Associations of the Angiotensin II Type 1 Receptor A\textsuperscript{1166}C and the Endothelial NO Synthase G\textsuperscript{894}T Gene Polymorphisms With Silent Subcortical White Matter Lesions in Essential Hypertension

Léon H.G. Henskens, MD; Abraham A. Kroon, MD, PhD; Martin P.J. van Boxtel, MD, PhD; Paul A.M. Hofman, MD, PhD; Peter W. de Leeuw, MD, PhD, FAHA

Background and Purpose—Silent white matter lesions (WMLs) may represent early target organ damage of the brain in patients with hypertension. Because these lesions may have a genetic background, we assessed the associations between polymorphisms of the renin-angiotensin system and the endothelial NO synthase (NOS3) genes and silent WMLs.

Methods—Ninety-three hypertensive individuals were studied. MRI of the brain was performed to obtain estimates of the total volume of subcortical and the extent of periventricular WMLs. Patients were genotyped for the angiotensinogen (M\textsuperscript{235}T), the angiotensin-converting enzyme (insertion/deletion [I/D]), the angiotensin II type 1 receptor (AGTR1 A\textsuperscript{1166}C), and the NOS3 (G\textsuperscript{894}T) genes. A linear regression model was used to assess the relationship of these gene polymorphisms with both subtypes of WMLs.

Results—When adjusted for age, diabetes mellitus, and blood pressure, subcortical WML volume was lowest in the presence of 1 or 2 AGTR1 C alleles (unstandardized $\beta = -38.8$ [95% CI, −66.1 to −11.4] and −112.6 [CI, −188.9 to −36.4], respectively), whereas it was highest in the presence of an NOS3 T allele (3.1 [CI, 3.6 to 58.4]). No interaction between these polymorphisms on WMLs could be demonstrated. No associations were present with the other polymorphisms, either with subcortical or periventricular lesions.

Conclusions—We found the AGTR1 A\textsuperscript{1166}C as well as the NOS3 G\textsuperscript{894}T polymorphisms to be associated with silent WMLs in the subcortical area. (Stroke. 2005;36:1869-1873.)

Key Words: hypertension  ■ nitric oxide synthase  ■ polymorphism  ■ renin-angiotensin  ■ white matter

Asymptomatic white matter lesions (WMLs) on MRI of the brain may represent an early sign of target organ damage in patients with hypertension.\textsuperscript{1} WMLs are thought to be caused by cerebral small-vessel disease (SVD), probably through endothelial dysfunction.\textsuperscript{2} Twin and family studies also suggest a strong genetic background.\textsuperscript{3,4} Among the potential candidate genes that can account for a genetic predisposition to WMLs, common genetic variants of the renin-angiotensin system (RAS) and the endothelial NO synthase (NOS3, previously known as eNOS) rank high.\textsuperscript{5-11} Homozygosity of the deletion variant of the angiotensin-converting enzyme insertion/deletion (ACE I/D) polymorphism and the presence of 1 or 2 C alleles of the A\textsuperscript{1166}C polymorphism of the angiotensin II type 1 receptor (AGTR1, previously known as AT\textsubscript{R}) gene indeed seem to increase the risk of WMLs.\textsuperscript{7-11} No evidence has been found for a role of the angiotensinogen (AGT) M\textsuperscript{235}T and the NOS3 G\textsuperscript{894}T polymorphisms.\textsuperscript{5,6,11}

Previously, other investigators distinguished periventricular WMLs from those in the subcortical area on the basis of vascularization patterns,\textsuperscript{12} susceptibility to (vascular) risk factors,\textsuperscript{13} and consequences (eg, cognitive impairment).\textsuperscript{14} Moreover, the Rotterdam Scan Study suggests that the genetic predisposition to WMLs is confined to specific brain areas (eg, to the subcortical but not the periventricular white matter).\textsuperscript{15} However, no data are available regarding the relationship between the aforementioned polymorphisms and this site specificity. Moreover, WMLs have been studied predominantly as a qualitative (eg, presence or absence) rather than as a quantitative trait (eg, lesion quantity). This prompted us to assess the associations between polymorphisms of the RAS and the NOS3 genes and silent WMLs, in terms of well-characterized subtypes and lesion quantity, in otherwise healthy hypertensive individuals between 30 and 80 years of age.

Materials and Methods

Subjects
A total of 105 consecutive hypertensive patients between 30 and 80 years of age who attended the outpatient clinic of the University...
Hospital Maastricht were enrolled in the present study. Hypertension was defined as a systolic blood pressure (BP) $\geq 140$ mm Hg, or a diastolic BP (DBP) of $\geq 90$ mm Hg, or both, as assessed on multiple occasions. Exclusion criteria were clinical evidence of ischemic or valvular heart disease, congestive heart failure, cerebrovascular accidents or transient ischemic attacks, chronic renal failure (serum creatinine $>150$ $\mu$mol/L), indication of secondary hypertension, and contraindications for MRI. As part of the local protocol, ambulatory BP monitoring (ABPM) was performed during 24 hours, and blood samples were drawn for routine laboratory investigations and genetic analysis. Brain MRI was performed for the present study only. All patients gave their written informed consent, and the study was approved by the local medical ethics committee.

### Risk Factors

Blood samples were drawn from fasting patients for assessment of serum creatinine, total cholesterol, and glucose levels. Hypercholesterolemia was defined as a total cholesterol level exceeding 6.5 $\text{mmol/L}$ or use of lipid-lowering drugs. Diabetes mellitus (DM) was considered to be present in case of fasting plasma glucose levels $>6.9 \text{mmol/L}$ or use of oral antidiabetic drugs or insulin.

Noninvasive ABPM (SpaceLabs 90217) was performed at the nondominant arm every 15 min during the day and every 30 min during the night. Antihypertensive medication was discontinued 3 weeks before ABPM. For analysis, average levels of 24-hour systolic BP (SBP), DBP, mean arterial pressure (MAP), and pulse pressure (PP) were calculated. At their visit to the hospital, patients' height, weight, and smoking status were obtained.

### WML Scoring

MRI scans were made on a 1.5-T Philips Intera NT. The scan protocol consisted of axial proton density (PD), axial T2-weighted fast spin-echo (FSE), and axial T2-weighted fluid-attenuated inversion recovery (FLAIR) sequences. Subcortical and periventricular WMLs were scored according to the Rotterdam Scan Study scale. All scans were analyzed off-line using custom software (Brain Image Analysis System). This program allowed a systematic inspection of side-by-side aligned axial PD, FSE, and FLAIR image stacks and manual demarcation of regions of interest (ROIs). Subcortical WMLs were scored using predefined ROI masks (ie, circles with a diameter of 2, 6, and 12 mm, respectively). Lesions were first identified on the FLAIR image and then confirmed on both other images at the same level. If a lesion was present on all 3 images, the mask that matched the ROI best was fitted over the lesion. After inspection and delineation of all subcortical WMLs in a stack, the program generated an output file with the number and size of all lesions at each level of the scan. To obtain the total subcortical WML volume for each patient, ROIs were inflated to spheres with the same diameter, with corresponding volumes of 4.2, 113, and 905 $\text{mm}^3$, respectively. Subcortical WMLs were processed by 1 medical investigator (M.P.J.v.B.) after satisfactory intraclass correlations between 0.81 and 0.98 had been reached, based on the independent assessments of subsequent series of 10 random stacks by this investigator and an experienced neuroradiologist (P.H.). Periventricular WML severity, ranging between 0 and 3, was scored for frontal and occipital regions (“caps”) and the medial periventricular lining (“bands”) separately, which were then summed to an overall periventricular WML score.

### Genetic Analysis

DNA was extracted from whole blood with the use of the QIAamp Blood Kit (Qiagen Inc.). The ACE I/D polymorphism was detected using the technique described by Rigat et al. A second polymerase chain reaction was performed to avoid misidentification of ID as DD. Genotyping of the AGT M$^{235}$T, the AGTR1 A$^{1166}$C, and the NOS3 G$^{894}$T polymorphisms was performed using a multilocus genotyping assay for candidate markers of cardiovascular disease risk (Roche Molecular Systems Inc.) and has been described in detail previously. Genetic analyses were performed after assessment of the MRI scans, so the investigators who scored WMLs were blinded for the genotypes.

### Statistical Analysis

Deviation from Hardy–Weinberg equilibrium was assessed using $\chi^2$ statistics comparing expected against observed genotype frequencies. Allele frequencies were estimated by gene counting. Because of skewed distribution of WMLs, associations between periventricular or subcortical WMLs and risk factors or demographics were determined by means of Spearman’s correlations and the nonparametric Mann–Whitney U test. Subsequently, WML data were log transformed to achieve normality before further analysis. Univariate and multivariate regression analyses were performed to evaluate the relationship between genotypes and WMLs, with adjustment for age, DM, and ambulatory BP. For that purpose, dummy variables were created using the homozygous wild-type genotype as reference category. All covariates were forced into the model simultaneously (multiple linear regression, enter procedure). In addition, for those models that reached statistical significance, the influence of the separate alleles on WMLs were assessed similarly.

Interactions between 2 polymorphisms with respect to WMLs were assessed using a linear regression model that included the alleles at risk, the interaction term between them, and other covariates when applicable. Unless indicated otherwise, data were expressed as medians with interquartile ranges. A 2-tailed $P$ value $<0.05$ was considered statistically significant. Because of the exploratory nature of the present study, no corrections for multiple testing were applied. Statistical analyses were performed using SPSS 11.5 for Windows (SPSS Inc.).

### Results

#### Patient Characteristics

Among the 105 patients available, MRI data of 9 patients were inadequate because of claustrophobia (1), movement artifacts (4), or premature withdrawal from the study (4). Another 3 patients withdrew consent for genetic investigations, leaving 93 patients for analysis. Patients who entered the study did not differ in baseline characteristics from those who did not.

Characteristics of the study population are summarized in Table 1. Twenty patients (22%) had no or only 1 small subcortical WML on their MRI scan, whereas periventricular WMLs were present in 61 patients (66%). Age was associated with higher subcortical WML volume and periventricular WML score ($r=0.587$ and $P<0.001$, and $r=0.485$ and $P<0.001$, respectively). Twenty-four-hour SBP, MAP, and PP correlated with periventricular WMLs ($r=0.360$ and $P=0.001$, $r=0.231$ and $P=0.027$, and $r=0.463$ and $P<0.001$, respectively), whereas 24-hour PP correlated with subcortical WMLs ($r=0.294$; $P=0.004$). There were no significant associations between WML categories regarding other demographics or risk factors.

#### Genetic Analysis

In 7 patients, a second analysis was necessary to obtain all the genotypes. Genotype and allele frequencies of all polymorphisms (Table 2) were in Hardy–Weinberg equilibrium. There were no statistically significant associations between the polymorphisms of the RAS or NOS3 genes and periventricular WML score. The same was true for the ACE I/D and the AGT M$^{235}$T polymorphisms, with respect to subcortical WML volume. However, the AGTR1 A$^{1166}$C and the NOS3 G$^{894}$T polymorphisms were significantly associated with sub-
TABLE 1. Characteristics of the Study Population (n=93)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Sex, % male</th>
<th>Age, y</th>
<th>Smoking, %</th>
<th>BMI, kg/m²</th>
<th>Glucose, mmol/L</th>
<th>DM, %</th>
<th>Cholesterol, mmol/L</th>
<th>HC, %</th>
<th>Creatinine, mmol/L</th>
<th>24-hour SBP, mm Hg</th>
<th>24-hour DBP, mm Hg</th>
<th>24-hour MAP, mm Hg</th>
<th>24-hour PP, mm Hg</th>
<th>Heart rate, bpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>60.2</td>
<td>55 (46, 64)</td>
<td>29.3</td>
<td>28.0 (25.0, 31.0)</td>
<td>5.6 (5.3, 6.1)</td>
<td>15.1</td>
<td>5.4 (4.9, 6.0)</td>
<td>24.7</td>
<td>85.0 (71.0, 96.0)</td>
<td>151 (136, 166)</td>
<td>91 (85, 100)</td>
<td>112 (103, 121)</td>
<td>57 (49, 65)</td>
<td>75 (67, 82)</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; HC, hypercholesterolemia.

Data are presented as medians with interquartile ranges, unless indicated otherwise.

Discussion

In the present study, we found the AGTR1 A1166C as well as the NOS3 G894T polymorphisms to be associated with silent WMLs in the subcortical white matter. When age, DM, and BP were accounted for, lesion volume was lowest in the presence of an AGTR1 C allele and in patients with the CC genotype, whereas it was highest in the presence of an NOS3 T allele.

The associations reported on here contradict the observations of most previous studies on the AGTR1, NOS3, and ACE gene polymorphisms. First, the C allele and the CC genotype of the AGTR1 A1166C polymorphism have been associated with the severity of periventricular WMLs or an increased risk of incident ischemic stroke.20 Furthermore, evidence suggests that the C allele is an independent cardiovascular risk factor.20 Consequently, one would expect the C allele rather than the A allele to be associated with subcortical WML volume. Second, others did not find an association between the NOS3 G894T variant and WMLs on MRI and computed tomography in patients with clinically evident cerebral SVD.5 Studies on ischemic stroke also failed to detect a relationship with this polymorphism or even reported an increased risk in patients homozygous for the G allele.23 Finally, we were not able to replicate previous associations of the ACE D allele and the DD genotype with ischemic stroke24 or WMLs7–10 in terms of lesion subtype and severity.

Inconsistencies in association studies can be attributed mainly to multiple hypothesis testing, heterogeneous study populations, and inadequate power.25 Whereas the first 2 situations are not likely to explain our findings because we performed this study with a clear a priori hypothesis and selected our population using strict inclusion criteria, the third situation potentially does. Although our study population is small, the cohort design enabled us to investigate the impact of genetic markers. This could help in identifying those patients who are at greatest risk and who may need (although this has yet to be proven) more aggressive treatment. Moreover, recent evidence suggests that the use of intermediate phenotypes and a “high quality” of phenotyping allow smaller populations to be studied.5,26 Silent WMLs are an intermediate phenotype for cerebral SVD and ischemic stroke.26,27 Therefore, we phenotyped WMLs according to subtype (subcortical or periventricular), and severity (lesion volume) and independently of the results of genotyping. By doing so, we improved the quality of phenotyping. Nevertheless, the small study population remains a limitation of the present study, and confirmation in larger cohorts including case-control studies is needed.

Other potential sources of inconsistency between our data and those in the literature are the use of different rating scales for quantifying WMLs and differences in disease status. With respect to the latter, most studies focused on patients with clinical evidence of stroke rather than on silent disease. Because stroke is characterized by a continuing increased risk of death,26 selection bias may have occurred by early death of patients carrying a high-risk allele. Indeed, several association studies observed lower frequencies of high-risk alleles in stroke patients compared with healthy controls.6,23 On the other hand, studies on the AGT M235T polymorphism including ours were all negative.6,7,11 Remarkably, the latter studies focused all on silent WMLs. Thus, it is possible that the
influence of genetic factors on the course of the disease (eg, from silent abnormalities to clinically evident lesions) varies.

The mechanisms behind the associations reported here remain speculative, especially because the functional aspects of the genetic polymorphisms are not clear yet. On the other hand, it is possible that these polymorphisms are in linkage disequilibrium with another unidentified functional mutation nearby. In keeping with this, our group and others recently provided indirect evidence of a functional role. The AGTR1 C and the NOS3 T alleles were found to be associated with increased sensitivity to angiotensin II and reduced bioavailability of NO, respectively.18,29

We and others15 provide evidence that the genetic predisposition to WMLs is confined to specific brain areas. Very recently, the Rotterdam Scan Study showed an increased risk to WMLs is confined to specific brain areas. Very recently, the Rotterdam Scan Study showed an increased risk to WMLs, which are supported by additional evidence. For instance, the periventricular white matter seems less resistant to the influence of hypertension and other vascular risk factors. Furthermore, these data illustrate the differences in the susceptibility of the cerebral white matter lesions in essential hypertension. Hypertension. 2002;39:343–347.


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In the article by Henskens et al1, “Associations of the angiotensin II type 1 receptor A1166C and the endothelial NO synthase G894T gene polymorphisms with silent subcortical white matter lesions in essential hypertension,” which appeared in the September 2005 issue of Stroke, there was incorrect data presented in the results section of the abstract and in Table 3. The unstandardized \( \beta \) of the NOS3 T allele is 31.1 (CI, 3.6 to 58.4), not 3.1 (CI, 3.6 to 58.4) as given in the article. The authors apologize for this error.