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# Angiotensinogen Gene Promoter Haplotype and Microangiopathy-Related Cerebral Damage

## Results of the Austrian Stroke Prevention Study

Helena Schmidt, MD; Franz Fazekas, MD; Gert M. Kostner, PhD;  
Cornelia M. van Duijn, PhD; Reinhold Schmidt, MD

**Background and Purpose**—Microangiopathy-related cerebral damage (MARCD) is a common finding in the elderly. It may lead to cognitive impairment and gait disturbances. Arterial hypertension and age are the most important risk factors. We assessed the association between MARCD and sequence alterations in the promoter region of the angiotensinogen (AGT) gene.

**Methods**—We studied 410 randomly selected community-dwelling individuals aged 50 to 75 years. MARCD was defined as early confluent or confluent white matter hyperintensities or lacunes on a 1.5-T MRI. The AGT promoter was analyzed by temporal temperature gradient gel electrophoresis and automated sequencing.

**Results**—We detected 4 polymorphic sites, at positions  $-6$ ,  $-20$ ,  $-153$ , and  $-218$ . They created 5 haplotypes, which we coded as A ( $-6:g, -20:a, -153:g, -218:g$ ), B ( $-6:a, -20:c, -153:g, -218:g$ ), C ( $-6:a, -20:c, -153:a, -218:g$ ), D ( $-6:a, -20:a, -153:g, -218:g$ ), and E ( $-6:a, -20:a, -153:g, -218:a$ ). MARCD was seen in 7 subjects (63.6%) carrying 2 copies of the B haplotype (B/B), in 12 subjects (38.7%) carrying 1 copy of the B haplotype in the absence of the A haplotype (B+/A $-$ ), but in only 70 subjects (19.0%) in the remaining cohort ( $P < 0.001$ ). The odds ratios for the B/B and the B+/A $-$  genotypes were 8.0 (95% CI, 2.1 to 31.1;  $P = 0.003$ ) and 1.8 (95% CI, 0.8 to 4.2;  $P = 0.14$ ) after adjustment for possible confounders.

**Conclusions**—The B haplotype of the AGT promoter in the absence of the wild-type A haplotype might represent a genetic susceptibility factor for MARCD. (*Stroke*. 2001;32:405-412.)

**Key Words:** angiotensins ■ genetics ■ magnetic resonance imaging ■ small-vessel disease

Microangiopathy-related cerebral damage (MARCD) is a common MRI observation in elderly persons and includes white matter changes and lacunar infarcts.<sup>1,2</sup> Although these findings may be recognized in otherwise normal individuals, they are likely to become associated with cognitive impairment and gait disturbances as they progress.<sup>3,4</sup> Identification of individuals prone to the development of such brain lesions and early control of causal factors could reduce the risk of these common clinical problems of the elderly. Thus far, it is unclear which factors other than advancing age and arterial hypertension predispose individuals to MARCD.<sup>2-4</sup> The significance of genetic influences was demonstrated by an investigation of World War II veteran twins. This investigation reported a probandwise concordance rate for extensive white matter lesions of 61% in monozygotic and of 38% in dizygotic twins compared with a prevalence of 15% in the entire population. The estimated heritability of lesion volume was 73%.<sup>5</sup> The consistent

association between MARCD and arterial hypertension suggests that genes involved in the regulation of blood pressure may contribute to this strikingly high heritability.<sup>6,7</sup>

The renin-angiotensin system (RAS) is a major regulator of blood pressure. Plasma angiotensinogen (AGT) synthesized by the liver is processed to angiotensin II (Ang II) by the serial action of renin and angiotensin-converting enzyme. Importantly, the plasma level of AGT is rate limiting in this cascade.<sup>8</sup> Positive correlation between plasma AGT concentration, RAS activity, and blood pressure in humans and in animal models supports this assumption.<sup>8-11</sup> Production of AGT in the liver is regulated mainly at the transcriptional level.<sup>11,12</sup> Two common polymorphisms in the promoter region at position  $-6:g \rightarrow a$  and  $-20:a \rightarrow c$  have been previously described and were shown to alter the transcriptional efficiency of the AGT gene.<sup>11-16</sup> Genetic linkage between the AGT locus and essential hypertension has been repeatedly reported,<sup>9,17</sup> but there are also studies in Chinese and Finnish

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From the Institute of Medical Biochemistry and Medical Molecular Biology (H.S., G.M.K.), Department of Neurology (F.F., R.S.), and MRI Center (F.F., R.S.), Karl-Franzens University, Graz, Austria, and Department of Genetic Epidemiology and Biostatistics, Erasmus University, Rotterdam, Netherlands (C.M. van D.).

Correspondence to Helena Schmidt, MD, Institute of Medical Biochemistry, Karl-Franzens University, Harrachgasse 21, A-8010 Graz, Austria. E-mail [helena.schmidt@kfunigraz.ac.at](mailto:helena.schmidt@kfunigraz.ac.at)

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populations that do not confirm these results.<sup>18–20</sup> Similarly, conflicting data have been described for the association between the T235 AGT gene variant and hypertension.<sup>9,21–27</sup> These controversial findings suggest ethnic variation in the genetics of hypertension, yet differences in the definition of the phenotype may also be responsible for these inconsistencies.

In the present study we investigated the association between AGT gene polymorphisms and MARCD in a cohort of community-dwelling middle-aged and elderly individuals. The study focused on variants in the promoter region of the gene since they are likely to influence AGT expression and may prove to be functionally important.

## Subjects and Methods

### Study Population

The study population consisted of participants of the Austrian Stroke Prevention Study, a single-center, prospective, cohort study on the cerebral effects of vascular risk factors in the normal elderly population of the city of Graz, Austria. The study was approved by the Medical Ethics Committee of Karl-Franzens University. Written informed consent was obtained from all study participants. We randomly selected 8193 individuals aged 50 to 75 years stratified by sex and 5-year age groups from the official community register. Between September 1991 and March 1994, individuals received a written invitation containing a full description of the purpose of the investigation to participate in the study. Overall, 2794 of the invited subjects returned a card stating their willingness to participate. Recruitment into the study was stopped after enrollment of 2000 eligible participants. They were all white and of Central European origin. Individuals were excluded from the study if they had a history of neuropsychiatric disease, including previous cerebrovascular attacks and dementia, or an abnormal neurological status determined on the basis of a structured clinical interview and a physical and neurological examination. A random age- and sex-stratified sample of 200 nonresponders was interviewed by telephone and did not differ in terms of length of education, occupational status, and history of vascular risk factors, including arterial hypertension, diabetes mellitus, and cardiac disease. Every fourth study participant, or in case of refusal the next participant, was invited to enter phase II of the study, which included MRI and Doppler sonography. Since 1993 DNA samples of phase II subjects had been collected. From a total of 498 phase II participants, 458 volunteered to undergo a MRI study. The current study cohort consists of those 410 individuals who underwent both MRI examination and genotyping of the AGT gene. There were 214 women (52.2%) and 196 men (47.8%). The mean age of this cohort was  $60.1 \pm 6.0$  years. The study sample did not differ from the remaining Austrian Stroke Prevention Study cohort in terms of age, sex, educational and occupational status, and risk factors for stroke.

### Vascular Risk Factors

The diagnosis of major risk factors for stroke, including arterial hypertension, diabetes mellitus, and cardiovascular disease, was determined by the history of the individual and appropriate laboratory findings. A detailed description of the laboratory methods used and the definition of these risk factors are given elsewhere.<sup>28</sup>

### DNA Analysis

Genomic DNA extracted from peripheral blood was polymerase chain reaction (PCR) amplified with the following oligonucleotides: AGT-PROM5: 5'-GC-Clamp-CTTGGCCCCGACTCCTGCAAAC-3' and AGTPROM3: 5'-CCCCGGCTTACCTTCTGCTGTA-3' in 40 cycles consisting of 1 minute at 94°C, 1 minute at 65°C, and 2 minutes at 72°C.

The PCR products (354 bp long, containing a part of the AGT promoter and exon 1, from -268 to +41 nucleotide, as well as a 40-bp-long GC clamp) were genotyped by temporal temperature

gradient gel electrophoresis (TTGE) with the use of the Dcode Universal mutation detection system (Bio-Rad Laboratories). TTGE is a sensitive method for the detection of virtually all polymorphisms, whether new or already known, and their precise combination within the amplified fragment in a single step without the need for further processing of the samples by, eg, restriction enzyme digestion.<sup>29</sup> Melting domain map was calculated with the MacMelt computer algorithm (Bio-Rad Laboratories). PCR products were electrophoresed on 9% polyacrylamide gels containing 8 mol/L urea at 130 V with a temperature gradient of 57°C to 66°C, at a heating rate of 1.5°C/h. Heterozygous DNA samples were used as positive controls on each gel to check gel resolution efficiency. At least 3 samples within each of the 15 distinct banding pattern groups seen on TTGE were sequenced on an ABI 373 automated sequencer (Perkin Elmer/Applied Biosystems).

### Magnetic Resonance Imaging

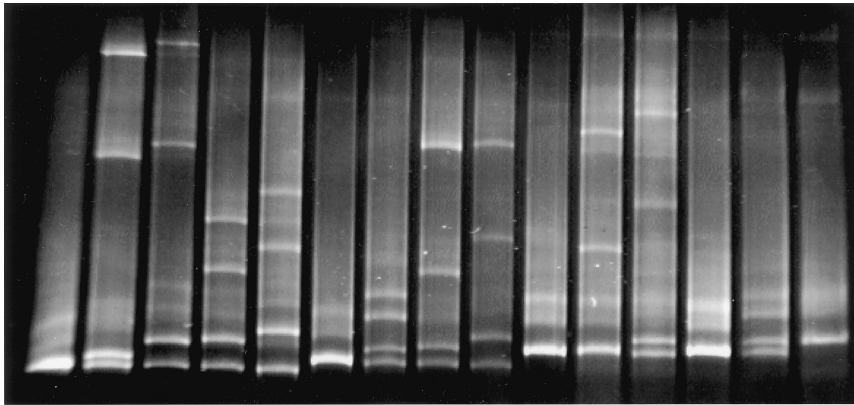
MRI was performed on 1.5-T superconducting magnets (Gyroscan S 15 and ACS, Philips) with the use of T2-weighted (repetition time, 2000 to 2500 ms; echo time, 30 to 60 ms) sequences in the transverse plane. T1-weighted images (repetition time, 600 ms; echo time, 30 ms) were generated in the sagittal and transverse planes. Slice thickness was 5 mm, and the matrix size used was 128×256 pixels. All scans were read by an experienced investigator without knowledge of the clinical and laboratory data. The scans were evaluated for white matter hyperintensities (WMH) and lacunar lesions. WMH were graded according to our scheme as absent, punctate, early confluent, and confluent.<sup>30</sup> Caps and periventricular lining were disregarded because they probably represent normal anatomic variants.<sup>31,32</sup> Lacunes were focal lesions involving the basal ganglia, internal capsule, thalamus, or brain stem not exceeding a maximum diameter of 10 mm. Assessment of intrarater variability for WMH grading and for presence of lacunar lesions was done in a subset of 70 randomly selected study participants and yielded  $\kappa$  values of 0.90 and 0.86, respectively. After the scans were read, individuals were considered to have MARCD if they presented with early confluent or confluent WMH or lacunes or any combination of these findings. Punctate WMH were not included in the definition of MARCD because these foci cannot definitely be attributed to cerebral ischemia according to histopathological correlations.<sup>32</sup>

### Statistical Analysis

We used the Statistical Package for Social Sciences (SPSS/PC+, version 8.0.0; SPSS Inc) for data analysis. Categorical variables among the genotypes were compared by the  $\chi^2$  test or by Fisher's exact test. Assumption of normal distribution for continuous variables was tested by Lilliefors statistics. Normally distributed variables were compared by 1-way ANOVA and nonnormally distributed variables by the Kruskal-Wallis test. To estimate the relationship between genotype and MARCD, we first performed an unadjusted comparison of the frequency of MARCD by genotypes. Logistic regression modeling was then done to assess the relative contribution of a given genotype on the presence of these brain lesions. We considered the dichotomized variables sex, hypertension, diabetes, and cardiac disease, the categorical variable smoking, and the continuous variables age, total cholesterol, and fibrinogen as possible confounders in the model. The analyses were also done with systolic and diastolic blood pressure in place of hypertensive status. Odds ratios (ORs) and 95% CI were calculated from the  $\beta$  coefficients and their SEs.

## Results

We screened a part of the AGT gene promoter and exon 1 (from -268 to +41 nucleotide related to the transcription start) for the presence of point mutations using TTGE in 410 elderly, neurologically asymptomatic subjects. TTGE showed 15 different banding patterns indicating the presence of 15 genotypes within this population. There were 5 banding patterns containing 1 homoduplex band (homozygotes) and



**Figure 1.** TTGE banding patterns of the 15 AGT promoter genotypes related to the haplotypes. A detailed description of TTGE conditions is given in Subjects and Methods.

A / A A / B A / C A / D A / E B / B B / C B / D B / E C / C C / D C / E D / D D / E E / E

AGT Promoter Genotypes

10 banding patterns containing 2 homoduplex and 2 heteroduplex bands (heterozygotes) (Figure 1). This is in accordance with the presence of 5 alleles combined in 15 genotypes. We designated the 5 alleles on the basis of their gel positions as A, B, C, D, and E alleles. The frequencies of the alleles A (wild-type) to E were 0.567, 0.151, 0.043, 0.138, and 0.101, respectively. The alleles and genotypes were in Hardy-Weinberg equilibrium ( $\chi^2_{\text{obt}}=2.7, P>0.99; \chi^2_{\text{crit}}=23.68, df=14$ ). We sequenced at least 3 samples within each genotype group. Samples with the C/C or D/D genotypes were all sequenced because they could not be unequivocally designated on the basis of TTGE alone. All samples within 1 TTGE banding pattern group showed identical results on sequencing. Altogether we sequenced each allele at least 15 times (3 times in homozygous state and 12 times as a component of a heterozygous genotype). We detected 4 polymorphic sites at positions -6:g/a, -20:a/c, -153:g/a, and -218:g/a in our cohort. Respective allele frequencies were 0.57 (-6:g) and 0.43 (-6:a), 0.81 (-20:a) and 0.19 (-20:c), 0.95 (-153:g) and 0.05 (-153:g), and 0.90 (-218:g) and 0.10 (-218:a). The alleles and the genotypes at the single-nucleotide polymorphisms were in Hardy-Weinberg equilibrium, as demonstrated by the respective  $\chi^2_{\text{obt}}$  and probability value (-6:  $\chi^2_{\text{obt}}=0.05, P>0.95$ ; -20:  $\chi^2_{\text{obt}}=0.12, P>0.90$ ; -153:  $\chi^2_{\text{obt}}=0.25, P>0.98$ ; -218:  $\chi^2_{\text{obt}}=0.44, P>0.95; \chi^2_{\text{crit}}=5.991, df=2$ ). Each of the 5 alleles contained a distinct combination of these polymorphic nucleotides and represented a haplotype (Table 1). Except for these polymorphisms, there was no deviation from the published AGT promoter sequence.<sup>33</sup>

**TABLE 1. Nucleotide Sequence at Polymorphic Sites in the 5 Haplotypes**

Position	Haplotypes				
	A	B	C	D	E
-6	g	a	a	a	a
-20	a	c	c	a	a
-153	g	g	a	g	g
-218	g	g	g	g	a

MARCD was seen in 89 subjects (21.7%). A total of 59 individuals (14.4%) had early confluent or confluent WMH, 16 (3.9%) had lacunar lesions, and 14 (3.4%) had both types of brain abnormalities. Subjects with MARCD were older ( $62.6 \pm 5.7$  years versus  $59.4 \pm 6.0$  years;  $P<0.0001$ ) and had a higher frequency of hypertension (50.6% versus 27.5%;  $P<0.0001$ ), higher systolic ( $144.7 \pm 22.8$  versus  $136.9 \pm 19.3$  mm Hg;  $P=0.004$ ) and diastolic ( $87.6 \pm 9.6$  versus  $85.1 \pm 10.6$  mm Hg;  $P=0.015$ ) blood pressure, and a higher frequency of cardiac disease (49.4% versus 34.4%;  $P=0.009$ ) than their counterparts without MARCD.

The frequency of MARCD in the different genotype subsets defined by the single-nucleotide polymorphisms is shown in Table 2. Only the -20:c allele in homozygotic state was significantly associated with an increased prevalence of MARCD ( $P=0.017$ ). A weak linear association between this polymorphism and MARCD was also present ( $P=0.04$ ). The association between the -6:a polymorphism and MARCD was borderline ( $P=0.054$ ). The other 2 polymorphic sites were not associated with MARCD.

Next we investigated the association of MARCD with the 15 genotypes reconstructed from the haplotypes. Overall, there was a significant association between the genotypes and MARCD ( $P=0.017$ ). Subsequently, we performed pairwise comparisons between the A/A genotype as reference group and the other genotypes to further elucidate their association with MARCD (Figure 2). Homozygotes for the B haplotype had the highest frequency of MARCD, while homozygotes for the C, D, and E haplotype showed very similar MARCD frequency as A/A carriers (Figure 2A). MARCD prevalence was also similar in all A haplotype carriers, including those with the A/B genotype (Figure 2B). However, there existed a trend toward higher MARCD frequency in individuals with 1 copy of the B haplotype in the absence of the A haplotype (B/C, B/D, B/E) (Figure 2C). The remaining genotypes, C/D, C/E and D/E, had MARCD frequencies similar to those of the wild-type A/A genotype (data not shown).

On the basis of these findings, we pooled the subjects into 3 investigational subsets. The first group consisted of the B homozygotes (B/B subset). The second group consisted of those B heterozygotes who carried the B haplotype in the absence of the wild-type A haplotype (B/C, B/D, and B/E)

**TABLE 2. Association of MARCD With AGT Promoter Polymorphisms**

	Genotypes			<i>P</i> *	<i>P</i> †
Position -6	gg (n=131)	ga (n=203)	aa (n=76)		
MARCD	28 (21.4%)	37 (18.2%)	24 (31.6%)	0.054	0.16
Position -20	aa (n=267)	ac (n=127)	cc (n=16)		
MARCD	53 (19.8%)	28 (22.0%)	8 (50%)	0.017	0.04
Position -153	gg (n=376)	ga(n=33)	aa (n=1)		
MARCD	82 (21.8%)	7 (21.2%)	0 (0%)	0.868	...
Position -218	gg (n=330)	ga (n=77)	aa (n=3)		
MARCD	71 (21.5%)	17 (22.1%)	1 (33.3%)	0.878	...

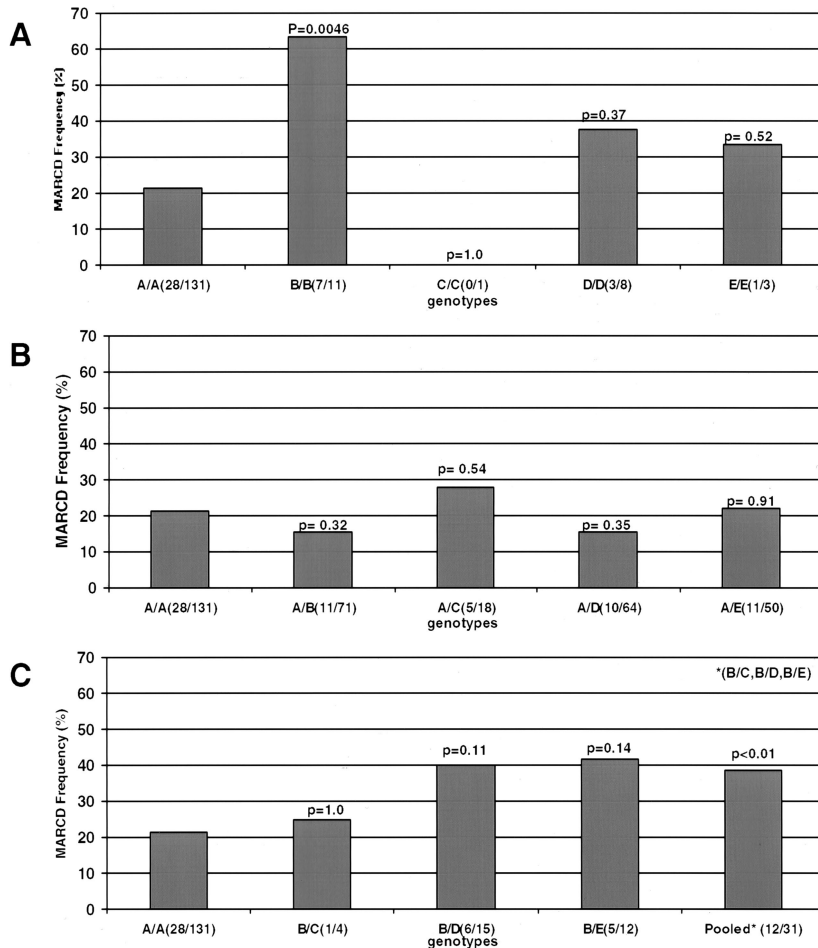
\* $\chi^2$  test.

†Mantel-Haenszel test for linear association.

(B+/A- subset). The third group contained the remaining genotypes (A/A, A/B, A/C, A/D, A/E, C/C, C/D, C/E, D/D, D/E, and E/E) and was designated as the reference cohort (RC subset). Distribution of demographics and vascular risk factors among the 3 investigational subsets is shown in Table 3.

Overall, MARCD was seen in 7 subjects (63.6%) in the B/B group, in 12 subjects (38.7%) in the B+/A- group, but in only 70 subjects (19.0%) in the RC group (Fischer's exact test,  $P < 0.001$ ; Mantel-Haenszel test for linear association,  $P < 0.001$ ). The age-adjusted ORs for MARCD relative to the RC subset were 7.6 (95% CI, 2.1 to 27.7) in the B/B and 2.2 (95% CI, 1.0 to 4.9) in the B+/A- subset.

To evaluate the extent to which the B+/A- genotype is associated with MARCD, we performed logistic regression analysis. The AGT genotype remained a significant predictor of MARCD ( $P = 0.0035$ ) after adjustment was made for age, sex, hypertension, diabetes, cardiac disease, smoking, plasma fibrinogen, and total cholesterol (Table 4). The respective ORs for the B/B and B+/A- genotypes remained unchanged when systolic (OR, 8.6; 95% CI, 2.26 to 32.7; OR, 1.9; 95% CI, 0.84 to 4.3) or diastolic blood pressure (OR, 8.6; 95% CI, 2.25 to 32.7; OR, 1.9; 95% CI, 0.83 to 4.3) levels instead of hypertension status were included in the model.



**Figure 2.** Frequency of MARCD among the AGT promoter genotypes reconstructed from the haplotypes. In each panel MARCD frequencies are compared with wild-type A homozygotes. A, Comparison with homozygotes for the B, C, D, and E haplotypes. B, Comparison with heterozygote A haplotype carriers. C, Comparison with subjects carrying at least 1 copy of the B haplotype in the absence of the A haplotype. Probability values represent  $\chi^2$  or Fisher's exact test results.

**TABLE 3. Demographics and Risk Factors Among the AGT Promoter Haplotype Subsets**

Variable	Genotypes			P
	RC (n=368)	B+/A- (n=31)	B/B (n=11)	
Age, y	59.9±5.98	62.8±6.1	61.1±6.8	0.042*
Male sex, n	175 (47.6%)	14 (45.2%)	7 (63.6%)	0.549*
Hypertension, n	114 (31.1%)	14 (45.2%)	5 (45.5%)	0.178†
Systolic blood pressure, mm Hg	138.0±19.9	143.9±24.2	141.4±22.0	0.391*
Diastolic blood pressure, mm Hg	85.4±10.4	87.6±11.1	86.8±10.1	0.5141*
Diabetes mellitus, n	21 (5.7%)	3 (9.7%)	0 (0%)	0.469†
Fasting glucose, mmol/L	5.16±1.14	5.50±2.03	4.74±0.40	0.399*
Cardiac disease, n	132 (36.0%)	20 (64.5%)	2 (18.2%)	0.003†
Smoking				
Ex-smokers, n	49 (13.4%)	2 (6.5%)	1 (9.1%)	
Current smokers, n	112 (30.5%)	8 (25.8%)	4 (36.4%)	0.695†
Body mass index, kg/m <sup>2</sup>	26.5±3.6	26.7±3.5	25.2±2.8	0.622*
Triglycerides, mmol/L	1.62±0.92	1.91±1.58	1.39±0.44	0.811*
Cholesterol, mmol/L	5.91±1.00	6.28±1.17	6.15±1.17	0.041‡
LDL cholesterol, mmol/L	3.86±0.92	4.20±1.00	4.16±0.99	0.076‡
HDL cholesterol, mmol/L	1.33±0.42	1.24±0.42	1.34±0.34	0.833*
Fibrinogen, mg/dL	301.5±79.3	308.6±84.7	301.6±60.5	0.503*

\*Kruskal-Wallis test.

† $\chi^2$  test.

‡One-way ANOVA.

Finally, we investigated the association between MARCD and the B haplotype in subgroups defined by age, sex, and hypertension status. MARCD frequency in subjects aged  $\leq 60$  years was 22 (11.1%) in the RC, 4 (40%) in the B+/A-, and 4 (66.7%) in the B/B group ( $P=0.001$ ). The respective frequencies were 48 (28.4%), 8 (38.1%), and 3 (69%) in subjects aged  $>60$  years ( $P=0.09$ ). MARCD prevalence in normotensive subjects was 35 (13.8%) in the RC, 5 (29.4%) in the B+/A-, and 4 (66.7%) in the B/B group ( $P<0.001$ ). The respective frequencies in the hypertensive group were 35 (30.7%), 7 (50%), and 3 (60%) ( $P=0.06$ ). The prevalence of MARCD in men was 35 (20.0%) in the RC, 5 (35.7%) in the B+/A-, and 4 (57.1%) in the B/B group ( $P=0.03$ ). Among women, the respective frequencies were 35 (18.1%), 7 (41.2%), and 3 (75.0%) ( $P=0.002$ ).

### Discussion

We report 4 new findings. First, we identified the presence of 5 novel haplotypes reconstructed from 4 polymorphisms at

the AGT gene promoter. Second, we found that the -20:c allele, which was shown to alter transcriptional efficiency of the AGT promoter in vitro,<sup>16</sup> is significantly associated with MARCD. Third, we described that 1 of the 5 haplotypes, designated as the B haplotype (nucleotide sequence at polymorphic positions -6:a, -20:c, -153:g, -218:g) predicts MARCD considerably better than the -20:c single-nucleotide allele. Fourth, the association between the B haplotype and MARCD was independent of hypertension. Our study was conducted in a homogeneous European population, making bias due to population admixture unlikely.

We found that homozygotes for the B haplotype had an 8-fold increased risk for MARCD. Persons carrying 1 copy of the B haplotype in the absence of the A haplotype showed a trend toward higher risk for MARCD. There was a significant linear association between B haplotype copy number and MARCD, suggesting a gene-dose effect. This gene-dose effect could also be observed in the subgroups of younger and older individuals, in men and in women,

**TABLE 4. Independent Predictors for MARCD in the Logistic Regression Model**

Variable	SE	df	P	OR	95% CI	
AGT genotype		2	0.005			
B+/B+ genotype	2.081	0.692	1	0.003	8.01	2.1–31.1
B+/A- genotype	0.614	0.420	1	0.144	1.84	0.8–4.2
Age, y	0.077	0.022	1	0.001	1.08	1.03–1.13
Hypertension	0.818	0.265	1	0.002	2.26	1.35–3.81

Adjustment was made for diabetes, cardiac disease, smoking, fibrinogen, cholesterol, and sex.

and also among hypertensive and normotensive subjects. Given their relation to protein levels, promoter polymorphisms are expected to have strongest effects in homozygotes and milder effects in heterozygotes. Our data support the presence of a gene-dose effect only for the haplotype but not for the single-polymorphic sites. Persons carrying the B haplotype in combination with the wild-type A haplotype did not show a higher risk for MARCD, indicating that the A haplotype might protect against the deleterious effect of the B haplotype. The observed association was not mediated by hypertension, since it remained virtually unchanged when adjustment was made for age and hypertension or for age alone.

With respect to the statistical assessment of the association between the AGT genotypes reconstructed from the haplotypes and MARCD, it is important to emphasize that the relationship was significant when a single comparison of MARCD frequency among the 15 genotypes was performed. We used pairwise comparisons between the wild-type A/A genotype and the other genotypes to further explore the association of each genotype with MARCD. We expected a priori that carriers of the B and the C haplotypes have higher MARCD frequencies than carriers of the other haplotypes. This was based on *in vitro* and *in vivo* data describing functional importance for the  $-20:c$  and the  $-6:a$  mutations,<sup>13–16</sup> both of which are only present in the B and C haplotypes. Our observation of a significant association between the  $-20:c$  allele and MARCD lends further support to this assumption. The results of the pairwise comparisons are not statistically significant after Bonferroni correction with the very conservative significance level of 0.0036. Adjustment for multiple testing is, however, difficult when haplotypes are studied because these are statistically dependent observations as a result of linkage disequilibrium. It is noteworthy that the strength of the association increased by using the haplotype in place of the single-nucleotide markers, as expected if a true causal relation is involved. Yet, despite the plausibility of the association between the B haplotype and MARCD, we cannot exclude with certainty that this is a chance finding. At this point it is important to note that our results apply strictly to a single cohort, and larger, probably concerted studies are needed to confirm these findings. The current investigation was exploratory. Overall, our findings show that the haplotype allows a more sensitive analysis of the association than the polymorphic sites alone. There are several explanations for this observation. It may be that the combination of the previously described sequence alterations in the B haplotype is functionally important or that the B haplotype captures an unknown sequence alteration functionally related to MARCD. The B haplotype may also be in linkage disequilibrium, with a functional polymorphism underlying the association.

On the basis of *in vitro* and *in vivo* data, a causal relationship between AGT genotype and hypertension seems plausible.<sup>9–27</sup> However, we have seen that the B haplotype is associated with MARCD independent of arterial hypertension. This suggests that it may operate through the local rather than through the systemic RAS.

Genetic variations at the AGT locus might alter tissue AGT expression. In preeclampsia, the expression of AGT in decidual arteries was associated with the T235 variant.<sup>34</sup> It is noteworthy that a strong linkage disequilibrium between the B haplotype and the T235 variant existed in our cohort. All B/B and 28 of the 31 B+/A– subjects were homozygous for the T235 allele. The remaining 3 B+/A– subjects were heterozygous for the M235T polymorphism.

Conceivably, the association between the AGT B haplotype and MARCD might be mediated by an altered expression of AGT in the brain,<sup>35</sup> which in turn leads to an altered local availability of AGT. Studies investigating AGT expression in the brain dependent on the haplotype have been initiated in our laboratory. If tissue RAS activity is also regulated by the AGT level, as is systemic RAS, then changes in AGT concentration may result in a higher level of Ang II at this site. It is known that Ang II acts on a variety of cell types in the brain.<sup>36,37</sup> In the present context the effect of Ang II on vascular smooth muscle cells is of particular interest. Ang II is a potent regulator of vascular tone and can lead to vasoconstriction and vasodilation in the cerebral arterioles, depending on the species studied.<sup>38–43</sup> Notably, MARCD was found to be related not only to hypertension but also to intermittent hypotensive episodes.<sup>6,7</sup> Ang II promotes vascular smooth muscle cell hyperplasia and hypertrophy.<sup>44–48</sup> It was shown to enhance the activity of NADH/NADPH oxidase and extracellular superoxide dismutase activity in the vessel wall.<sup>49,50</sup> It is also thought to alter the production of extracellular matrix proteins in the vessels.<sup>51,52</sup> Therefore, alterations in the local availability of Ang II might result in imbalance of physiological processes such as brain perfusion, autoregulation of cerebral blood flow, the oxidative state of the vessel wall, or function of the blood-brain barrier. Each of these processes might be involved in the development of MARCD.

In summary, we found that a certain haplotype of the AGT gene is significantly associated with MARCD in a community-dwelling cohort of elderly individuals. If larger studies can replicate our results, then this haplotype might serve as a genetic marker for the identification of individuals prone to develop these lesions and their clinical consequences. An association of small-vessel disease-related cerebral damage with genetic variants in the RAS system independent of arterial hypertension might not only extend our etiologic understanding of these brain lesions but might also point to possible favorable effects of drugs acting on the RAS system beyond those expected from lowering blood pressure alone.

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