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# Angiotensinogen Single Nucleotide Polymorphisms, Elevated Blood Pressure, and Risk of Cardiovascular Disease

Amar A. Sethi, Børge G. Nordestgaard, Marie-Louise M. Grønholdt, Rolf Steffensen, Gorm Jensen, Anne Tybjærg-Hansen

**Abstract**—In this study of 10 690 individuals, associations with elevated blood pressure, ischemic heart disease, and ischemic cerebrovascular disease were determined for two noncoding [A(−20)C, G(−6)A] and two coding (T174M, M235T) single nucleotide polymorphisms, analyzed alone and in combination (haplotypes). Participants from the general population with (n=4950) and without (n=4234) elevated blood pressure were compared (study 1), as were participants from the general population without ischemic heart disease and ischemic cerebrovascular disease (n=7965) and cases with either ischemic heart disease (n=1850, study 2) or ischemic cerebrovascular disease (n=848, study 3). Finally, 22-year follow-up of 9184 individuals from the general population examined risk of ischemic heart disease (study 4) and ischemic cerebrovascular disease (study 5). Individuals with −6AA, 174TT, or 235TT had plasma angiotensinogen levels increased by 80 ng/mL ( $P=0.01$  and  $0.05$  for women and men) compared with individuals with −6GG, 174TT, or 235 MM. In women, this difference was associated with an odds ratio of elevated blood pressure of 1.25 (1.03 to 1.51), which increased to 1.63 (1.05 to 2.51) in postmenopausal women receiving hormone replacement therapy. The promoter single nucleotide polymorphisms alone or as haplotypes did not predict the continuous variables of systolic, diastolic, or pulse pressure in cross section or the risk of ischemic heart disease or ischemic cerebrovascular disease in either gender in case-control or prospective studies. Individuals with −6AA, 174TT, or 235TT in the angiotensinogen gene have increased plasma angiotensinogen levels and moderately increased risk of elevated blood pressure (women only) but unaltered blood pressure examined as a continuous variable and unaltered risk of ischemic heart disease and ischemic cerebrovascular disease. (*Hypertension*. 2003;41:1202-1211.)

**Key Words:** blood pressure ■ hypertension, genetic ■ cardiovascular diseases ■ cerebrovascular disorders

Single nucleotide polymorphisms (SNPs) in the angiotensinogen gene have been associated with elevated angiotensinogen levels,<sup>1</sup> elevated blood pressure,<sup>1</sup> ischemic heart disease (IHD),<sup>2</sup> and ischemic cerebrovascular disease (ICVD).<sup>3</sup> In the Copenhagen City Heart Study,<sup>4</sup> we have previously shown that the M235T SNP (T-allele) was associated with elevated plasma angiotensinogen levels in both genders and a 30% increased risk of elevated blood pressure in women homozygous for 235T. However, the risk of IHD and ICVD was not associated with variation in genotype.<sup>5</sup>

SNPs in the transcription factor binding region (−25 to −1) of the angiotensinogen gene promoter have been reported to affect gene expression.<sup>6–8</sup> The −6A SNP has been associated with increased gene transcription,<sup>8,9</sup> elevated blood pressure, and increased risk of IHD in some<sup>10–12</sup> but not in all studies,<sup>13–15</sup> whereas the −20C SNP has been associated with increased gene transcription, elevated angiotensinogen levels, and elevated blood pressure in one<sup>7</sup> but not in other studies.<sup>1,10,15</sup> These conflicting results may be due to

small sample size in previous studies, which have included between 301 and 1232 participants.

Using Copenhagen City Heart Study control subjects (n=7965) and cases with IHD (n=1805) and with ICVD (n=848), we explored whether these promoter SNPs independently or in combination with the T174M and M235T SNPs as haplotypes predict systolic, diastolic, or pulse pressure in cross section, elevated blood pressure, IHD, or ICVD in 3 case-control studies, or IHD or ICVD in 2 prospective studies. Prospective analyses were also performed for different subtypes of these large entities, for example, myocardial infarction, angina pectoris, ischemic stroke, and transient ischemic attack.

## Methods

Three case-control and two prospective studies in the ethnically homogenous Danish population were conducted (Figure 1 in an online supplement available at <http://www.hypertensionaha.org>). Study 1 compared individuals from the Copenhagen City Heart Study with (n=4950) and without elevated blood pressure

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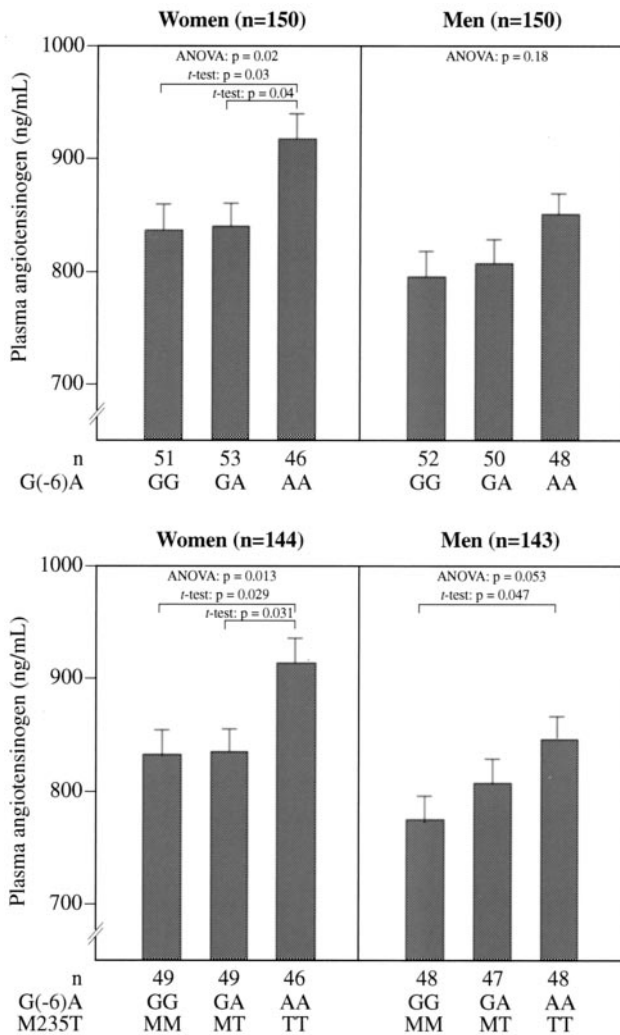
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**Figure 1.** Plasma angiotensinogen levels adjusted for age as a function of G(-6)A genotype (top) and G(-6)A and M235T genotype (bottom) are shown. Individuals taking medication known to affect blood pressure or taking estrogen or other hormones and individuals with suspected liver disease were excluded from analyses. All individuals carried 174TT.

(n=4234). In studies 2 and 3, individuals in the Copenhagen City Heart Study without IHD and ICVD (controls; n=7965) were compared with cases with either IHD (n=1805; study 2) or ICVD (n=848; study 3). Each of these case groups was a combination of cases from the Copenhagen City Heart Study and patients recruited at Copenhagen University Hospital. Studies 4 and 5 were conducted prospectively within the Copenhagen City Heart Study (n=9184). Incident IHD and ICVD cases were recorded in the follow-up period from 1976 through 1997 by reviewing the Danish National Hospital Discharge Register, the Danish National Register of Causes of Death, and medical records from hospitals and general practitioners.<sup>5,16</sup> Individuals with IHD or ICVD diagnosed before entry into the Copenhagen City Heart Study were excluded in the analysis of IHD risk (n=15) and ICVD risk (n=31), respectively.

The Copenhagen City Heart Study is a prospective cardiovascular population study recruiting participants randomly from the Danish Central Population Register to obtain a representative sample of the adult Danish general population.<sup>4,5,16</sup> The Danish Ethics Committee for Copenhagen and Frederiksberg approved this study (No. 100.2039/91), and all subjects gave informed consent.

During 1991 to 1993, 992 patients from the greater Copenhagen area were referred to Copenhagen University Hospital, Rigshospita-

let, for coronary angiography.<sup>5</sup> The study was approved by the Danish Ethics Committees for Copenhagen County (No. KA93125). Similarly, we identified patients with ICVD among patients referred from 1994 to 1999 for outpatient ultrasonography of the carotid artery at Copenhagen University Hospital, Rigshospitalet.<sup>5</sup> The study was approved by the Ethics Committee of Copenhagen and Frederiksberg (No. KF01-375/94, KF01-372/94).

### DNA Analyses

The A(-20)C and G(-6)A promoter SNPs were diagnosed by PCR<sup>7,8</sup>: sense primer, 5'CTCTCCAGCCTGTGCACAG3'; antisense primer, 5'GACAAGACCAGAAGGAGCTGA3'. The PCR product (158 bp) was digested with either Eco01091 (A[-20]C), resulting in SNP-specific bands of 130 bp (A-allele) or 67 bp and 63 bp (C-allele) (common bands of 27 and 1 bp), or with MvaI (G[-6]A), resulting in SNP-specific bands of 133 bp (G-allele) or 78 bp and 55 bp (A-allele) (common band, 25 bp). Individuals with rare haplotypes were sequenced to confirm the diagnoses.

### Other Analyses

Plasma angiotensinogen levels were measured in 174TT individuals from the Copenhagen City Heart Study, excluding individuals taking medication or with conditions that potentially affect angiotensinogen levels. Individuals with the genotype 174TT were selected because individuals with the haplotype 235T, 174T had higher risk of elevated blood pressure when compared with the haplotype 235M, 174T.<sup>4</sup> Therefore, among the 6786 individuals homozygous for 174TT in our study population, we randomly selected 300 men and women (40 to 67 years old) distributed equally between the 2 genders and among homozygotes, heterozygotes, and noncarriers of M235T.

Elevated blood pressure was systolic blood pressure  $\geq 140$  mm Hg and/or diastolic blood pressure  $\geq 90$  mm Hg<sup>17</sup> or treatment with antihypertensive medication. Stratification of elevated blood pressure into mild, moderate, and severe was as previously defined. Blood pressure was measured by trained technicians, using a London School of Hygiene sphygmomanometer on the left arm, after 5 minutes of rest, and with the subject in the sitting position.<sup>4</sup>

### Haplotype Estimation and Linkage Disequilibrium

Previous information of linkage disequilibrium and observed haplotypes between T174M and M235T<sup>4</sup> was used together with already published data about linkage disequilibrium between the four examined loci<sup>10</sup> to propose the construction of observed haplotypes (Table 1). However, to get an objective and thus reliable estimation of haplotype constructions, we also used a linkage utility program (<http://linkage.rockefeller.edu>), which lists all possible haplotype combinations by frequency, reflecting their estimated probability in our study population. These estimations are based on genotype frequencies of each of the four polymorphisms and their pairwise linkage disequilibrium.<sup>18</sup> In all our statistical analyses, we used the six most frequent haplotypes estimated by the linkage utility program (Table 1).

Pairwise linkage disequilibrium between the four angiotensinogen SNPs were tested by the linkage utility program, which for each pair of SNPs estimated allele and haplotype frequencies with and without allelic association. The pairwise linkage disequilibrium coefficient D was calculated as  $D = P_{11} - p_1q_1$ , where  $P_{11}$  is the observed frequency of the 1/1 haplotype,  $p_1$  is the frequency of the "1" allele at locus 1 in the general population, and  $q_1$  is the population frequency of the "1" allele at locus 2.<sup>19</sup> The "1" allele at each locus is defined as the most common of the alleles at that locus. The extent of disequilibrium was expressed as the  $D' = D/D_{max}$ .<sup>20</sup>

### Statistical Analyses

All statistical analyses were performed stratified by gender and with the use of the SPSS program. A 2-sided probability value  $< 0.05$  was considered significant.

ANOVA and a t test were used to examine continuous variables as a function of genotype or haplotype. Interaction between age, body

**TABLE 1. Observed and Estimated Haplotype Frequencies for A(-20)C, G(-6)A, T174M, and M235T SNPs in 9184 Individuals From the General Population**

| Haplotype | A(-20)C | G(-6)A | T174M | M235T | Frequency, %  |            |
|-----------|---------|--------|-------|-------|---------------|------------|
|           |         |        |       |       | Observed (n)  | Estimated* |
| H1        | -       | -      | -     | -     | 58.8 (10 809) | 58.8       |
| H2        | -       | +      | -     | +     | 23.5 (4310)   | 23.4       |
| H3        | +       | +      | +     | +     | 12.1 (2225)   | 12.1       |
| H4        | +       | +      | -     | +     | 4.0 (738)     | 4.0        |
| H5        | -       | -      | -     | +     | 1.0 (176)     | 1.0        |
| H6        | -       | +      | -     | -     | 0.5 (98)      | 0.5        |
| H7        | -       | +      | +     | +     | 0.1 (25)      | 0.1        |
| H8        | +       | +      | -     | -     | 0.01 (1)      | 0.01       |
| H9        | +       | +      | +     | -     | 0             | 0.001      |
| H10       | -       | -      | +     | -     | 0             | 0.0002     |
| H11       | +       | -      | +     | -     | 0             | 0.0001     |
| H12       | -       | -      | +     | +     | 0             | 0          |
| H13       | -       | +      | +     | -     | 0             | 0          |
| H14       | +       | -      | -     | -     | 0             | 0          |
| H15       | +       | -      | -     | +     | 0             | 0          |
| H16       | +       | -      | +     | +     | 0             | 0          |

\*Linkage utility program used for the estimation of haplotypes.

mass index, alcohol consumption, and genotype or haplotype were examined by means of ANCOVA.

Risk of elevated blood pressure, IHD, and ICVD were examined by logistic regression analysis in age-adjusted, multifactorial-adjusted, and matched analyses.

Log-rank tests and Kaplan-Meier curves were used to examine prospective data. Cox regression analysis was used to calculate relative risks (95% CI) adjusted for age at entry into the Copenhagen City Heart Study.

An expanded Methods section can be found in an online supplement available at <http://www.hypertensionaha.org>.

## Results

Characteristics of participants in studies 1 to 5 are shown in Table 2. Table 3 shows relative genotype frequencies in the general population. Genotype frequencies did not differ significantly from those predicted by the Hardy-Weinberg equilibrium (A[-20]C,  $P>0.95$ ; G[-6]A,  $P>0.30$ ; T174M,  $P>0.95$ ; M235T,  $P>0.70$ ) and were similar to frequencies observed in other studies of whites.<sup>10,13,15</sup> Relative allele frequencies of -20C and -6A in the general population were 0.16 and 0.40. Linkage disequilibrium was observed in all pairwise combinations of the four SNPs, A(-20)C, G(-6)A, T174M, and M235T ( $\chi^2$  tests,  $P<0.000$ ; Figure II in an online supplement available at <http://www.hypertensionaha.org>); the degree of linkage disequilibrium between the -6 and 235 loci was 98%.

Table 1 shows observed and estimated haplotype frequencies for 16 possible haplotypes of the A(-20)C, G(-6)A, T174M, and M235T SNPs in 9184 individuals from the general population. Statistical analyses below are performed for the A(-20)C and G(-6)A SNPs alone and for the 6 most frequent haplotypes.

Plasma angiotensinogen levels were measured in 300 individuals from the general population in either gender.<sup>4</sup> All of these 300 individuals were homozygous for 174T. Individuals on medications known to affect blood pressure or treated with estrogen or other hormones and individuals with suspected liver disease were excluded from the analyses. Plasma angiotensinogen levels were increased by  $\approx 80$  ng/mL in women with -6AA versus -6GG and -6GA ( $P=0.03$ ;  $P=0.04$ ) (Figure 1). In men, the association was similar but nonsignificant. However, if individuals were double noncarrier, double heterozygous, or double homozygous for -6A and 235T, the overall association with angiotensinogen levels became significant in both genders ( $P=0.01$  and  $P=0.05$  in women and men, respectively) (Figure 1). Angiotensinogen levels did not vary as a function of A(-20)C genotype in either gender (data not shown).

Systolic, diastolic, or pulse pressure did not differ significantly as a function of genotype for the promoter SNPs or as a function of haplotypes in women or men (Figure 2). We did not find evidence for plausible interaction.

On logistic regression analysis adjusted for age, women with -6AA had an odds ratio for elevated blood pressure of 1.25 (95% CI, 1.03 to 1.51) compared with women with the -6GG genotype (Figure 3). In accordance with this, women carrying the AATT haplotypes of the -20, -6, 174, and 235 loci had an odds ratio for elevated blood pressure of 1.13 (1.02 to 1.26) compared with the AGTM haplotype (Figure 3). Similar odds ratios were found in multifactorial-adjusted analyses (data not shown) and in matched analyses in which each case was matched by gender, age, alcohol consumption, body mass index, and antihypertensive medication with one control (data not shown). In men, neither promoter genotype

TABLE 2. Characteristics of Participants

| Characteristic                     | Study 1             |                  | Studies 2 and 3      |          |          | Studies 4 and 5 |
|------------------------------------|---------------------|------------------|----------------------|----------|----------|-----------------|
|                                    | Without Elevated BP | With Elevated BP | Without IHD and ICVD | IHD      | ICVD     | Total CCHS      |
| <b>Women</b>                       |                     |                  |                      |          |          |                 |
| Number of individuals              | 2536                | 2542             | 4556                 | 588      | 345      | 5084            |
| Age, y                             | 51±0.3              | 66±0.2           | 57±0.2               | 64±0.5   | 68±0.5   | 46±0.2          |
| Body mass index, kg/m <sup>2</sup> | 24±0.1              | 26±0.1           | 25±0.1               | 26±0.2   | 25±0.3   | 25±0.1          |
| Cholesterol, mmol/L                | 5.9±0.03            | 6.7±0.02         | 6.2±0.02             | 6.6±0.06 | 6.8±0.07 | 6.3±0.02        |
| HDL cholesterol, mmol/L            | 1.7±0.01            | 1.7±0.01         | 1.7±0.01             | 1.5±0.02 | 1.6±0.03 | 1.7±0.01        |
| Apolipoprotein A1, mg/dL           | 148±0.54            | 154±0.57         | 151±0.42             | 148±1.2  | 154±2.4  | 151±0.40        |
| Lipoprotein (a), mg/L              | 288±7.25            | 359±8.26         | 316±5.68             | 526±26.4 | 365±57.6 | 323±5.52        |
| Triglycerides, mmol/L              | 1.5±0.02            | 1.9±0.02         | 1.6±0.02             | 2.1±0.09 | 2.0±0.07 | 1.7±0.01        |
| Alcohol consumption, g/wk          | 71±1.7              | 68±1.9           | 71±1.4               | 56±4.4   | 66±7.2   | 70±1.3          |
| Systolic blood pressure, mm Hg     | 119±0.2             | 155±0.4          | 136±0.3              | 148±1.3  | 153±1.8  | 137±0.3         |
| Diastolic blood pressure, mm Hg    | 75±0.2              | 90±0.2           | 82±0.2               | 84±0.7   | 87±0.9   | 82±0.2          |
| Elevated blood pressure, %         | 0.0                 | 100.0            | 46.8                 | 58.0     | 68.4     | 49.9            |
| Diabetes mellitus, %               | 1.5                 | 3.4              | 2.0                  | 8.5      | 8.2      | 2.4             |
| Smokers, %                         | 49.8                | 41.9             | 45.6                 | 43.7     | 53.3     | 46.1            |
| Postmenopausal, %                  | 57.1                | 93.4             | 72.6                 | 91.5     | 98.6     | 75.2            |
| Hormonal replacement therapy, %    | 22.4                | 18.0             | 20.5                 | 13.3     | 18.9     | 20.3            |
| <b>Men</b>                         |                     |                  |                      |          |          |                 |
| Number of individuals              | 1698                | 2408             | 3409                 | 1217     | 503      | 4111            |
| Age, y                             | 51±0.4              | 61±0.3           | 55±0.3               | 62±0.3   | 65±0.4   | 45±0.2          |
| Body mass index, kg/m <sup>2</sup> | 25±0.1              | 27±0.1           | 26±0.07              | 27±0.1   | 26±0.2   | 26±0.1          |
| Cholesterol, mmol/L                | 5.7±0.03            | 6.1±0.02         | 5.9±0.02             | 6.2±0.03 | 6.3±0.05 | 6.0±0.02        |
| HDL cholesterol, mmol/L            | 1.4±0.01            | 1.4±0.01         | 1.4±0.01             | 1.2±0.01 | 1.3±0.02 | 1.4±0.01        |
| Apolipoprotein A1, mg/dL           | 128±0.60            | 131±0.51         | 130±0.43             | 128±0.7  | 127±1.8  | 130±0.39        |
| Lipoprotein (a), mg/L              | 286±8.55            | 295±7.56         | 281±5.94             | 471±15   | 363±35.8 | 291±5.67        |
| Triglycerides, mmol/L              | 1.8±0.04            | 2.3±0.04         | 2.1±0.03             | 2.4±0.05 | 2.2±0.06 | 2.1±0.03        |
| Alcohol consumption, g/wk          | 148±3.7             | 176±3.8          | 167±3.0              | 146±6.7  | 155±10.2 | 164±2.7         |
| Systolic blood pressure, mm Hg     | 123±0.2             | 153±0.4          | 139±0.4              | 147±1.0  | 153±1.6  | 140±0.3         |
| Diastolic blood pressure, mm Hg    | 78±0.2              | 92±0.2           | 86±0.2               | 86±0.5   | 88±0.9   | 86±0.2          |
| Elevated blood pressure, %         | 0.0                 | 100.0            | 54.9                 | 48.0     | 62.5     | 58.6            |
| Diabetes mellitus, %               | 2.9                 | 5.6              | 3.7                  | 9.3      | 12.1     | 4.5             |
| Smokers, %                         | 56.2                | 49.3             | 52.1                 | 43.0     | 56.2     | 52.4            |
| Postmenopausal, %                  | —                   | —                | —                    | —        | —        | —               |
| Hormonal replacement therapy, %    | —                   | —                | —                    | —        | —        | —               |

The number of individuals may differ slightly between covariates according to availability of data. BP indicates blood pressure; IHD, ischemic heart disease; ICVD, ischemic cerebrovascular disease; and CCHS, Copenhagen City Heart Study.

nor haplotypes could predict risk of elevated blood pressure in age-adjusted, multifactorial-adjusted, or matched analyses.

When analyzing data stratified by menopausal status and hormone replacement therapy, we found that the observed risk increment in women was confined to postmenopausal women receiving hormone replacement therapy carrying the -6AA genotype with an age-adjusted odds ratio for elevated blood pressure of 1.63 (1.05 to 2.51) when compared with women with the -6GG genotype (Figure 4). Correspondingly, postmenopausal women receiving hormone replacement therapy carrying the haplotype AATT had an odds ratio of elevated blood pressure of 1.35 (1.05 to 1.72) compared

with the AGTM haplotype (Figure 4). We did not detect other plausible evidence for interaction.

Women homozygous for the -6AA had an odds ratio for mild elevated blood pressure of 1.33 (1.04 to 1.70) compared with women with the -6GG genotype (Figure 5). This was not found in men or for moderate or severe elevated blood pressure in either gender. In accordance with this finding, women carrying the haplotype AATT versus the AGTM haplotype had an odds ratio of 1.16 (1.00 to 1.33) for mild elevated blood pressure. Furthermore, when compared with the AGTM haplotype, women carrying the CAMT haplotype had an odds ratio for

TABLE 3. Genotype Frequencies in 9184 Individuals From the General Population

| Genotype | A(-20)C |              | G(-6)A   |      |              | Thr174Met |      |              | Met235Thr |      |              |
|----------|---------|--------------|----------|------|--------------|-----------|------|--------------|-----------|------|--------------|
|          | n       | Frequency, % | Genotype | n    | Frequency, % | Genotype  | n    | Frequency, % | Genotype  | n    | Frequency, % |
| AA       | 6467    | 70           | GG       | 3311 | 36           | TT        | 7072 | 77           | MM        | 3226 | 35           |
| AC       | 2472    | 27           | GA       | 4354 | 47           | TM        | 1976 | 22           | MT        | 4447 | 48           |
| CC       | 245     | 3            | AA       | 1519 | 17           | MM        | 136  | 1            | TT        | 1511 | 17           |

moderate elevated blood pressure of 1.36 (1.07 to 1.72) and an odds ratio for severe elevated blood pressure of 2.51 (1.11 to 5.67) if they carried the AGTT haplotype. This was not true for men.

Neither the A(-20)C and G(-6)A SNPs nor the haplotypes predicted risk of IHD or ICVD in age-adjusted case-control analyses in either gender (Figures 6 and 7). This was also true for multifactorial-adjusted and for matched analyses, in which each case was matched with two controls by gender, age, total cholesterol, elevated blood pressure, and smoking (data not shown). Although men heterozygous for G(-6)A had an apparently decreased risk of ICVD (odds ratio, 0.77; 95% CI, 0.62 to 0.95), this turned statistically insignificant in multifactorial-adjusted analysis (0.88; 0.69 to 1.12). Likewise, men heterozygous for A(-20)C also had a marginally decreased risk of ICVD (0.79; 0.63 to 0.99), which turned insignificant in multifactorial analysis (0.78; 0.60 to 1.02). No plausible significant interactions were found.

The promoter SNPs alone or as haplotypes also could not predict risk of acute myocardial infarction (data not shown).

During 153 412 and 155 722 person-years, 847 and 407 IHD and ICVD events were observed in the Copenhagen City Heart Study, respectively. This results in an incidence rate of 55 IHD cases per 10 000 person-years and 26 ICVD cases per 10 000 person-years. The incidence of IHD and ICVD did not differ between A(-20)C and G(-6)A genotypes in either gender. This was also true for the subgroup analyses of myocardial infarction, angina pectoris, ischemic stroke, and transient ischemic attack (data not shown). Similarly, age-adjusted relative risks were nonsignificant for all comparisons between A(-20)C and G(-6)A genotypes for the large entities IHD and ICVD as well as for subgroup analyses of myocardial infarction, angina pectoris, ischemic stroke, and transient ischemic attack in both genders. Finally, incidence of IHD, ICVD, and for the different subgroups did not differ among the 6 common haplotypes, and we also did not observe any significant age-adjusted relative risk among haplotypes.

### Discussion

In this study, we provide data suggesting that women and men with -6AA, 174TT, and 235TT (versus -6GG, 174TT, and 235 MM) in the angiotensinogen gene have increased plasma angiotensinogen levels. This difference was associated with a 63% increase in risk of elevated blood pressure (as a dichotomized variable) in postmenopausal women receiving hormone replacement therapy. In contrast, the -6A and -20C promoter SNPs alone or as haplotypes did not predict systolic or diastolic blood pressure (as a continuous variable), pulse pressure, or risk of IHD or ICVD in either gender.

### Plasma Angiotensinogen Levels

Both women and men with -6AA, 174TT, and 235TT (versus -6GG, 174TT, and 235 MM) had higher angiotensinogen levels. A study in Japanese found no association between the promoter SNPs and plasma angiotensinogen levels.<sup>7</sup> Possible explanations for this divergence could include that we only measured angiotensinogen levels in individuals homozygous for 174T and differences in allele frequencies and linkage disequilibrium caused by different ethnic backgrounds. However, our results are in accordance with previous findings that the -6A SNP affects basal transcription *in vitro* and is associated with increased transcription of the angiotensinogen gene.<sup>8</sup>

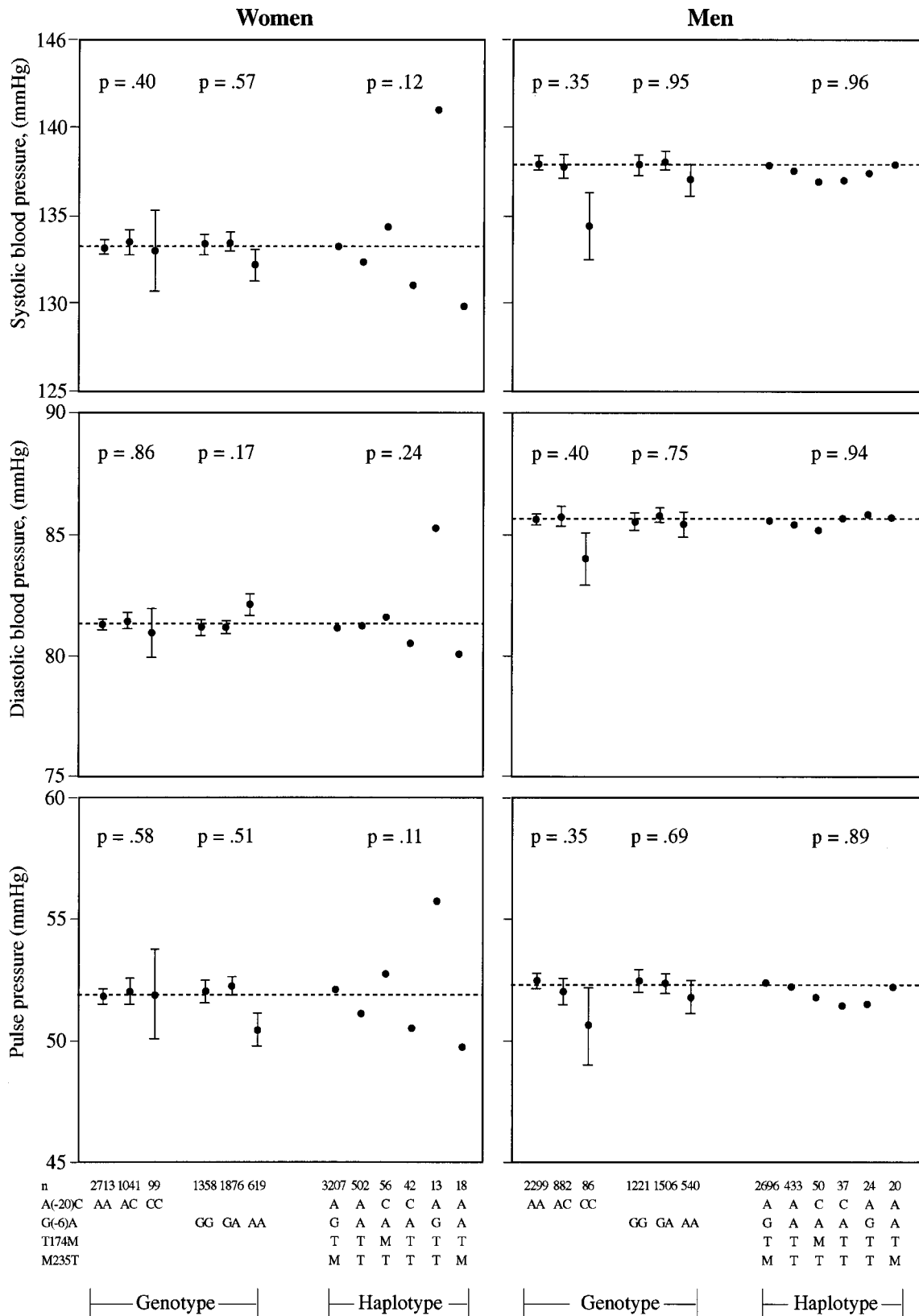
Although we did not observe an association between A(-20)C and plasma angiotensinogen levels, it is worth emphasizing that this SNP along with another promoter SNP, A(-217)G, have been suggested to affect basal promoter activity of the angiotensinogen gene.<sup>9,21</sup> Therefore, we cannot exclude that the positive association observed with G(-6)A might also reflect the effect of other polymorphisms located in the regulatory region of the angiotensinogen gene.<sup>22</sup>

### Blood Pressure as a Continuous Variable

None of the A(-20)C or G(-6)A genotypes and none of the haplotypes were associated with significant variation in systolic, diastolic, or pulse pressure in either gender. All individuals taking medications known to affect blood pressure were excluded in these analyses. Our finding is in accordance with previous studies showing no association of the G(-6)A SNP with interindividual variation in blood pressure levels.<sup>13</sup> Similar results were previously found for 235T, which is in nearly complete linkage disequilibrium with -6A in our study.<sup>4</sup> Although a Japanese study (n=274) showed a significant correlation between blood pressure increase and -6A,<sup>11</sup> we were unable to replicate this in 7120 individuals. A possible explanation for this could be the large difference in allele frequencies of -6A between the Japanese (84%) and Danish populations (40%).

### Blood Pressure as a Dichotomous Variable

Although a previous study of whites found an association between the G(-6)A SNP and elevated blood pressure,<sup>10</sup> our study is the first to show that this association may be gender-specific, depending on menopausal status and hormone replacement therapy. Failure of previous gender-stratified studies<sup>13-15</sup> to show an association may be due to small sample size and/or lack of stratification by gender. A(-20)C has previously been shown to modify an estrogen-responsive element located in the regulatory region of the



**Figure 2.** Systolic, diastolic, and pulse pressure as a function of angiotensinogen genotypes and haplotypes are shown. Mean±SEM values are shown for the A(-20)C (far left) and G(-6)A genotypes (left). Average haplotype effects are shown to the right. Broken lines show mean values for all women and men, respectively, in the general population. Individuals taking medications known to affect blood pressure (ie, antihypertensive medications or medication for angina pectoris, heart failure, or cardiac arrhythmias) were excluded from analyses. Probability values are for ANOVA; because all were nonsignificant, no post hoc 2-genotype comparisons were examined.

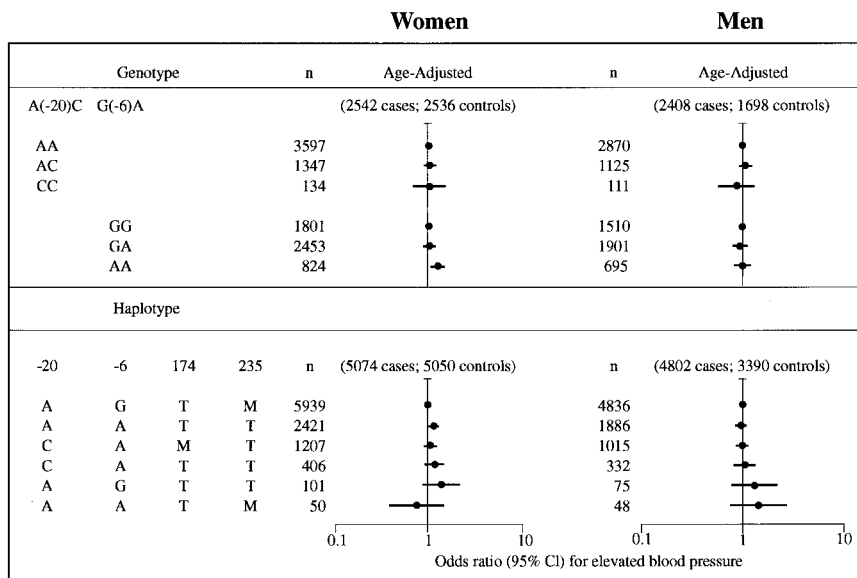


Figure 3. Risk of elevated blood pressure as a function of A(-20)C and G(-6)A genotypes (top) and as a function of haplotypes (bottom), using logistic regression, are shown (study 1).

angiotensinogen gene.<sup>9</sup> Whether G(-6)A has a role to play in modifying the estrogen-responsive element is unknown, but others have shown that 235T, which is in complete linkage disequilibrium with -6A, is associated with greater stimulation of angiotensinogen secretion in plasma after estrogen administration in humans, suggesting an important role of exogenous estrogen administration.<sup>23</sup> Thus, although the interactive relation between G(-6)A and A(-20)C on transcriptional regulation of the angiotensinogen gene remains to be examined, our data suggest that G(-6)A has an important role to play in postmenopausal women treated with hormone replacement therapy. Furthermore, the 235T genotype has been associated with preeclampsia and pregnancy-induced elevated blood pressure<sup>24</sup> in the homozygote state; keeping in mind that -6A is in near complete (98%) linkage disequi-

librium with 235T, this would appear to support our gender-specific finding. The absolute size of the association of genotype with angiotensinogen levels is very similar in women and men: ≈80 ng/mL higher in -6A, 235T homozygotes compared with -6G, 235M. However, the mean angiotensinogen level in women and men in our study was 861 ng/mL and 811 ng/mL, respectively (P=0.007), suggesting that genotype might have an effect on risk of elevated blood pressure in women, but not in men.

That the increased risk for elevated blood pressure overall in women homozygous for -6AA was seen only in those with mild elevated blood pressure and not in those with moderate and severe elevated blood pressure could be due to the smaller sample size for the analyses of moderate and severe versus mild elevated blood pressure.

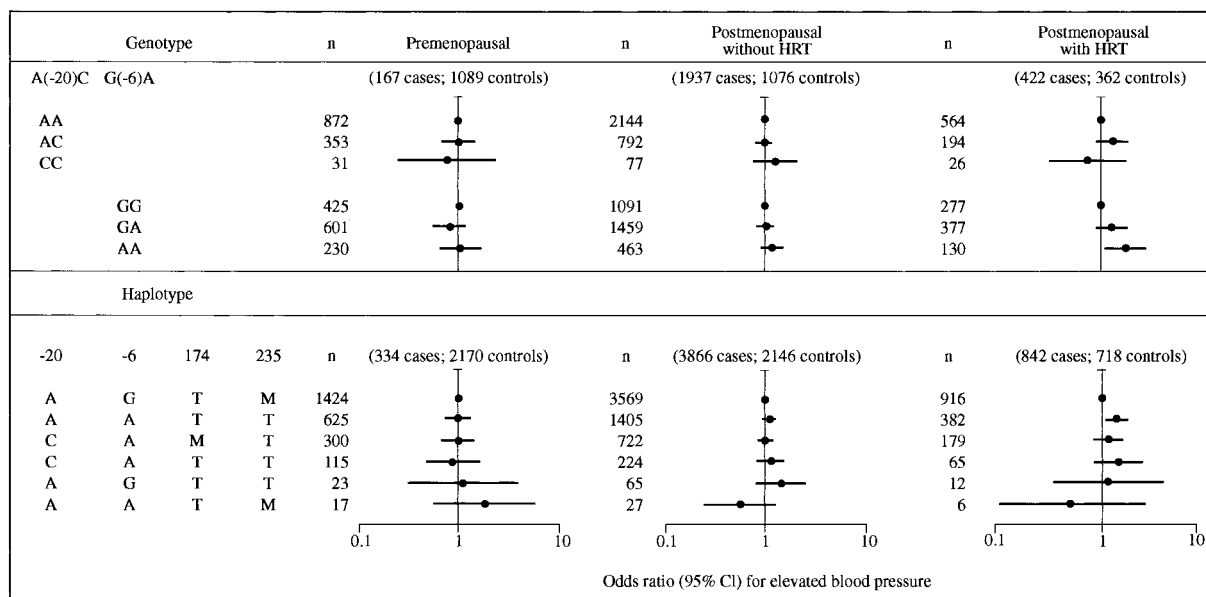
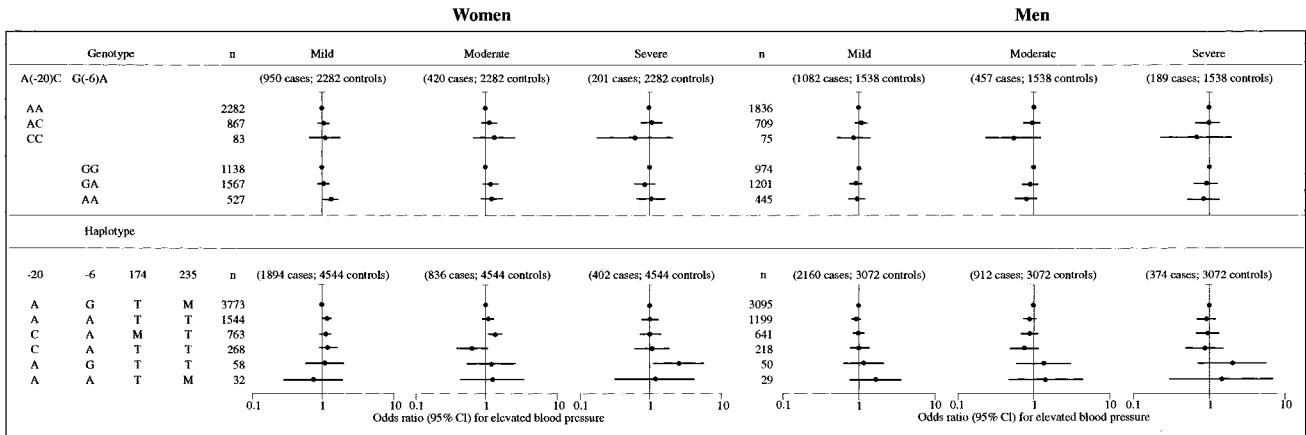


Figure 4. Risk of elevated blood pressure as a function of A(-20)C and G(-6)A genotypes (top) and as a function of haplotypes (bottom), using logistic regression, in women stratified for menopausal status and hormone replacement therapy, are shown.



**Figure 5.** Risk of mild, moderate, and severe elevated blood pressure as a function of A(-20)C and G(-6)A genotypes (top) and as a function of haplotypes (bottom), using logistic regression, are shown (study 1). Individuals taking medications known to affect blood pressure (ie, antihypertensive medications or medication for angina pectoris, heart failure, or cardiac arrhythmias) were excluded from analyses.

The significant findings for moderate elevated blood pressure and severe elevated blood pressure in women carrying the CAMT and AGTT haplotypes, respectively, could also be chance findings because of smaller sample sizes for these stratified analyses. In support of this interpretation, no previous reports have examined risk of elevated blood pressure stratified into mild, moderate, and severe elevated blood pressure for the angiotensinogen gene SNPs, and thus these results are not confirmed elsewhere.

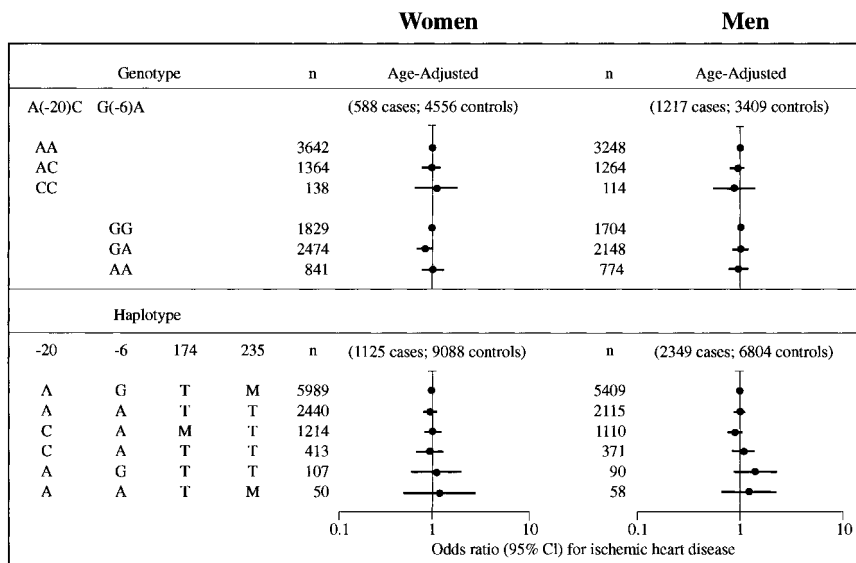
**Risk of IHD**

Both case-control and prospective studies from the Copenhagen City Heart Study suggest that neither promoter SNPs alone nor haplotypes are associated with increased risk of IHD. A recent study including 614 cases and control subjects of both genders found an increased risk of coronary heart disease in individuals homozygous for -6A SNP on univariate analyses; however, this increase in risk disappeared when adjusted for classic risk factors.<sup>12</sup>

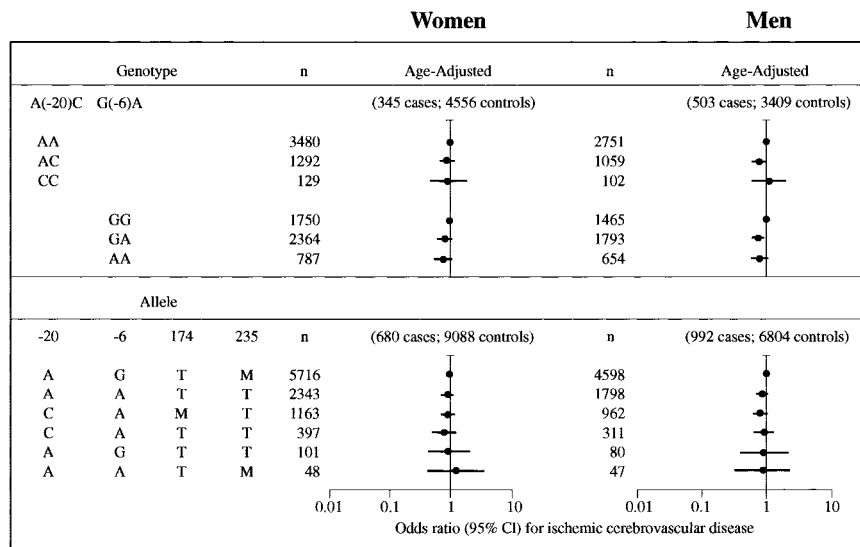
**Risk of ICVD**

Previous reports suggested local expression of the angiotensinogen gene and its transcription regulators in the brain<sup>25</sup> and development of hypertension when the protein is overexpressed in the brain.<sup>26</sup> Both case-control and prospective studies from the Copenhagen City Heart Study suggest that neither promoter SNPs alone nor haplotypes are associated with increased risk of ICVD. In the case-control studies, men heterozygous for G(-6)A and A(-20)C had decreased risk of ICVD when adjusted for age alone; however, these findings disappeared in the multifactorial analyses, were not observed in women, and could not be confirmed in the prospective studies and therefore suggest chance findings.

No previous reports of the promoter SNPs have explored risk of ICVD, but recently we published a report showing no association between the T174M and M235T angiotensinogen SNPs and ICVD.<sup>5</sup> The current study is in agreement with our previous results, when keeping in mind that -6A is in 98% linkage disequilibrium with 235T in our sample.



**Figure 6.** Risk of IHD as a function of A(-20)C and G(-6)A genotypes (top) and as a function of haplotypes (bottom), using logistic regression, are shown (study 2).



**Figure 7.** Risk of ICVD as a function of A(-20)C and G(-6)A genotypes (top) and as a function of haplotypes (bottom), using logistic regression, are shown (study 3).

**Mechanism**

The end product of the renin-angiotensin system, angiotensin II, may increase blood pressure by stimulating renal sodium reabsorption and vasoconstriction. Our results imply that since one of the promoter SNPs, G(-6)A, in the homozygous form is associated with a 10% increase ( $\approx 80$  ng/mL) in plasma angiotensinogen levels, this higher throughput in the renin-angiotensin system raises angiotensin II levels and hence blood pressure. Our data suggest that this may be most pronounced in postmenopausal women receiving hormone replacement therapy. It is therefore tempting to speculate that chronic stimulation of the renin-angiotensin system by estrogen could result in different physiological responses, depending on the G(-6)A genotype. Although our results suggest that -6A and 235T in the homozygote state is associated with a moderately increased risk of elevated blood pressure, this association seems too weak to be detected as an increased risk of IHD or ICVD.

**Limitations**

Blood pressure was only measured once in our study sample. Since intraindividual variation in blood pressure may be considerable, we believe that this represents a limitation of our study. There are several possible explanations as to why there is no association with blood pressure as a continuous variable, whereas there is a moderate association with risk of dichotomized elevated blood pressure in women: (1) The finding for the dichotomous variable is a chance finding. However, the association between genotype and angiotensinogen levels was also most pronounced in women; furthermore, this finding has been confirmed in several other studies, including meta-analyses for the M235T SNP.<sup>27,28</sup> (2) Misclassification of blood pressure as a dichotomous variable is probable among individuals with blood pressure  $\approx 140/90$  mm Hg, the cutoff point; however, individuals with very high or very low blood pressure are less likely to be misclassified. In contrast to this, when exploring blood pressure as a continuous variable, misclassification will occur on all values. Although categorization of a continuous variable leads to loss of information, this is the only well-

understood manner in which clinicians treat their patients. (3) The association between the SNP and the continuous variables of systolic and diastolic blood pressure could be so weak that only when combining these two variables into a dichotomous variable, one is able to demonstrate significant association with elevated blood pressure. Also important is the fact that people with clinically recognized hypertension, those who are treated for the condition, are excluded in the analysis of blood pressure as a continuous variable but included in the analysis of elevated blood pressure as a dichotomous variable.

Although genotype determination of T174M and M235T was made in the same PCR reaction enabling unequivocal haplotype determination for these two loci, our analyses of all 4 SNPs (A[-20]C, G[-6]A, T174M, and M235T) together naturally cannot be based on unequivocal but only on estimated haplotypes. All individuals can be classified uniquely in terms of his or her 4-loci genotype, but haplotype determination cannot be done uniquely in individuals heterozygous for more than one of the examined loci. These individuals present a difficulty, but omitting them from consideration in an analysis of linkage disequilibrium and haplotype estimation can lead to bias and loss of information for the considered test. Furthermore, it has been shown that exclusion of individuals in whom the phase cannot be uniquely determined a priori results in skewed information about linkage disequilibrium and hence haplotype frequencies.<sup>18</sup> Therefore, a linkage utility program with maximum log likelihood methods that allow these individuals into the analyses was used by us to construct haplotypes used in statistical analyses.

When studying the average haplotype effects on blood pressure and cardiovascular disease, one must keep in mind that statistical analyses were based on each individual divided into two independent haplotypes, each with a set of covariates. This is controversial when adjusting for one or more risk factors in the statistical analyses; however, when confidence intervals were calculated by simple unadjusted  $\chi^2$  estimation, confidence intervals were similar to those shown in Figures 3 through 7.

## Perspectives

Although the individual risk associated with the two SNPs in homozygous women is  $\approx 30\%$ , the attributable risk, that is, the fraction of elevated blood pressure in the population that can be attributed to these SNPs, is  $\approx 5\%$  in our female population. This is due to the high prevalence of these alleles in the population and is comparable to the 6% fraction of ischemic heart disease attributable to diabetes mellitus. Future research should be directed toward other genes, because additional genetic factors besides the angiotensinogen gene must be taken into account in modulating risk of elevated blood pressure. This may allow us to define a subset of individuals in whom a special genotype combination is associated with increased risk or especially amenable to a specific kind of antihypertensive treatment.

## Acknowledgments

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## Methods

### Overview

Three case-control and two prospective studies in the ethnically homogenous Danish population were conducted (Figure I). Study 1 compared individuals from the Copenhagen City Heart Study with (n=4950) and without elevated blood pressure (n=4234). In Studies 2 and 3, individuals in the Copenhagen City Heart Study without IHD and ICVD (=controls;n=7965) were compared with cases with either IHD (n=1805;Study 2) or ICVD (n=848;Study 3). Each of these case-groups were a combination of cases from the Copenhagen City Heart Study and patients recruited at Copenhagen University Hospital. Studies 4 and 5 were conducted prospectively within The Copenhagen City Heart Study (n= 9184). All participants had attended the 3<sup>rd</sup> examination from 1991-94 where DNA was collected. Incident ischemic heart disease and ischemic cerebrovascular disease cases were recorded in the follow-up period from 1976 through 1997. Individuals with IHD or ICVD diagnosed before entry into The Copenhagen City Heart Study were excluded in the analysis of IHD risk (n=15) and ICVD risk (n=31), respectively.

### Participants

#### *The Copenhagen City Heart Study*

The Copenhagen City Heart Study is a prospective cardiovascular population study recruiting women and men randomly from the Danish Central Population Register to obtain a representative sample of the adult Danish general population<sup>1-3</sup>. Individuals invited were stratified into 5-year age groups from 20 to 95 years. In the first examination (in 1976-1978) a total of 19,329 individuals were invited of whom 14,223 (74%) participated. In the second examination (in 1981-1983) the original cohort supplemented with 500 individuals in the 20- to 25-year-old group was

invited, of which 12,698 (70%) participated. During the third examination (in 1991-94) the cohort supplemented with 3,000 individuals in the 20- to 49-year-old group was invited, and 10,135 (61%) participated. Less than 1% were Black or Asian. Subjects reported on smoking status, use of medication, physical activity, weekly alcohol consumption (g/wk), diabetes mellitus, menopausal status (women) and hormonal replacement therapy (women). At the 3<sup>rd</sup> examination blood samples were drawn for DNA extraction: 9259 gave blood for DNA isolation. The Danish ethical committee for Copenhagen and Frederiksberg approved this study (No.:100.2039/91), and all subjects gave informed consent.

Information on IHD and ICVD was collected until the end of 1997 by reviewing the Danish National Hospital Discharge Register, the Danish National Register of Causes of Death, and medical records from hospitals and general practitioners<sup>1,2</sup>. Experienced cardiologists determined whether cases had IHD (WHO International Classification of Diseases, Eighth Edition, codes 410-414) based on previous myocardial infarction or characteristic symptoms of stable angina pectoris according to the guidelines of the European Society of Cardiology (based on location, character, and duration of pain and the relation of pain to exercise)<sup>4</sup>. Myocardial infarction (WHO International Classification of Diseases, Eighth Edition, code 410) was diagnosed by experienced cardiologists on the basis of presence of at least two of the following: characteristic chest pain, elevated levels of cardiac enzymes, and electrocardiographic results consistent with a myocardial infarction. Cases with ICVD (codes 432-435) were diagnosed by experienced neurologists on the basis of sudden onset of focal neurological symptoms lasting = 24 hours (ischemic stroke), focal neurological symptoms lasting < 24 hours (transient ischemic attack), or transient monocular blindness (amaurosis fugax). Patients with cerebral hemorrhage were excluded.

### *Patients from Copenhagen University Hospital*

During 1991-1993, 992 patients from the greater Copenhagen area were referred to Copenhagen University Hospital, Rigshospitalet, for coronary angiography. Among these, 948 received a diagnosis of IHD from experienced cardiologists because of characteristic symptoms of stable angina pectoris plus at least one of the following<sup>1,2</sup>: severe stenosis on coronary angiography (>70 % stenosis of at least one coronary vessel or >50 % stenosis of the left main coronary artery), myocardial infarction, or a positive exercise electrocardiography test. Less than 1% were Black or Asian. The study was approved by the Danish ethical committees for Copenhagen County (No.:KA93125).

We identified ICVD patients among patients referred from 1994 to 1999 for outpatient ultrasonography of the carotid artery at Copenhagen University Hospital, Rigshospitalet. Experienced neurologists and vascular surgeons diagnosed ICVD based on sudden onset of focal neurological symptoms together with carotid artery stenosis =50% on the symptomatic or most stenotic side<sup>2</sup>. Patients either had ischemic stroke, transient ischemic attack, or amaurosis fugax. Cerebral hemorrhage was excluded by CT-scan. Less than 1 % were Black or Asian. The study was approved by the ethical committee of Copenhagen and Frederiksberg (No.:KF01-375/94, KF01-372/94).

### **DNA analyses**

The A(-20)C and G(-6)A promoter SNPs were diagnosed by PCR<sup>5,6</sup>: sense primer, 5'CTCTCCAG CCTGTGCACAG3'; antisense primer, 5'GACAAGACCAGAAGGAGCTGA3'. The PCR product (158 bp) was digested with either Eco0109I (A(-20)C) resulting in SNP specific bands of 130bp (A-allele), or 67bp and 63bp (C-allele) (common bands of 27 and 1bp), or with

MvaI (G(-6)A) resulting in SNP specific bands of 133bp (G-allele), or 78bp and 55bp (A-allele) (common band 25bp). Individuals with rare haplotypes were sequenced to confirm the diagnoses.

### **Other analyses**

Plasma angiotensinogen levels were measured in 174TT individuals from the Copenhagen City Heart Study excluding individuals on medication or with conditions that potentially affect angiotensinogen levels. Individuals with the genotype 174TT were selected because individuals with the haplotype 235T, 174T had higher risk of elevated blood pressure when compared with the haplotype 235M, 174T<sup>3</sup>. Therefore, among the 6786 individuals homozygous for 174TT in our study population, we randomly selected 300 men and women (40 to 67 years old) distributed equally between the 2 genders and among homozygotes, heterozygotes, and noncarriers of M235T.

Elevated blood pressure was systolic blood pressure  $\geq 140$  mmHg and/or diastolic blood pressure  $\geq 90$  mmHg<sup>7</sup>, or treatment with antihypertensive medication. Elevated blood pressure was stratified into mild (systolic blood pressure  $\geq 140$  mmHg and  $< 160$  mmHg and/or diastolic blood pressure  $\geq 90$  mmHg and  $< 100$  mmHg), moderate (systolic blood pressure  $\geq 160$  mmHg and  $< 180$  mmHg and/or diastolic blood pressure  $\geq 100$  mmHg and  $< 110$  mmHg), and severe (systolic blood pressure  $\geq 180$  mmHg and/or diastolic blood pressure  $\geq 110$  mmHg). Pulse pressure was systolic minus diastolic blood pressure. Blood pressure was measured by trained technicians using a London School of Hygiene sphygmomanometer on the left arm, after 5 minutes rest, and with the subject in the sitting position<sup>3</sup>.

## Haplotype estimation and linkage disequilibrium

Previous information of linkage disequilibrium and observed haplotypes between T174M and M235T<sup>3</sup> was used together with already published data about linkage disequilibrium between the four examined loci<sup>8</sup> to propose the construction of observed haplotypes (Table 1). However, in order to get an objective and thus reliable estimation of haplotype constructions we also used a linkage utility program (<http://linkage.rockefeller.edu>), which lists all possible haplotype combinations by frequency, reflecting their estimated probability in our study population. These estimations are based upon genotype frequencies of each of the four polymorphisms and their pairwise linkage disequilibrium<sup>9</sup>. In all our statistical analyses we have used the six most frequent haplotypes estimated by the linkage utility program (Table 1).

Pairwise linkage disequilibrium between the four angiotensinogen SNPs were tested by the linkage utility program, which for each pair of SNPs estimated allele and haplotype frequencies with and without allelic association. The pair-wise linkage disequilibrium coefficient  $D$  was calculated as follows:

$$D = P_{11} - p_1q_1$$

where  $P_{11}$  is the observed frequency of the 1/1 haplotype,  $p_1$  is the frequency of the “1” allele at locus 1 in the general population and  $q_1$  is the population frequency of the “1” allele at locus 2<sup>10</sup>.

The “1” allele at each locus is defined as the most common of the alleles at that locus. The extent of disequilibrium was expressed as the  $D' = D/D_{\max}$ <sup>11</sup>.

## Statistical analyses

All statistical analyses were performed stratified by gender and using the SPSS program. A two-sided  $p$ -value  $<0.05$  was considered significant.

Analysis of variance (ANOVA) and t-test examined continuous variables as a function of genotype or haplotype. Interaction between age, body mass index, alcohol consumption and genotype or haplotype was explored by analysis of covariance (ANCOVA).

Average effects of eight haplotypes in the population at large ( $\alpha_i$ ,  $i = p, q, r, s, t, u, v, x$ ) on systolic, diastolic, and pulse pressure were estimated by the following equation<sup>12</sup>, exemplified for the p haplotype:

$$\alpha_p = \frac{f_{pp} \overline{E_{p/p}} + \frac{1}{2} f_{pq} \overline{E_{p/q}} + \frac{1}{2} f_{pr} \overline{E_{p/r}} + \frac{1}{2} f_{ps} \overline{E_{p/s}} + \frac{1}{2} f_{pu} \overline{E_{p/u}} + \frac{1}{2} f_{pt} \overline{E_{p/t}} + \frac{1}{2} f_{px} \overline{E_{p/x}}}{f_{pp} f_{pq} f_{pr} f_{ps} f_{pu} f_{pt} f_{px}} - \mu,$$

where  $f_{ep}$  is the haplotype frequency estimated by gene counting,  $f_{pp}, f_{pq}, f_{pr}, f_{ps}, f_{pu}, f_{pt}$ , and  $f_{px}$  are expected Hardy-Weinberg genotype frequencies, while  $\overline{E_{i/i}}$  is average blood pressure for individuals with genotype  $i/i$  ( $= p/p, p/q, p/r, p/s, p/u, p/t, p/x$ ), and  $\mu$  is mean blood pressure of the population.

Risk of elevated blood pressure, IHD, and ICVD were examined by logistic regression analysis allowing for age only or for age, body mass index, diabetes mellitus, smoking, total cholesterol, and menopausal status. Multifactorial analyses also adjusted for 1) alcohol consumption, antihypertensive medication, physical activity, and hormonal replacement therapy on elevated blood pressure, 2) for elevated blood pressure, high-density lipoprotein cholesterol, and triglycerides on IHD and ICVD, and 3) for apolipoprotein AI, lipoprotein (a), and hormonal replacement therapy on IHD. Logistic regression analyses exploring risk of elevated blood pressure in women stratified for menopausal status and hormonal replacement therapy were only adjusted for age, as were the analyses investigating risk of mild, moderate, and severe elevated blood pressure.

Interaction between the above mentioned risk factors and genotype or haplotypes was explored in models including age, genotype or haplotype, the risk factor in question, and an interaction term of the two latter factors.

Log-rank tests and Kaplan-Meier curves examined prospective data. Cox regression analysis was used to calculate relative risks (95% CI) adjusted for age at entry into The Copenhagen City Heart Study.

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## Figure Legends

### Figure I.

Study design. A) Case-control studies B) Prospective studies. Total number of individuals differ slightly between panel A and B due to availability of covariates.

### Figure II.

Schematic diagram of the angiotensinogen gene showing exon and intron length, genomic distance between the four SNPs, pairwise linkage disequilibrium coefficient (D), and degree of linkage disequilibrium in bracket.

