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Short Title: *Vangjeli REN-5312C/T Variant and Blood Pressure*

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Abstract

Background: Studies of knockout and transgenic mice have demonstrated key roles for genes encoding components of the Renin Angiotensin System (RAS) in blood pressure (BP) regulation. However, whether or not polymorphisms in these genes contribute to the causation of essential hypertension in humans is still a matter of debate.

Methods and Results: We performed an experiment with dense tagging single nucleotide polymorphism (SNP) coverage of four genes encoding proteins that control the overall activity of the cascade, namely renin (REN), angiotensinogen (AGT), angiotensin converting enzyme (ACE) and angiotensin converting enzyme 2 (ACE2), in two Irish populations. Both clinic and 24-hour ambulatory BP measurements were available from population I (n=387), whereas just clinic BP was measured in population II (n=1024). Of the 23 polymorphisms genotyped, only a single renin gene polymorphism, REN-5312C/T, showed consistent statistically significant associations with elevated diastolic pressures. Carriage of one REN-5312T allele was associated with the following age and sex adjusted increments in diastolic pressures (mean [95% confidence interval], mmHg); Population I, clinic 1.5[0.3, 2.8], daytime 1.4[0.4, 2.4], night-time 1.3[0.4, 2.3], and Population II, clinic 1.1[0.1, 2.1]. Haplotypic analyses and multivariate stepwise regression analyses were in concordance with individual SNP analyses.

Conclusions: The REN-5312T allele had previously been shown to result in increased in vitro expression of the renin gene. We have now shown, in two independent populations, that carriage of a REN-5312T allele is associated with elevated diastolic BP. These data provide evidence that renin is an important susceptibility gene for arterial hypertension in Caucasians.

Key words: Renin Angiotensin System, Renin, Polymorphism, Gene, Blood Pressure

Introduction

The Renin Angiotensin System (RAS) plays important roles in the regulation of blood pressure (BP) and electrolyte balance, and also in the pathogenesis of atherosclerosis.[1-3] In the first and rate limiting step of the RAS, renin (REN) catalyses the cleavage of angiotensinogen (AGT) into angiotensin I. Angiotensin I can then be further catalysed to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II raises BP through potent vasoconstriction and sodium retention[1, 2] and it also promotes cell growth, fibrosis and inflammation in cardiovascular and renal tissues.[3] Recently, however, some important additional pathways in the RAS have been elucidated.[4] These include the discovery that there is a second angiotensin converting enzyme (ACE2) which catalyses the conversion of angiotensin I and angiotensin II, to angiotensin(1-9) and angiotensin(1-7), respectively.[5-7] Angiotensin(1-9) is an inactive nonapeptide, while angiotensin(1-7) appears to act as a natural antagonist for angiotensin II, in that it has potent vasodilator, natriuretic, antigrowth and endothelium protective properties.[8, 9]

Studies of knockout and transgenic mice have confirmed the role of genes encoding proximal RAS components in BP regulation. Targeted disruption of the AGT, REN and ACE genes in mouse models resulted in decreased pressures,[10-12] while disruptions in ACE2 resulted in higher BP.[13] A double transgenic rat, expressing both the human renin gene and the human angiotensinogen gene, develops severe early hypertension and end-organ damage, and usually dies by the eighth week of life.[14] Hence it appears that the overall activity of the cascade, both in the circulation and in renal and vascular tissues, is critically dependant on levels of angiotensinogen protein and renin enzymatic activity, and on the balance of activity between the ACE and ACE2 enzymes.

While candidate gene studies have provided good evidence that polymorphisms in AGT and ACE result in altered plasma protein concentrations, whether or not polymorphisms in RAS genes are associated with BP level in humans and contribute to the causation of essential

hypertension is still a matter of debate.[15] Many published reports of positive associations have not been replicated in subsequent studies.[16] Small sample sizes, limited statistical power, population heterogeneity, and inaccurate phenotypic assessments are all likely to have contributed to this situation. In addition most studies have tested the association of single genes, or even single polymorphisms, with BP level or hypertension. In studying the genetics of complex traits such as hypertension, the assumption that a single genetic mutation is a necessary and sufficient cause of disease is unwise – instead we have to think of a web of causation involving multiple and complex pathways.[17] Susceptibility to hypertension is likely to entail many genes acting in an additive or interactive manner.

The RAS represents a perfect example where the cumulative effects of a number of variations in the genes encoding components of the cascade, acting together with environmental factors, could result in considerable alteration in activity of the system. We previously reported an association between a renin gene distal enhancer polymorphism (REN-5312C/T) and BP level in a group of Irish bank workers.[18] The aim of this study was to confirm and extend this finding, through performing an experiment with dense tagging single nucleotide polymorphism (SNP) coverage of all four genes that are thought to control RAS activity. We studied the associations of individual SNPs and haplotypes with BP level both in the original population, and in a second large Irish population. We also performed multivariate stepwise regression analyses, examining for additive effects of genes and environmental factors on BP level.

Methods

Population descriptions

Population I: This cohort of 815 current and retired Caucasian bank employees and their spouses were free of diagnosed hypertension and vasoactive drugs when recruited to the Allied

Irish Bank Study.[19] At the baseline (phase I) examination, conducted between 1989 and 1991, age, gender, smoking habit, alcohol consumption, salt intake, past medical history, current drug treatments, height, weight, clinic and 24-hour ambulatory blood pressures were recorded. Between 1996 and 2001, after a mean interval of 8.4 years, 441 subjects responded to a written invitation to undergo repeated assessments. At this time, only four subjects were taking antihypertensive medication, and these discontinued therapy one week prior to the phase II assessments. At the phase II examination, all phase I measurements were repeated, and in addition blood was drawn for biochemical measurements and for the extraction of DNA. We excluded 9 participants because of technically unsatisfactory ABPM recordings, and 45 participants in whom DNA extraction was unsuccessful. Thus the total number of participants included in the present analysis was 387. All subjects provided written informed consent to each phase of the study. The study protocols of the two phases were approved by the Beaumont Hospital Research Ethics Committee.

Population II: A second cohort of 1024 current and retired Caucasian bank employees and their spouses aged between 18 and 80 years were recruited to the Allied Irish Bank study between June 2003 and June 2004. Age, gender, smoking habit, alcohol consumption, salt intake, past medical history, current drug treatments, height, weight and clinic blood pressure were recorded. Blood samples were drawn for biochemical measures and for the extraction of DNA. All subjects provided written informed consent. The study protocol was approved by the Beaumont Hospital Research Ethics Committee.

Blood Pressure and Laboratory Measurements

Sitting clinic BP was measured from the right arm using a mercury sphygmomanometer (population I) or a regularly calibrated validated automated sphygmomanometer (Omron HEM-705CP) (population II). Blood pressure was measured in the brachial artery 3 times at 5-minute

intervals. The mean of the last 2 measurements was recorded as representative of clinic BP. Ambulatory BP measurements in population I were made every half-hour throughout a 24-hour period using validated oscillometric 90202 or 90207 SpaceLabs recorders (SpaceLabs, Wokingham, Berkshire, UK). Mean daytime (mean of all measurements between 0900 and 2100 hours), and night-time (mean of all measurements between 0100 and 0600 hours) systolic and diastolic pressures were calculated for each individual for each phase. For population 1, both the phase I and phase II, clinic, daytime and night-time, BP measurements were included in the analyses models.

Non-fasting total cholesterol, triglycerides and glucose were measured using standard enzymatic methods on a Roche/Hitachi 912 automated analyser (Roche Diagnostics, Basel Switzerland) in population I, and a Cholestech LDX lipid analyser (Cholestech Corp, California, USA) in population II.

Identification of Gene Sequence Variants and Genotyping.

Genomic DNA was extracted from leukocytes from both populations by a salting out procedure.[20] For the REN and ACE2 genes, DNA from 20 subjects (10 normotensive and 10 hypertensive) was screened for mutations in the known promoter regions (REN; -750 to +47, and ACE2; -1224 to +121) and in the protein coding regions (10 REN exons, 18 ACE2 exons and at least 40bp of flanking intronic regions). The human renin gene distal enhancer region (-5868 to -5226) was also screened for mutations. As previously described, this was achieved by a combination of ion-pairing reversed-phase partially denaturing high-performance liquid chromatography and direct sequencing.[18] Ten REN gene SNPs and three ACE2 SNPs were detected, and these were genotyped in population I. Using the multiple-marker haplotype r^2 criterion, implemented as Criterion 11 of the TagIT software package (http://www.ucl.ac.uk/tcga/software/)[21] five REN tag SNPs and two ACE2 tag SNPs were found

to capture 85% of the variation (r^2=0.85) within these genes in population I. Hence only these were genotyped in population II.

SNPs in the coding and intronic regions of the ACE and AGT genes were selected using HapMap (www.hapmap.org data release #22). Fifteen tag SNPs in the AGT gene and five tag SNPs in the ACE gene, each with a frequency of at least 5% in the Centre d'Etude du Polymorphisme Humain Caucasian (CEPH) HapMap population, were identified using "pairwise tagging" in the Haploview Tagger software, with an r^2 cutoff of 0.85. [22] All 20 SNPs were genotyped in both populations. Further LD analysis revealed that 8 SNPs (4 pairs) in the AGT gene, and 2 SNPs (1 pair) in the ACE gene, had R^2 values of >0.85 within both populations. Hence, in order to reduce redundancy, the SNP from each pair with the most missing genotypic data was excluded. Hence only 11 AGT tag SNPs and 4 ACE tag SNPs were included in the final analyses. In addition to the SNPs selected using HapMap, the commonly studied ACE insertion/deletion (I/D) variant was genotyped and included in the analysis.

Genotyping of SNPs was performed by KBiosciences (Herts, U.K.) using modified TaqMan assays (www.kbiosciences.co.uk). Genotyping of the ACE I/D variant was adapted from the method by Viswanathan et al[23] and is described in detail in the supplementary methods section. Briefly, it was performed by PCR amplification using a flanking primer pair to detect the insertion (I = 490bp) and deletion (D= 190bp) alleles and confirmed using a second PCR designed to recognise the insertion specific sequences (335bp).

Statistical analysis

Statistical analyses were performed using Plink (version 1.04, http://pngu.mgh.harvard.edu/purcell/plink/)[24, 25] and the Stata statistical package (version 8.2, StataCorp, College Station, Texas) .

Phenotypic data are expressed as mean ± SD, as median [interquartile range], or as numbers (percentages). Alcohol intake followed a lognormal distribution, and was log-transformed prior to inclusion in any analysis. Two way ANOVAs and Chi-squared tests were used to compare phenotypic variables across the two populations and genders.

Departure from Hardy–Weinberg equilibrium was tested by Chi-squared tests. Linear regression analysis was used to determine whether individual RAS polymorphisms predicted clinic and ambulatory systolic and diastolic BP levels after adjustment for age and sex. Additive genetic models were used. For any variants found to be significantly associated with BP, likelihood ratio testing was performed so as to compare the fits of additive and dominant genetic models. The primary objective of this study was to replicate our previous finding of an association between the REN-5312T allele and BP level. Hence a p-value of $p<0.05$ was considered significant for all analyses concerning this SNP within population II. In recognition of the risks of multiple comparisons, all other analyses were performed in two independent populations, and Bonferroni corrections were to be applied where consistent associations were found in both populations with p-values <0.05. Study power was in excess of 90% to detect 2 mmHg systolic and 1 mmHg diastolic BP increments (ambulatory daytime and night-time pressures for population I, clinic pressures for population II) associated with genetic variants where the minor allele frequency was 20% or greater.

Haplotypes were inferred for each subject from the SNPs in each gene using the "--hap-phase" function in Plink. Testing for associations of haplotype with BP levels was performed, by including sex, age, and predicted individual haplotypes in linear regression models, weighted according to haplotype probability. Rare haplotypes (<5% frequency) were pooled for analysis.

Finally, multiple regression analysis with backwards step-wise removal of non-significant variables was performed seeking additive effects of genes and environmental factors on BP level.

Age, sex, salt addition at meals, alcohol intake, body mass index (BMI) and all RAS gene polymorphisms were all initially included in the models. The five phenotypic variables, already established as influencing BP level, were retained in the models throughout. However, in successive steps, the least significant genetic variables were excluded, such that the final models only retained genetic variables with $p<0.05$.

Results

Population Characteristics

42% of population I and 52% of population II were female (Table 1). By comparison with population I, participants in population II were older ($p<0.001$), and were more likely to be current or ex smokers ($p<0.01$) and to add salt to meals ($p<0.001$). Both total cholesterol ($p<0.001$) and triglycerides ($p<0.001$) were lower in population II. In both populations, female participants were younger ($p=0.01$), and were less likely to smoke cigarettes than males ($p>0.05$). Self reported alcohol intake was lower amongst female participants ($p<0.001$), as was BMI ($p=0.001$), total cholesterol ($p<0.001$) and triglycerides ($p<0.001$). Clinic pressures were also lower in females than in males in both populations (systolic $p<0.001$, and diastolic $p<0.001$), but the between sex difference was less marked in population II (population x sex interaction $p=0.02$).

Associations of individual RAS polymorphisms with BP

All polymorphisms in the four genes, REN, AGT, ACE and ACE2, were found to be in HWE, and minor allele frequencies in the two populations were similar (see supplementary table 1).

Mean age and sex-adjusted differences in systolic and diastolic pressures associated with carriage of one minor allele for each RAS polymorphism are illustrated in figures 1 and 2 respectively. Of the 23 polymorphisms (22 tag SNPS and 1 ACE I/D) genotyped, only the distal

enhancer renin gene polymorphism, REN-5312C/T, showed consistent statistically significant associations with elevated pressures. Carriage of one REN-5312T allele was associated with the following age and sex adjusted increments in diastolic pressures (mean [95% confidence interval], mmHg); Population 1, clinic 1.5[0.3, 2.8], daytime 1.4[0.4, 2.4], night-time 1.3[0.4, 2.3], and Population 2, clinic 1.1[0.1, 2.1]. Diastolic blood pressures of younger and older, males and females, in the two populations are illustrated according to REN-5312C/T genotype in Figure S1. While REN-5312C/T heterozygotes consistently demonstrated higher pressures than CC homozygotes, the pressures of TT homozygotes were not always further elevated above the values of the CT heterozygotes. Despite these findings being more in keeping with a dominant effect, formal likelihood ratio testing showed equivalence of age and gender adjusted additive and dominant genetic models, in both populations, for all measures of diastolic pressures. Carriage of one REN-5312T allele was also associated with statistically significant age and sex adjusted increments in systolic pressures in population I; clinic 2.1[0.0, 4.1], daytime 2.0[0.6, 3.4], and night-time 3.6[0.9, 2.3]. Clinic systolic pressures tended to be higher in population II participants carrying a REN-5312T allele 0.7[-1.1, 2.6], but this difference was not statistically significant.

Associations of RAS haplotypes with blood pressure

Estimated RAS gene haplotype frequencies were similar in the two populations (see Table S2). Ambulatory and clinic pressures were found to differ with renin gene haplotype (figures S2 and S3). This was in large part attributable to the age and sex adjusted estimates for BP of the TCAAG haplotype being significantly higher than the corresponding pressure estimates of the remaining haplotypes – this haplotype alone carries the REN-5312T variant. Similar to the analyses of the associations of individual polymorphisms with BP levels, only diastolic pressures

were consistently statistically significantly elevated amongst carriers of the TCAAG haplotype in the REN gene versus non-carriers of this haplotype in the two populations.

Stepwise regression analysis of the associations of environmental factors and RAS polymorphisms with blood pressure levels

Multiple regression analysis of diastolic BP in both populations showed that REN-5312C/T was significantly and independently related to both clinic and ambulatory pressures, after adjustment for traditional covariates including sex, age, salt usage, alcohol intake and BMI (Table 2). The REN-5312C/T SNP was the only polymorphism that remained in all models for DBP in the two populations. Consistent with the findings for individual SNP analysis, this same SNP remained in the models for daytime and night time SBP in population I, but not in the model for clinic pressures for either population.

Discussion

This study has shown for the first time the potential importance of the renin gene in the regulation of blood pressure level in humans. In two independent populations, carriage of one REN-5312T allele was associated with an increment in diastolic BP ranging from 1.1 to 1.8 mmHg, depending on whether clinic or ambulatory pressures were the end-point, and on whether univariate, multiple regression analysis or haplotype analyses were utilized. Importantly, pressure differences of this magnitude have been shown to be associated with increases in the incidence of stroke and heart attack[26, 27]. Systolic pressures also tended to be higher in subjects carrying one or two REN-5312T alleles, but the increments were not consistently statistically significant.

None of the RAS polymorphisms, other than REN-5312C/T, were found to have consistent statistically significant associations with BP level in both Irish populations. For the 12 variants with a minor allele frequency of 20% or greater, we had 90% power to detect 2mmHg

systolic and 1mmHg diastolic blood pressure differences in both populations. It is therefore unlikely that these particular polymorphisms contribute to significant blood pressure differences in North-Western European Caucasian populations. However it would not be correct to generalize these findings to other ethnic groups. In addition true clinically relevant effects of RAS variants on BP may not have been detected, because of insufficient study power for variants with minor allele frequencies less than 20%. Furthermore because of the SNP selection and tagging strategies adopted for this study, coverage of all variants in the four genes was not complete - only SNPs in the coding, promoter and enhancer regions of the REN and ACE2 genes were studied, and while variants in both the coding and intronic regions of the AGT and ACE genes were included, those in the promoter and enhancer regions were not. Lastly, because of the size of the two populations, this study only sought direct associations of polymorphisms and environment with BP level – there was insufficient power to look for gene-gene interactions, or for gene-environment interactions. Hence these complexities remain to be examined within much larger cohorts.

In the past, several studies reported associations between renin restriction fragment length polymorphisms and essential hypertension in a range of ethnic groups.[28-31] However others reported no association between the same renin gene variants.[29, 32-35] A missense mutation in exon 9 (10501G/A) was associated with both hypertension and elevated PRA in a Japanese population (212 hypertensives vs. 209 controls).[36] Haplotypes composed of the intron 1 BglI variant and the exon 9 10501G/A SNP were found to be associated with hypertension in 329 hyperlipidaemic US Caucasian men and women (140 hypertensives, 141 normotensives, and 48 hypertensive patients who had suffered a stroke) aged 40 to 70 years. [37] The exon 9 10501G/A SNP was associated with hypertension in a group of 689 Gulf Arabs from the United Arab Emirates (326 hypertensives and 363 age- and gender-matched controls).[38] Zhu and colleagues reported C-4021T and C-3212T to be associated with hypertension in African Americans but not

in European Americans.[39] A SNP in intron 4 (54620025A>C) was associated with elevated BP and hypertension in two populations of Spanish women aged greater than 40 years old (total n=1,418).[40] Most recently Rana and colleagues reported that neither the Intron 4 G-131T SNP nor the Thr68Thr REN gene SNPs were individually associated with elevated BP in a group of 611 male and 656 female age-matched Caucasian Americans. However they did find a significant epistatic interaction between the REN Intron 4 SNP and the ACE intron 24 (G-6A) SNP.[41] Overall few of the previous candidate gene studies addressing the question of whether renin polymorphisms influence BP level have been replicated, and none have provided evidence of functionality.

Renin catalyses the first and rate limiting step of the RAS cascade. A 466-base pair element (nucleotides -5777 to -5312) has been reported to activate the renin promoter approximately 60-fold in primary cultures of human chorionic cells.[42, 43] Fuchs and colleagues went on to describe 45% greater rates of renin gene transcription in the presence of a -5312T allele rather than a -5312C allele.[43] Hence it is not surprising that we have now shown in two populations that this polymorphism is associated with elevated BP. We previously described that plasma renin activity was similar in hypertensive REN-5312T allele carriers and hypertensive CC homozygotes.[18] Hence it does not appear that the REN-5312C/T polymorphism influences the highly regulated secretion of active renin from kidney juxtaglomerular cells into the systemic circulation, and it appears much more likely that functionality is mediated by altered local tissue renin levels.

If renin is an important susceptibility gene for arterial hypertension, one might question why this was not identified in several recent whole genome association studies (WGASs) that were conducted in considerably larger populations.[44-46] Three of these studies listed SNPs in chromosome 1 as being amongst their top hits, but none of these were close to the renin gene

region, at 1q32. The genotyping chips used in these WGASs did include several renin gene SNPs, but none specifically genotyped REN-5312C/T. Previous work from our group has suggested that REN-5312C/T is not in strong linkage disequilibrium with any other renin SNPs, even the nearby REN 5'-flank -5434 SNP.[18] Unfortunately, as REN-5312C/T has not been genotyped as part of the HapMap project, the lack of linkage disequilibrium surrounding this SNP cannot be verified. Furthermore, while the usage of very small p-values ($p < 5 \times 10^{-7}$), necessitated because of the level of multiple testing within WGASs does protect against false positive findings, it markedly reduces power to detect small but true effects. The limited power of these WGASs has been acknowledged, and it is well recognised that many more common variants are likely to exist with effects on blood pressure. It is an advantage of gene-centric studies that the p-value that is regarded as significant does not have to be reduced so dramatically, and power to detect true associations is retained.

In conclusion, the REN-5312T allele had previously been shown to result in increased in vitro expression of the renin gene. We have now shown, in two independent populations, that carriage of a REN-5312T allele is associated with elevated diastolic BP. These data provide evidence that renin is an important susceptibility gene for arterial hypertension in Caucasians.

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Conflict of Interest Disclosures: NC, AVS and the Royal College of Surgeons in Ireland hold a patent concerning renin genotype, blood pressure lowering and prognosis.

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Table 1. Characteristics of the Participants of Populations I and II

	Population I†		Population II	
	Males (n=224)	Females (n=163)	Males (n=493)	Females (n=531)
Variable				
Age, years	46 ± 9*	42 ± 10	48 ± 14	46 ± 14
Current/Ex smoker, %	71 (32)	45 (28)	217 (44)	206 (39)
Alcohol intake, units/week	15[8-24]	6[2-11]	12[6-20]	5[2-9]
Salt usage at table, %	128 (57)	98 (60)	375 (76)	397 (75)
Diabetes mellitus, %	2 (1)	1 (1)	10 (2)	6 (1)
Body Mass index, kg/m^2	27 ± 3	24 ± 4	27 ± 4	25 ± 4
Clinic SBP‡, mmHg	130 ± 17	116 ± 19	126 ± 17	120 ± 19
Clinic DBP‡, mmHg	82 ± 11	74 ± 11	79 ± 9	75 ± 9
Daytime SBP‡, mmHg	131 ± 12	120 ± 12		
Daytime DBP‡, mmHg	82 ± 8	75 ± 8		
Night- time SBP‡, mmHg	112 ± 12	104 ± 10		
Night-time DBP‡, mmHg	67 ± 8	61 ± 7		
Total cholesterol, mmol/L	5.7 ± 1.2	5.3 ± 1.2	5.1 ± 1.1	5.0 ± 0.9
Triglycerides, mmol/l	1.4 ± 0.9	1.0 ± 0.6	1.7 ±1.1	1.3 ± 0.7

*Data expressed as mean ± SD, median [interquartile range] or as number (%).
†Population I participant information refers to the phase II assessment.
‡SBP = systolic blood pressure. DBP = diastolic blood pressure. mmHg = millimetres of mercury

Table 2. Multiple regression analysis of ambulatory and clinic, systolic and diastolic pressures in the two populations.

	Systolic BP				Diastolic BP			
	Population I			Population II	Population I			Population II
	Day	Night	Clinic	Clinic	Day	Night	Clinic	Clinic
Age (per year)	*0.19 $^{0.000}$	0.19 $^{0.000}$	0.67 $^{0.000}$	0.56 $^{0.000}$	0.09 $^{0.001}$	0.23 $^{0.000}$	0.32 $^{0.000}$	0.16 $^{0.000}$
Sex (female = 0, male = 1)	-7.69 $^{0.000}$	-6.23 $^{0.000}$	-5.59 $^{0.000}$	-1.46 $^{0.222}$	-4.13 $^{0.000}$	-3.41 $^{0.000}$	-4.17 $^{0.000}$	-2.52 $^{0.000}$
BMI (per kg/m^2)	0.05 $^{0.001}$	0.05 $^{0.001}$	0.13 $^{0.000}$	0.88 $^{0.000}$	0.02 $^{0.133}$	0.02 $^{0.119}$	0.05 $^{0.001}$	0.63 $^{0.000}$
Alcohol (per unit/week)	0.14 $^{0.000}$	0.05 $^{0.130}$	0.17 $^{0.001}$	0.31 $^{0.000}$	0.07 $^{0.004}$	0.01 $^{0.657}$	0.08 $^{0.018}$	0.10 $^{0.003}$
Salt usage at table (no = 0, yes = 1)	2.42 $^{0.003}$	0.62 $^{0.448}$	1.40 $^{0.244}$	1.17 $^{0.349}$	1.21 $^{0.044}$	0.06 $^{0.916}$	0.71 $^{0.358}$	-0.08 $^{0.906}$
ACE Intron9 6329T					1.13 $^{0.009}$		1.46 $^{0.008}$	

AGT Intron1 459T			6.92 $^{0.001}$	6.66 $^{0.001}$	7.30 $^{0.007}$	
AGT Intron1 751T			-4.93 $^{0.019}$	-6.63 $^{0.001}$	-7.79 $^{0.003}$	
AGT Intron2 6384C			5.70 $^{0.005}$	6.79 $^{0.000}$	6.57 $^{0.011}$	
AGT Intron2 6436T			-7.11 $^{0.013}$	-7.34 $^{0.007}$	-8.63 $^{0.018}$	
REN 5flank 5312T	2.49 $^{0.001}$	2.88 $^{0.000}$	1.65 $^{0.002}$	1.68 $^{0.001}$	1.81 $^{0.009}$	1.67 $^{0.008}$

*Data expressed as differences in blood pressure [p-value]

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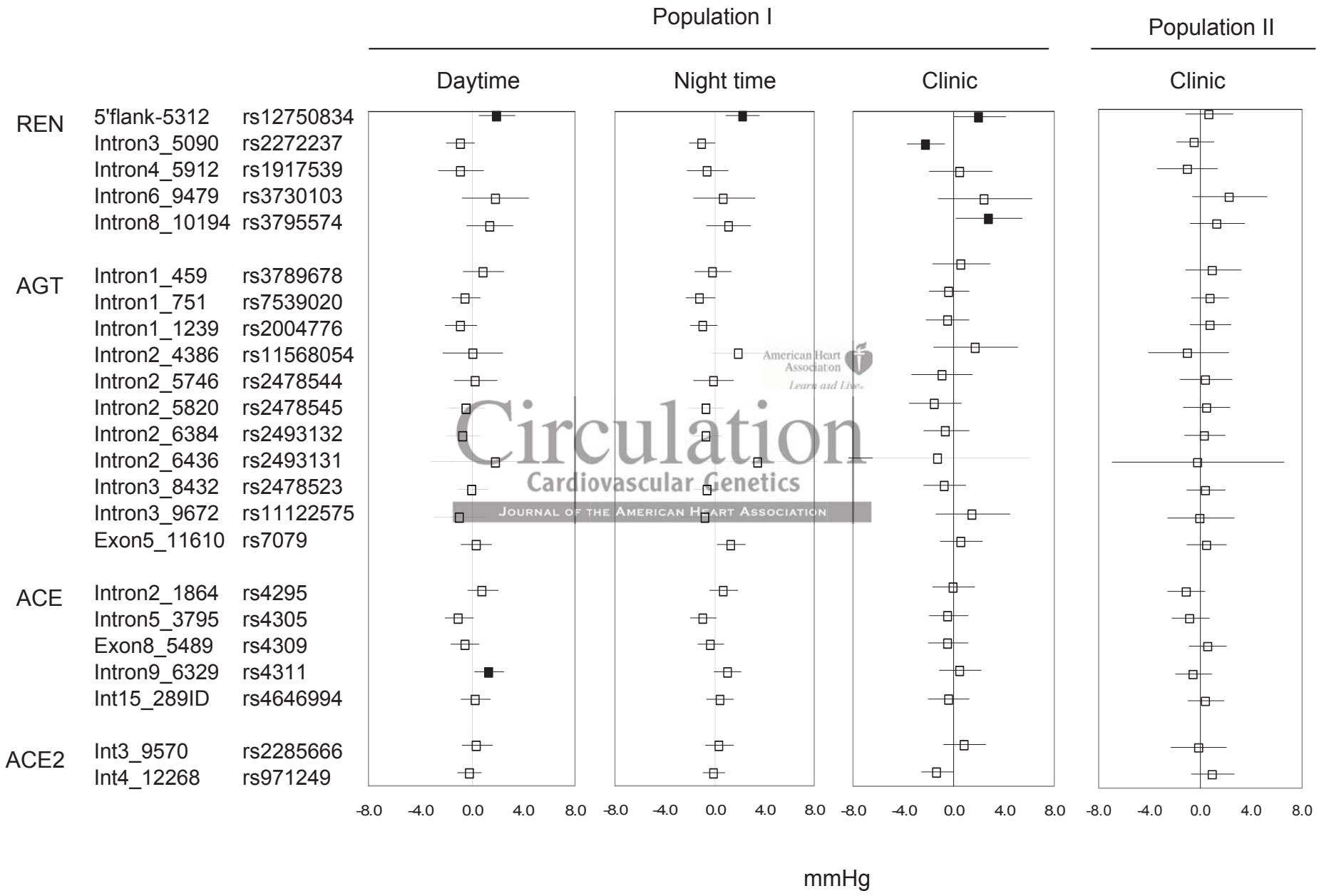
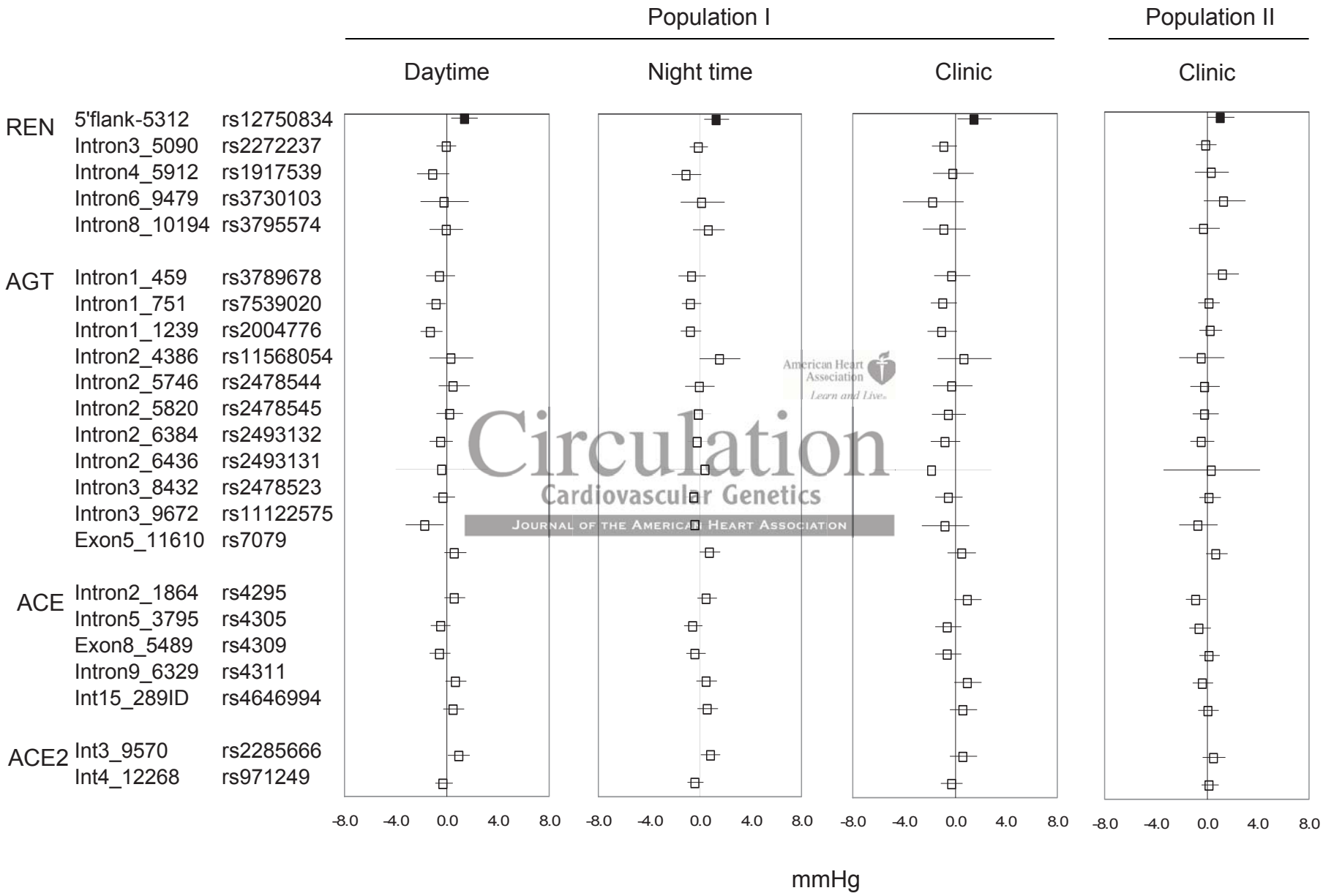
Figure Legends:

Figure 1. Differences in systolic blood pressure (mean difference and 95% confidence interval, mmHg) per copy of minor allele for each polymorphism in the two populations. Significant results are indicated by filled symbols.

Figure 2. Differences in diastolic blood pressure (mean difference and 95% confidence interval, mmHg) per copy of minor allele for each polymorphism in the two populations. Significant results are indicated by filled symbols.





SUPPLEMENTAL MATERIAL

Supplemental methods

Genotyping of the ACE insertion/deletion polymorphism

Ace genotypes were determined by PCR amplification using a Flanking primer pair to detect the Insertion (I = 490bp) and Deletion (D= 190bp) alleles and confirmed using a second PCR designed to recognise the insertion specific sequences (335bp). Flanking PCR samples were amplified in a 15µl PCR reaction containing 25ng DNA, 2.5 pmol of each primer (FLKF: CTGGAGACCACTCCCATCCTTTCT, FLKR: GATGTGGCCATCACATTCGTCACGAT; synthesized by MWG Biotech Ag, Germany), 200 µM dNTPs, 2.5mM $MgCl_2$, 3µl GoTaq™ Flexi Buffer, and 0.625U Go *Taq*™ DNA Polymerase (Promega). Amplifications were carried out in 96well plates on a DNA Engine™ (MJ Research, UK) for 1 cycle of 5 min at 95 °C followed by 30 cycles of 30sec at 95°C, 30sec at 58°C and 30 sec at 72°C with a final elongation step of 72°C for 5 min. Confirmatory PCR samples were amplified in a 15µl PCR reaction containing 25ng DNA, 0.125 pmol of each primer (MISF:TGGGACCACAGCGCCCGCCACTAC, MISR: TCGCCAGCCCTCCCATGCCCATAA), 200 µM dNTPs, 1.25mM $MgCl_2$, 3µl GoTaq™ Flexi Buffer, and 0.625U Go *Taq*™ DNA Polymerase (Promega). Amplifications were carried out for 1 cycle of 5 min at 95 °C followed by 31 cycles of 30sec at 95°C, 30sec at 68°C and 30 sec at 72°C with a final elongation step of 72°C for 5 min. PCR products were pooled, separated by electrophoresis on 12% polyacrylamide gels and visualized by ethidium bromide staining.

Genotyping quality control

All genotyping included at least 5% duplicates. Where initial genotyping results were inconclusive, genotyping was repeated once, as was genotyping of at least 10% of successful assays. Genotype concordance, both for within assay and between assay duplications, was in

excess of 99% for all polymorphisms. The final genotyping success rate was in excess of 99% for all SNPs.

Supplemental Tables

Table S1: Allele frequencies and p-values for the Chi-squared test for departure from Hardy-Weinberg Equilibrium for each SNP in both populations

			Population I		Population II	
SNP	**Rs number***	**Minor allele**	**MAF**	**HWE p-value**	**MAF**	**HWE p-value**
REN_5'flank_-5312	rs12750834	A	0.18	0.399	0.18	0.831
REN_Intron3_5090	rs2272237	G	0.40	0.516	0.38	0.505
REN_Intron4_5912	rs1917539	G	0.11	0.716	0.11	0.867
REN_Intron6_9479	rs3730103	G	0.05	0.249	0.07	0.794
REN_Intron8_10194	rs3795574	T	0.10	0.671	0.13	0.892
AGT_Intron1_459	rs3889728	T	0.15	0.555	0.13	0.743
AGT_Intron1_751	rs2004776	T	0.40	1.000	0.40	0.813
AGT_Intron1_1239	rs7539020	T	0.28	0.703	0.28	0.856
AGT_Intron2_4386	rs11568054	A	0.06	0.283	0.05	1.000
AGT_Intron2_5745	rs2478544	G	0.13	0.085	0.14	1.000
AGT_Intron2_5820	rs2478545	A	0.18	0.802	0.19	1.000
AGT_Intron2_6384	rs2493132	C	0.26	0.850	0.28	0.938
AGT_Intron2_6436	rs2493131	T	0.01	1.000	0.01	1.000
AGT_Intron3_8432	rs2478523	G	0.33	0.565	0.33	0.829
AGT_Intron3_9672	rs11122575	G	0.09	0.154	0.08	0.537
AGT_Exon5_11610	rs7079	T	0.32	0.074	0.33	0.319
ACE_Intron2_1864	rs4295	C	0.36	0.238	0.37	0.381
ACE_Intron5_3795	rs4305	A	0.44	0.159	0.45	0.404
ACE_Exon8_5489	rs4309	T	0.44	0.883	0.42	0.845

ACE_Intron9_6329	rs4311	T	0.46	0.139	0.48	1.000
ACE_Intron15_289bp_ID	rs4646994	I	0.46	0.883	0.46	0.658
ACE2_Intron3_9570R	rs2285666	A	0.16	1.000	0.18	0.559
ACE2_Intron4_12268R	rs971249	A	0.40	0.479	0.40	0.365

*Rs number = dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) reference number

MAF = minor allele frequency

HWE = Hardy Weinberg Equilibrium

NS = not significant

Table S2: Haplotype frequencies in population I and population II

Gene	Haplotypes	Haplotype frequency	
		Population I	Population II
REN	CGAAG	0.41	0.38
	CCAAG	0.21	0.21
	TCAAG	0.18	0.17
	CCGAG	0.10	0.10
	CCAGT	0.05	0.06
	CCAAT	0.05	0.07
AGT	CCCGCGTCAAT	0.31	0.32
	CCCGCGTCAAG	0.20	0.20
	TTTGCGTCGAG	0.14	0.13
	CTCGGACCGAG	0.11	0.11
	CTTGCGCCAGG	0.08	0.08
	CCCACGTCAAG	0.05	0.05
	CTTGCACCGAG	0.05	0.05
	rare	<0.05	<0.05
ACE	GGTCI	0.42	0.40
	CACTD	0.34	0.36
	GGCTD	0.09	0.10
	GACCD	0.06	0.06
	rare	<0.05	<0.05

ACE2	GG	0.43	0.44
	GA	0.41	0.39
	AG	0.16	0.17

Supplemental Figures and Figure Legends

Figure S1. Distribution of diastolic blood pressures (daytime, night-time and clinic, means and standard deviations) according to REN-5312C/T genotype amongst younger and older males and females in the two populations. Numbers within each box pertain to number of subjects per genotype group while those above the boxes pertain to standard deviation of blood pressure measurements.

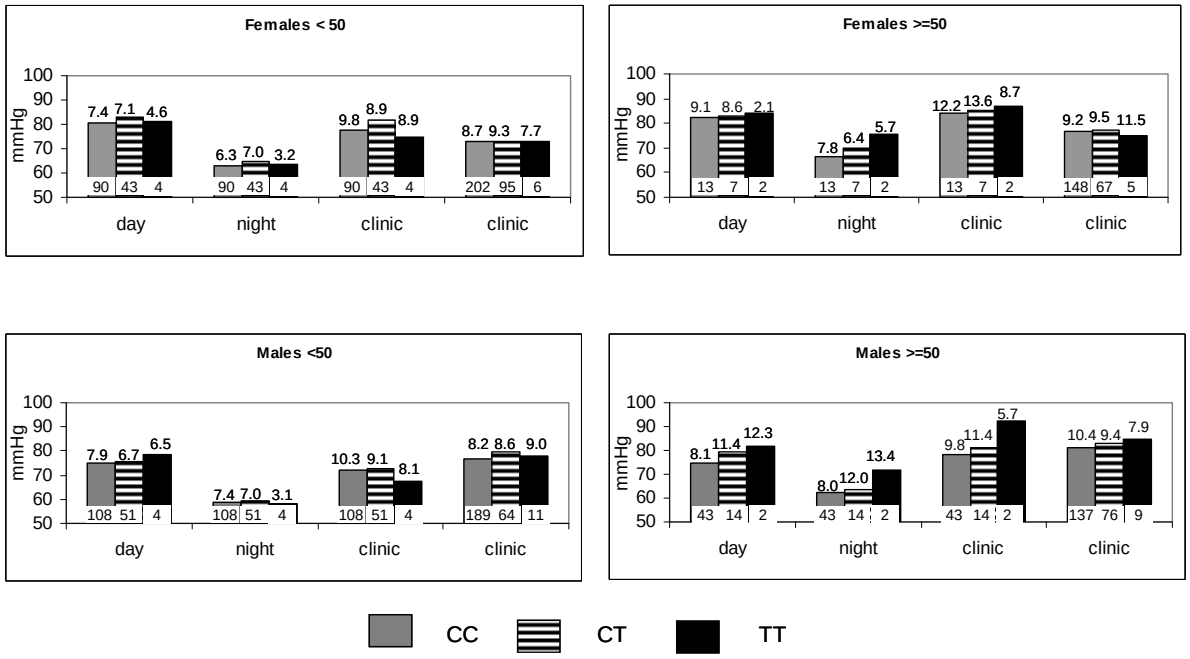


Figure S2. Differences in systolic blood pressure (mean difference 95% CI, mmHg,) between carriers and non-carriers of each haplotype in population I and population II. Statistically significant results are indicated by filled symbols. P values represent the overall p-value for the gene.

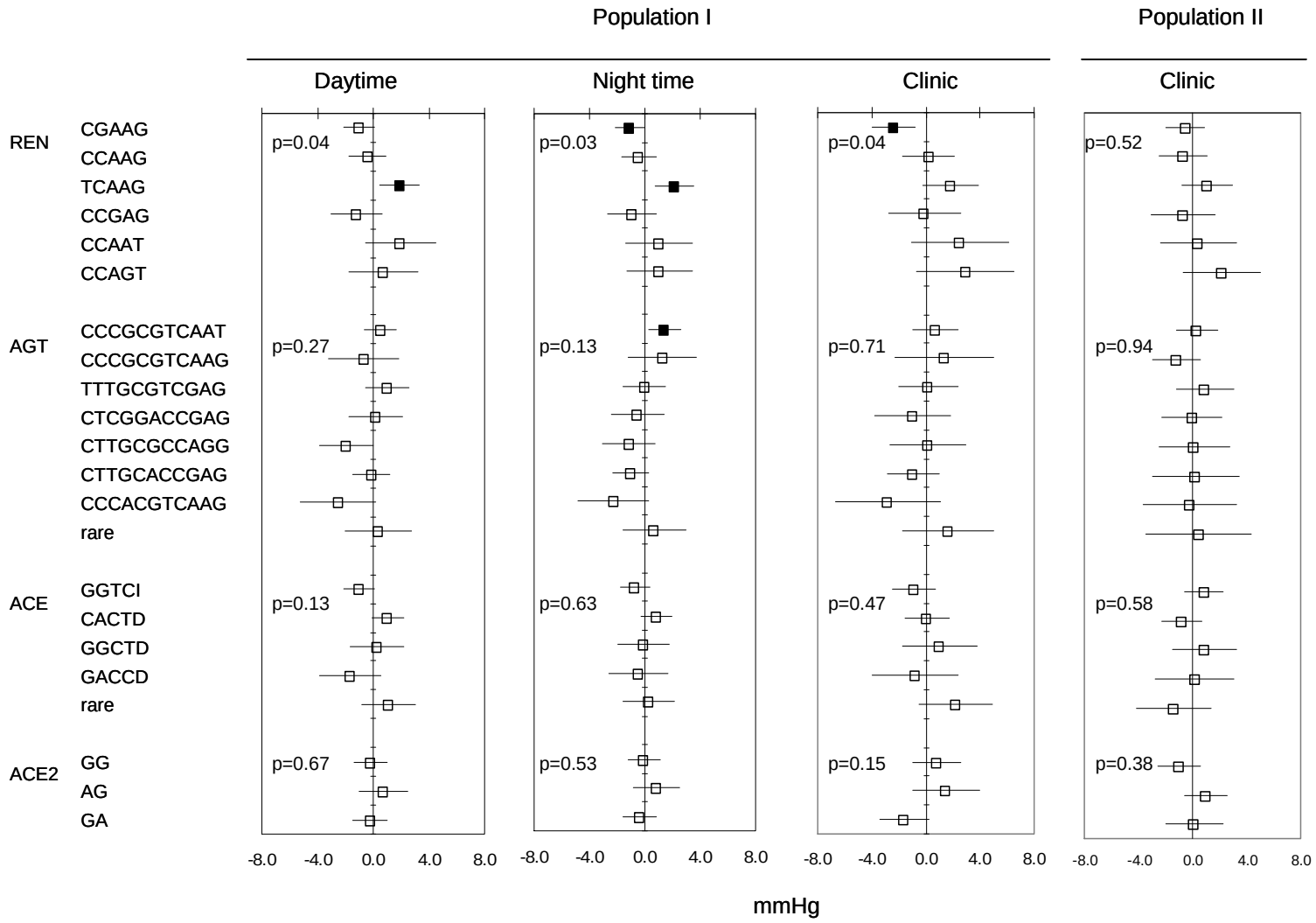


Figure S3. Differences in diastolic blood pressure (mean difference 95% CI, mmHg,) between carriers and non-carriers of each haplotype in population I and population II. Statistically significant results are indicated by filled symbols. P values represent the overall p-value for the gene.