.3±0.3	25.7±0.8	25.3±0.3	25.1±0.3	0.58	0.40
Waist-to-hip ratio†	0.84±0.1	0.85±0.01	0.84±0.01	0.84±0.01	0.79	0.51
Triceps skinfold (cm)	1.61 (1.50-1.72)	1.68 (1.39-1.97)	1.61 (1.50-1.72)	1.60 (1.50-1.70)	0.82	0.72
Biochemical measurements unadjusted						
Serum total cholesterol (mmol/liter)	5.09±0.05	5.18±0.23	5.09±0.05	5.11±0.03	0.88	0.73
Serum HDL-cholesterol (mmol/liter)	1.29±0.02	1.28±0.08	1.29±0.02	1.29±0.01	0.99	0.95
Serum triglycerides (mmol/liter)	1.69 (1.56-1.82)	1.62 (1.03-2.22)	1.69 (1.55-1.83)	1.77 (1.69-1.86)	0.53	0.27
Blood glucose (mmol/liter)	4.93 (4.85-5.01)	4.98 (4.60-5.35)	4.93 (4.84-5.01)	4.88 (4.83-4.95)	0.59	0.32
Biochemical measurements adjusted						
Serum total cholesterol (mmol/liter)	4.98±0.08	5.05±4.97	4.99±0.07	4.99±0.07	0.91	0.86
Serum HDL-cholesterol (mmol/liter)	1.27±0.03	1.27±0.06	1.27±0.03	1.28±0.02	0.75	0.45
Serum triglycerides (mmol/liter)	1.62 (1.39-1.85)	1.64 (1.06-2.21)	1.62 (1.38-1.85)	1.64 (1.43-1.86)	0.91	0.67
Blood glucose (mmol/liter)	5.00 (4.85-5.15)	5.01 (4.63-5.40)	5.00 (4.84-5.16)	4.94 (4.80-5.09)	0.42	0.19

Values are arithmetic means ± SE. Alternatively, in cases of skewed distributions, values are geometric means (95% confidence interval). To convert values for total and high-density lipoprotein (HDL) cholesterol to milligrams per deciliter, divide by 0.02586. To convert values for triglycerides to milligrams per deciliter, divide by 0.01129. To convert values for glucose to milligrams per deciliter, divide by 0.05551. Statistical parameters, which were obtained by PROC MIXED, were adjusted for centre, age, gender, smoking and alcohol consumption. Additional adjustments included: for blood pressure, body mass index and use of antihypertensive drugs; for body mass index, for the waist-to-hip ratio, for triceps skinfold, and for serum total and HDL cholesterol, use of lipid-lowering drugs; for serum triglycerides and blood glucose, use of lipid-lowering drugs and fasting status. P_{A-G} indicates the significance of the difference between A alleles carriers and GG homozygotes. *Average of 5 blood pressure readings obtained at one home visit. §The body mass index is weight in kilograms divided by the square of height in meters. †The waist-to-hip ratio is the smallest circumference at the waist divided by the largest circumference at the hip level.

TABLE 4.   Changes associated with transmission of haplotype H2-G/del in the Quantitative Transmission Disequilibrium Test

Trait	Effect size (95% confidence interval)	P
Clinical features		
Systolic blood pressure (mm Hg)[*]	2.800 (-2.056 to 7.657)	0.25
Diastolic blood pressure (mm Hg)[*]	1.777 (-2.095 to 5.648)	0.37
Body mass index (kg/m^2)[§]	0.090 (-14984 to 1.6787)	0.91
Waist-to-hip ratio[†]	0.016 (-0.009 to 0.042)	0.21
Triceps skinfold (cm)	0.129 (-0.189 to 0.439)	0.41
Biochemical measurements		
Serum total cholesterol (mmol/liter)	-0.302 (-0.754 to 0.149)	0.19
Serum HDL-cholesterol (mmol/liter)	-0.078 (-0.217 to 0.059)	0.26
Serum triglycerides (mmol/liter)	0.206 (-0.155 to 0.567)	0.26
Blood glucose (mmol/liter)	0.098 (-0.185 to 0.381)	0.49

The number of informative offspring was 1680. The effect sizes, which were obtained by the PROC MIXED procedure, were adjusted for centre, age, gender, smoking and alcohol consumption. Additional adjustments included: for blood pressure, body mass index and use of antihypertensive drugs; for body mass index, the waist-to-hip ratio, the triceps skinfold, and serum total and HDL cholesterol, use of lipid-lowering drugs; and for serum triglycerides and blood glucose, use of lipid-lowering drugs and fasting status. *Average of 5 blood pressure readings obtained at one home visit. §The body mass index is weight in kilograms divided by the square of height in meters. †The waist-to-hip ratio is the smallest circumference at the waist divided by the largest circumference at the hip level.

Discussion

The key finding of the present study was the non-replication of the association of blood pressure, hypertension, or any blood pressure-related phenotype with variation in the human SAH gene, as previously reported in case-control [25,26] and population studies [22,24]. Research into the SAH gene found its root in studies of genes, which are overexpressed in genetically hypertensive rats [34,35]. Analyses of several F2 cohorts [36,37] and the establishment of several congenic rat strains [35,38], initially confirmed that the SAH gene, located on rat chromosome 1, might contribute to blood pressure regulation in this rodent. However, more recent studies with fine-mapping in congenic strains separated the blood pressure quantitative trait loci on rat chromosome 1 from the SAH locus [39-41]. Thus, on balance, the evidence from animal experiments is inconsistent with respect to the association of the hypertension with the SAH locus.

Benjafield and colleagues [27] reported a case-control study including 121 hypertensive patients with two hypertensive parents and 178 normotensive subjects, whose parents were both normotensives. These Australian investigators did not find any association of hypertension with genetic variation in the SAH gene, including the -962ins/del polymorphism in the promoter. However, the frequency of the minor allele of the intron-1 polymorphism was higher in overweight (body mass index > 25 kg/m^2) than in normal weight hypertensive patients. Experimental evidence supports a possible role of SAH in lipid metabolism [22,42]. Indeed, the SAH protein shares homology with a number of metabolic enzymes, including bacterial acetylcoenzyme A (CoA) synthetase [43], and acyl-CoA ligases [44]. The SAH product also exerts acyl-CoA synthetase activity for medium-chain fatty acids [22].

In line with the putative role of SAH in lipid metabolism, Haketa and colleagues [26] in 287 hypertensive patients and 259 normotensive controls, reported association of plasma HDL-cholesterol with a SAH intron 3 variant as well as with a SNP in exon 8

of the MACS1 gene, which maps within a 150-kb region, including the SAH locus, on human chromosome 16p13.11. The PEGASE Study [25] included 651 hypertensive patients and 776 controls. Carriers of the *-1606A* allele had a slightly increased risk of hypertension (odds ratio, 1.28; *P*=0.049). Conversely, the odds ratio for carriers of the *-962del* polymorphism was 0.18. Moreover, after adjustment for body mass index, only the odds ratio associated with the deletion polymorphism remained significant (odds ratio, 0.77; *P*=0.028).

In contrast to the here decribed findings, two publications on large Japanese population [22,24] samples (n=4000 and n=1976, respectively) reported significant associations of the SAH region, with blood pressure, heart rate, fasting blood glucose and serum triglycerides. However, blood pressure was only significantly associated with a SAH intron-12 variant. We cannot exclude that ethnicity, lifestyle or other context-dependent factors might impact on the possible associations of blood pressure and blood pressure related phenotypes with genetic variation in SAH. The prevalence of hypertension in the Japanese population studies was 38.0%, whereas in our study it was 25.3%. However, we do not believe that under-representation of hypertensive patients in our random population sample might explain our null findings. Indeed, mean age was 21 years higher in the Japanese studies (59.8 vs 38.8 years). Moreover, a prevalence of hypertension of approximately 25% is in line with other population studies with a similar age distribution as ours [45]. A more plausible explanation of the negative findings in our study, as opposed to the positive results of some previous reports might be confounding by population stratification or admixture. Our family-based analyses suggested that population stratification did not influence our current results. Moreover, our study addresses some weaknesses of previous reports, such as small sample size, not accounting for lifestyle factors, and/or a low-quality or dichotomized blood pressure phenotypes.

In conclusion the evidence supporting an association of hypertension or hypertension-related phenotypes with the SAH gene is mainly based on animal experiments and remains equivocal in human studies.

Part 2

Blood pressure and metabolic phenotypes in relation to the *ADRB1 Arg389Gly* and *ADRA2B I/D* polymorphisms in a White population

Introduction

The β_1 (*ADRB1*) and α_{2B} (*ADRAB2*) adrenergic receptors play an important role in the regulation of blood pressure (BP), cardiovascular function and lipid metabolism. Their genes locate to chromosomes 10q24-26 and 2p13-q13, respectively. The *ADRB1 Arg389Gly* polymorphism leads to the substitution of the amino acid arginine by glycine near to the intracellular carboxyl terminal, which is involved in G-protein binding [46]. The *ADRA2B* insertion/deletion (Ins+910Del [*I/D*]) polymorphism involves the deletion of three glutamic acids from a repeat element in the third intracellular loop of the protein [47]. *ADRB1 Arg* allele carriers have increased adenyl cyclase activity in response to agonists, such as isoproterenol, epinephrine and norepinephrine [46]. The *ADRA2B* deletion results in a loss of agonist-promoted desensitization [48].

Case-control studies [46] and continuous trait analyses in patients with coronary heart disease [49] demonstrated association of the *ADRB1 Arg389Gly* polymorphism with hypertension [46] or with systolic [46] or diastolic [46,49] BP. However, none of these studies involved families randomly recruited from a general population. Furthermore, our group previously demonstrated that in Chinese men the *ADRA2B I* allele was associated with higher BP, but with lower body mass index and waist-to-hip ratio, and with less insulin resistance [50]. Several studies in diabetic [51] and nondiabetic [47,52] patients reported association of the *ADRA2B I/D* polymorphism with metabolic phenotypes. However, with the exception of our small Chinese Study (n=481) [50], none of these previous reports one *ADRA2B*

involved a family-based population sample or assessed the population stratification as a possible explanation of significant phenotype-genotype associations.

In the present study, we investigated in a family-based cohort possible association of the *ADRB1 Arg389Gly* and *ADRA2B I/D* polymorphisms with BP and metabolic phenotypes, analyzed as continuous traits. For analysis, we applied a variance decomposition method, which is robust to population stratification or admixture.

Methods

Study population

The ethics committee of the University of Leuven approved the Flemish Study on Environment, Genes, and Health Outcomes (FLEMENGHO) [53]. The FLEMENGHO project was conducted according to the principles outlined in the Helsinki declaration for investigations in human subjects [30]. From August 1985 until November 1990, a random sample of the households living in a geographically defined area in northern Belgium was investigated with the goal to recruit an equal number of participants in each of 6 subgroups by sex and age (20 to 39, 40 to 59, and ≥60 years). All household members ≥20 years of age were invited to take part until the quota of their sex–age group had been fulfilled. Further, from June 1996 until December 2006, nuclear families including children who were ≥10 years of age were recruited using the former participants (1985 to 1990) as index persons. The participation rate averaged 64.3%. The participants, and in case of underaged offspring, their parents or custodians, gave informed consent. Of these 2165 subjects, we excluded 284 from analysis, because their DNA could not be extracted or genotyped or failed to amplify (n=42), because of inconsistency in Mendelian segregation (n=22), because participants were younger than 18 years (n=200), or because not all measurements required for the present analysis was available (n=10) or because a phenotype was

more than 3SD away from the population mean (n=10). Thus, the number of subjects statistically analyzed totalled 1881.

Phenotypes

At the enrolment home visit, trained nurses measured BP and anthropometric characteristics. They also administered a questionnaire to collect information about each subject's medical history, smoking and drinking habits, and intake of medications. BP and pulse rate was the average of 5 consecutive readings. Hypertension was defined as a blood pressure of ≥140 mm Hg systolic or ≥90 mm Hg diastolic or as the use of antihypertensive drugs. Body mass index was weight in kilograms divided by the square of height in meters. The waist-to-hip ratio was the smallest circumference at the waist divided by the largest circumference at the hip. Venous blood samples, collected in the late afternoon, were analysed for serum total and HDL cholesterol by automated enzymatic methods.

Determination of genotypes

Genomic DNA was extracted from white blood cells, using standard kits (Qiagen, Hilden, Germany). We genotyped the *ADRB1 Arg389Gly* and *ADRA2B I/D* polymorphisms by PCR and restriction fragment length polymorphism analysis (RFLP).

PCR amplification was carried out in a 20-μL volume, containing 10 ng genomic DNA, 10 pmol/L of each primer, 2.5 mmol/L $MgCl_2$, 200 μmol/L of each dNTPs, and 1U AmpliTaq polymerase (Rapidozym, Berlin, Germany). The PCR was performed initially for 3 min at 94°C and followed by 35 cycles, each with 45 sec at 94°C, 45 sec at 65° and 68°C (depending on primer sequence), 60 sec at 72°C and final elongation of 10 min at 72°C.

For the *Arg389Gly* variant (rs1801253) of the adrenergic β_1−receptor (*ADRB1*, NM_000684), PCR products were digested, using the restriction enzyme *Scr*FI. For

the adrenergic α_{2B}–receptor (*ADRA2B*, NM_000682), the Ins/Del (rs45605534) was determined by high percentage agarose gel electrophoresis (3.5 %).

Statistical methods

For statistical analysis, we used SAS software, version 9.1.3 (SAS Institute, Cary, NC). We compared means and proportions by the standard normal z-test and the χ^2 statistic, respectively. We searched for possible covariates of the phenotypes under study, using stepwise multiple regression analysis with the *P*-value for independent variables to enter and stay in the models set at 0.10. Depending on the phenotypes, the variables considered for entry into the models were sex, age, age squared, body mass index, use of β-blockers, diuretics, smoking (0,1), and alcohol consumption (0,1), and in women use of oral contraceptive and hormonal replacement therapy.

In population-based analyses, we tested phenotype-genotype associations, using the PROC MIXED procedure of the SAS package, while adjusting for covariables and family clusters. We tested for heterogeneity, using appropriate interaction terms. To remain consistent with previous studies [53], we analyzed the ADRAB1 polymorphism comparing ArgArg homozygotes with Gly carriers and the ADRA2B polymorphism both by genotypes and by contrasting II homozygotes with the D allele carriers. In family-based analyses, we evaluated the within- and between-family components of phenotypic variance, using the orthogonal model proposed by Abecasis et al. [33]. We implemented the QTDT in SAS, using a mixed model with similar adjustments as in the population-based analyses. The within-family component of phenotypic variance reflects the genetic effect and is robust to population stratification.

Results

Characteristics of the participants

The study population consisted of 1802 relatives from 175 families and 79 unrelated subjects. Mean values were 45.5 years for age and 124.7 mmHg and 76.9 mmHg for systolic and diastolic BP, respectively. The study sample included 525 (27.9%) hypertensive patients, of whom 281 were on antihypertensive drugs. The 920 male and 961 female participants had a mean age of 45.3 and 45.7 years, respectively. Women compared to men had lower BP and less frequently reported smoking and alcohol consumption (Table 1). The frequencies of the *ADRB1* genotypes (*ArgArg* 56.2%, *ArgGly* 36.9%, and *GlyGly* 6.9%; *P*=0.66) and the *ADRA2B* genotypes (*II* 45.7%, *ID* 41.7%, and *DD* 12.5%; *P*=0.05) did not deviate from Hardy-Weinberg proportions.

Population-based association study

Table 2 shows the multivariate-adjusted phenotype-genotype associations for the two polymorphisms in the population-based analyses, which accounted for family clusters. Based on results of stepwise multiple regression, we adjusted all models for age, sex, current smoking and alcohol intake, the use of diuretics and β-blockers, and the use of oral contraceptive or hormonal replacement. We additionally adjusted systolic and diastolic blood pressures, pulse rate and serum lipids for the quadratic term of age and body mass index. We additionally adjusted indexes of obesity, and serum lipids for the use of lipid lowering drugs. Both before and after adjustment for those covariables (Table 2), *ADRB1 Arg* homozygotes, compared with *Gly* allele carriers, had higher diastolic BP (79.4 versus 78.4 mmHg, *P*=0.012) and higher serum HDL cholesterol (1.33 versus 1.29 mmol/L, *P*=0.020). Otherwise, none of the phenotype-genotype associations for both genes under study reached statistical significance. Furthermore, sensitivity analyses by sex and

30

excluding subjects on antihypertensive drugs, did not reveal significant heterogeneity. Considering both SNPs together, with or without their interaction, did not significantly improve the fit of the mixed models. This was also the case, when we applied an additive model for the *ADRA2B I/D* polymorphism (*P*≥0.24).

Family-based association study

The family-based analyses included 175 pedigrees, of which 72 spanned more than two generations. We adjusted the family-based analyses as in Table 2. For both the *ADRB1 Arg389Gly* and *ADRA2B I/D* polymorphisms, the orthogonal model did not reveal significant population stratification for any phenotype-genotype association (*P*≥0.23 and *P*≥0.32, respectively). The effect size (± SE) of the transmission of the *ADRB1 Gly* allele from parents to informative offspring was −0.33 mmHg (n=906; *P*=0.64) for diastolic BP and −0.045 mmol/L (n=906; *P*=0.07) for HDL-cholesterol.

Table 1. Baseline characteristics of women and men

	Women (n=961)	Men (n=920)
Anthropometrics		
Age, years	45.3±15.3	45.7±15.1
Body mass index, kg/m^2	25.4±4.5	26.0±3.7
Body weight, kg	66.7±12.2	79.6±12.2
Waist-to-hip ratio	0.79±0.07	0.90±0.07
Hemodynamic measurements		
BP systolic, mm Hg	127.4±14.1	122.1±15.8
BP diastolic, mm Hg	78.6±10.1	75.2±9.5
Pulse rate, bpm	69.7±9.3	66.8±9.4
Serum cholesterol		
Total cholesterol, mmol/L	5.47±1.05	5.41±1.09
HDL cholesterol, mmol/L	1.56±0.39	1.21±0.31
Lifestyle factors		
Smokers, n (%)	254 (26.4)	280 (30.4)
>5 Alcoholic beverages per day, n (%)	125 (13.0)	360 (39.1)
Hypertension (%)	245 (25.5)	280 (30.4)
Antihypertensive agents, n (%)	122 (13.3)	159 (16.6)
Diuretics, n (%)	73 (7.6)	39 (4.2)
β-Blockers, n (%)	100 (10.4)	73 (7.9)
ACE inhibitors, n (%)	8 (0.8)	12 (1.3)
Lipid lowering agents, n (%)	49 (5.1)	27 (2.9)

Values are mean ± SD or number of subjects (%). Gender differences were significant (P<0.05) except for age (P=0.61) and total cholesterol (P=0.24). BP was the average of 5 consecutive readings obtained at one home visit. Hypertension was a BP of at least 140 mm Hg systolic or 90 mm Hg diastolic or use of antihypertensive drugs.

TABLE 2. Phenotype-genotype associations in population-based analyses

	ADRB1 Arg389Gly		P	ADRA2B I/D		P
	GlyArg+GlyGly (n=808)	ArgArg (n=1073)		ID+DD (n=1026)	II (n=855)	
Clinical features						
Systolic pressure (mm Hg)*	128.3±0.9	128.9±0.8	0.31	128.7±0.8	128.6±0.8	0.83
Diastolic pressure (mm Hg)*	78.4±0.6	79.4±0.6	0.01	79.0±0.6	78.9±0.6	0.90
Pulse rate (bpm)	66.6±0.6	66.8±0.6	0.58	66.4±0.6	67.1±0.6	0.12
Body mass index (kg/m^2)†	26.5±0.2	26.5±0.3	0.72	26.4±0.2	26.7±0.2	0.12
Body weight (kg)	75.5±0.8	75.9±0.8	0.39	75.5±0.8	76.1±0.8	0.25
Waist-to-hip ratio‡	0.854±0.004	0.851±0.004	0.24	0.852±0.004	0.854±0.004	0.44
Serum cholesterol (mmol/L)§						
Total	5.49±0.07	5.45±0.07	0.46	5.44±0.07	5.51±0.07	0.16
HDL	1.29±0.02	1.33±0.02	0.02	1.33±0.02	1.31±0.02	0.62

Values are least squared means ± SE. Based on the results of stepwise multiple regression, all models were adjusted for sex, age, smoking and alcohol consumption, and use of diuretics, β-blockers and female sex hormones. We additionally adjusted blood pressure, pulse rate and serum cholesterol for the quadratic term of age and serum cholesterol also for the use of lipid-lowering drugs. * Average of 5 blood pressure readings obtained at one home visit. † The body mass index is weight in kilograms divided by the square of height in meters. ‡ The waist-to-hip ratio is the smallest circumference at the waist divided by the largest circumference at the hip level. § To convert values for total and high-density lipoprotein (HDL) cholesterol to milligrams per deciliter, divide by 0.02586.

Discussion

The key finding of the present study was that in the absence of population stratification diastolic BP and serum HDL cholesterol were significantly lower in *ADRB1 Gly* allele carriers than in *ArgArg* homozygotes. However, we could not replicate the association of BP and the metabolic phenotypes with *ADRA2B I/D* polymorphism.

The β_1- and the α_{2B}- adrenergic receptors are members of a large superfamily of membrane receptors which mediate their activity trough interaction with 1 of a series of guanosine nucleotide-binding regulatory proteins (G proteins). Several tissues, including adipocytes, cardiac myocytes and kidney cells, express *ADRB1*. Its activation increases BP, stimulates lipolysis [54] enhances myocardial contractility [46], and stimulates the release of renin [46]. In transfected Chinese hamster fibroblasts, the *ADRB1 Arg389Gly* variant affects the receptor's ability to bind the Gs molecule [55]. Several studies have investigated a possible impact of the Arg389Gly polymorphism of the β1-AR on the resting hemodynamics and hypertension/blood pressure. There is some uncertainty regarding the pathogenic effect of the variant, as studies have demonstrated and other have failed to show an association between the Arg389Gly variant and hypertension in a case-control setting and increased blood pressure in quantitative trait analyses [46]. In line with our current findings, the *Arg389Gly* polymorphism in *ADRB1* might be a potential pharmacogenetic target. Indeed *ADRB1 ArgArg* homozygotes are more sensitive to treatment with β-blocker drugs, such as metoprolol, atenolol, and the β_1-adrenergic receptor agonist dobutamine. This effect is,however, disputed. The β_1-adrenergic receptor is also an obvious candidate in the pathogenesis of obesity because of its influence on lipolysis [46]. Stimulation of the β_1-adrenergic receptor results in decreased circulating leptin levels, and a relationship between body weight reduction and decreased β_1-adrenergic receptor expression has also been demonstrated. However, most studies examining the effect of this variant in

34

relation to body weight have been negative. However, in a study of 931 white American women, the Arg allele was associated with higher BMI and body weight [46]. We hypothesize that facilitated signal transduction after stimulation of the the β_1-adrenergic receptor [46] might explain the higher serum HDL cholesterol level in *ADRB1 ArgArg* homozygotes.

The α_{2B}-adrenergic receptors when activated, act as negative feedeback controllers, reducing sympathetic outflow from the central nervous system and decreasing the blood pressure. Several reports in Caucasians revealed association of various cardiovascular and obesity-related phenotypes with ADRA2B I/D polymorphism. Although functional, the evidence linking the *ADRA2B I/D* polymorphism with BP and metabolic phenotypes remains equivocal. In our previous family-based study in Chinese [50], we found associations only in men, but not in women. Other studies of the *ADRA2B I/D* polymorphism mainly involved selected groups of patients. For istance, Heinonen et al. showed that homozygosity for the *ADRA2B* deletion was assoiated with reduced based metabolic rate in nondiabetic obese Finns (n=166) [47]. Sivenious et al. reported a greater increase in body weight during a 10 year follow up in nondiabetic subjects (n=210) homozygous for the deletion [52]. In addition to selection, small sample size and the possibility of undetected population stratification limit the interpretation of most *ADRA2B* study in humans.

In conclusions, our study showed association of diastolic BP and HDL cholesterol with the *ADRB1 Arg389Gly* polymorphism in the absence of population stratification, whereas the *ADRA2B* polymorphism does probably not contribute much to the variation of BP and metabolic phenotypes in unselected White Europeans.

General discussion

At best, common variants identified to date likely account for a very small fraction of the variance in the blood pressure trait in the general population. The lack of consistent reproducibility of these studies highlights the difficulties in genetic investigation of multifactorial traits. It seems apparent that firm conclusions require consistent replication of results in large and rigorously characterized populations that are well suited for detecting alleles imparting small effects.

Populations should include cohorts of unrelated individuals as well as family-based studies that would allow use of tests of transmission disequilibrium [11]. These latter tests minimize the chance of false-positive associations arising from population admixture of individuals of different genetic backgrounds [33]. Indeed a potential problem with the analysis of genetic effects in independent individuals is the possibility of confounding by ethnicity. This can occur if the distribution of alleles at the candidate locus varies by race/ethnicity and race/ethnicity is independently associated with the trait. However, in a large scale genetic-epidemiologic study, obtaining good quality information on race/ethnicity is difficult. Even for two children who report the same race/ethnicity, their gene pools may still differ due to subtle mixtures in their respective ancestries. Thus, inclusion of a "race/ethnicity" variable in the analysis does not guarantee protection from confounded estimates of genetic effects. To avoid this confounding, one should use a family-based design in which candidate genotypes for the parents of each study subject are determined.

Accurate measurement of the blood pressure phenotype is of paramount importance in studies on the genetic determination of complex trait such as hypertension and related-hypertension phenotypes [57]. The EPOGH, a study involving seven centres in six European countries has been conducted with emphasis on intensive phenotyping of continuous traits under strict quality

control to construct a common database and bank of biological samples, which is representative for several European populations [28].

Due to the very large number of genetic variants in the human genome and the lack of detailed knowledge about the molecular and biochemical processes involved in aetiology of complex diseases, many spurious associations might have been found and reported. In the part 1 of the present thesis, we evaluated the possible association between SAH gene variants and blood pressure phenotype. We concluded that the previous positive reports should be regards as equivocal. Therefore, closer attention to study design and use of bioinformatics data has the potential to limit the rate of false positive reports. Chance, bias and confounding factors have to be taken into account (Table 1) [58].

Table 1 Examples of ways in which chance, bias and confounding can lead to false positive associations between genetic variants and disease states or traits or drug response

Chance
- Due to multiple association studies performed with publication of only those that show positive results (multiple testing together with publication bias)
- Due to testing of multiple markers (each with low prior probability of causing disease) and failure to adjust significance levels accordingly or otherwise interpret results appropriately
- Multiple testing due to reporting secondary and post hoc (subgroup) analyses as if they related to *a priori* hypotheses then selectively reporting only analyses that reach statistical significance

Bias
- Due to artefact (differences between cases/controls unrelated to cause of disease) such as differences in handling or storage between cases and controls
- Due to systematic error introduced in selection of cases and controls for study
- Due to systematic error introduced by differences in genotyping between cases and controls

Confounding
- Due to population stratification
- Due to differences in distribution of genetic and environmental risk factors for disease under study between cases and controls (limited, in theory, by ''Mendelian randomisation''[10])
- Due to linkage disequilibrium (LD) or 'allelic association' between marker under study and true disease susceptibility variant

---

Moreover consistency of association across studies is a useful indicator of causal association, when present. However, problems in interpreting failure to replicate findings limits the utility of this criterion and argues in favour of investment in large, integrated study designs that can perform internal checks in a single population. Strength of association is also a useful indicator of causal association but over-estimates due to bias are frequent.

Even if single studies are conducted and reported without bias and with full transparency, the cumulative evidence may still be biased if availability of information is driven from selective reporting or other publications biases [59]. Therefore it is important to encourage journals and investigators to publish high-quality null results. Selection biases can also be reduced by the establishment of consortia of multiple teams that have explicit policies of analysing all eligible data from all participating teams. The development and support of multidisciplinary groups with expertise in bioinformatics, (genetic) epidemiology/statistics and experimental biology is important for the future success of this field. Current proposals to move away from a hypothesis testing paradigm of investigation to one of high-throughput (functional) genomics in which very large numbers of variants are related to a wide range of phenotypes underlines the need for an international consensus on a framework for the interpretation of genetic association studies and for the issues raised in this review to inform the design of these studies.

References

1. Snieder H, Harshfield GA, Treiber FA. Heritability of blood pressure and hemodynamics in African- and European-American youth. Hypertension 2003; 41:1196–1201.

2. Ward R. Familial aggregation and genetic epidemiology of blood pressure. In Laragh, J.H. and Brenner, B.M. (eds), Hypertension: Pathophysiology, Diagnosis and Management 1990. Raven Press, New York, 81–100.

3. Feinleib M, Garrison RJ, Fabsitz R, Christian JC, Hrubec Z, Borhani NO, Kannel WB, Rosenman R, Schwartz JT, Wagner JO. The NHLBI twin study of cardiovascular disease risk factors: methodology and summary of results. Am J Epidemiol 1977; 106: 284–285

4. Longini IM, Higgins MW, Hinton PC, Moll PC, Keller JB. Environmental and genetic sources of familial aggregation of blood pressure in Tecumseh. Michigan. Am J Epidemiol 1984; 120:131–144.

5. Rice T, Vogler GP, Perusse L, Bouchard C, Rao DC. Cardiovascular risk factors in a French Canadian population: resolution of genetic and familial environmental effects on blood pressure using twins, adoptees, and extensive information on environmental correlates. Genet Epidemiol 1989; 6: 571–588

6. Lifton RP, Gharavi AG, Geller DS. Molecular mechanisms of human hypertension. Cell 2001; 104:545–556.

7. Harrap SB. Where are all the blood-pressure genes? Lancet 2003;361:2149–2151.

8. Mein CA, Caulfield MJ, Dobson RJ, Munroe PB. Genetic of essential hypertension. Hum Mol Genet 2004; 13:R169–R175.

9. Binder A. A review of the genetics of essential hypertension. Curr Opin Cardiol 2007; 22:176-184.

10. Gong M, Hubner N. Molecular genetics of human hypertension. Clin Sci 2006; 110:315-326.

11. Spielman RS, Ewens WJ. The TDT and other family-based tests for linkage disequilibrium and association. Am J Hum Genet 1996; 59: 983–989.

12. Stoll M, Kwitek-Black AE, Cowley AW Jr, Harris EL, Harrap SB, Krieger JE, Printz MP, Provoost AP, Sassard J, Jacob HJ. New target regions for human hypertension via comparative genomics.Genome Res 2000; 10:473-82.

13. Rapp JP. Genetic analysis of inherited hypertension in the rat. Physiol Rev 2000; 80:135–172.

14. Takahashi N, Smithies O. Gene targeting approaches to analyzing hypertension. J Am Soc Nephrol 1999; 17:1598–1605.

15. Comuzzie AG, Allison DB. The search for human obesity genes. Science 1998; 280:1374–1377.

16. Guo SW, Lange K. Genetic mapping of complex traits: promises, problems, and prospects. Theor Popul Biol 2000; 57:1–11.

17. Altmuller J, Palmer LJ, Fischer G, Scherb H, Wjst M. Genomewide scans of complex human diseases: true linkage is hard to find. Am J Hum Genet 2001; 69:936–950.

18. Iwai N, Inagami T. Isolation of preferentially expressed genes in the kidneys of hypertensive rats. Hypertension 1991; 17:161-169.

19. Patel HR, Thiara AS, West KP, Lodwick D, Samani NJ. Increased expression of the SA gene in the kidney of the spontaneously hypertensive rat is localized to the proximal tubule. J Hypertens 1994; 12:1347-1352.

20. Yang T, Hassan SA, Singh I, Smart A, Brosius FC, Holzman LB, Schnermann JB, Briggs JP. SA gene expression in the proximal tubule of normotensive and hypertensive rats. Hypertension 1996; 27:541-551.

21. Samani NJ, Whitmore SA, Kaiser MA, Harris J, See CG, Callen DF, Lodwick D. Chromosomal assignment of the human SA gene to 16p13.11 and demonstration of its expression in the kidney. Biochem Biophys Res Commun 1994; 199:862-868.

22. Iwai N, Katsuya T, Mannami T, Higaki J, Ogihara T, Kokame K, Ogata J, Baba S. Association between SAH, an acyl-CoA synthetase gene, and hypertriglyceridemia, obesity, and hypertension. Circulation 2002; 105:41-47.

23. Fujino T, Kang MJ, Suzuki H, Iijima H, Yamamoto T. Molecular characterization and expression of rat acyl-CoA synthetase 3. J Biol Chem 1996; 271:16748-16752.

24. Iwai N, Mannami T, Tomoike H, Ono K, Iwanaga Y. An acyl-CoA synthetase gene family in chromosome 16p12 may contribute to multiple risk factors. Hypertension 2003; 41:1041-1046.

25. Telgmann R, Brand E, Nicaud V, Hagedorn C, Beining K, Schönfelder J, et al. SAH gene variants are associated with obesity-related hypertension in Caucasians: the PEGASE Study. J Hypertens 2007; 25:557-564.

26. Haketa A, Soma M, Nakayama T, Sato M, Kosuge K, Aoi N, Matsumoto K. Two medium-chain acyl-coenzyme A synthetase genes, SAH and MACS1, are associated with plasma high-density lipoprotein cholesterol levels, but they are not associated with essential hypertension. J Hypertens 2004; 22:1903-1907.

27. Benjafield AV, Iwai N, Ishikawa K, Wang WYS, Morris BJ. Overweight, But Not Hypertension, Is Associated with SAH Polymorphisms in Caucasians with Essential Hypertension. Hypertens Res 2003; 26:591-595.

28. Kuznetsova T, Staessen J.A., Kawecka-Jaszcz K., Babeanu S., Caviglia E, Filipovsky J, Nachev C, Nikitin Y, Peleska. J and O'Brieni E. Quality control of the blood pressure phenotype in the European Project on Genes in Hypertension. Blood Press Monit 2002; 7:215-224.

29. Staessen J, O'Brien E, Atkins N, Bulpitt CJ, Cox J, Fagard R, O'Malley K, Thijs L, Amery A. The increase in blood pressure with age and body mass index is overestimated by conventional sphygmomanometry. Am J Epidemiol 1992; 136:450-9.

30. 41st World Medical Assembly 1990 Declaration of Helsinki: recommendations guiding physicians in biomedical research involving human subjects. Bull Pan Am Health Organ 1990; 24:606-609.

31. Petrie JC, O'Brien ET, Littler WA, de Swiet M. Recommendations on blood pressure measurement by a working party of the British Hypertension Society. BMJ 1989; 293:611-615.

32. Staessen JA, Fagard R, Amery A. Life style as a determinant of blood pressure in the general population. Am J Hypertens 1994; 7:685-694.

33.   Abecasis G. R., Cardon L. R., and Cookson W. O. A general test of association for quantitative traits in nuclear families. Am J Hum Genet 2000; 66:279-292.

34. Lindpaintner K, Hilbert P, Ganten D, Nadal-Ginard B, Inagami T, Iwai N. Molecular genetics of the SA-gene: cosegregation with hypertension and mapping to rat chromosome 1. J Hypertens 1993; 11:19-23.

35. Iwai N, Tsujita Y, Kinoshita M. Isolation of a chromosome 1 region that contributes to high blood pressure and salt sensitivity. Hypertension 1998; 32:636-638.

36. Iwai N, Inagami T. Identification of a candidate gene responsible for the high blood pressure of spontaneously hypertensive rats. J Hypertens 1992; 10:1155-1157.

37. Harris EL, Dene H, Rapp JP. SA gene and blood pressure cosegregation using Dahl salt-sensitive rats. Am J Hypertens 1993; 6:330-334.

38. Frantz SA, Kaiser M, Gardiner SM, Gauguier D, Vincent M, Thompson JR, Bennett T, Samani NJ. Successful isolation of a rat chromosome 1 blood pressure quantitative trait locus in reciprocal congenic strains. Hypertension 1998; 32:639-646.

39. Cui ZH, Nemoto K, Kawakami K, Gonda T, Nabika T, Masuda J. Fine linkage mapping of the blood pressure quantitative trait locus region on rat chromosome 1. Hypertens Res 2002; 25:605-608.

40. Hübner N, Lee Y-A, Lindpaintner K, Ganten D, Kreutz R. Congenic substitution mapping excludes SA as a candidate gene locus for a blood pressure quantitative trait locus on rat chromosome 1. Hypertension 1999; 34:643-648.

41. Frantz S, Clemitson JR, Bihoreau MT, Gauguier D, Samani NJ. Genetic dissection of region around the Sa gene on rat chromosome 1: evidence for multiple loci affecting blood pressure. Hypertension 2001; 38:216-221.

42. Vessey DA, Kelley M. Purification and partial sequencing of the XL-I form of xenobiotic-metabolizing medium chain fatty acid: CoA ligase from bovine liver mitochondria, and its homology with the essential hypertension protein. Biochem Biophys Acta 1997; 1346:231-236.

43. Iwai N, Ohmichi N, Hanai K, Nakamura Y, Kinoshita M. Human SA gene locus as a candidate locus for essential hypertension. Hypertension 1994; 23:375-380.

44. Lodwick D, Norman RI, Feeney G, West KP, Samani NJ. Immunohistochemical localisation of SA protein [Abstract]. J Hypertens 1998; 16:S45.

45. Lamon-Fava S, Wilson PW, Schaefer EJ. Impact of body mass index on coronary heart disease risk factors in men and women. The Framingham Offspring Study. Arterioscler Thromb Vasc Biol 1996; 16:1509-1515.

46. Brodde OE. β-1 and β-2 adrenoceptor polymorphisms: Functional importance, impact on cardiovascular diseases and drug responses. Pharmacol Ther 2008; 117:1-29.

47. Heinonen P, Koulu M, Pesonen U, Karvonen MK, Rissanen A, Laakso M, et al. Identification of a three-amino acid deletion in the α2(B)-adrenergic receptor

that is associated with reduced basal metabolic rate in obese subjects. J Clin Endocrinol Metab 1999; 84: 2429-2433.

48. Small KM, Brown KM, Forbes SL, Liggett SB. Polymorphic Deletion of Three Intracellular Acidic Residues of the α2B-Adrenergic Receptor Decreases G Protein-coupled Receptor Kinase-mediated Phosphorylation and Desensitization. J Biol Chem 2001; 276: 4917-4922.

49. Defoor J, Martens K, Zielinska D, Matthijs G, Van Nerum H, Schepers D et al. The CAREGENE study: polymorphisms of the beta1-adrenoceptor gene and aerobic power in coronary artery disease. Eur Heart J 2006; 27: 808-816.

50. Zhang H, Li X, Huang J, Li Y, Thijs L, Wang Z, et al. Cardiovascular and metabolic phenotypes in relation to the ADRA2B insertion/deletion polymorphism in a Chinese population. J Hypertens 2005; 23:2201-2207.

51. Siitonen N, Lindström J, Eriksson J, Valle TT, Hämäläinen H, Ilanne-Parikka P, et al. Association between a deletion/insertion polymorphism in the alpha2B-adrenergic receptor gene and insulin secretion and Type 2 diabetes. The Finnish Diabetes Prevention Study. Diabetologia 2004; 47:1416-1424.

52. Sivenius K, Lindi V, Niskanen L, Laakso M, Uusitupa M. et al. Effect of a three-amino acid deletion in the alpha2B-adrenergic receptor gene on long-term body weight change in Finnish non-diabetic and type 2 diabetic subjects. Int J Obes Relat Metab Disord 2001; 25:1609-1614.

53. Li Y, Zagato L, Kuznetsova T, Tripodi G, Zerbini G, Richart T, Thijs L. et al. Angiotensin-converting enzyme I/D and alpha-adducin Gly460Trp polymorphisms: from angiotensin-converting enzyme activity to cardiovascular outcome. Hypertension 2007; 49:1291-1297.

54. Lafontan M, Berlan M. Fat cell adrenergic receptors and the control of white and brown fat cell function. J Lipid Res 1993; 34:1057-1091.

55. Mason DA, Moore JD, Green SA, Liggett SB. A gain-of-function polymorphism in a G-protein coupling domain of the human beta1-adrenergic receptor. J Biol Chem 1999; 274:12670-12674.

56. Sofowora GG, Dishy V, Muszkat M, Xie HG, Kim RB, Harris PA, Prasad HC, Byrne DW, Nair UB, Wood AJ, Stein CM. A common beta1-adrenergic receptor polymorphism (Arg389Gly) affects blood pressure response to beta-blockade. Clin Pharmacol Ther 2003; 73:366-371.

57. Sharma A, Jeunemaitre X. The future of genetic association studies in hypertension: improving the signal-to-noise ratio. J Hypertens 2000; 18:811–814.

58. Campbell H, Rudan I. Interpretation of genetic association studies in complex disease. The Pharmacogenomics Journal 2002; 2:349-360.

59. Ioannidis JP, Boffetta P, Little J, O'Brien TR, Uitterlinden AG, Vineis P, Balding DJ, Chokkalingam A, Dolan SM, Flanders WD, Higgins JP, McCarthy MI, McDermott DH, Page GP, Rebbeck TR, Seminara D, Khoury MJ. Assessment of cumulative evidence on genetic associations: interim guidelines. Int J Epidemiol 2008; 37:120-132.

Acknowledgements

I am deeply indebted to my supervisor, Prof. Edoardo Casiglia, for all his support, patience and encouragement not only throughout my PhD programme but also my entire University studies. I also gratefully acknowledge my promoter, Prof Achille Pessina, for giving me the nice opportunity to participate in this international PhD programme.

I also would like to thank Prof Jan Staessen for the possibility to analyse the EPOGH and FLEMENGHO database in Leuven and for his advices on data interpretation, statistical analyses, and manuscripts' draft. It was a great privilege and a real pleasure to collaborate with such enthusiastic and open-minded scientist.

The encouragement and support of many people have contributed to this thesis. I express my gratitude to all the EPOGH investigators. Especially, I owe my greatest thankfulness to Tatiana Kuznetsova for her support, for her friendship, and her incredible kindness including all her family during these years.

Finally my greatest debt of thanks must be to my family.

List of EPOGH investigators

Belgium (Leuven) — E. Balkestein, H. Celis, E. Den Hond, R. Fagard, J. Gąsowski, T. Kuznetsova, Y. Li, T. Nawrot, M. Plusquin, L. Thijs, J.A. Staessen, J.G. Wang; *Czech Republic* (Pilsen and Prague) — O. Beran, J. Filipovský, L. Goláñ, T. Grus, G. Grusová, M. Jáchymová, J. Seidlerova, Z. Marečková, J. Peleška, V. Svobodová, M. Tichá; *Poland* (Cracow) — A. Adamkiewicz-Piejko, M. Cwynar, T. Grodzicki, K. Kawecka-Jaszcz, W. Lubaszewski, A. Olszanecka, K. Stolarz, B. Wizner, W. Wojciechowska, J. Zyczkowska; *Romania* (Bucharest) — S. Babeanu, D. Jianu, C. Sandu, D. State, M. Udrea; *Russian Federation* (Novosibirsk) — T. Kuznetsova, S.

46