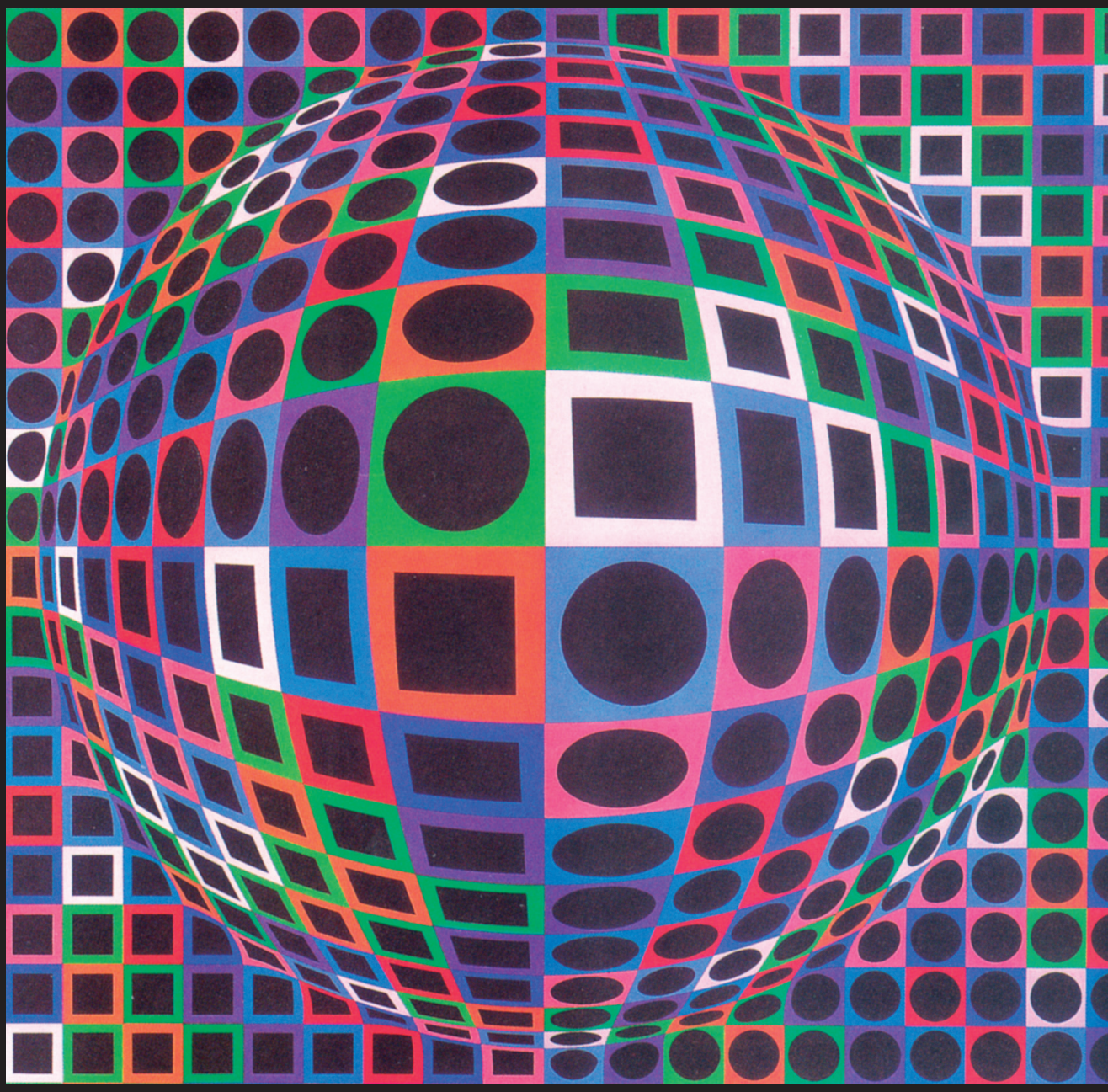
Genetic Determinants of Cerebral Small Vessel Disease



Helena Schmidt

Cover: Taken from the painting “Vega-Gyongiy-2“ by Victor Vasarely
Born in Hungary in 1906 Victor Vasarely is perhaps the best known and most influential artist of the Op Art movement.
Vasarely was a master at making flat surfaces appear 3-dimensional. He achieved this effect by meticulously arranging large numbers of geometric shapes in such a way as to create the illusion of movement or depth.
Vasarely‘s work is highly collectable and can be seen in galleries and museums worldwide. Victor Vasarely died in 1997.
According to the artist, „In the last analysis, the picture-object in pure composition appears to me as the last link in the family ‚paintings,‘ still possessing by its shining beauty, an end in itself. But it is already more than a painting, the forms and colors which compose it are still situated on the plane, but the plastic event which they trigger fuses in front of and in the plane. It is thereby an end, but also a beginning, a kind of launching pad for future achievements.“

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Helena Schmidt

Acknowledgements

The work presented in this thesis was conducted at the Institute of Molecular Biology and Biochemistry of the Medical University of Graz, Austria and at the Genetic Epidemiology Unit, Department of Epidemiology & Biostatistics of the Erasmus Medical Center, Rotterdam, The Netherlands.

The studies described in this thesis were supported by the Austrian Science Fund, project P13180-MED and project P15440, by the Jubileumsfond of the Austrian National Bank, project 7776, by the Franz Lanyar Foundation of the Karl-Franzens University, Graz and by the Netherlands Heart Organization 2000B117.

The publication of this thesis was supported by the Institute of Molecular Biology and Biochemistry, Medical University of Graz, by the Department of Epidemiology & Biostatistics of the Erasmus Medical Center, the Netherlands as well as by ServoLab, Graz, Austria; Promega, Wien, Austria and Sanofi-Aventis, Wien, Austria.

Cover design: Helena Schmidt, Graz, Austria
Cover: "Vega-Gyongiy" by Victor Vasarelly,
Printed by: Universitätsdruckerei Klampfer, Weiz, Austria
ISBN: 3-200-00440-1

Genetic Determinants of Cerebral Small Vessel Disease

Genetische determinanten van cerebrale microangiopathie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
prof.dr. S.W.J. Lamberts
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 14 september 2005 om 11.45 uur

door

Helena Schmidt
geboren te Budapest, Hungary

PROMOTIECOMMISSIE

Promotor	:	prof.dr.ir. C.M. van Duijn
Overige leden	:	prof.dr. M.M.B Breteler prof.dr. F.Fazekas prof.dr. B.A.Oostra
Copromotor	:	dr. Y. S. Aulchenko

Science makes people reach selflessly for the truth and objectivity; it teaches people to accept reality, with wonder and admiration, not to mention the deep awe and joy that the natural order of things brings to the true scientist.

Lisa Meitner, Austrian Physicist (1878-1968)

Köszönettel es szeretettel
szüleimnek,
férjemnek, Reinholdnak és
kislányomnak, Veronikának

Publications based on the studies described in this thesis

Chapter 2

R.Schmidt, F.Fazekas, P.Kapeller, H.Schmidt, HP.Hartung. MRI white matter hyperintensities Three-year follow-up of the Austrian Stroke Prevention Study. Neurology 1999;53:132-139.

R.Schmidt, C.Enzinger, S.Ropele, H.Schmidt, F.Fazekas. Progression of cerebral white matter lesions: 6-year results of the Austrian Stroke Prevention Study. Lancet 2003;361:2046-48

Chapter 3.1

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Chapter 3.2

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Chapter 3.3

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Chapter 4.1

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Chapter 4.2

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Chapter 4.3

H.Schmidt, R.Schmidt, K.Niederkorn, S.Horner, P.Becsagh, B.Reinhart, M.Schumacher, V.Weinrauch, GM.Kostner. Beta-fibrinigen gene polymorphism (C148-->T) is associated with carotid atherosclerosis.Results of the Austrian Stroke Prevention Study. Arterioscler Thromb Vasc Biol 1998;18:487-492

Contents

General Introduction

1

Cerebrovascular disease is a leading cause of disability and mortality in the elderly. The term describes a highly heterogeneous group of disorders manifesting as parenchymal brain damage due to cerebral vascular pathology. Based on the size of the vessel affected one distinguishes cerebral large- and small vessel disease, i.e. atherosclerosis and arteriolosclerosis.

Histopathological studies show marked differences at the tissue level between these two processes. Atherosclerotic lesions are characterized by the presence of lipid laden macrophages, so called foam cells in the subendothelial areas in the early fatty streaks. Over years these lesions progress by accumulating smooth muscle cells and extracellular matrix proteins into advanced atherosclerotic plaques featuring extensive fibrosis, necrotic and calcified tissue with cholesterol crystals (1). Both epidemiological and experimental studies underscore the central role lipids play in the development of atherosclerosis (1). The main histopathological features of arteriolosclerotic lesions is a thickening of the vessel wall due to vascular smooth muscle cell hyperplasia and fibrohyalinosis (2,3). Major and widely accepted risk factors for small vessel disease are age and hypertension (4). The development of arteriolosclerosis seems to be independent of lipids and lipoproteins.

From the genetic perspective both athero- and arteriolosclerosis are conceptualized as complex traits. The risk of an individual to develop these pathologies depends on an interaction between exposure to environmental factors and inherited susceptibility. Efforts are undertaken to identify the genetic factors as their knowledge delivers information about etiology and pathomechanisms of these disorders and may facilitate the development of preventive and therapeutic strategies on an individual basis. The clinical presentation of both atherosclerosis as well as arteriolosclerosis is heterogeneous and ranges from strokes with abrupt onset and clear-cut focal neurological deficits to slowly progressive signs and symptoms including cognitive impairment, gait disturbances and falls, incontinence and mood changes. Large and small vessel disease can also be clinically silent (5). The non-uniform phenotypes resulting from common vessel pathology clearly hampers genetic research of these traits.

The heterogeneity can partly be reduced by the introduction of strict diagnostic criteria as well as by the use of intermediate, subclinical phenotypes as surrogate markers. A frequently used subclinical paradigm of cerebral large vessel disease (cLVD) is carotid intima media thickness (IMT) or the presence of atherosclerotic plaques as assessed by carotid Doppler sonography (6), while severe subcortical white matter hyperintensities and lacunar infarcts detected by magnet resonance imaging (MRI) are used as subclinical manifestations for cerebral small vessel disease (cSVD) (7). The development of uniform definitions and terminology for both cerebral large and small disease and its clinical sequelae is very much needed. Such "common language" creates a first step in reducing the inconsistency of current genetic association studies in cerebrovascular disease. The heritability estimates of carotid atherosclerosis vary from 20 to 40% (6). The heritabilty of cSVD is

significantly higher, in the range of 55 to 73% (8-10).

Compared to the large body of literature available on carotid atherosclerosis, less is known about cSVD. This is partly due to the fact that detection of this phenotype is based on MRI which is a less easily accessable and more cost intensive examination than sonography. Only a small number of the large population-based studies incorporated brain MRI in their protocol. The prevalence of cSVD based on these studies is estimated to be 10-20% in the elderly population (11). It has been suggested that these lesions, in the past referred as leukoaraioses, progress gradually over time and ultimately may result in subcortical arteriosclerotic encephalopathy with concomitant cognitive decline (12). The rate and the determinants of cSVD progression as well as the clinical consequences of cSVD remain a major focus of research interest for those working on this field.

The aim of this thesis was to contribute to a better understanding of the development of cSVD by studying the evolution of these lesions over time and by identifying genetic factors determining the risk for cSVD. Large and small vessel disease are clearly unique entities, nonetheless they are frequently associated with each other, suggesting that they share some common pathways in their development. One approach that may help to increase our understanding of cerebrovascular disease in general is to search for genetic factors which concur in athero- and arteriolosclerosis but also to identify genetic factors which are unique for one of these two vessel pathologies. We took such an approach in the setting of the Austrian Stroke Prevention Study (ASPS), 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.

In Chapter 2 we describe rate and determinants of WMH progression over a period of 3-year (Chapter 2.1) and 6-year (Chapter 2.2) follow up.

In Chapter 3 we focus on the genetic determinants of cSVD. In chapter 3.1 we give an overview about the genetics of cSVD based on data available on the topic in year 2000 and suggest a model to select candidate genes for cSVD. Next we present the results of association studies investigating the role of candidate genes in cSVD. First we describe the association between cSVD and the apolipoprotein E isoforms 2, 3 and 4 (Chapter 3.2), as well as between cSVD and the paraoxonase 1 polymorphisms at position 54 and 191 (Chapter 3.3). In a separate chapter, Chapter 3.4 we pool a series of three manuscripts evaluating the role of angiotensinogen (AGT) gene promoter haplotypes in cSVD. In the first manuscript we describe the presence of five novel haplotypes at the AGT promoter and report a positive association between the B-haplotype and cSVD (Chapter 3.4.1). In the second manuscript we review the possible role of AGT in cSVD based on the literature and present our own first functional data (Chapter 3.4.2). In the third manuscript we investigate the effect of the B-haplotype on the transcriptional activity of the AGT gene and study the evolutionary relatedness of the different AGT haplotypes (Chapter 3.4.3).

In Chapter 4 we present three studies on the genetics of carotid atherosclerosis. In Chapter 4.1. and Chapter 4.2 we investigate if carotid atherosclerosis is associated with polymorhisms in the paraoxonase and the AGT gene, which we identified as risk factors for cSVD (Chapter 3). In Chapter 4.3 we describe the association between beta-fibrinogen promoter polymorphism and carotid atherosclerosis, a polymorphism which was not associated with cSVD in our cohort.

Finally, in Chapter 5 we discuss our main findings and summarise their possible implications for future research.

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Progression of White Matter Lesions

2

MRI White Matter Hyperintensities Three-Year Follow-Up of the Austrian Stroke Prevention Study

2.1

MRI white matter hyperintensities

Three-year follow-up of the Austrian Stroke Prevention Study

R. Schmidt, MD; F. Fazekas, MD; P. Kapeller, MD; H. Schmidt, MD; and H.-P. Hartung, MD

Article abstract—*Objective:* To determine the rate, clinical predictors, and cognitive consequences of MRI white matter hyperintensity evolution over 3 years. *Methods:* In the setting of the Austrian Stroke Prevention Study, 1.5-T MRI was performed at baseline and at a 3-year follow-up in 273 community-dwelling elderly (mean age, 60 ± 6.1 years) without neuropsychiatric disease. At each visit individuals underwent a structured clinical interview and examination, EKG, echocardiography, extensive laboratory workup, and demanding neuropsychological testing. MR images were read by three independent raters, and the change of white matter hyperintensities from baseline was assessed by direct image comparison. The change was graded as absent, minor, or marked. Minor change was defined as a difference of no more than one to four punctate lesions between both scans. A change was considered to be marked if there was a difference of more than four abnormalities or a transition to early-confluent and confluent lesions. *Results:* Combined ratings indicated lesion progression in 49 individuals (17.9%). Lesion progression was minor in 27 participants (9.9%) and was marked in 22 (8.1%). Regression of white matter hyperintensities did not occur. Diastolic blood pressure (odds ratio, 1.07/mm Hg) and early-confluent or confluent white matter hyperintensities at baseline (odds ratio, 2.62) were the only significant predictors of white matter hyperintensity progression. Lesion progression had no influence on the course of neuropsychological test performance over the observational period. *Conclusions:* White matter hyperintensities progress in elderly normal subjects. Our data may be used as a reference for future observational and interventional studies on white matter hyperintensity progression in various CNS diseases. The lack of an association between lesion progression and cognitive functioning needs to be explored further.

NEUROLOGY 1999;53:132–139

White matter hyperintensities (WMH) are a common MRI observation in the elderly.[1] When located in the deep and subcortical white matter they mostly reflect ischemic damage and correspond to focal rarefaction of myelin, loss of fibers, and sometimes even lacunar infarctions according to histopathologic correlations.[2-6] Arteriolosclerosis is thought to be the most important causative factor in the evolution of such abnormalities.[4] Main predictors of WMH are advancing age[2,7-13] and arterial hypertension.[2,7,8,11,13-15] Since 1986 it has been suggested that WMH progress gradually over time with the accumulation of vascular risk factors, and ultimately may result in extensive subcortical arteriosclerotic encephalopathy with concomitant cognitive decline.[2] Indeed, numerous correlative studies described subtle neuropsychological deficits in elderly nondemented individuals with such lesions.[12,16-20] Moreover, in a small sample of 26 healthy persons, it has been reported that individuals with WMH experienced a greater decline in cognitive performance than those with normal MR images over an 18-month period.[21] Although these results support the suggested increase of WMH over time,[2] there have been no prospective studies to date that have actually determined the rate and speed of progression, and have attempted to delineate the risk factors for and cognitive sequelae of this process. We studied the natural history of WMH during a 3-year period in a large, well-defined elderly cohort.

Methods. *Individuals and study design.* The study population consisted of participants of the Austrian Stroke Prevention Study, a single-center, prospective follow-up study on the cerebral effects of vascular risk factors in the normal elderly population of the city of Graz, Austria. The study is purely descriptive. We selected randomly a sample of 8,193 individuals age 50 to 75 years stratified by gender and 5-year age groups from the official community register. Between September 1991 and March 1994, individuals received a written invitation to participate in the study that described the purpose of the investigation. Overall, 2,794 of the invited returned a card stating their willingness to participate. Recruitment into the study was stopped after enrollment of 1,998 eligible participants. Individuals were excluded from the study if they had a history of neuropsychiatric disease, including previous cerebrovascular attacks and dementia, or an abnormal neurologic examination determined on the basis of a structured clinical interview and a physical and neurologic ex-

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Funded by the Austrian Science Fund (F.F.), project P13180-MED.

Presented in part at the 50th annual meeting of the American Academy of Neurology, Minneapolis, MN.

Received September 9, 1998. Accepted in final form February 13, 1999.

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amination. A random age- and sex-stratified sample of 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. All study participants underwent three blood pressure readings, EKG, echocardiography, and laboratory testing including blood cell count and a complete blood chemistry panel. Every fourth study participant or the next was invited to enter phase II of the study, which included MRI, Doppler sonography, SPECT, and neuropsychological testing. From a total of 498 phase II participants, 458 volunteered to undergo MRI. During the second study panel, 3 years after baseline, 345 phase II attendees agreed to be reexamined following the same protocol. From the 113 individuals that could not be reexamined, 7 died and another 7 experienced a stroke, which is an end point in our study. Seventy-two subjects underwent all other examinations but refused to undergo a second MR image because they experienced claustrophobic sensations during the initial evaluation. The remaining 27 individuals were contacted by telephone but did not want to undergo the extensive diagnostic workup a second time. The current study cohort is comprised of those 273 study participants, with a baseline and 3-year follow-up MRI. There were 142 women and 131 men. The mean age was 60 ± 6.1 years (median, 60.0 years). The sample consisted exclusively of whites of central European origin, and the length of education ranged from 9 to 18 years (mean, 11.7 years). At the time of examination, 48% of study participants were retired, 12% were blue-collar and 29% were white-collar workers, and 11% were housewives or housemen. No unemployed individual participated in the study. Overall, 76.6% of study participants were married, 8.4% were unmarried, 8.4% were divorced, and 6.6% were widowed. The individuals who participated in the follow-up MRI study did not differ from those who dropped out in terms of age, gender, educational and occupational status, and risk factors for stroke.

Vascular risk factors. Historic information and laboratory findings at baseline and follow-up were considered for risk factor diagnosis. Arterial hypertension was considered present if an individual had a history of arterial hypertension with repeated blood pressure readings higher than 160/95 mm Hg, if an individual was treated for arterial hypertension, or if the two readings at the examinations exceeded this limit. Diabetes mellitus was coded present if an individual was treated for diabetes at the time of examination or if the fasting blood glucose level at one examination exceeded 140 mg/dL. Cardiac disease was assumed to be present if there was evidence of cardiac abnormalities known to be a source for cerebral embolism,[22] evidence of coronary heart disease according to the Rose questionnaire,[23] or appropriate EKG findings[24] (Minnesota codes: I, 1 to 3; IV, 1 to 3; or V, 1 to 2), or if an individual presented signs of left ventricular hypertrophy on echocardiogram or EKG (Minnesota codes: III, 1; or IV, 1 to 3). Study participants were asked whether they ever smoked and if they currently smoked. The body mass index (BMI; in kilograms per square meters) was determined at both examinations. The means of systolic and diastolic blood pressure, fasting blood sugar, and BMI of baseline and follow-up measurements were calculated and used for data analyses.

Laboratory measurements. During both examinations a lipid status, including the level of triglycerides, total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol, as well as Lp(a) lipoprotein, was determined for each study participant. Thirty minutes after venipuncture the coagulated blood samples were centrifuged at 1,600 *g* for 10 minutes, then the serum was transferred to plastic tubes and analyzed within 4 hours. Triglycerides and total cholesterol were determined enzymatically using commercially available kits (Uni-Kit III "Roche" and MA-Kit 100 "Roche," Hoffman-La-Roche, Vienna, Austria). HDL cholesterol was measured by using the TDx REA Cholesterol assay (Abbott, Vienna Austria). LDL cholesterol was calculated by the equation of Friederwald. Lp(a) lipoprotein concentration was determined by the electroimmunodiffusion method using a reagent kit containing monospecific anti-Lp(a) antiserum and the Rapidophor M3 equipment (Immuno AG, Vienna, Austria). The levels of apolipoprotein B and A-I were assessed with an immunoturbidometric method utilizing polyclonal antibodies and a laser nephelometer (Behringwerke AG, Marburg, Germany). We also measured the plasma fibrinogen concentration of study participants according to the Clauss method, using the prescription and reagents of Behringwerke AG. For data analysis we used the means of the baseline and follow-up lipid and fibrinogen values.

Magnetic resonance imaging. MRI was performed on 1.5-T supraconducting magnets (Gyroscan S 15 and ACS, Philips, Eindhoven, The Netherlands) using proton density- and T2-weighted sequences (repetition time [TR]/echo time [TE], 2,000 to 2,500 msec/30 to 90 msec) in the transverse orientation. T1-weighted images (TR/TE, 600/30 msec) were generated in the sagittal plane. The slice thickness was 5 mm and the matrix size was 128 × 256 pixels. At baseline and the 3-year follow-up, the MRI protocols were identical. The scanning plane was always determined by a sagittal and coronal pilot to ensure consistency in image angulation throughout the study. The baseline and follow-up scans of each study participant were read independently by three experienced investigators blinded to the clinical data of study participants. Blinding of the readers for the date of the examinations was impossible because the format of hard copies changed from baseline to follow-up. Only proton density-weighted images were used for WMH reading. WMH were specified and graded according to our scheme[8,25] into absent (grade 0), punctate (grade 1), early-confluent (grade 2), and confluent (grade 3) abnormalities. The number of WMH was recorded and categorized into zero, one to four, five to nine, and more than nine lesions. We disregarded caps and "pencil-thin" periventricular lining because they represent normal anatomic variants.[5,26] Change of WMH in grade and number from baseline was determined by direct scan comparison. The change in number was again categorized into zero, one to four, five to nine, and more than nine lesions. Regression or progression of WMH was then graded as absent, minor, or marked. A change from baseline by one to four punctate lesions was defined as minor. If there was a difference of more than four lesions, or a transition to early-confluent or confluent WMH, the change was considered to be marked.

Examples for minor and marked progression of abnormalities are shown in figures 1 and 2. The final rating of

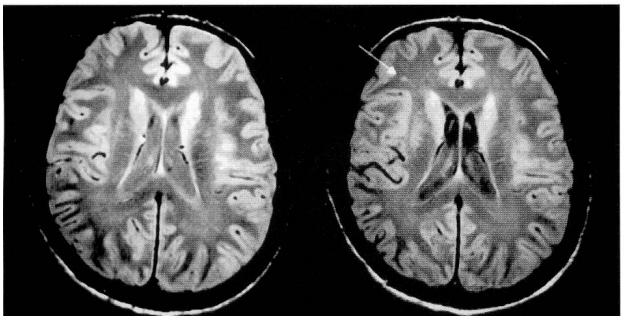

Figure 1. Minor white matter hyperintensity progression in a 59-year-old female study participant. The baseline scan was normal (left). One punctate white matter abnormality in the frontal subcortical white matter (arrow) was noted at the 3-year follow-up examination (right).

WMH evolution relied on the majority judgment of the three assessors. In case of complete disagreement, consensus was found in a joint reading session. During the baseline examination, four participants were found to have silent thromboembolic infarcts and 18 had lacunes, defined as focal lesions involving the basal ganglia, the internal capsule, the thalamus, or the brainstem not exceeding a maximum diameter of 10 mm. These individuals were not excluded from additional study.

Neuropsychological testing. During the baseline and 3-year follow-up, an identical neuropsychologic test battery assessing memory and learning abilities, conceptional reasoning, attention, and speed as well as visuopractical skills was administered to every subject. For tests of memory and learning as well as conceptional reasoning we administered validated parallel forms at follow-up. The tests employed have been widely used in the German-speaking area and were always applied in the same order and under same laboratory conditions. Bäumler's "Lern-und Gedächtnistest"[27] assessed for learning capacity and intermediate memory. It is a highly demanding, paper–pencil procedure and consists of six subtests. Three subtests (word and digit association tasks, and story recall) screen for verbal memory, and two subtests (trail and design recall) screen for visuospatial memory. The sum of weighted scores from these subtests and of an image recognition paradigm result in the total learning and memory performance score. The stimulus sets of the word association task (German–Turkish word pairs), the story (facts about construction of a library), and design recall (core symbol and frame), and the recognition paradigm (objects) consist of 20 items each. A trail in an abstracted city map serves as the trail recall test. These sets of stimuli were presented to the person being tested for 1 minute. Two minutes were given for learning the 13 items of the digit association task (three-digit telephone numbers and names of extension holders). During a learning phase the six sets of stimuli are subsequently presented to the person being tested. The recall phase starts immediately thereafter and follows the same order. The delay between presentation and recall for a given subtest ranges between 7 and 11 minutes. The Wisconsin Card Sorting test[28] was used as a measure of conceptional reasoning. Adhering to Millner's criteria,[29] the measures computed were categories completed, perseverative errors, and total errors. Attention and speed were assessed with the Alters–Konzentrations–Test of Gatterer,[30] form B of the Trail Making Test,[31] the Digit Span Test from the Wechsler Adult Intelligence Scale–Revised,[32] and a complex reaction time task.[33] The Alters–Konzentrations–Test is a cancellation test that has been designed particularly for use in elderly populations. The test is composed of 5 lines with 11 symbols each. The target symbol is a semicircle with the base on the bottom and a black quadrant on the right. Distractors are semicircles that are either positioned differently or positioned with the black quadrant on the left. The persons being tested were instructed to work as quickly and as accurately as possible. The variables used for analysis were the time needed to finish the test and the number of correct responses. The reaction time task was performed on a computerized system that tested the subject's ability to react selectively to a specific combination of a visual and acoustic signal by pressing a button as quickly as possible. The computer records the number of correct responses and the reaction time. Visuopractical skills were evaluated with the Purdue Pegboard Test.[34]

Statistical analyses. We used the Statistical Package of Social Sciences (PC+) (version 8.0.0; SPSS Inc., Chicago, IL) for data analysis. The degree of agreement for WMH rating at baseline and for WMH progression rating among observers was expressed by the means of kappa statistics. According to Fleiss,[35] a kappa value less than 0.40 reflects poor agreement, a value between 0.40 and 0.75 indicates fair to good agreement, and a kappa value higher than 0.75 reflects excellent agreement. The kappa statistic was calculated for the agreement between each pair of raters. Categoric variables among the subgroups of individuals with various degrees of WMH progression were compared using the Mantel–Haenszel test for linear trend. Assumptions of normal distribution for continuous variables were tested by Lillifors statistics. Normally distributed continuous variables were compared with one-way analysis of variance (ANOVA), and the Kruskall–Wallis test was used to compare abnormally distributed variables. Multiple logistic regression analysis was used to assess the relative and independent contribution of demographics, vascular risk factors, and baseline MRI findings on total WMH progression. We simultaneously entered age, sex, and all variables for which the p value was less than 0.10 after univariate testing. The selected factors were used as independent variables, and the presence or absence of WMH progression was used as a dependent variable. Odds ratios and 95% CIs were calculated from the beta coefficients and their standard errors. ANOVA for repeated measures, with

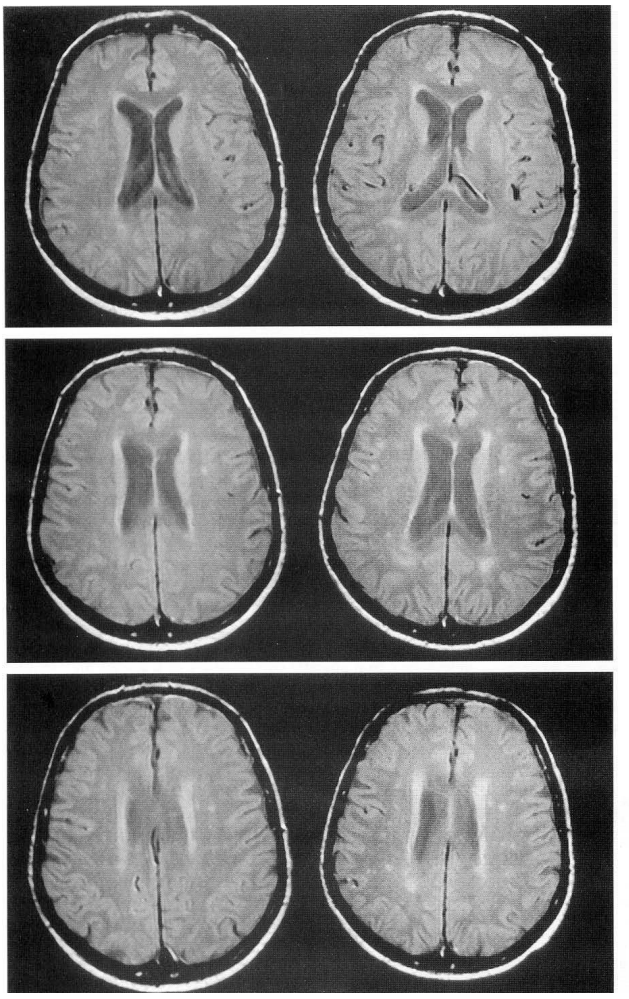

Figure 2. Marked white matter hyperintensity progression in a 67-year-old female study participant. The composite shows baseline scans (left) and the corresponding follow-up studies (right). Several punctate foci were seen at baseline. After 3 years multiple, new, and partly confluent abnormalities occurred.

adjustment for age and duration of education, was applied to evaluate the effect of WMH progression × time on neuropsychological test performance.

Results. At baseline, 176 individuals (64.5%) had WMH. Punctate, early-confluent, and confluent changes were noted in 142 (52.0%), 25 (9.2%), and 9 (3.3%) participants. There were 90 individuals (33.0%) with one to four abnormalities, 36 individuals (13.2%) with five to nine abnormalities, and 50 individuals (18.3%) with more than nine abnormalities. The interrater agreement for WMH grade at baseline ranged from 0.63 to 0.70, and for WMH number it ranged from 0.66 to 0.68. Table 1 shows the frequency and extent of WMH change at follow-up as indicated by each rater and by combined judgment. As can be seen from the table, WMH regression did not exceed four lesions, and there was never a consensus on evidence of regression among raters. Progression occurred by one grade at most. Combined judgment indicated an increase by one to four WMH in 32 individuals (11.7%); more than four lesions developed in 14 study participants (5.2%) throughout the observational period. Overall, any progression in grade or number was noted in 49 study participants (17.9%). Progression was minor in 27 individuals (9.9%) and marked in 22 (8.1%). The kappa values for any progression ranged from 0.52 between raters 1 and 3 to 0.58 between raters 1 and 2. The agreement between raters for marked WMH progression was excellent (kappa range, 0.76 to 0.83)

Table 1 *Frequency and degree of white matter hyperintensity (WMH) change after 3 years*

Variable	Rater 1, n (%)	Rater 2, n (%)	Rater 3, n (%)	Combined, n (%)
WMH grade				
−1	8 (2.9)	1 (0.4)	0	0
0	249 (91.2)	248 (90.8)	254 (93.0)	253 (92.7)
+1	16 (5.9)	24 (8.8)	16 (5.9)	20 (7.3)
WMH number				
−1 to 4	14 (5.1)	2 (0.7)	0	0
0	199 (72.9)	214 (78.4)	222 (81.3)	224 (82.1)
+1 to 4	47 (17.2)	39 (14.3)	31 (11.4)	32 (11.7)
+5 to 9	11 (4.0)	9 (3.3)	4 (1.5)	7 (2.6)
>9	2 (0.7)	9 (3.3)	4 (1.5)	7 (2.6)
WMH progression				
Total	60 (22.0)	57 (20.9)	48 (17.6)	49 (17.9)
Minor	41 (15.0)	33 (12.1)	27 (9.9)	27 (9.9)
Marked	19 (7.0)	24 (8.8)	21 (7.7)	22 (8.1)

whereas there was only poor interrater reliability for rating minor progression (kappa range, 0.29 to 0.41). As shown in table 2, individuals with progressing lesions were older, had higher diastolic blood pressure, higher fibrinogen levels, and demonstrated higher grades and numbers of WMH at baseline MRI. Also, presence of arterial hypertension tended to be more common in these individuals, and they had a trend toward higher systolic blood pressure. When entering these variables simultaneously into a logistic regression model, diastolic blood pressure and evidence of grade 2 or grade 3 WMH at baseline remained the only significant and independent predictors of lesion progression (table 3). The same variables were found to be significantly related to progression when considering only marked progression in the analysis.

Throughout the observational period, test performance improved on the subtest "perseverative errors" ($p = 0.001$) and "total errors" ($p = 0.02$) of the Wisconsin Card Sorting

Table 2 *Selected demographic variables, risk factors, and baseline MRI findings in individuals without, with minor, and with marked white matter hyperintensity progression*

	Progression			
Variable	Absent (n = 224)	Minor (n = 27)	Marked (n = 22)	*p* Value
Age, y, mean ± SD	59.5 ± 6.1	62.2 ± 5.9	62.1 ± 5.5	0.02*
Sex, male, n (%)	118 (52.7)	10 (37.0)	14 (63.6)	0.83†
Hypertension, n (%)	84 (37.5)	10 (37.0)	13 (59.1)	0.09†
Systolic blood pressure, mm Hg, mean ± SD	137.9 ± 18.1	142.6 ± 20.1	146.8 ± 19.0	0.06‡
Diastolic blood pressure, mm Hg, mean ± SD	84.6 ± 8.0	86.2 ± 8.8	89.3 ± 10.3	0.03‡
Fibrinogen, mg/dL, mean ± SD	286.8 ± 65.4	300.0 ± 59.2	303.5 ± 49.9	0.03*
Baseline MRI findings				
Grade, n (%)				
0	87 (38.8)	9 (33.3)	1 (4.5)	
1	117 (52.2)	14 (51.9)	11 (50.0)	
2	16 (7.1)	2 (7.4)	7 (31.8)	
3	4 (1.8)	2 (7.4)	3 (13.6)	<0.00001†
Number, n (%)				
0	87 (38.8)	9 (33.3)	1 (4.5)	
1 to 4	77 (34.4)	8 (29.6)	5 (22.7)	
5 to 9	27 (12.1)	4 (14.8)	5 (22.7)	
>9	33 (14.7)	6 (22.2)	11 (50.0)	0.00001†

* Kruskal–Wallis test.
† Mantel–Haenszel linear trend test.
‡ One-way analysis of variance.

Table 3 *Logistic regression analysis: Predictors of white matter hyperintensity (WMH) progression*

Variable	β	SE	*df*	*p* Value	Odds ratio	95% CI
Age, y	0.048	0.032	1	0.13	1.05/y	0.99–1.12
Sex, male	−0.221	0.337	1	0.51	0.80	0.42–1.53
Hypertension	−0.358	0.452	1	0.43	0.70	0.30–1.65
Systolic blood pressure, mm Hg	−0.007	0.016	1	0.65	0.99/mm Hg	0.96–1.02
Diastolic blood pressure, mm Hg	0.064	0.032	1	<0.05	1.07/mm Hg	1.01–1.14
WMH grade 2 or 3						
At baseline	0.965	0.388	1	0.04	2.62	1.05–6.55
WMH number >4	0.615	0.389	1	0.11	1.85	0.57–5.99

Test whereas it declined on the Trail Making Test because the study participants needed more time to finish the test ($p = 0.02$) during the follow-up examination. Progression of WMH had no influence on the course of cognitive functioning. A subanalysis for only marked WMH progression did not alter the results. (The neuropsychological test results in individuals without and with WMH progression have been filed with the National Auxiliary Publication Service.) The power of the statistical analyses of neuropsychological results was low. The highest value for a given test (Purdue Pegboard Test, preferred hand) was 42%.

Discussion. In our cohort of neurologically asymptomatic middle-age and older individuals, 17.9% of the participants showed a progression of deep and subcortical WMH during a 3-year time period. The progression was minor in 9.9% and marked in 8.1% of individuals. Regression of WMH did not occur. Some bias toward a higher progression rating might have occurred in this study due to the raters' awareness of the time sequence of scans. Unfortunately, blinding for the date of scanning was not possible because the format of hard copies changed between the baseline and the follow-up MRI examinations.

The interrater reliability for a diagnosis of marked progression was excellent, but there existed only poor agreement among the assessors for rating minor progression in the range of one to four punctate foci. It is evident that subtle changes from baseline might be missed more readily by a single evaluator than marked changes. Yet we cannot exclude that in some cases minor progression was assumed wrongly as a result of slight differences in image quality or angulation, even though this was a prospectively designed study with a constant MRI protocol. The low interrater agreement for minor WMH progression should be considered in future intervention trials that plan to use progression of white matter lesions as an outcome measure.

Before our study at least two investigations reported data on WMH progression in small samples.[36,37] One study was published in abstract form and was comprised of 60 healthy elderly. The authors described the mean increase of WMH on a semiquantitative 18-point scale over a 5-year period, but did not report the actual frequency of participants with progressing white matter changes.[36] This made a direct comparison with our results impossible. The second investigation reported that 8 of 14 normal or mild to moderately demented individuals showed an increase in WMH over an observational period of 2 years.[37]

We found that diastolic blood pressure and the extent of WMH at baseline were the only significant and independent predictors of lesion progression. There existed no relationship with age or other major risk factors for stroke. Very similar results have also been reported by the previous investigations on the evolution of WMH in healthy and mild to moderately demented individuals.[36,37] Age was not associated with lesion progression, although virtually all studies on risk factors for WMH found that advancing age is their most important predictor.[1,2,7-13] This is not contradictory, however, because increasing age implies a higher probability of lesion accumulation but does not necessarily affect the the speed of lesion progression.

Multivariate statistical analysis demonstrated that our study participants with early-confluent or confluent abnormalities during the first MRI examination had a 2.6-fold increased risk for additional lesion progression than their counterparts with either normal scans or only punctate changes. The relationship was independent of other major risk factors for stroke, which provides additional evidence for other predisposing factors than those generally held responsible for atherothrombotic brain infarction to play an important role in the pathogenesis and evolution of MRI white matter abnormalities. Our observation is also in line with pathohistologic findings demonstrating that only more extensive abnormalities reflect a true ischemic process, which is likely to progress, whereas punctate foci represent a plethora of minimal cerebral changes that cannot be attributed unequivocally to brain ischemia.[2,5,38] Nonischemic pathologic correlates of punctate WMH are enlarged spaces around arterioles[2,5,37] and venules.[5] In some cases even ganglionic cell heterotopia was noted.[37]

Although most previous investigations on neuropsychological consequences of WMH described subtle cognitive impairment at a higher lesion load,[10,12,16-20] we did not find any association between lesion pro-

gression and cognitive decline in the current study. This applies to all cognitive domains, including attention and speed of mental processing—the two intellectual functions most severely affected in the presence of white matter lesions in normal subjects.[10,12,16,18-20] There was also no association between WMH progression and test performance when we excluded the subset of individuals with only minor progression. Several methodological issues need to be discussed before interpreting these results. The size of the subgroup with WMH progression was small and the variability of neuropsychological test results was considerable, which resulted in an insufficient statistical power to detect small effects. Also, the relatively short time of follow-up in a neurologically normal sample with a low tendency for cognitive decline might have contributed to our negative findings. It is of note, however, that we did not even see a trend toward more pronounced impairment over time on any of the cognitive measures in individuals with lesion progression. It might well be that the extent of abnormalities in study participants with progression was still below the threshold reported to affect cognitive functioning,[19] or that expansion and increasing number of lesions indeed play only a subordinate role for the development of WMH-related neuropsychological dysfunction. Other characteristics of evolving lesions, such as their location, may be much more important. The small size of the study subset with WMH progression precluded a further breakdown of the current cohort. Data pooling from several centers will probably be necessary to allow a more detailed assessment of the association between WMH progression and cognitive functioning.

Note. Readers can obtain 1 page of supplementary material from the National Auxiliary Publications Service, 248 Hempstead Turnpike, West Hempstead, NY 11552. Request document no. 05489. Remit with your order, not under separate cover, in US funds only, $15.00 for photocopies or $5.00 for microfiche. Outside the United States and Canada, add postage of $4.50 for the first 20 pages and $1.00 for each 10 pages of additional material thereafter, or $1.75 for the first microfiche and $1.00 for each fiche thereafter. *There is a $25.00 invoicing charge on all orders filled before payment.*

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Progression of Cerebral White Matter Lesions: 6-Year Follow-Up of the Austrian Stroke Prevention Study

2.2

Progression of cerebral white matter lesions: 6-year results of the Austrian Stroke Prevention Study

Reinhold Schmidt, Christian Enzinger, Stefan Ropele, Helena Schmidt, Franz Fazekas

More than half of all elderly people have some degree of cerebral white matter lesions. However, the rate of progression of these lesions is uncertain. We aimed toassess the progression of lesions in community-dwelling volunteers aged 50–75 years without neuropsychiatric disease. We used MRI to grade and measure the total volume of white matter lesions in 296 volunteers at baseline, 3 years, and 6 years. 58 participants with no lesions and 123 with punctate abnormalities at baseline had a low tendency for lesion progression, whereas 14 participants with early confluent and nine with confluent lesions underwent median increases of 2·7 cm^3 (IQR 0·5–5·9) and 9·3 cm^3 (7·1–21·0), respectively, in lesion volume at 6 years. Lesion grade at baseline was the only significant predictor of lesion progression ($p<0·0001$). Punctate white matter lesions are not progressive and are thus benign, whereas early confluent and confluent white matter abnormalities are progressive, and thus malignant.

Lancet 2003; 361: 2046–48

Some degree of cerebral white matter change is almost endemic in elderly people. Lesions progress,[1–3] but inwhich individuals is unclear. At the extreme end of the spectrum, full-blown dementia corresponds with diffuse demyelination and arteriolosclerosis.[4] We aimed to assess the volume change of white matter lesions during 6 years in a community-dwelling cohort.

We randomly selected 2007 individuals aged 50–75 years without neuropsychiatric disease from our community register. 509 study participants underwent brain MRI at baseline. Follow-up examinations were done at 3 years and at 6 years. Sampling procedure and clinical assessment have been described.2 296 volunteers underwent baseline MRI and at least one follow-up examination. 271 people had a 3-year and 204 a 6-year follow-up assessment. 191 participants had all three MRI examinations. At baseline and at each follow-up 1·5 T-scanners from the same manufacturer (Philips Medical Systems; Eindhoven, Netherlands) and identical protocols were used.

C E identified white matter lesions, drew their outlines onto an overlaid transparency, and graded them into punctate, early confluent, or confluent lesions (figure). Blinding for the date of examinations was impossible since the format of hard cop

ies changed between baseline and follow-up. Follow-up scans were compared with the baseline scan, and the lesions drawn as before. Scan series were reviewed by C E and R S and a consensus reached for borderline grades. The volume of lesions was quantified independently from visual analysis without knowledge of the sequence of the investigation by a trained technician with the DISPImage program (version 4.8), with the hard copy as reference. Maximum intra-rater coefficient of variation was 6·4%. We analysed the scans of 50 randomly selected participants on two separate occasions to establish the error in volumetric assessment.[3] We set the error range as the 95% CI of the most pronounced difference between repeated measurements: –1·59 to 1·81 cm^3. We constructed a generalised estimation equation (GEE) model including a-priori defined demographic and major vascular risk factors to establish predictors of white matter lesion progression. In a second GEE model we added the baseline grade of white matter lesions.

At 3-years' and 6-years' follow-up the median increase of lesion volume was 0 cm^3 (IQR 0–0·3) and 0·1 cm^3 (0–0·7), respectively. Only 25 (9%) and 35 (17%) participants had lesions whose increase in volume exceeded possible measurement error (1·81cm3) at 3-years' and 6-years' follow-up, respectively. All drops in lesion volume were within the range of measurement variability.

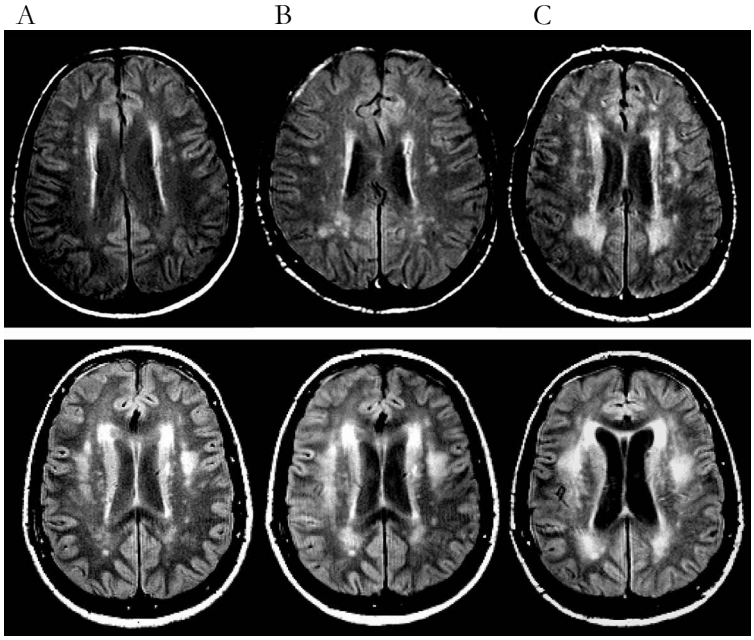
Older age (=0·07, 95% CI 0·02–0·12, p=0·008) and arterial hypertension (=1·14, 0·47–1·81, p=0·001) were significant predictors of white matter lesion progression. There was a non-significant negative relation for diabetes, but there were only 13 people with diabetes in the study. The first model accounted for 10% of the variance in change of lesion volume. The addition of the baseline grade of white matter lesions increased the explained proportion of variance in volume change to 28·5%. In this second model, lesion grade was the only significant predictor of lesion progression (=1·78; 95% CI 1·39–2·12, p<0·0001); the relation with age and hypertension was no longer significant.

The table shows the change in volume from baseline of each grade of lesion. Participants who had no lesions at baseline had negligible changes in volume at both follow-up examinations. In none was progression of lesion load greater than 1·81 cm^3, even after 6 years' follow-up. The change in lesion volume in participants with punctate lesions was only slightly higher than in the no-lesion group. By contrast, individuals with early confluent and confluent lesions at baseline had substantial median increases in lesion volume (table and figure). All participants with confluent lesions had lesion progression beyond measurement variability at 6 years' follow-up.

Our results show that baseline grading of lesions predicts their progression better than age and hypertension and is an intermediate factor in the association between these risk factors and progression. Since few participants had early confluent and confluent lesions our results should be confirmed in larger investigations. Our results might have been affected by a low response rate for enrolment into

the study, a high drop-out rate, and our inability to blind grading assessments for the date of examination.

We have shown previously that punctate white matter lesions often represent widened perivascular spaces without substantial ischaemic tissue damage, whereas early confluent and confluent lesions correspond to incomplete ischaemic destruction, often with focal transition to true infarcts with advanced microangiopathy.[5] Our results show that punctate white matter lesions are not ischaemic, not progressive, and thus benign; whereas early confluent and confluent lesions areischaemic, progressive, and thus malignant. The clinical relevance of white matter lesion progression is unclear. Clinical stability in people with no or punctate lesions and reduction of functional abilities in individuals with coalescent lesions remains to be shown.



Baseline lesion grades in each group (A-C) and change in white matter lesion volume in a 64-year-old woman (D-F) Arrows show punctate (A), early confluent (B), and confluent (C) lesions. Early confluent white matter lesions at baseline (D). In the single slice shown in this figure the volume of lesions increased by 2.6cm^3 between baseline and 3-year follow-up (E) and by 8.1cm^3 between baseline and 6-year follow-up (F).

Baseline white matter lesion grade	None (n=91)	Punctate (n=164)	Early confluent (n=28)	Confluent (n=13)
3-year follow-up				
Participants, number	85 (93%)	149 (91%)	25 (89%)	12 (92%)
Volume change from baseline, cm3, median (IQR)	0·0 (0·0–0·0)	0·1 (0·0–0·4)	0·7 (0·1–3·1)	4·8 (1·9–8·8)
Progression >1·81 cm3, number	0	7 (5%)	9 (36%)	9 (75%)
6-year follow-up				
Participants, number	58 (64%)	123 (75%)	14 (50%)	9 (69%)
Volume change from baseline, cm3, median (IQR)	0·0 (0·0–0·0)	0·2 (0·0–1·1)	2·7 (0·5–5·9)	9·3 (7·1–21·0)
Progression >1·81 cm3, number	0	18 (15%)	8 (58%)	9 (100%)
Change in white matter lesion volume in each group				

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Genetic Determinants of Cerebral Small Vessel disease

3

Genetic Aspects of Microangiopathy-Related Cerebral Damage

3.1

Genetic aspects of microangiopathy-related cerebral damage

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Summary. Microangiopathy related cerebral damage (MARCD) includes early confluent and confluent white matter hyperintensities (WMH) and lacunar lesions. It is expected to be the result of interactions between multiple genetic and environmental factors. The estimated proportion of genetic factors contributing to the interindividual variation seen in WMH volume is 73%. This estimate points to a significant genetic component in WMH development. In the setting of the Austrian Stroke Prevention Study we search for genes being associated with the presence, severity and progression of MARCD using the candidate gene approach. Defining susceptibility genes may allow to better identify individuals at high risk for MARCD and to target preventive measures.

Definition of MARCD

Microangiopathy-related cerebral damage (MARCD) includes early confluent and confluent white matter changes as well as lacunar infarcts. (Schmidt et al., 1997). The definition is based on histopathological findings demonstrating that both of these changes are associated with arteriolosclerosis (Awald et al., 1986; Kirkpatrick and Hayman, 1987; Fazekas et al., 1993; Braffman et al., 1988; Van Swieten et al., 1991). They also share common risk factors and have the highest probability to progress (Schmidt et al., 1999). Punctate WMH which include a plethora of minimal cerebral abnormalities that can not unequivocally be attributed to cerebral ischemia according to pathohistologic correlations (Fazekas et al., 1991) are excluded from this definition. Although MARCD may be recognized in otherwise normal individuals it is likely to become associated with cognitive impairment and gait disturbances as it progresses (Pantoni and Garcia, 1995; Longstreth et al., 1998). So far epidemiological studies could establish arterial hypertension and age as risk factors for this type of brain abnormalities (Van Swieten et al., 1991; Breteler et al., 1994; Schmidt et al., 1997).

Is there evidence that MARCD is influenced by genes?

A recent investigation of Carmelli et al. suggests a substantial genetic background in MARCD. This study on World War II veteran twins has shown a high heritability index ($h^2 = 0.73$) for white matter hyperintensity volume (Carmelli et al., 1998). This means that up to 73% of the interindividual variation seen in WMH volume in this population of twins can be explained by genetic factors. The probandwise concordance rates for extensive white matter lesions, defined as 0.5% of total intracranial volume, were 61% in monozygotic and 38% in dizygotic twins at a prevalence of 15% for the entire study population. This gives a relative risk of 4 for monozygotic and 2.5 for dizygotic twins compared to the risk of the general population. These data show that MARCD as one might expect is a multifactorial disorder. Both genetic and environmental factors influence its presence and severity. Although a heritability index in general should be interpreted cautiously, the high estimate for WMH volume stresses the need for further investigations on genetic factors in relation to these brain changes.

Aim of genetic analyses in complex traits

Identification of genes operating in complex disorders should facilitate our understanding of pathomechanisms leading to these disorders. On the other side genetic epidemiological studies should aim at defining subsets of people in the population particularly susceptible to certain disorders. In this context the presence of interactions between genetic and environmental factors is an important aspect of complex disorders. It is hypothesized that due to interactions the effect of environmental factors will depend on the genetic constitution of individuals. However, this also means, that the effect of genes can be modified by environmental factors. This basic concept has major implications for both preventive and therapeutic measures. Defining individuals susceptible to MARCD based on their inherited factors will help to target prevention and therapy to those who will probably have the highest benefit.

Identification of genes by candidate gene approach

Identification of genetic factors contributing to a complex trait is not an easy task. The presence of incomplete penetrance, phenocopy, genetic heterogeneity and interactions between genes represent the basic problems for investigations. Nonparametric linkage studies (allele sharing methods) on nuclear families and association studies in unrelated individuals are the two approaches already used with success for the identification of genes in such disorders. (For description and comparision of the two methods see reviews published by Lander and Schork, 1994 and by Weeks and Lathrop, 1995.) For

Table 1. Working model of MARCD for candidate gene approach

I. Arteriolosclerosis	II. Brain parenchymal damage
Systemic factors	Vulnerability to injury
— Aging	Ability to repair
— Blood pressure	
— Blood chemistry	
Local factors	
— Vessel wall tone	
— Vessel permeability	

MARCD only association studies have been conducted so far. These studies are most powerful when they examine functional polymorphisms in candidate genes. Selection of candidate genes requires some knowledge about pathomechanisms involved in the examined disorder. Table 1 shows our basic assumption about possible pathways leading to MARCD. We hypothesize that MARCD is initiated by arteriolosclerotic changes in the subcortical vessels leading to ischemic damage in the neighboring brain parenchyma. Susceptibility of an individual to MARCD will depend on the extent of arteriolosclerosis but also on the vulnerability of the brain parenchyma to ischemia and the ability of the brain to repair parenchymal damage. Principally any gene encoding a protein involved in any of these processes can be regarded as a possible candidate gene for MARCD. The effect of genes involved in arteriolosclerosis might be partly mediated by systemic parameters like blood pressure or levels of blood chemicals. Conventional risk factors indicated by epidemiological studies provide a helpful hint for the selection of these candidate genes. However genes which are associated with MARCD independently of the already described or suspected risk factors are at least equally interesting. Genes expressed locally at the site of MARCD by the vessel wall or by the brain parenchyma belong into this group of candidates. Selection of these candidate genes should rather be based on pathohistological or on in vitro studies than on epidemiological studies.

Genetic risk factors for MARCD: Results of the Austrian Stroke Prevention Study

Since 1994 we have built up a DNA bank of the Austrian Stroke Prevention Study participants aiming to identify genetic factors associated with MARCD in the elderly. The first candidate gene we have studied was the apolipoprotein E (apoE) gene.

We found a highly significant positive association between the apoE2 isoform and MARCD (Schmidt et al., 1997). This was the first report on a genetic factor, which may contribute to the development of such brain abnormalities. The observed association was suprising because it was present with

the apoE2 isoform, which is known to be related to a favorable atherosclerosis risk factor profile with reduced total and LDL cholesterol levels.

A possible explanation for the positive association between apoE2 and small vessel disease related brain lesions might be the involvement of apoE2 in arteriolosclerosis. Couderc et al. described higher cerebrovascular morbidity at younger ages associated with the e2 allele (Couderc et al., 1993). These authors suggested a potentiation of the effect of other risk factors like diabetes, hypertension and obesity by the e2 allele. However, in our study the presence of the e2 allele, as expected favorably influenced the lipid profile of study participants and lowered the frequency of cardiac disease without affecting the rate of arterial hypertension and diabetes mellitus. Therefore other mechanisms than those exerted by conventional risk factors must be responsible for the increase of such brain changes in elderly persons with the e2 allele. Greenberg et al. found that both the e4 allele and the e2 allele were associated with cerebral amyloid angiopathy (Greenberg et al., 1998). They proposed a different role for the two alleles, with the apoE4 isoform enhancing amyloid deposition into the vessel wall, and the apoE2 isoform rather leading to vasculopathy. When dividing the study collective into a group of patients with cerebral amyloid angiopathy with or without vasculopathy, the authors found that the frequency of the e2 allel was indeed significantly higher in those with vasculopathy.

ApoE might have various effects on the vessel wall. It reduces foam cell formation, by mediating cholesterol efflux from macrophages (Mahley, 1988; Kruth et al., 1994). It suppresses lipoprotein oxidation and therefore oxidation induced endothelial toxicity (Myata and Smith, 1996). Moreover it inhibits lymphocyte proliferation and may therefore limit inflammation in the vessel wall (Mahley, 1988). ApoE has also been shown to supress platelet aggregation (Riddel et al., 1997). A recent report from Ishigami et al. demonstrated that apoE is able to reduce vascular smooth muscle cell proliferation and migration induced by platelet derived growth factor or oxidised LDL (Ischigami et al., 1998). These findings are particularly interesting since smooth muscle cells seem to play an important role in small vessel disease. The effect of apoE on smooth muscle cells was mediated by MAP kinase. It is yet unknown whether members of the LDL-receptor family, like LRP or VLDL receptor are involved in this process. Also, no isoform specific results are so far available.

Nevertheless, apoE also plays an important role in neuroregeneration and remyelinisation (Snipes et al., 1986; Ignatius et al., 1987; Boyles et al., 1989; Pitas et al., 1987). Mahley suggested that the model of peripheral nerve injury might also apply to a more general model of response to injury and repair taking place in various tissues including the central nervous system (Mahley, 1988) which expresses apoE and its various receptors (Pitas et al., 1987). The reparative process requires the binding of apoE to the LDL-receptor and apoE2 has about a 100 fold reduced binding capacity to this receptor compared to apoE3. If this nerve injury-repair model plays a role in the response of the white matter to ischemic injury then a deleterous role for apoE2 is possible in the development of MARCD.

Interpretation of positive association

A positive association between a genetic marker and a disease must be interpreted with caution. A causal relationship is a tempting hypothesis in case of good candidate genes. However, causality can not be ascertained by association studies. A strong support for causality is if the results can be replicated in different ethnic groups. Complementary studies including immunohistochemistry or cell and molecular biological investigations are needed to explain the pathomechanisms. Other reasons for positive association have to be considered as well. The major alternative is that the observed association is due to linkage dysequilibrium. In this case the investigated polymorphism can be regarded as a marker for the presence of a causal mutation in its vincinity. This causal mutation might be located in a neighboring gene and not necessarily in the gene under study. Positive association due to linkage dysequilibrium is an important finding. It helps to localise a gene of interest to a very short segment on the chromosome. The real problem in association studies is that the positive association might be an artefact due to population admixture. To reduce the risk for this kind of error, an association study should be conducted within genetically homogenous populations. Drawing controls from a subpopulation genetically distinct from the cases may lead to spurious results. Therefore methods using internal controls have been developed (TDT, HRR test) to reduce the risk of these kinds of artefacts.

Acknowledgment

Genetic studies of the Austrian Stroke Prevention Study are supported by Austrian Science Fund, project P 13180 MED.

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Apolipoprotein E Polymorphism and Silent Microangiopathy-Related Cerebral Damage

3.2

Apolipoprotein E Polymorphism and Silent Microangiopathy-Related Cerebral Damage

Results of the Austrian Stroke Prevention Study

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Background and Purpose Microangiopathy-related cerebral damage (MARCD) includes white matter abnormalities and lacunar infarctions and represents a common MRI observation in subjects above 50 years of age. The risk factors of such brain abnormalities are not fully determined. The goal of this study was to determine whether the genetic heterogeneity of apolipoprotein E (apoE) contributes to the occurrence of MARCD.

Methods Brain MRI (1.5 T) was performed in 280 individuals (ages 50 to 75 years) without neuropsychiatric disease randomly selected from the official register of residents of the city of Graz, Austria. All study participants underwent apoE genotyping, carotid Doppler sonography, electrocardiography, echocardiography, and a complete blood chemistry panel. MARCD was defined as evidence of early confluent and confluent white matter hyperintensities or lacunes. Carotid atherosclerosis was graded on a five-point scale ranging from not present (0) to complete occlusion (5).

Results MARCD occurred in 61 individuals (21%). The distribution of apoE genotypes differed significantly between subjects with and without MARCD ($P=.036$). Subjects with such findings more commonly had the $\epsilon2/\epsilon3$ genotype (24.6% versus 10%) at similar frequencies of genotypes containing the $\epsilon4$ allele. The $\epsilon2/\epsilon3$ genotype was associated with lower levels of total cholesterol ($P=.0009$), LDL cholesterol ($P=.00001$), and apolipoprotein B ($P=.00001$). Also, there was a nonsignificant trend toward less cardiac disease. Other major vascular risk factors and carotid abnormalities were similar among the various genotypes. Multiple logistic regression analysis created a model of significant MARCD predictors, including age (odds ratio [OR], 1.1 per year), hypertension (OR, 3.4), and the apoE $\epsilon2/\epsilon3$ genotype (OR, 3.0).

Conclusions These data suggest an association between the apoE $\epsilon2/\epsilon3$ genotype and MARCD despite favorable effects on the lipid profile and cardiac disease. (***Stroke*. 1997;28:951-956.**)

Key Words • apolipoproteins • lacunar infarction • magnetic resonance imaging • stroke prevention • white matter

Apolipoprotein E acts as a ligand for the "remnant" (apoE) and the LDL (apoB/E) receptor and plays a crucial role in maintaining plasma cholesterol homeostasis. It is a polymorphic protein, with apoE $\epsilon2$, $\epsilon3$, and $\epsilon4$ as common isoforms. The catabolism of lipoproteins appears to be modulated by the apoE and apoB/E receptor affinity of apoE. ApoE $\epsilon2$ binds defectively, resulting in receptor upregulation and subsequent decrease of plasma cholesterol. ApoE $\epsilon4$, by contrast, is associated with increasing cholesterol levels because this isoform accelerates hepatic remnant uptake by apoE receptors, thereby downregulating the number of apoB/E receptors.[1]

Previous work has indicated that the apoE $\epsilon4$ allele is associated with early development of coronary heart disease and arteriosclerosis.[2] There exist only three investigations on the importance of the apoE polymorphism for the evolution of cerebral ischemia.[3-5] All of them suggested some role of the genetic heterogeneity of apoE for the occurrence of strokes, but it remained undetermined which genotype carries the highest risk. Two studies observed high frequencies of the $\epsilon4$ allele along with a low frequency of the $\epsilon3$ allele in stroke patients.[3,4] Couderc et al,[5] however, found that it was the $\epsilon2$ allele that may be associated with higher cerebrovascular morbidity at younger ages. These authors suggested a potentiation of other risk factors, including diabetes, hypertension, and obesity, in the presence of the $\epsilon2$ allele as a possible mechanism. In light of these results, we conducted the present investigation to determine whether the apoE polymorphism may also be involved in the development of MARCD, which is a common MRI observation in the elderly and includes white matter abnormalities and lacunar lesions.[6] The predisposing factors of clinically silent MARCD are widely unknown. However, their exploration may hold important preventive implications, since MARCD probably identifies a group of individuals at high risk for clinically overt cerebrovascular disease.[7,8]

Received October 29, 1996; final revision received February 10, 1997; accepted February 25, 1997.

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Subjects and Methods

Subjects and Design

Individuals aged 50 to 75 years and stratified by sex and 5-year age groups were randomly selected from the official register of residents of the city of Graz, Austria. They received a written invitation to participate in the Austrian Stroke Prevention Study, a single-center prospective follow-up study in our community. The study has been approved by the Medical

Selected Abbreviations and Acronyms

apo = apolipoprotein
ECG = electrocardiogram
MARCD = microangiopathy-related cerebral damage
PCR = polymerase chain reaction
WMH = white matter hyperintensities

Ethics Committee of the Karl-Franzens University of Graz. Written informed consent was obtained from all study participants. The rationale and design of the Austrian Stroke Prevention Study have been previously described.[9] Briefly, the objective of the study is to examine the frequency of cerebrovascular risk factors and their effects on cerebral morphology and function in the normal elderly population. The inclusion criteria for the study were no history of neuropsychiatric disease (including previous cerebrovascular disease and dementia) and a normal neurological examination. From a total of 8193 individuals invited between September 1991 and March 1994, a sample of 2794 subjects agreed to participate, with 1998 individuals fulfilling the inclusion criteria. All study participants underwent a structured clinical interview, physical and neurological examinations, three blood pressure readings, ECG, and echocardiography, as well as laboratory testing including blood cell count and a complete blood chemistry panel. Every fourth study participant was then invited to enter phase II of the Austrian Stroke Prevention Study, which included MRI, Doppler sonography, single-photon emission CT, and neuropsychological testing. Mini-Mental State Examination (MMSE)[10] and Mattis' Dementia Rating Scale (MDRS)[11] scores were obtained from all study participants. Since 1993, we also performed apoE genotyping in all phase II attendees. The current study cohort consists of those 280 individuals consecutively selected since 1993 who underwent both brain MRI and apoE genotyping. All but four study participants had MMSE and MDRS scores above 24 and 137, respectively, the cutoff points thought to be indicative of dementia.[10,11]

Vascular Risk Factors

Diagnosis of vascular risk factors was based on the individual's history and appropriate laboratory findings.[12] Arterial hypertension was considered present if a subject had a history of arterial hypertension with repeated blood pressure readings above 160/95 mm Hg or if the readings at examination exceeded this limit. Diabetes mellitus was coded as present if a subject was being treated for diabetes at the time of the examination or if the fasting blood glucose level at examination exceeded 140 mg/dL. Cardiac disease was assumed to be present if there was evidence of cardiac abnormalities known to be a source for cerebral embolism,[13] evidence of coronary heart disease according to the Rose questionnaire[14] or appropriate ECG findings[15] (Minnesota codes I: 1 to 3, IV: 1 to 3, or V: 1 to 2), or if an individual presented signs of left ventricular hypertrophy on echocardiogram or ECG (Minnesota codes III: 1 or IV: 1 to 3). Study participants were asked if they ever smoked and if they currently smoked.

Laboratory Measurements

A lipid status including the level of triglycerides, total cholesterol, and LDL and HDL cholesterol, as well as lipoprotein(a), was determined for each study participant. Thirty minutes after venipuncture, the coagulated blood samples were centrifuged at 1600*g* for 10 minutes, and the serum was transferred to plastic tubes and analyzed within 4 hours. Trigycerides and total cholesterol were enzymatically determined using commercially available kits (Uni-Kit III "Roche" and MA-Kit 100 "Roche," Hoffman-La Roche). HDL cholesterol was measured by the use of the TDx REA cholesterol assay (Abbott). LDL cholesterol was calculated by the equation of Friedewald. The lipoprotein(a) concentration was determined by the electroimmunodiffusion method using a reagent kit containing monospecific anti-lipoprotein(a) antiserum and the Rapidophor M3 equipment (Immuno AG). The levels of apoB and apoA-I were assessed by an immunoturbidometric method utilizing polyclonal antibodies and a laser nephelometer (Behringwerke AG). The plasma fibrinogen concentration of study participants was measured according to the Clauss method using the prescription and reagents of Behringwerke AG.

ApoE Genotyping

High-molecular-weight DNA was extracted from peripheral whole blood using Qiagen genomic tips. ApoE genotyping was done according to the method of Hixson and Vernier.[16] A 244-bp-long fragment of the apoE gene was amplified using the two oligonucleotide primers F6 (5′-TAA GCT TGG CAC GGC TGT CCA AGG A-3′) and F4 (5′-ACA GAA TTC GCC CCG GCC TGG TAC AC-3′). PCR was performed on 0.8 μg of genomic DNA in a buffer containing 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L MgCl2, 10% DMSO, 0.2 mmol/L of each dNTP, 800 ng/μL of each primer, and 2 U of DynaZyime II DNA polymerase (Finnzymes Oy) in a final volume of 50 μL. After 5 minutes at 94°C, amplification was carried out in 30 cycles, each consisting of 1 minute at 94°C, 1 minute at 60°C, and 2 minutes at 72°C. A final step of elongation was performed at 72°C for 10 minutes. Amplification was assessed by electrophoresis of 5 mL of the PCR product on 1.5% agarose gel stained with ethidium bromide. The PCR products (15 mL) were digested with 20 U of Cfo1 (Promega Corp) in the supplied buffer over 3 hours at 37°C. After digestion, samples were electrophoresed on 20% nondenaturating polyacrylamide gel for 1.5 hours at 180 V. Gels were stained with ethidium bromide (1.0 μg/mL) and photographed under UV transillumination. The most common ϵ3 allele is cut by Cfo1 at codon 158, the ϵ4 allele is cut twice by the addition of a second restriction site at position 112, and the less frequent ϵ2 allele lacks either recognition site. The restriction enzyme digestion results in DNA fragments characteristic of the different alleles. The ϵ2 allele results in fragments 91 and 83 bp long; the ϵ3 allele in fragments 91, 48, and 35 bp long; and the ϵ4 allele in fragments 72, 48, and 35 bp long. The 38-bp fragment is common in all alleles. Fragments smaller than 35 bp are no longer precisely seen on the gel. The six different genotypes can be easily determined by the banding pattern of the allele-specific fragments.

Carotid Duplex Scanning

Color-coded equipment (Diasonics, VingMed CFM 750) was used to determine atherosclerotic vessel-wall abnormalities of the carotid arteries. All B-mode and Doppler data were transferred to a Macintosh personal computer for processing and storage on optical disks. The imaging protocol involved scanning of both CCA and ICA in multiple longitudinal and transverse planes and has been previously described.[9] The examinations were performed by one experienced physician. Image quality was assessed and graded as good (CCA and ICA clearly visible and ICA detectable over a distance of >2 cm), fair (CCA and ICA sufficiently visible and ICA detectable over a distance of at least 2 cm), and poor (CCA and ICA insufficiently visible or ICA detectable over a distance of <2 cm). Three examinations were of poor quality and were excluded from further analysis. Measurements of maximal plaque diameter were done in longitudinal planes, and the extent of atherosclerosis was graded according to the most severe visible changes in the CCA and ICA as 0, normal; 1, vessel-wall thickening (>1 mm); 2, minimal plaque (<2 mm); 3, moderate plaque (2 to 3 mm); 4, severe plaque (>3 mm); and 5, lumen completely obstructed.

Magnetic Resonance Imaging

MRI was performed on 1.5-T superconducting magnets (Gyroscan S 15 and ACS, Philips) using proton-density and T2-weighted (repetition time [TR], 2000 to 2500 ms; echo time [TE], 30 to 90 ms) sequences in the transverse orientation. T1-weighted images (TR, 600 ms; TE, 30 ms) were generated in the sagittal plane. 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 WMH and lacunar lesions. WMH were graded according to our scheme as absent, punctate, early confluent, and confluent.[17] Assessment of intrarater variability yielded a value of κ=0.9 for WMH grading.[18] Caps and periventricular lining were disregarded as they probably represent normal anatomic variants.[19,20] Lacunes were focal lesions involving the basal ganglia, the internal capsule, the thalamus, or brain stem not exceeding a maximum diameter of 10 mm. Punctate WMH were not included in the definition of MARCD because these foci represent a plethora of minimal cerebral abnormalities that cannot unequivocally be attributed to cerebral ischemia according to histopathologic correlations.[19]

Statistical Analysis

We used the Statistical Package for Social Sciences (SPSS/PC+) for data analysis. Categorical variables among the different apoE genotypes were compared by χ^2 test. Assumption of normal distribution for continuous variables was tested by Kolmogorov-Smirnov statistics. Comparisons of continuous variables were done with Student's *t* test and one-way ANOVA. Multiple logistic regression was used to assess the relative contribution of apoE genotypes and vascular risk factors in the presence of MARCD. We simultaneously entered age; presence of arterial hypertension, diabetes mellitus, and cardiac disease; plasma fibrinogen levels; and the apoE genotypes to create a model of significant MARCD predictors. The selection of variables other than the apoE genotypes followed a recent study of Pantoni and Garcia,[21] which reviewed previous MRI studies on risk factors for leukoaraiosis. Odds ratios and 95% confidence intervals were calculated from the β coefficients and their standard errors.

Results

The allele frequencies calculated from the genotypes of the 280 study participants were 0.076, 0.81, and 0.11 for the ϵ2, ϵ3, and ϵ4 allele, respectively. The ϵ2/ϵ3, ϵ3/ϵ3, ϵ2/ϵ4, ϵ3/ϵ4, and ϵ4/ϵ4 genotypes were noted in 37 (13.2%), 184 (65.7%), 6 (2.1%), 51 (18.2%), and 2 (0.7%) subjects, respectively. MARCD occurred in a total of 61 study participants (21.8%). Early confluent and confluent WMH were seen in 49 (17.5%) and lacunes in 15 (5.4%) individuals. There were 3 subjects (1.1%) with both types of ischemic brain changes. As can be seen from Table 1, subjects with MARCD were older and more commonly suffered from arterial hypertension than their counterparts without such brain abnormalities. The frequency of MARCD varied significantly among participants depending on their apoE genotype (Table 2). MARCD was twice as commonly encountered in individuals with the ϵ2/ϵ3 than in those with the ϵ3/ϵ3 or ϵ3/ϵ4 genotype. The small number of subjects carrying the ϵ2/ϵ4 and ϵ4/ϵ4 alleles precluded meaningful statistical analyses of these subsets. One of the 6 individuals with the ϵ2/ϵ4 genotype had lacunar lesions, and 1 of the 2 homozygotes for ϵ4 had confluent WMH. The comparison of demographics, vascular risk factors, and duplex scanning results among apoE genotypes is shown in Table 3. As can be seen from this table, the ϵ2/ϵ3 genotype was associated with significantly lower serum concentrations of total cholesterol, LDL cholesterol, and apoB. This group also tended to have less cardiac disease. There were no between-group differences for duplex scanning results. Overall, atherosclerotic plaques were noted in 19 (52.8%), 103 (56.6%), and 32 (62.7%) subjects with the ϵ2/ϵ3, ϵ3/ϵ3, and ϵ3/ϵ4 genotypes, respectively (P=.62). When multiple logistic regression was used to assess the relative contribution of the apoE ϵ2/ϵ3, ϵ3/ϵ3, and ϵ3/ϵ4 genotypes on MARCD occurrence, the apoE ϵ2/ϵ3 genotype was found to be significantly and independently associated with these cerebral abnormalities in addition to age and arterial hypertension (Table 4). No other putative risk factors were found to be significantly related to such brain abnormalities.

TABLE 1. Demographics and Risk Factors in Subjects Without and With MARCD

	MARCD Absent (n=219)	MARCD Present (n=61)	*P*
Age, y	59.9±5.9	63.3±6.0	.0001*
Sex, male, n (%)	108 (49.3)	35 (57.4)	.27†
Hypertension, n (%)	59 (29.9)	34 (55.7)	.00002†
Diabetes, n (%)	10 (4.6)	3 (4.9)	.91†
Cardiac disease, n (%)	87 (39.7)	29 (47.5)	.27†
Ever smoking, n (%)	100 (45.7)	24 (39.3)	.38†
Current smoking, n (%)	39 (17.8)	9 (14.8)	.58†
Triglycerides, mg/dL	144.7±86.4	154.0±94.5	.47*
Total cholesterol, mg/dL	231.2±40.3	224.8±38.0	.26*
LDL cholesterol, mg/dL	153.2±35.5	145.0±33.5	.11*
HDL cholesterol, mg/dL	49.5±14.8	50.1±15.7	.79*
Lipoprotein(a), mg/dL	25.4±28.7	21.4±28.8	.35*
ApoB, mg/dL	145.6±34.6	140.1±33.1	.27*
ApoA-I, mg/dL	171.8±31.7	170.7±29.8	.80*
Fibrinogen, mg/dL	312.6±75.7	318.4±67.5	.77*

*Student's *t* test; †χ^2 test.

Discussion

In our study population, the frequencies of apoE alleles were consistent with expected rates in white populations.[22,23] This also applied for the rate of early confluent and confluent white matter changes, the most common type of MARCD in current investigation. The Cardiovascular Health Study assessed white matter abnormalities in 3301 elderly subjects and found early confluent and confluent lesions in 14.2% of study participants.[24] This is close to the 17.5% rate that we observed. We confirm previous investigations demonstrating that MARCD occurs with advancing age and arterial hypertension.[6,17,25-29] The association between such brain abnormalities and presence of the apoE ϵ2 allele is a novel finding.

Histopathologic studies demonstrated that early confluent and confluent white matter abnormalities represent areas of perivascular demyelination, mild to moderate loss of fibers, and gliosis.[30-32] They

TABLE 2. Frequency of MARCD Among ApoE Genotypes

MARCD	ϵ2/ϵ3 (n=37)	ϵ3/ϵ3 (n=184)	ϵ3/ϵ4 (n=51)	*P**
Absent, n (%)	22 (59.5)	151 (82.1)	40 (78.4)	
Present, n (%)	15 (40.5)	33 (17.9)	11 (21.6)	.009

*χ^2 test.

TABLE 3. Demographics, Risk Factors, and Duplex Results Among ApoE Genotypes

	ε2/ε3 (n=37)	ε3/ε3 (n=184)	ε3/ε4 (n=51)	P
Age, y	61.9±5.8	60.7±6.1	59.5±5.7	.15*
Sex, male, n (%)	18 (48.6)	90 (48.9)	30 (58.8)	.44†
Hypertension, n (%)	15 (40.5)	58 (31.5)	19 (37.3)	.52†
Diabetes, n (%)	1 (2.7)	10 (5.4)	2 (3.9)	.91†
Cardiac disease, n (%)	11 (29.7)	78 (42.4)	24 (47.1)	.35†
Ever smoking, n (%)	12 (32.4)	84 (45.7)	24 (47.1)	.30†
Current smoking, n (%)	5 (13.5)	33 (17.9)	9 (17.6)	.81†
Triglycerides, mg/dL	155.3±89.7	140.1±80.0	160.3±116.0	.50*
Total cholesterol, mg/dL	206.4±37.9	234.2±39.7	234.5±36.5	.0009*
LDL cholesterol, mg/dL	123.8±27.4	156.7±35.1	155.5±31.4	.00001*
HDL cholesterol, mg/dL	52.5±18.4	50.2±14.8	46.7±13.0	.19*
Lipoprotein(a), mg/dL	19.8±21.4	26.3±31.6	23.3±23.8	.65*
ApoB, mg/dL	114.7±24.6	148.1±34.0	154.4±31.7	.00001*
ApoA-I, mg/dL	174.2±34.4	173.6±31.6	165.2±28.5	.22*
Fibrinogen, mg/dL	320.7±76.4	313.9±74.4	302.7±59.7	.48*
Duplex scanning, n (%)‡				
Grade 0	16 (44.4)	68 (37.4)	18 (35.3)	
Grade 1	1 (2.8)	11 (6.0)	1 (2.0)	
Grade 2	11 (30.6)	68 (37.4)	21 (41.2)	
Grade 3	4 (11.1)	25 (13.7)	10 (19.6)	
Grade 4	4 (11.1)	10 (5.5)	1 (2.0)	.50†

*One-way ANOVA; †χ^2 test.
‡Three duplex scans (1 in the ε2/ε3 and 2 in the ε3/ε3 subset) were excluded because of poor quality; no individual had grade 5 abnormalities.

commonly contain central lacunes.[33,34] Several groups of researchers reported that these changes are associated with arteriolosclerosis,[30-32,35] with one study describing a strong correlation between vessel-wall thickness and extent of white matter abnormalities, as would be expected if small-vessel disease is involved in the etiology of such lesions.[36] The common pathogenetic mechanism and the histological similarities between more extensive WMHs and lacunes prompted us to compile the two types of abnormalities for analysis in the present investigation. Punctate foci in the white matter were not considered because they were seen to include a plethora of parenchymal changes that commonly do not relate to ischemia. Nonischemic histopathologic findings associated with punctate white matter foci are enlarged spaces around arterioles but also around venules.[19,30,32] In some cases, even ganglion-cell heterotopia was noted.[19]

The mechanisms leading to an association between the apoE ε2 allele and MARCD are unclear. One of three studies[3-5] on the role of apoE in stroke also more commonly encountered ε2 carriers in patients than in control subjects.[5] The authors suggested potentiation of other cerebrovascular risk factors rather than a direct deleterious effect of the apoE2 isoform to be the cause for their observation. In the present study, we show that the presence of the apoE ε2 allele favorably influenced the lipid profile of study participants and lowered the frequency of cardiac disease without affecting the rate of arterial hypertension and diabetes mellitus. Therefore, mechanisms other than risk factor–dependent ones must be responsible for the increase of MARCD in elderly persons carrying the ε2 allele. Even though we did not find any association between extracranial carotid atherosclerosis and the apoE polymorphism, one cannot exclude that apoE2 exerts a selective atherogenetic effect on intracranial small vessels. A direct atherogenetic role of apoE2 has been suggested by previous investigations, showing an overrepresentation of this isoform associated with lower-limb atheromatosis in the absence of dyslipidemia.[37] Another explanation for the higher prevalence of MARCD in individuals with the ε2/ε3 genotype might be ε2-related impairment of repair mechanisms, particularly of remyelination processes. Immunohistochemical studies in rats have

TABLE 4. Logistic Regression Analysis: Predictors of MARCD

Variable	β	SE	df	P	Odds Ratio	95% Confidence Interval
Age, y	0.11	0.03	1	.0002	1.11	1.05-1.18
Hypertension	1.23	0.33	1	.0002	3.43	1.83-6.44
Diabetes	−0.41	0.71	1	.57	0.67	0.17-2.56
Cardiac disease	0.10	0.34	1	.77	1.10	0.57-2.11
Fibrinogen, mg/dL	−0.0008	0.02	1	.74	1.00	0.99-1.00
ApoE genotype*			2	.04		
ε2/ε3	1.10	0.42	1	.01	3.00	1.35-6.69
ε3/ε4	0.33	0.42	1	.44	1.39	0.62-3.10

*The apoE ε3/ε3 genotype was used as reference for computation of the odds ratios.

shown that apoE is synthesized and secreted in greatly elevated amounts during selective demyelination and remyelination, suggesting that this lipoprotein has a vital function during normal and pathological turnover of myelin cholesterol in the central nervous system.[38-41] We know from postmortem studies that ischemia-related demyelination represents the most common histopathologic substrate of the type of MRI changes seen in our study participants.[25-27] A reparative potential of apoE in demyelinating diseases is also emphasized by observations in patients with multiple sclerosis, who have significant apoE elevations in their cerebrospinal fluid during clinical remission when remyelination occurs.[42] ApoE is thought to participate in the storage of lipids produced by neuronal damage and in the reutilization of the stored lipids during regeneration.[43,44] It transports myelin and cell debris lipids to macrophages and probably also to myelin-producing oligodendrocytes in the vicinity of the injury.[44] Binding of apoE to specific receptors that have been detected on the surface of macrophages and oligodendrocytes is essential for these processes.[45] Since apoE $\epsilon 2$ binds defectively to these receptors, it may inhibit repair mechanisms, which ultimately may result in more extensive parenchymal damage after cerebral ischemia.

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MRI Cerebral White Matter Lesions and Paraoxonase PON1 Polymorphisms

3.3

MRI Cerebral White Matter Lesions and Paraoxonase *PON1* Polymorphisms

Three-Year Follow-Up of the Austrian Stroke Prevention Study

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Abstract—White matter lesions (WMLs) on magnetic resonance imaging (MRI) scans of older persons are thought to be caused by cerebral small-vessel disease. As they progress, these brain abnormalities frequently result in cognitive decline and gait disturbances, and their predictors are incompletely understood. Genetic risk factors have been implicated but remain undetermined so far. We examined whether 2 common polymorphisms of the paraoxonase (*PON1*) gene leading to a methionine (*M* allele)–leucine (*L* allele) interchange at position 54 and an arginine (*B* allele)–glutamine (*A* allele) interchange at position 191 are associated with the presence and progression of WMLs. We studied 264 community-dwelling subjects without neuropsychiatric disease (ages 44 to 75 years). All underwent vascular risk factor assessment, brain MRI, and *PON1* genotyping. MRI scanning was repeated after 3 years. The extent and number of WMLs were recorded by 3 independent readers. Progression of WMLs was assessed by direct scan comparison. The final rating relied on the majority judgment of the 3 readers. The *LL*, *LM,* and *MM* genotypes were noted in 111 (42.0%), 118 (44.7%), and 35 (13.3%) subjects, respectively; the *AA, AB,* and *BB* genotypes occurred in 146 (55.3%), 98 (37.1%), and 20 (7.8%) individuals, respectively. Carriers of the *LL* genotype showed a nonsignificant trend toward more extensive WMLs and more frequently demonstrated lesion progression over the 3-year observation period ($P=0.03$). The polymorphism at position 191 had no effect. Logistic regression analysis yielded age (odds ratio, 1.08/y), diastolic blood pressure (odds ratio, 1.05/mm Hg), and *LL* paraoxonase genotype (odds ratio, 2.65) to be significant predictors of WML progression. These data suggest that the *LL PON1* genotype at position 54 influences the extent and progression of WMLs in elderly subjects. **(*Arterioscler Thromb Vasc Biol.* 2000;20:1811-1816.)**

Key Words: white matter lesions ■ cerebral small-vessel disease ■ paraoxonase ■ genetics

Magnetic resonance imaging (MRI) shows cerebral white-matter lesions (WMLs) in a large proportion of individuals above the age of 50 years.[1] Histopathological studies have demonstrated that these changes occur in the presence of arteriolosclerosis and are correlated with widening of the perivascular spaces, perivascular demyelination, or lacunar infarcts.[2] Such abnormalities may be recognized in otherwise-normal individuals but are likely to become associated with cognitive impairment and gait disturbances as they progress.[1] Identification of individuals prone to the development and progression of WMLs is important, because early control of causal factors in high-risk groups could reduce their clinical consequences, which are a major source of disability in the elderly population. So far, it is unclear which factors other than advancing age[1,3,4] and arterial hypertension[1,5,6] predispose individuals to this type of ischemic brain damage. A significant contribution of genetic influence has only recently been demonstrated in a US study on World War II veteran twins.[7] That investigation reported a probandwise concordance rate for severe WMLs of 61% in monozygotic twins and of 38% in dizygotic twins compared with a prevalence rate of 15% for the entire population. The estimated heritability of WML volume was 73%.

The strong association of WMLs with aging and a previous study of our own demonstrating an inverse relationship between lesion extent and plasma levels of antioxidants[8] suggest that genes involved in oxidative defense could play a role in the etiology of such brain abnormalities. Free-radical formation increases significantly with aging,[9] and increased lipid peroxidation and oxidative stress due to excess free-radical activity and impaired antioxidant defenses have been associated not only with large- but also with small-vessel disease,[10,11] the most likely cause of WMLs in the elderly.[2] Paraoxonase has antioxidative potential[12] and could thus protect against both macrovascular and microvascular diseases, even though they represent distinct vascular pathologies. So far, there have been several publications relating paraoxonase to pathological phenotypes of large-vessel dis-

Received September 27, 1999; revision accepted January 20, 2000.

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ease, such as coronary heart disease[13–15] and carotid atherosclerosis[16]; a single study found an association with small-vessel disease–related diabetic retinopathy.[17] The paraoxonase gene is located at q21 to q22 on the long arm of chromosome 7.[18] The ability of paraoxonase to detoxify organophosphorus compounds has been known for years, and its enzyme activity had been determined earlier by the use of the pesticide paraoxon. White populations have a triphasic distribution of paraoxonase activity toward paraoxon,[19] which is caused by an amino acid substitution at position 191. Glutamine (*A* allele) is replaced by arginine (*B* allele) in its high-activity isoform.[18] The *B* allele has been shown to be associated with coronary heart disease.[13–15] Another frequent polymorphism at position 54 leads to a methionine (*M* allele)–leucine (*L* allele) interchange.[18] The 2 polymorphisms are in linkage disequilibrium with leucine at position 54, giving rise to arginine at position 191.[18] We explored whether these genetic variants influence the occurrence and progression of WMLs in a large cohort of randomly selected, community-dwelling middle-aged and elderly individuals who have been followed up over a time period of 3 years.

Methods

Individuals and Study Design

The study population consisted of participants in the Austrian Stroke Prevention Study. The rationale and design of this study have been previously described.[20] In brief, 1998 participants without a history or signs of neuropsychiatric disease were randomly selected from the official community register. They underwent 3 blood pressure readings, an ECG, and echocardiography, as well as laboratory testing including blood cell count and a complete blood chemistry panel. Every fourth, or in case of refusal, the next, study participant was invited to enter phase 2 of the study, which included MRI and Doppler sonography. From a total of 498 phase 2 participants, 458 volunteered to undergo an MRI study. At the second study panel, 3 years after baseline, we were able to contact 386 participants of the original MRI sample or their proxies. Seven subjects had died and 7 had experienced a stroke, which was an end point in our study. There were 27 subjects who did not want to undergo the extensive diagnostic work-up a second time. A total of 21 individuals had moved from the city, and 51 subjects could not be reached on the occasion of 3 phone calls and did not respond to a written invitation. The remaining 345 phase 2 attendees agreed to be reexamined, but 72 individuals refused to have a second MRI scan because they had experienced claustrophobic sensations at the initial evaluation. Blood sampling for DNA extraction was done in all but 9 individuals. The current study cohort therefore comprises those 264 participants who underwent MRI scanning at baseline and at the 3-year follow-up and assessment of the *PON1* polymorphisms. There were 128 women and 136 men. The mean±SD age was 59.9±6.1 years (median, 60.0 years) at baseline. The sample consisted exclusively of white subjects of central European origin; the length of education ranged from 9 to 18 years, with a mean of 11.7 years. The individuals who participated in the follow-up MRI study did not differ from those who dropped out in terms of age, sex, educational and occupational status, and risk factors for stroke. This 3-year follow-up of the Austrian Stroke Prevention Study is not prospective in design because no data on *PON1* polymorphisms for 25% of the original group of subjects who volunteered to undergo a first MRI were available.

Vascular Risk Factors

Historical information and laboratory findings at baseline and follow-up were considered for diagnosis of arterial hypertension, diabetes mellitus, and cardiac disease, including embologenic abnormalities, coronary heart disease, and left ventricular hypertrophy. We also assessed smoking habits and body mass index (BMI). Lipid status including the levels of plasma triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol, and Lp(a) lipoprotein, as well as measurement of plasma fibrinogen, was determined for each study participant at both examinations. A detailed description of the definitions of risk factors and of the laboratory methods used has been published previously.[16] The means of systolic and diastolic blood pressures, fasting blood sugar, BMI, lipid, and fibrinogen values on baseline and follow-up measurements were calculated and used for data analyses.

Isolation of DNA and Genotype Analysis

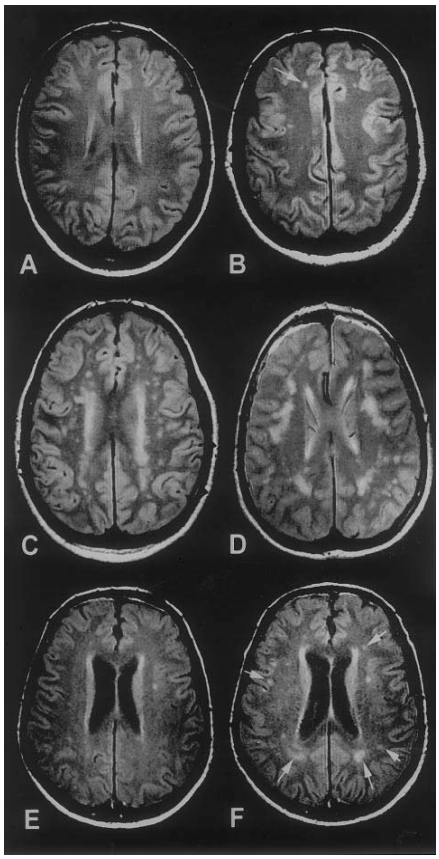
High-molecular-weight DNA was extracted from peripheral whole blood by using Qiagen genomic tips (Qiagen Inc) according to the protocol supplied by the manufacturer. Genotyping of the Met-Leu54 polymorphism was done by polymerase chain reaction (PCR) amplification of a 170-bp-long fragment and using the primers described by Humbert et al.[18] The PCR products were cleaved by *Nla*III in the presence of BSA at 37°C for 3 hours. The digested products were analyzed on a 15% polyacrylamide gel, stained with ethidium bromide, and examined under UV transillumination. The *L* allele corresponded to the nondigested 170-bp-long fragment, whereas the *M* allele corresponded to 126- and 44-bp fragments. A similar protocol was used for genotyping the Gln-Arg191 polymorphism and also using the primers described by Humbert et al.[18]

Magnetic Resonance Imaging

MRI was performed on 1.5-T superconducting magnets (Gyroscan S 15 and ACS, Philips) with proton-density and T2-weighted (repetition time [TR]/echo time [TE], 2000–2500/30–90 ms) sequences in the transverse orientation. T1-weighted images (TR/TE, 600/30 ms) were generated in the sagittal plane. Slice thickness was 5 mm and the matrix size used was 128×256 pixels. The MRI protocols at baseline and at the 3-year follow-up were identical. The scanning plane was always determined by a sagittal and coronal pilot to ensure consistency in image angulation throughout the study. The baseline and follow-up scans of each study participant were read independently by 3 experienced investigators who were blinded to the clinical data of study participants. Blinding of the readers for the date of the examinations was impossible because the format of hard copies had changed from baseline to follow-up. According to our scheme, WMLs included abnormalities in the subcortical region and deep white matter as well as irregular periventricular lesions extending into the deep white matter.[3] They were graded into absent (grade 0), punctate (grade 1), early confluent (grade 2), and confluent (grade 3) abnormalities.[3] The number of WMLs was recorded and categorized into 0, 1 to 4, 5 to 9, and >9 lesions. κ Values for interrater agreement regarding WML grade at baseline and at 3 years ranged from 0.63 to 0.70 and from 0.66 to 0.68, respectively, in regard to the number of lesions. We disregarded caps and pencil-thin periventricular linings because they represent normal anatomic variants.[21,22] We also disregarded a smooth "halo" surrounding the lateral ventricles because it is nonischemic in etiology, according to histopathological correlations.[21] A change of WMLs in grade or number from baseline was determined by direct scan comparison. The final rating of WML progression relied on majority judgment of the 3 assessors. In case of complete disagreement, consensus was found in a joint reading session. The interrater agreement for WML progression ranged from 0.58 to 0.68. The Figure displays examples for each WML grade and for WML progression. We also recorded lacunes. They were defined as focal lesions isointense to cerebrospinal fluid and involving the basal ganglia, the internal capsule, the thalamus, or the brain stem and not exceeding a diameter of 10 mm.

Carotid Artery Duplex Scanning

Color-coded equipment (Diasonics, Vingmed CFM 750) was used to determine vessel wall abnormalities of the carotid arteries in all participants. The imaging protocol, grading of atherosclerotic changes, and the associations between duplex findings and paraoxonase genotypes in our study population have been reported previously.[16] In the current study, we describe the presence of atherosclerotic changes among the genotype subsets and adjust for this variable when assessing the influence of the paraoxonase polymorphisms on WMLs.



Examples for each WML grade (A to D) and for WML progression (E, F). A, Grade 0=absent; B, grade 1=punctate (arrow); C, grade 2=early confluent; and D, grade 3=confluent lesions. E, Baseline scan in a 67-year-old study participant, showing few punctate white matter foci in the centrum semiovale. F, Follow-up study in the same subject after 3 years, demonstrating multiple, new, and partly confluent white matter changes (arrows).

Statistical Analysis

We used the Statistical Package for the Social Sciences (SPSS 8.0) for data analysis. Categorical variables among the paraoxonase genotypes were compared by the χ^2 test. Assumptions of a normal distribution for continuous variables were compared by Lilliefors statistics. Normally distributed continuous variables were compared by 1-way ANOVA, whereas the Kruskal-Wallis test was used for comparison of nonnormally distributed variables. Allele frequencies were calculated by the gene counting method, and Hardy-Weinberg equilibrium was assessed by the χ^2 test. The relative contribution of the paraoxonase genotypes to the presence of WMLs at baseline and to WML progression at the 3-year follow-up was assessed by multiple logistic regression analysis. Forward-selection stepwise regression analysis was used to create a model of independent predictors of MRI findings. At each step, each variable not yet in the model was assessed for its contribution to the model, with the most significant variable to be added. This process continued until no further variable made a significant ($P<0.05$) contribution to the model. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated from the β coefficients and their SEs.

Results

The *LL*, *LM,* and *MM* genotypes were noted in 111 (42.0%), 118 (44.7%), and 35 (13.3%) individuals, respectively. The *AA*, *AB*, and *BB* genotypes occurred in 146 (55.3%), 98 (37.1%), and 20 (7.8%) individuals, respectively. These frequencies are similar to those reported in other European populations.[18,23] The genotypes of both polymorphisms were in Hardy-Weinberg equilibrium. There existed a moderate association between the 2 polymorphisms, with arginine at position 191 being, with 1 exception, always concurrent with leucine at position 54.

Table 1 compares demographic variables and risk factors among the *LL*, *LM,* and *MM* genotype subsets. Individuals with the *MM* genotype had slightly higher fasting glucose levels than did those with the *LM* or *LL* genotype, and there was a nonsignificant trend for lower triglyceride levels in *LM* carriers. These between-group differences diminished further after correction for antidiabetic and lipid-lowering treatment. There existed no significant difference among the *AA*, *AB*, and *BB* genotype subsets when the demographic variables and risk factors listed in Table 1 were compared (data not shown). Duplex scanning showed atherosclerotic changes of the carotid arteries in 71 (64.0%) subjects with the *LL* genotype but in only 56 (47.5%) *LM* and 18 (51.4%) *MM* carriers ($P=0.04$). Carotid artery abnormalities were also seen in 81 (55.5%) *AA*, 51 (52.0%) *AB*, and 13 (65.0%) *BB* carriers ($P=0.56$).

At baseline, 171 (64.8%) participants had WMLs on MRI. After 3 years, progression of abnormalities had occurred in 47 (17.8%) subjects. Regression of abnormalities was never observed by the majority of readers. A breakdown of WML findings by paraoxonase genotypes is given in Table 2. As shown in the Table, the *PON1* polymorphisms had no significant influence on baseline results. Yet subjects homozygous for the *L* allele at position 54 showed a trend toward higher grades of WMLs at the initial MRI examination than did their counterparts with either the *LM* or *MM* genotype. Progression of lesions over 3 years occurred at a significantly higher frequency in the *LL* genotype subset. There was a significant association between WML findings at baseline and the presence of carotid atherosclerosis ($P=0.017$). The association was mainly due to a higher frequency of grade 2 or 3 WMLs in subjects with carotid changes than in those with a normal sonographic examination (5.9% versus 16.6%). Progression of WMLs was not related to the presence of carotid atherosclerosis. At baseline, lacunes were found in a total of 17 subjects and were always seen in the basal ganglionic/thalamic region. At the 3-year follow-up, new lacunes had evolved in 8 subjects. There was no significant difference in the distribution of subjects with lacunes at baseline and with evolving lacunes at follow-up among the genotypes, but the numbers in the comparative subsets were small.

Logistic regression analysis yielded an unadjusted OR of 2.38 (95% CI, 1.25 to 4.53; $P=0.008$) for progression of WMLs in the *LL* genotype relative to the 2 other genotypes. The OR after adjustment for age and sex was 2.55 (95% CI, 1.32 to 4.96; $P=0.005$). Evaluation for the effect of the

TABLE 1. Demographics and Risk Factors Among Paraoxonase Leu-Met54 Genotypes

Variable	*LL* (n=111)	*LM* (n=118)	*MM* (n=35)	*P*
Age, y	59.6±5.9	60.1±6.4	60.4±5.6	0.73*
Sex, male, n (%)	55 (49.5)	61 (51.7)	20 (57.1)	0.74†
Hypertension, n (%)	46 (41.4)	39 (33.1)	17 (48.6)	0.19†
Diabetes, n (%)	7 (6.3)	5 (4.2)	3 (8.6)	0.58†
Fasting glucose, mg/dL	95.3±20.9	92.4±14.2	101.8±30.1	0.07*
Cardiac disease, n (%)	50 (45.0)	47 (39.8)	13 (37.1)	0.61†
Smoking status				
Never, n (%)	66 (59.5)	64 (54.2)	20 (57.1)	...
Former, n (%)	34 (30.6)	37 (31.4)	11 (31.1)	...
Current, n (%)	11 (9.9)	17 (14.4)	4 (11.4)	0.87†
BMI, kg/m^2	27.0±3.6	26.6±3.8	27.4±4.4	0.60*
Triglycerides, mg/dL	161.6±142.9	129.3±61.0	151.0±71.3	0.06‡
Cholesterol, mg/dL	233.9±39.5	233.3±37.4	227.7±35.9	0.69‡
LDL cholesterol, mg/dL	151.3±36.0	154.2±34.2	147.7±30.5	0.59‡
HDL cholesterol, mg/dL	53.3±16.9	53.1±15.0	48.7±15.5	0.22*
Lipoprotein(a), mg/dL	25.0±28.8	23.1±25.0	28.9±33.1	0.88*
ApoB, mg/dL	137.4±31.5	133.7±27.9	137.8±33.0	0.60‡
ApoA-I, mg/dL	178.4±31.9	177.0±29.0	173.1±32.0	0.68‡
Fibrinogen, mg/dL	283.7±54.4	293.9±69.4	286.2±60.3	0.64*

*Kruskal-Wallis test; †χ^2 test; ‡1-way ANOVA.

Gln-Arg191 polymorphism demonstrated that WML progression occurred more commonly in *BB* carriers, but these differences with respect to the *AA* and *BB* genotypes were nonsignificant. The Gln-Arg191 polymorphism did not modulate the effect of the *LL* genotype on WML progression because lesion progression was seen at almost identical frequency in 11(23.9%) subjects in the *LL*/*AA* group and in 5 (26.3%) individuals in the *LL*/*BB* group. When we used forward stepwise regression analysis to create a model of predictors of WML progression, the *LL* genotype remained in this model in addition to age and diastolic blood pressure. Age entered the model first, the *LL* genotype second, and diastolic blood pressure third (Table 3). All other variables, including sex, BMI, systolic blood pressure, fasting glucose level, diabetes, smoking status, cardiac disease, blood lipids, plasma fibrinogen level, and carotid atherosclerosis did not enter the model. When carotid atherosclerosis was forced into the model, the OR for the association between the *LL* genotype and WML progression did not materially change.

Discussion

We found that homozygosity for the *L* allele at position 54 tended to be associated with the extent of WMLs at baseline and predicted WML progression in addition to advancing age and diastolic blood pressure. As in the Cardiovascular Health Study,[24] we have seen an association between WMLs and

TABLE 2. Paraoxonase Genotypes and MRI WMLs: Baseline Findings and 3-Year Progression

	LL (n=111)	*LM* (n=118)	*MM* (n=35)	*P**	*AA* (n=146)	*AB* (n=98)	*BB* (n=20)	*P**
WML grade, n (%)								
0	38 (34.2)	44 (37.3)	11 (31.4)		54 (37.0)	32 (32.7)	7 (35.0)	
1	52 (46.8)	66 (55.9)	22 (62.9)		78 (53.4)	52 (53.1)	10 (50.0)	
2	16 (14.4)	6 (5.1)	2 (5.7)		11 (7.5)	11 (11.2)	2 (10.0)	
3	5 (4.5)	2 (1.7)	0	0.08	3 (2.1)	3 (3.1)	1 (5.0)	0.93
WML number, n (%)								
0	38 (34.2)	44 (37.3)	11 (31.4)		54 (37.0)	32 (32.7)	7 (35.0)	
1–4	31 (27.9)	41 (34.7)	14 (40.0)		53 (36.3)	29 (29.6)	4 (20.0)	
5–9	22 (19.8)	16 (13.6)	5 (14.3)		20 (13.7)	18 (18.4)	5 (25.0)	
>9	20 (18.0)	17 (14.4)	5 (14.3)	0.70	19 (13.0)	19 (19.4)	4 (20.0)	0.46
Progression, n (%)								
Absent	83 (74.8)	104 (88.1)	30 (85.7)		120 (82.2)	83 (84.7)	14 (70.0)	
Present	28 (25.2)	14 (11.9)	5 (14.3)	0.03	26 (17.8)	15 (15.3)	6 (30.0)	0.29

*χ^2 test.

TABLE 3. Final Logistic Regression Model of WML Progression

Variable	β	SE	df	P	OR	95% CI
Age, y	0.08	0.03	1	0.005	1.08/y	1.02, 1.15
LL genotype	0.97	0.34	1	0.004	2.65	1.35, 5.18
Diastolic blood pressure, mm Hg	0.05	0.02	1	0.02	1.05/mm Hg	1.01, 1.09

carotid atherosclerosis at baseline, with carotid atherosclerosis being common among *LL* carriers. Yet the relationship between the Leu-Met54 polymorphism and WML progression occurred independently of extracranial carotid disease. We failed to detect a significant association between the Gln-Arg191 polymorphism and WML progression, although progression was more common in *BB* than in *AB* and *AA* carriers. The frequency of lesion progression in subjects with the combination of the *LL*/*BB* genotypes was virtually identical to that in the study participants with the combined *LL*/*AA* genotypes. This indicates that the Gln-Arg191 polymorphism has no effect on the natural course of WMLs per se and does not modulate the effect of the *L* allele.

In line with these results, Garin et al[25] found that the Leu-Met54 polymorphism is of central importance to paraoxonase function because it influences the serum activity and concentration of the enzyme, whereas the 191 variant has only little effect. This finding, together with the inconsistent results of other studies on the association between the Gln-Arg 191polymorphism and coronary heart disease in genetically distinct populations,[26] suggests that the *PON1* polymorphism at position 191 is not causal but rather may be in linkage disequilibrium with a functional sequence variant in the vicinity. Whether the Leu-Met54 polymorphism represents this variant cannot be elucidated in allelic association studies. *PON1* belongs to a multigene family including *PON2* and *PON3* at the same locus on chromosome 7.[27] Two polymorphisms in the *PON2* gene have only recently been described. Their functional importance is not yet fully determined, but like the *PON1* polymorphisms, they were linked to coronary heart disease.[28]

Several studies support the role of paraoxonase in atherogenesis. The enzyme is linked to HDL and may be partly responsible for the antioxidative effect of this lipid fraction.[12,19] Paraoxonase decreases lipid peroxide accumulation on LDL,[29] a process that takes place in the subendothelial space.[30] In line with this presumed location of action, paraoxonase was found to be present in interstitial fluid in an enzymatically active form.[31] Results in *PON1* knockout mice have demonstrated that HDL from PON1-deficient mice is unable to prevent LDL oxidation in a coculture model of the arterial wall.[32] Paraoxonase immunoreactivity is seen in atherosclerotic lesions, and its intensity increases with their progression.[33] There exists much less information on the association between paraoxonase and microvascular disease, which is the most likely cause of WMLs.[2] Yet a study of Kao et al[17] investigated the role of *PON1* polymorphisms in small-vessel disease–related diabetic retinopathy and found that the *LL* genotype at position 54 was associated with this condition, whereas there existed no effect of the Gln-Arg191 polymorphism. These results are consistent with our findings on WMLs. Importantly, in the context of our study results, Primo-Parmo et al[27] reported PON1 expression in adult mouse brain and described sequence homologues isolated from a postnatal human brain cDNA library.

Conceivably, the functional significance of the *PON1* polymorphism at position 54 is due to its effect on enzyme activity and concentration caused by altered gene expression.[34] Unfortunately, we have no frozen serum from our study participants and therefore could not measure these variables in our study group.

In summary, our data suggest a moderate modulatory effect of the Leu-Met54 polymorphism of the PON1 gene on the extent and progression of small-vessel disease–related MRI WMLs. Identification of individuals at risk for an unfavorable evolution of these brain changes is important, because increasing lesion load commonly results in cognitive decline and other neurological signs and symptoms such as gait disturbances and a tendency to falls.[1,35]

Acknowledgment

The study was funded by the Austrian Science Fund, project P13180-MED.

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The Role of Angiotensinogen in Cerebral Small Vessel Disease

3.4

Angiotensinogen Gene Promoter Haplotype and Microangiopathy-Related Cerebral Damage

3.4.1

Angiotensinogen Gene Promoter Haplotype and Microangiopathy-Related Cerebral Damage

Results of the Austrian Stroke Prevention Study

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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, −218g), 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.[1,2] 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.[3,4] 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.[2–4] 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%.[5] 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.[6,7]

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.[8] Positive correlation between plasma AGT concentration, RAS activity, and blood pressure in humans and in animal models supports this assumption.[8–11] Production of AGT in the liver is regulated mainly at the transcriptional level.[11,12] Two common polymorphisms in the promoter region at position −6:g→a and −20:a→c have been previously described and were shown to alter the transcriptional efficiency of the AGT gene.[11–16] Genetic linkage between the AGT locus and essential hypertension has been repeatedly reported,[9,17] but there are also studies in Chinese and Finnish

Received July 10, 2000; final revision received October 5, 2000; accepted November 8, 2000.

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populations that do not confirm these results.[18–20] Similarly, conflicting data have been described for the association between the T235 AGT gene variant and hypertension.[9,21–27] 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 ± 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.[28]

DNA Analysis

Genomic DNA extracted from peripheral blood was polymerase chain reaction (PCR) amplified with the following oligonucleotides: AGTPROM5: 5′-GC-Clamp-CTTGGCCCCGACTCCTGCAAACT-3′ and AGTPROM3: 5′-CCCCCGGCTTACCTTCTGCTGTA-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.[29] 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.[30] Caps and periventricular lining were disregarded because they probably represent normal anatomic variants.[31,32] 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 κ 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.[32]

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 χ^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 β 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

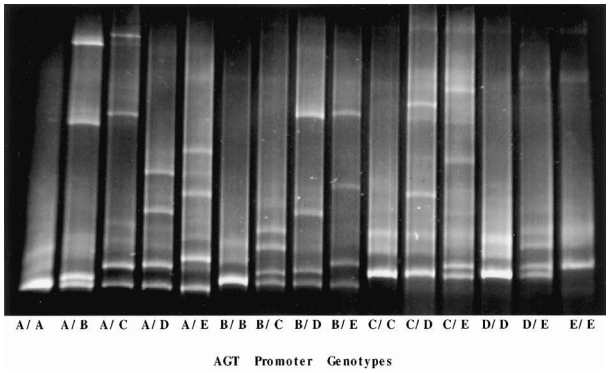

Figure 1. TTGE banding patterns of the 15 AGT promoter genotypes related to the haplotypes. A detailed description of TTGE conditions is given in Subjects and Methods.

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 (χ^2_{obt}=2.7, P>0.99; χ^2_{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 χ^2_{obt} and probability value (−6: χ^2_{obt}=0.05, P>0.95; −20: χ^2_{obt}=0.12, P>0.90; −153: χ^2_{obt}=0.25, P>0.98; −218: χ^2_{obt}=0.44, P>0.95; χ^2_{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.[33]

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

	Haplotypes				
Position	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±5.7 years versus 59.4±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±22.8 versus 136.9±19.3 mm Hg; P=0.004) and diastolic (87.6±9.6 versus 85.1±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			*P**	*P*†
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	…

*χ^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.

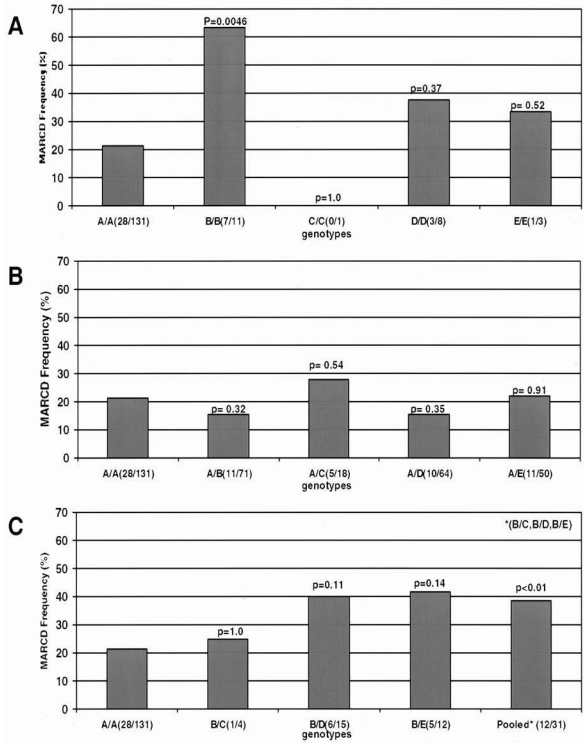

Figure 2. Frequency of MARCD among the AGT promoter genotypes reconstructed from the haplotypes. In each panel MARCD frequencies are compared with wild-type A homozygotes. A, Comparison with homozygotes for the B, C, D, and E haplotypes. B, Comparison with heterozygote A haplotype carriers. C, Comparison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

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

Variable	Genotypes RC (n=368)	B+/A− (n=31)	B/B (n=11)	*P*
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^2	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.
†χ^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 ≤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,[16] 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,[13–16] 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 dysequilibrium. 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 dysequilibrium, 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.[9–27] 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.[34] It is noteworthy that a strong linkage dysequilibrium 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,[35] 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.[36,37] 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.[38–43] Notably, MARCD was found to be related not only to hypertension but also to intermittent hypotensive episodes.[6,7] Ang II promotes vascular smooth muscle cell hyperplasia and hypertrophy.[44–48] It was shown to enhance the activity of NADH/NADPH oxidase and extracellular superoxide dismutase activity in the vessel wall.[49,50] It is also thought to alter the production of extracellular matrix proteins in the vessels.[51,52] 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.

Acknowledgments

This study was supported by the Austrian Science Foundation, project P13180-med (to Dr R. Schmidt), the Jubileumsfond of the Austrian National Bank, project 7776 (to Dr H. Schmidt), and the Franz Lanyar Foundation of Karl-Franzens University (to Dr H. Schmidt). The excellent technical assistance of Johann Semmler and Anita Gradert is appreciated.

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Microangiopathy-Related Cerebral Damage and Angiotensinogen Gene: from Epidemiology to Biology

3.4.2

Microangiopathy-related cerebral damage and angiotensinogen gene: from epidemiology to biology

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Summary. 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 best accepted risk factors for MARCD. Genes involved in blood pressure regulation, like genes encoding the proteins of the renin-angiotensin system (RAS) therefore represents good candidate genes for MARCD. Plasma angiotensinogen level is a major determinant of the RAS activity. Positive correlation between angiotensinogen gene expression and RAS activity, as well as blood pressure were observed. Common mutations described in the AGT promoter were able to alter AGT expression in cell culture. We described that 4 frequent mutations at the AGT promoter are combined in 5 haplotypes coded as A (−6:g, −20:a, −152:g, −217:g), B (−6:a, −20:c, −152:g, −217:g), C (−6:a, −20:c, −152:a, −217:g), D (−6:a, −20:a, −152:g, −217:g), and E (−6:a, −20:a, −152:g, −217:a). The B haplotype was significantly associated with MARCD in the cohort of the Austrian Stroke Prevention Study ($p = 0.005$). The association was independent of hypertension, which pinpointed to a possible role of the local RAS in this relationship. Investigation of the promoter activity of the AGT gene in astrocytes suggests that expression of this gene may be modulated by the haplotype.

Definition of the Phenotype

Microangiopathy-related cerebral damage (MARCD) includes early confluent and confluent white matter changes as well as lacunar infarcts (Schmidt H et al., 2000). The definition is based on histopathological findings demonstrating that both of these changes are associated with arteriolosclerosis (Awald et al., 1986; Fazekas et al., 1993; Van Swieten et al., 1991a). MARCD is a common MRI observation in elderly persons and may lead to cognitive impairment and gait disturbances as it progresses (Bots et al., 1993). Identification of individuals prone to the development of MARCD and early control of causal factors could reduce the risk of these common clinical

problems of the elderly. It is unclear which factors other than advancing age and arterial hypertension predispose individuals to MARCD (Van Swieten, 1991b; Schmidt R et al., 1997)

Evidence for genetic factors in MARCD

MARCD is probably a multifactorial disorder with both genetic and environmental factors influencing its presence and severeity. A recent investigation of Carmelli et al. (1998) supports the presence of a substantial genetic background in MARCD. This study on World War II veteran twins has shown a heritability index of 0.73 for white matter hyperintensity (WMH) volume meaning that up to 73% of the interindividual variation seen in WMH volume can be explained by genetic factors. The probandwise concordance rates for extensive white matter lesions, defined as ≥0.5% of total intracranial volume, were 61% in monozygotic and 38% in dizygotic twins at a prevalence of 15% for the entire study population. This gives a relative risk estimate of 4 for monozygotic and 2.5 for dizygotic twins compared to the risk of the general population. Altough a heritability index in general should be interpreted cautiously and its value strictly applies only to the investigated population, the high estimate for WMH volume stresses the need for further investigations on genetic factors in relation to these brain changes.

Angiotensinogen as a candidate gene for MARCD

The consistent association between MARCD and arterial hypertension (Pantoni et al., 1995; Van Swieten et al., 1991) suggests that genes involved in the regulation of blood pressure may contribute to the strikingly high heritability in this phenotype. The renin-angiotensin system (RAS) is a major regulator of blood pressure. Plasma angiotensinogen (AGT) synthesized by the liver is processed to angiotensin II (AT-II) by the serial action of renin and angiotensin converting enzyme (ACE). The plasma level of AGT is rate limiting in this cascade (Lynch et al., 1991). Plasma AGT concentration is positively correlated with RAS activity and blood pressure in humans and in animal models (Lynch et al., 1991; Kim et al., 1995; Yang, 1999).

Structure of the human AGT gene

The mature human AGT (MW 61.400 D) protein is 452 aminoacides long and has a 14% carbohydrate content. Present data suggest that beside being the extracellular reservoir of angiotensin peptides, AGT exerts no other function (Lynch et al., 1991). The AGT gene is located on Chr1q. It spans 12kb and contains 5 exons (Fukamizu et al., 1990). The second exon contains 859 nucleotides and encodes the signal sequence and angiotensin I peptide. This exon carries two frequently investigated polymorphisms, namely the T174M

and the M235T, which are both located downstream of the renin cutting site. Sequence analysis of the 5′-flanking region of the human AGT gene revealed the existence of several putative regulatory elements like AGCE2 (human angiotensinogen core promoter element 2), AGCE1 (human angiotensinogen core promoter element 1), 5′-AGCE2, glucocorticoid responsive elements, estrogen responsive element, heat shock responsive element, cAMP responsive elements (Kim, 1995; Yanai et al., 1996, 1997a, b).

AGT expression in the liver

The primary site of AGT synthesis is the liver. The basal transcription of the human AGT gene in liver cells is controlled by a suprisingly short region from −32 to +44 bp (Yanai et al., 1996). It has been shown that deleting the fragment from −16 to +44 bp reduces promoter activity by 95%, even though the TATA box is located outside of this region. The AGCE1 element (from position −9 to −25 relative to the transcriptional start site) between the TATA box and the transcriptional start site was identified as a key regulator of AGT expression. In vitro substituted mutations within AGCE1 had a more profound effect on AGT gene transcription than mutations in the TATA box (Kim et al., 1995; Yanai et al., 1997). The commonly observed, naturally occurring sequence alterations at position −6 and −20, have indeed been shown to alter transcriptional efficiency of the AGT promoter (Jeunemaitre et al., 1992; Yanai et al., 1997; Inoue et al., 1997; Zhao et al., 1999).

Angiotensinogen expression in the brain

AGT is expressed in several organs (fat, brain, vascular wall, in small amounts in lung, kidney, ovary, testis, adrenal gland, heart, spinal cord) and cell types (adipocytes, astrocytes, fibroblasts, vascular smooth muscle cells) (Paul et al., 1993). Not only AGT but also other components of the RAS system including renin, ACE and angiotensin receptors are present in these tissues. The major source of AGT in the brain seems to be the astrocyte (Bunnemann et al., 1992; Wright et al., 1992). This is supported by the facts that AGT mRNA appears in the fetal brain as astrocytes develop and that cell lines derived from human and rat astrocytomas are able to produce AGT. Cerebrospinal fluid AGT is also derived from astrocytes as AGT does not cross the blood brain barrier. There is however a large variation in AGT expression among astrocytes according to their location in the brain.

Positive association exists between angiotensinogen polymorphisms and MARCD

In our previuos investigation we could observe a positive association between T235 variant and the presence of MARCD in the cohort of Austrian Stroke Prevention Study (Schmidt R et al., 2001). The T235 allele also enhanced the

risk for the progression of small vessel disease related brain abnormalities. The association was present in T235 homozygotes but not in heterozygotes. We hypothesized that the association between the T235 allele and MARCD is not causal but the T235 mutation rather represents a marker being in linkage dysequilibrium with a causal mutation in its vincinity. Our focus of interest were variants located in the promoter region of the AGT gene as they were shown to alter the expression of angiotensinogen. Using TTGE (temporal temperature gradient electrophoreses) for screening the AGT promoter for mutations, we detected 4 polymorphic sites at positions −6:g/a, −20:a/c, −152:g/a, and −217:g/a in our cohort (Schmidt H et al., 2001). The 4 polymorphic alleles were present in form of 5 haplotypes which we coded as A (−6:g, −20:a, −152:g, −217:g), B (−6:a, −20:c, −152:g, −217:g), C (−6:a, −20:c, −152:a, −217:g), D (−6:a, −20:a, −152:g, −217:g), and E (−6:a, −20:a, −152:g, −217:a). Among the single nucleotide polymorphic alleles the −20:c in homozygous state was associated with a significantly higher risk for MARCD (OR = 2.5, p = 0.04), while the association between MARCD and −6:a allele was borderline significant (OR: 1.5, p = 0.054). The B haplotype which carries both the −6:a and the −20:c alleles, showed an even stronger effect on MARCD frequency than any of the single nucleotide alleles. The relative risk for B homozygotes was 8 fold increased (p = 0.003), and also B heterozygotes showed a trend to increased MARCD risk (OR:1.8, p = 0.1). There was a significant linear association between B haplotype copy number and MARCD risk suggesting the presence of a gene-dose effect for the haplotype. A similar gene-dose relationship was not seen for the single nucleotide polymorphic alleles. In summary, using haplotypes instead of the single nucleotide markers increased the strength of the association as expected if a true causal relationship exists.

The association between AGT promoter haplotype and MARCD is independent of hypertension

Interestingly, the association between B haplotype and MARCD was, contrary to our primary hypotheses, not mediated by hypertension. The presence of hypertension rather masked the effect of the haplotype due to the higher baseline risk for MARCD in this group. In the light of the strong correlation between angiotensinogen expression, plasma level and RAS activity this finding suggested that the association between MARCD and AGT promoter haplotype is mediated by an altered activity of the local rather than the systemic RAS. In preeclampsia it has been shown that the M235T variant can modulate the AGT expression in decidual arteries (Morgan et al., 1997).

Possible pathological pathways linking the AGT promoter haplotype to MARCD

Conceivably, the association between the AGT B haplotype and MARCD might be mediated by an altered expression of AGT in the brain, which in turn

may lead to an altered local availability of AGT. Our preliminary results on the haplotype dependent promoter activity of the AGT gene showed that the B haplotype leads to an increased promoter activity in astrocytes (unpublished data). If tissue RAS activity is also regulated by the AGT level as is the systemic RAS, then changes in AGT concentration may result in an altered level of AT-II at this site. AT-II acts on a variety of cell types in the brain (Bunnemann et al., 1992; Wright et al., 1992). In the present context its effect on vascular smooth muscle cells is of particular interest. AT-II, a potent regulator of vascular tone can lead to vasoconstriction but also to vasodilation in the cerebral arterioles (Wei et al., 1978; Whalley et al., 1988; Haberl et al., 1990, 1996). AT-II promotes vascular smooth muscle cell hyperplasia and hypertrophy (Taubman et al., 1989; Weber et al., 1994; Marrero et al., 1995; Ushio-Fukai et al., 1998). It modulates NADH/NADPH oxidase and extracellular superoxide dismutase activity in the vessel wall (Rajagopalan et al., 1996; Fukai et al., 1999). It was also reported to alter the production of extracellular matrix proteins in the vessels (Kakinuma et al., 1998; Ford et al., 1999). Therefore, alterations in local AT-II availability might result in imbalance of physiological processes like brain perfusion, autoregulation of cerebral blood flow, the oxidative state of the vessel wall, or blood brain barrier function. Each of these processes might be involved in the development of MARCD.

In summary, if our results can be replicated in independent studies, then the B haplotype might serve as a genetic marker for the identification of individuals prone to develop microangiopathy-related brain damage and its clinical consequences. The etiological understanding of the association of small vessel disease related cerebral damage with genetic variants in the RAS system independently of arterial hypertension might also point to possible favorable effects of drugs acting on the RAS system beyond what can be expected from lowering blood pressure alone.

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Angiotensinogen Promoter B-Haplotype Associated with Cerebral Small Vessel Disease Enhances Basal Transcriptional Activity

3.4.3

Angiotensinogen Promoter B-Haplotype Associated With Cerebral Small Vessel Disease Enhances Basal Transcriptional Activity

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Background and Purpose—Previously, we described the presence of 5 haplotypes (A to E) at the angiotensinogen (*AGT*) promoter and reported a significant association between the B-haplotype (nucleotide substitutions −6:G→A and −20:A→C compared with the wild-type A-haplotype) and magnetic resonance imaging correlates of cerebral small vessel disease (cSVD). The association was independent of hypertension, suggesting a brain-specific effect of this haplotype. In the current study, we investigated transcriptional activities of the 5 promoter haplotypes in astrocytes, the main source of cerebral *AGT*, and in hepatocytes, the main source of systemic *AGT*, as well as determined the evolutionary relatedness of the promoter haplotypes.

Methods—Transcriptional activity depending on the haplotypes and the −6:A and −20:C substitutions was measured in transiently transfected A172 and HepG2 cells. We genotyped 5 new single nucleotide polymorphisms (SNPs) at the *AGT* gene and measured linkage disequilibrium (LD) among SNPs and the promoter haplotypes. An evolution-based haplotype tree was constructed.

Results—The B-haplotype increased transcriptional activity in both cell types. Its effect was stronger in astrocytes than in hepatocytes (2.4±0.09-fold, $P<0.001$ versus 1.6±0.06-fold, $P=0.014$). Importantly, in astrocytes the combination of the −6:A and the −20:C was mandatory for increased activity, whereas in hepatocytes the −20:C on its own was sufficient. Strong LD between the 5 new SNPs and the promoter haplotypes allowed the reconstruction of 9 haplotypes over the *AGT* gene. Cladistic analyses suggest that the B-haplotype represents an ancient promoter variant.

Conclusions—Combination of the −6:A and −20:C substitutions in the B-haplotype may promote the development of cSVD by enhancing cerebral angiotensinogen expression. **(*Stroke*. 2004;35:2592-2597.)**

Key Words: angiotensinogen ■ gene expression regulation ■ genetics ■ white matter

Cerebral small-vessel disease (cSVD) is an important cause of stroke, cognitive decline, and dementia.[1–2] cSVD can be visualized by magnetic resonance imaging of the brain as early confluent and confluent white matter hyperintensities and lacunar infarcts. The estimated heritability of cSVD is 73%, suggesting that genetic factors play an important causative role.[3] Arterial hypertension is the major risk factor for cSVD, which also has a high heritability in most populations.[4]

The renin-angiotensin system (RAS) is an important regulator of blood pressure. Plasma angiotensinogen (*AGT*) synthesized by the liver is processed to angiotensin II (AT-II) by the serial action of renin and angiotensin-converting enzyme. Liver production of *AGT* is regulated mainly at the transcriptional level.[5–6] Sequence alterations at position −6 and −20 have been shown to affect transcriptional efficiency of the promoter.[7–11] There is a positive correlation between plasma *AGT* concentration, RAS activity, and blood pressure.[5,6,12,13] The *AGT* gene has been implicated in hypertension by association and linkage studies.[13–15]

Previously, we described 5 haplotypes (A wild-type to E) at the *AGT* promoter created by nucleotide substitutions at positions −6, −20, −152, and −217 relative to the transcription start (Figure 1).[16] Homozygosity for the B-haplotype (−6:A, −20:C, −152:G, −217:G) conferred an 8-fold increased risk for cSVD in our cohort. The association was independent of hypertension, suggesting that the effect of the haplotype is mediated through the local RAS of the brain rather than through the systemic RAS.

The B-haplotype differs from the wild-type A at positions −6 (G→A) and at −20 (A→C). We hypothesize that either one of these single nucleotide polymorphisms (SNPs) or their combination alters promoter activity of the *AGT* gene in the brain,

Received May 6, 2004; final revision received June 17, 2004; accepted July 5, 2004.

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***Stroke* is available at http://www.strokeaha.org** **DOI: 10.1161/01.STR.0000144646.96121.d2**

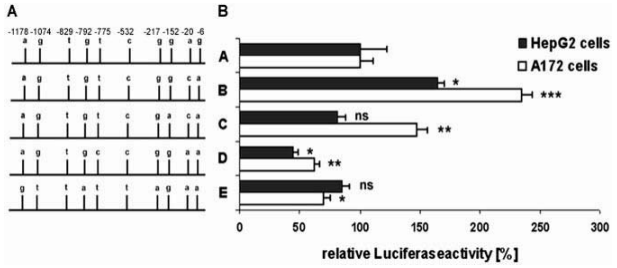

Figure 1. Comparison of the transcriptional activities of *AGT* promoter haplotypes in astrocytes and hepatocytes. A, Position and nucleotide sequences at polymorphic sites in the promoter haplotypes (−1222 to +44). B, Transcriptional activities of the promoter haplotypes in HepG2 cells (dark bars) and A172 cells (white bars). Transcriptional activities are presented as percentage of the activity of the A-haplotype. Each measurement was performed in triplicates and repeated in at least 3 independent experiments. Values are the means±SEM. Transcriptional activities of the B-, C-, D-, and E-haplotypes were compared with the A-haplotype pairwise (Student *t* test, **P*<0.05, ***P*<0.01, ****P*<0.001; NS indicates not significant).

which may then promote development of cSVD through increased cerebral RAS activity. To test this hypothesis, we investigated *AGT* promoter activity dependent on the promoter haplotypes and on the single −6:A and −20:C substitutions in astrocytes, which represent the major source of cerebral *AGT*, and in hepatocytes, which represent the major source of systemic *AGT*.[5]

Because it is also possible that the association between the B-haplotype and cSVD is not functional but is rather caused by linkage disequilibrium (LD), we genotyped 5 additional sequence variations scattered over the *AGT* gene and estimated the magnitude of LD between the new SNPs and the promoter haplotypes. Based on these data, we performed a cladistic analysis and investigated the evolutionary relatedness of the haplotypes.

Materials and Methods

Cell Culture

A172 cells were maintained in DMEM containing 10% fetal bovine serum, 4.5 g/L glucose, 100 000 U/L penicillin, and 0.1 g/L streptomycin, and HepG2 cells were maintained in DMEM containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, 100 000 U/L penicillin, and 0.1 g/L streptomycin.

Construction of Plasmids

Genomic DNA from subjects homozygous for the A-, B-, C-, D-, or E-haplotypes was amplified by polymerase chain reaction (PCR) between positions −1222 and +44 (AF424741) and cloned into *Xho*I/*Hind*III sites of the pGL3-basic reporter vector (Promega) upstream of the firefly luciferase gene.

The reporter vector including the A-haplotype was used to introduce the −6:A (MutAB-6) and the −20:C substitutions (MutAB-20) by oligonucleotide-directed mutagenesis. To introduce the −6:A mutation, a fragment spanning positions −120 to +10 (primers: 5′-GATCCCAGCCTGTGGTCTGGCCAAGTGA-3′ and 5′-AGCAGCTTCTTCCCCTGGCC-3′) and a fragment spanning positions −53 to +44 (primers 5′-GCTCCATCCCCACCCCTCA-3′ and 5′-ATTTAAAGCTTCGGCTTACCTTCTGCTGTAG-3′) were created. The 2 fragments were then combined by amplification with the primers 5′-GATCCCAGCCTGTGGTCTGGCCAAGTGA-3′ and 5′-ATTTAAAGCTTCGGCTTACCTTCTGCTGTAG-3′. The −20:C mutation was introduced similarly except that a fragment spanning position −120 to −14 was created in the first step (primers 5′-GATCCCAGCCTGTGGTCTGGCCAAGTGA-3′ and 5′-TCACGAGGCCCTATTTATAGCTGAG-3′). The combined fragments were subcloned into the *Pfl*MI/*Hind*III sites of the plasmid carrying the A-haplotype. All inserts were verified by DNA sequencing.

Transient Transfection Assays

Cells were plated at a density of 4×10^5 cells/60-mm dish and transfected 24 hours later by calcium phosphate coprecipitation with the reporter constructs (10 μg). In each experiment, phRG-TK control plasmid (20 ng) was cotransfected to normalize for transfection efficiency. Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega) on a LUMAT LB9501 luminometer (Berthold). All measurements were performed in triplicate and have been repeated in at least 3 independent experiments. Results are expressed as the ratio of firefly to renilla luciferase activity (±SEM). Statistical significance was tested by the Student *t* test and 1-way ANOVA (SPSS for Windows 10.0.5.0).

Subjects and Design

The study population consisted of participants of the Austrian Stroke Prevention Study, a single-center, prospective, follow-up study on the cerebral effects of vascular risk factors in the normal elderly population of Graz, Austria. Study design, sampling procedure, and definition of vascular risk factors have been previously described.[16,17] The present study consisted of those 662 individuals who underwent both magnetic resonance imaging analyses and DNA sampling. There were 357 women and 305 men. The mean age was 63.1 years. The sample consisted exclusively of whites of central European origin. The study was approved by the ethical committee of the University of Graz. All subjects gave informed consent to participate in the study.

Mutation Screening and Genotyping

We screened the promoter region upstream of the haplotypes (positions −704 to −245bp) and the 3′-UTR (positions +11407 to +11712bp) for SNPs. Genomic DNA was PCR-amplified using the oligonucleotides: fragment 1 (spanning position −532): 5′-GC-clamp-GGTCATGTGAAACTTACCATTTGC-3′ and 5′-CAAAGAGCCTACCCTGTGCAAAG; fragment 2 (spanning position −386): 5′-GC-clamp-AAGTTTGCAGGAGTCGGGGCCAAG and 5′-GCTGAAGGTCACACATCCCATGAGC; and fragment 3 (spanning positions +11407 and +11712bp):5′-GC-clamp-CACCTAAAACTTCAAAGGACTGC-3′ and 5′-CATTGCCTTCGGTTTGTATTTAG-3′. The sequence of the GC-clamp was 5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCG-3′. PCR products were screened for the presence of mutations by TTGE (Dcode Universal mutation detection system; Bio-Rad Laboratories). Melting domain map was calculated with the MacMelt algorithm (Bio-Rad Laboratories). Heterozygous DNA samples were used as positive controls on each gel to check gel resolution efficiency. At least 3 samples with a distinct banding pattern on TTGE were sequenced (CEQ 2000XL DNA analyses system; Beckman Coulter). The M235T polymorphism was genotyped using PCR-refraction fragment length polymorphism.[18]

Statistical Analysis

The haplotype tree was inferred with the maximum likelihood method, using DNAML 3.573c (Phylip; http://evolution.genetics.

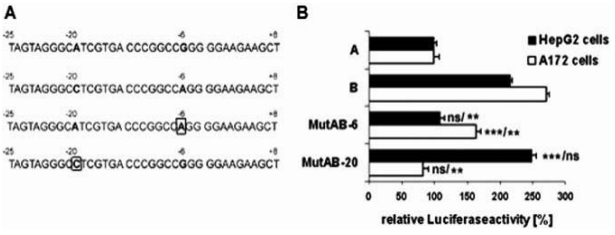

Figure 2. Effect of the −6G and −20C substitutions on the transcriptional activities of *AGT* promoter in astrocytes and hepatocytes. A, Nucleotide sequences of the A- and B-haplotypes and of MutAB-6 and MutAB-20 constructs within the core promoter region (−25 to +8bp). Nucleotide exchanges in MutAB-6 and MutAB-20 are marked with a frame. The remaining sequence of MutAB-6 and MutAB-20 corresponds to that of the A-haplotype (−1222 to +44 bp). B, Promoter activities of the MutAB-6 and MutAB-20 constructs in HepG2 cells (dark bars) and A172 cells (white bars). Each measurement was performed in triplicate and repeated in at least 3 independent experiments. Transcriptional activities are presented as percentage of the A-haplotype activity (mean±SEM). The first probability value compares mutated constructs versus A-haplotype, and the second probability value compares mutated constructs versus B-haplotype (Student *t* test, **P*<0.05, ***P*<0.01, ****P*<0.001; NS indicates not significant).

washington.edu/phylip.html).[19] We assumed equal base frequency and a transition to transversion ratio of 2.0. Haplotype frequencies were estimated by the expectation-maximization method (Arlequin v. 2001; http://lgb.unige.ch/arlequin/).[20]

Pairwise LD was characterized by D′ (normalized Lewontin measure of LD) and by the probability value from the test of significance of LD, and was estimated by GOLD software package (http://www.sph.umich.edu/csg/abecasis/GOLD/).[21,22] The relation between physical distance and LD was assessed using the booLD software (http://www.geneticepi.com/Research/software/software.html).[23]

Results

Transcriptional Activities of the Promoter Haplotypes

Basal transcriptional activities of the haplotypes in astrocytes (A172 cells) and hepatocytes (HepG2 cells) are shown in Figure 1. Among the 5 haplotypes, the B-haplotype had the highest promoter activity in both cell types (hepatocytes: 1.6±0.06-fold, P=0.014; astrocytes, 2.4±0.09-fold increase, P<0.001 compared with the A-haplotype). The C-haplotype showed similar promoter activity as the A-haplotype in hepatocytes (P=0.5) and a 1.5±0.09-fold increased activity in astrocytes (P=0.007). The D-haplotype decreased promoter activity by ≈50% in both cell lines (HepG2, P=0.02; A172, P=0.003). The E-haplotype did not change promoter activity in hepatocytes (P=0.5) but decreased activity by ≈30% in astrocytes (P=0.01).

Transcriptional Activities of the MutAB-6 and the MutAB-20 Constructs

To elucidate the individual effect of the −6:A and −20:C substitutions distinguishing the B-haplotype from the A-haplotype, we transiently transfected A172 and HepG2 cells with the reporter constructs containing either only the −6:A substitution (MutAB-6) or the −20:C substitution (MutAB-20) (Figure 2A). The promoter activities of these constructs were compared with the promoter activities of both the A- and B-haplotypes. In astrocytes, MutAB-6 had a significantly increased promoter activity (1.6±0.07-fold, P<0.001) compared with the A-haplotype, but its transcriptional activity was still significantly lower than that of the B-haplotype (P<0.001). MutAB-20 had similar promoter activity in astrocytes (P=0.3) as the A-haplotype. By contrast, in hepatocytes promoter activity of MutAB-6 was similar to that of the A-haplotype (P=0.2), whereas MutAB-20 had a 2.5±0.06-fold increased promoter activity (P<0.001) (Figure 2B). This is comparable with the effect of the B-haplotype in these cells (P=0.8)

Pairwise LD and Haplotype Analyses

We genotyped our cohort for the SNPs −532C→T and −386G→A in the promoter region, and for the SNPs +11535:C→A and +11553C→delC in the 3′-UTR as well as for the M235T polymorphism. Allele and genotype proportions for all the SNPs were in Hardy–Weinberg equilibrium (Table 1). The allele frequencies in our cohort were similar to the previously published frequencies in a sample of white subjects (Utah CEPH pedigrees) (Table 1).[24]

The magnitude of LD among the SNPs is shown in Figure 3. There was no decline of LD with physical distance (constant LD versus exponential decline models, P>0.1). Average LD between the SNPs as assessed by D′ was 0.841.

Including the 5 newly investigated SNPs and the 4 SNPs determining the promoter haplotypes, we inferred 9 haplotypes over the *AGT* gene in our cohort. These haplotypes coded from H1 to H9 based on their frequencies are described in Table 2. The evolutionary relatedness of these haplotypes in form of a haplotype tree is presented in Figure 4. Overall, 80% of the promoter B-haplotype was present on H4, 12% on H8, and 8% on H9, both closely related to H4. The H4 *AGT* haplotype is equivalent to the published

TABLE 1. Distribution of the *AGT* Polymorphisms

Position	Base Change*	N ASPS†	HWE ASPS	Frequency ASPS	Frequency SLC
−532	C→T	513	0.63	0.11	0.09
−386	G→A	650	0.87	0.02	0.04†
−217	G→A	638	0.77	0.11	0.16
−152	G→A	638	0.96	0.03	—
−20	A→C	638	0.95	0.18	0.16
−6	G→A	638	0.59	0.43	0.42
4072	T→C	643	0.26	0.44	0.44
11535	C→A	642	0.64	0.32	0.32
11553	C→delC	642	0.96	0.01	—

*Allele with lower frequency in ASPS sample comes second.

†Frequency is based on data of Jeunemaitre et al (1992).

ASPS indicates Austrian Stroke Prevention Study; N ASPS, number of genotypes unambiguously established in the ASPS; HWE ASPS, p-values from the test for Hardy–Weinberg equilibrium in the ASPS; SLC, Salt Lake City.

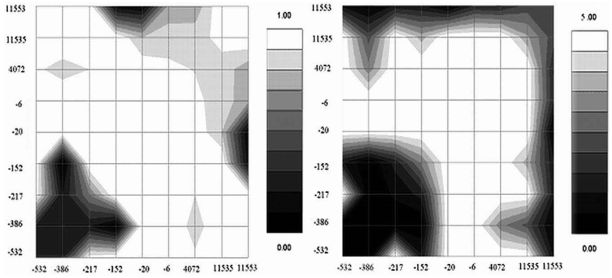

Figure 3. Pairwise LD in *AGT*, evaluated by D′ (left) and minus logarithm of the probability value coming from the test of LD significance (right).

chimpanzee sequence (AF193457.1), suggesting that the B-haplotype represents an ancient promoter variant.

Discussion

In the current study, we show that the *AGT* promoter B-haplotype, which was associated with cSVD in our previous investigation,[16] significantly enhances basal transcriptional activity of the *AGT* gene. The association was independent of hypertension, indicating that it might be mediated through an altered cerebral *AGT* expression. We found that the B-haplotype increases promoter activity more strongly in astrocytes, which are the major source of cerebral *AGT*, than in hepatocytes, which are the major source of systemic *AGT*.

If tissue RAS is regulated at the *AGT* level as is the systemic RAS, then a higher *AGT* transcription in astrocytes may lead to elevated AT-II concentration in the brain. AT-II is a potent regulator of vascular tone,[25,26] it promotes vascular smooth muscle cell growth, and modulates the production of extracellular matrix proteins.[27–29] It enhances the activity of NADH/NADPH oxidase and of extracellular superoxide dismutase in the vessel wall.[30,31] Alteration in the local availability of AT-II may therefore result in an 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,[32] and by these mechanisms may lead to the development of cSVD.

The B-haplotype contains 2 nucleotide substitutions, −6:G→A and −20:A→C, compared with the wild-type A-haplotype. We found that in contrast to hepatocytes, the effect of the B-haplotype in astrocytes is dependent on the presence of both of these substitutions, because neither the *−6:A* nor the *−20:C* mutation alone was able to enhance promoter activity to the level observed for the B-haplotype. This is in line with our previous genetic epidemiological data, showing that the B-haplotype is stronger and more significantly associated with

TABLE 2. Frequencies of Haplotypes in the *AGT* Gene, as Estimated by the EM Algorithm

	AGT Haplotype Composition						
Designation	SNP −532	SNP −386	Promoter Haplotype	SNP 4072	SNP 11535	SNP 11553	Frequency
H1	C	G	A	T	T	C	0.299
H2	C	G	A	T	C	C	0.248
H3	C	G	D	C	C	C	0.119
H4	C	G	B	C	C	C	0.112
H5	T	G	E	C	C	C	0.102
H6	C	G	C	C	C	C	0.031
H7	C	G	A	C	C	C	0.022
H8	C	A	B	C	C	C	0.017
H9	C	G	B	C	T	C	0.011

Only the haplotypes with a frequency of 1% or more are shown. These haplotypes account for 95.9% of haplotypes observed in ASPS population.

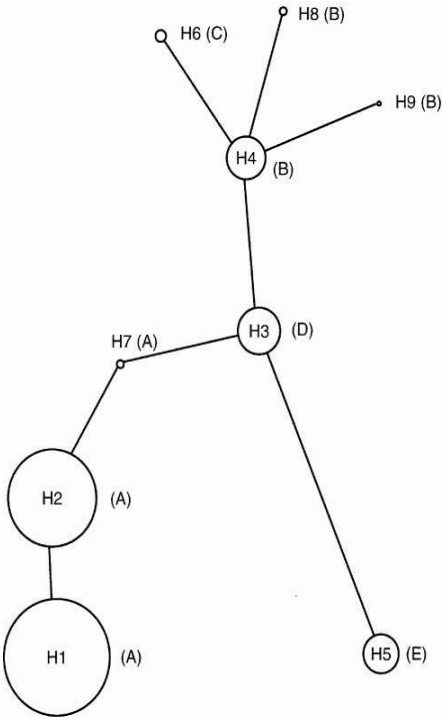

Figure 4. Haplotype tree for *AGT* haplotypes, based on the 9 SNPs. The size of the circle corresponds to the frequency of the haplotype. The letter in the brackets shows the promoter haplotype carried.

cSVD than either the *−20:C* or the *−6:A* alleles. An interaction between the −6:A and the −20:C mutation is biologically plausible because these 2 mutations are located in 2 distinct regulatory elements of the core promoter.[9,11]

The 5 promoter haplotypes showed a cell type-specific and differential effect on the transcriptional activity, suggesting that the effect of the −20:C and the −6:A mutations are further modulated by other sequence variations at the *AGT* promoter. This is in line with previous findings indicating that the sequence between positions −25 to −1 mediate the responsiveness of the core promoter to upstream cis-acting elements.[8]

A further, although indirect, support for the functional relevance of the B-haplotype is suggested by its ancient origin. It had been hypothesized that the *AGT* haplotype carrying the −6:A and the 235T mutations was advantageous during human evolution in the low-salt environment of Africa by allowing for a higher salt reabsorption through enhanced *AGT* expression and aldosterone secretion.[13,24] When our ancestors moved to the high-salt environment in Europe, however, this ancient haplotype may have become disadvantageous. The B-haplotype is in complete LD with the 235T variant, and the enhanced risk for cSVD associated with 235T variant might be caused by LD with the B-haplotype.[33] Importantly, although the B-haplotype contains the *−6:A* substitution and also perfectly predicts the presence of C at position 4072, which codes for the 235T variant, only 34% of the *235T* allele is associated with the B-haplotype and may therefore enhance the risk for cSVD.

A strength of our study is that we investigated the effect of naturally occurring promoter haplotypes on the transcriptional activity of the *AGT* gene, and that we dissected the individual effect of the −6:A and −20:C mutations present on the B-haplotype. We used astrocytes as a cellular model for cerebral *AGT* expression. It is important to realize, however, that other cell types, like vascular smooth muscle cells, endothelial cells, or eventually neurons, may also contribute to *AGT* production at the site of cSVD.

In conclusion, we showed that the combination of the −6:A and −20:C mutations in the B-haplotype enhances angiotensinogen promoter activity in astrocyte. This finding together with our previous epidemiological data suggests that increased cerebral RAS activity is involved in the cause of cSVD. Further studies are needed to investigate the role of the B-haplotype in independent populations, and to elucidate the biological role of the SNPs at the *AGT* gene in cSVD. A causal relation between B-haplotype and cSVD independent of hypertension points to intervention in the RAS as a possible treatment strategy in cSVD, a highly prevalent entity in elderly subjects for which no treatment has been proven to be effective until now.

Acknowledgments

This study was supported by the Austrian Science Foundation P15440 (H.S.) and P13180-med (R.S.), by the Franz Lanyar Foundation of the University of Graz (H.S.), and by the Netherlands Heart Organization 2000B117 (C.v.D.). The excellent technical assistance of Irmgard Poelzl and Johann Semmler is appreciated.

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Genetic Determinants of Carotid Atherosclerosis

4

Paraoxonase PON1 Polymorphism Leu-Met54 is Associated with Carotid Atherosclerosis

4.1

Paraoxonase PON1 Polymorphism Leu-Met54 Is Associated With Carotid Atherosclerosis

Results of the Austrian Stroke Prevention Study

Helena Schmidt, MD; Reinhold Schmidt, MD; Kurt Niederkorn, MD; Anita Gradert; Martin Schumacher, MD; Norbert Watzinger, MD; Hans-Peter Hartung, MD; Gert M. Kostner, PhD

Background and Purpose—Genetic polymorphism at the paraoxonase locus is associated with serum concentration and activity of paraoxonase and with increased risk for coronary heart disease. Two frequent polymorphisms present at the paraoxonase gene are the methionine (M allele) leucine (L allele) interchange at position 54 and the arginine (B allele) glutamine (A allele) interchange at position 191. This is the first study to determine the effect of these polymorphisms on carotid atherosclerosis.

Methods—The paraoxonase genotypes at positions 54 and 191 of 316 randomly selected individuals aged 44 to 75 years were determined by polymerase chain reaction–based restriction enzyme digestion. Carotid atherosclerosis was assessed by color-coded Duplex scanning and was graded on a 5-point scale ranging from 0 (normal) to 5 (complete luminal obstruction).

Results—The LL, LM, and MM genotypes at position 54 were noted in 137 (43.4%), 132 (41.8%), and 47 (14.9%) subjects; the AA, AB, and BB genotypes at position 191 occurred in 172 (54.4%), 124 (39.2%), and 20 (6.3%) individuals. The LL genotype was significantly associated with the presence and severity of carotid disease ($P=0.022$), whereas the 191 polymorphism had no effect. Logistic regression analysis with age and sex forced into the model demonstrated plasma fibrinogen (odds ratio [OR], 1.005 per mg/dL), LDL cholesterol (OR, 1.01 per mg/dL), cardiac disease (OR, 1.75), and the paraoxonase LL genotype to be significant predictors of carotid atherosclerosis. The ORs for the associations with age and sex were 1.09 ($P=0.0003$) and 1.66 ($P=0.052$) per year.

Conclusions—These data suggest that the paraoxonase LL genotype may represent a genetic risk factor for carotid atherosclerosis. **(*Stroke*. 1998;29:2043-2048.)**

Key Words: atherosclerosis ■ carotid arteries ■ genetics ■ paraoxonase

Carotid atherosclerosis is considered to be a major cause of ischemic stroke.[1] In recent years, oxidative stress has been demonstrated to play an important role in the pathogenesis of atherosclerosis.[2] Low-density lipoprotein (LDL) seems to be the major target of oxidative modification, making it particularly atherogenic.[3,4] Identification of factors protecting against oxidative modification of LDL are therefore of major interest. High-density lipoprotein (HDL) has been shown to have antioxidative potential; however, the mechanism(s) of its action is not known.[5] One mechanism might be the enzymatic removal of lipid peroxides accumulating on the LDL particle by enzymes present on HDL.[6] Paraoxonase is tightly associated with HDL and has been shown to reduce the accumulation of lipid oxidation products on LDL.[7,8]

The human serum paraoxonase is a 43- to 45-kDa protein. Its gene is located at q21 to q22 on the long arm of chromosome 7.[9] The amino acid sequence of paraoxonase is highly conserved among animal species, suggesting an important metabolic role for this enzyme.[10] The ability of paraoxonase to detoxify organophosphorous compounds has been known for years. Its activity was determined earlier by the use of paraoxon, a widely used pesticide. The physiological substrate of paraoxonase is yet unknown. Watson et al[11] reported recently that an oxidized phospholipid may represent a potential candidate. White populations have a triphasic distribution of serum paraoxonase activity towards paraoxon but not to other substrates such as phenylacetate.[7] This difference in enzyme activity is caused by an amino acid substitution at position 191. Glutamine (A allele) is replaced by arginine (B allele) in the high-activity isoform.[9] The B allele has been shown to be associated with coronary heart disease.[12–14] Another frequent polymorphism present at position 54 involves a methionine (M allele) leucine (L allele) interchange.[9] The 2 polymorphisms are in linkage disequilibrium, with leucine at position 54 giving rise for arginine at position 191.[9] The suspected role of paraoxonase in the

Received May 22, 1998; final revision received July 15, 1998; accepted July 21, 1998.

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protection of LDL against oxidative modification, and the positive association found between paraoxonase genotypes and coronary heart disease, prompted us to investigate the effect of both 54 and 191 polymorphisms on carotid atherosclerosis in a normal middle-aged and elderly population.

Subjects and Methods

Study Population

Individuals aged 44 to 75 years and stratified by sex and 5-year age groups were randomly selected from the official register of residents of the city of Graz, Austria. They received a written invitation to participate in the Austrian Stroke Prevention Study (ASPS), a single-center prospective follow-up study in our community. The study has been approved by the Medical Ethics Committee of the Karl-Franzens University of Graz. Written informed consent was obtained from all study participants. The rationale and design of the ASPS have been previously described.[15] Briefly, the objective of the study is to examine the frequency of cerebrovascular risk factors and their effects on carotid atherosclerosis, as well as on cerebral morphology and function, in the normal elderly. The inclusion criteria for the study were no history of neuropsychiatric disease and a normal neurological examination. From a total of 8193 individuals invited between September 1991 and March 1994, a sample of 2794 subjects agreed to participate, with 1998 individuals fulfilling the inclusion criteria. All study participants underwent a structured clinical interview, a physical and neurological examination, 3 blood pressure readings, ECG, and echocardiography, as well as laboratory testing including blood cell count and a complete blood chemistry panel. Every fourth study participant was then invited to enter phase II of the ASPS, which included Doppler sonography, MRI, SPECT, and neuropsychological testing. Since 1993, we started to establish a gene bank in all phase II attendees. The present study cohort consists of those 316 individuals who underwent both carotid duplex scanning and assessment of the paraoxonase polymorphisms. There were 158 women and 158 men. The mean age of this cohort was 60.0±6.1 years.

Vascular Risk Factors

Diagnosis of vascular risk factors relied on the individuals' histories and appropriate laboratory findings. Arterial hypertension was considered present if a subject had a history of arterial hypertension with repeated systolic/diastolic blood pressure readings >160/95 mm Hg or if the readings at examination exceeded this limit. Diabetes mellitus was coded present if a subject was treated for diabetes at the time of the examination or if the fasting blood glucose level at examination exceeded 140 mg/dL. Cardiac disease was assumed to be present if there was evidence of cardiac abnormalities known to be a source for cerebral embolism,[16] evidence of coronary heart disease according to the Rose questionnaire[17] or appropriate ECG findings[18] (Minnesota codes I: 1 to 3; IV: 1 to 3; or V: 1 to 2), or if an individual presented with signs of left ventricular hypertrophy on echocardiogram or ECG (Minnesota codes III: 1; or IV: 1 to 3).

Study participants were defined as smokers if they currently smoked >10 cigarettes a day. From current smokers and ex-smokers information was obtained as to the daily number of tobacco products smoked and smoking duration in years. The data on the amount of tobacco were converted into grams of tobacco consumed during the lifetime using the following conversion factors: 1 cigarette=1 g, 1 cheroot=3 g, 1 cigar=5 g. For measurements of hematocrit, blood was obtained from a large antecubital vein without stasis.

The body mass index was calculated as weight (kg)/height (m^2). The regular use of estrogen replacement therapy was recorded among all female study participants.

A lipid status including the level of triglycerides, total cholesterol, LDL and HDL cholesterol, as well as lipoprotein(a) [Lp(a)], apolipoprotein (apo) B, and apoA-I was determined for each study participant. Thirty minutes after venipuncture, the coagulated blood samples were centrifuged at 1600*g* for 10 minutes, and the serum was transferred to plastic tubes and analyzed within 4 hours. Triglycerides and total cholesterol were enzymatically determined using commercially available kits (Uni-Kit III "Roche" and MA-Kit 100 "Roche," Hoffman-La-Roche). HDL cholesterol was measured by the use of the TDx REA Cholesterol assay (Abbott). LDL cholesterol was calculated by the equation of Friedewald. The Lp(a) concentration was determined by the electroimmunodiffusion method using a reagent kit containing monospecific anti-Lp(a) antiserum and Rapidophor M3 equipment (Immuno AG). The levels of apoB and apoA-I were assessed by an immunoturbidometric method using polyclonal antibodies and a laser nephelometer (Behringwerke AG).

The plasma fibrinogen concentration was measured according to the Clauss method[19] using the prescription and reagents of Behringwerke AG.

Isolation of DNA and Genotype Analysis

High-molecular-weight DNA was extracted from peripheral whole blood using Qiagen genomic tips (Qiagen Inc) according to the protocol of the manufacturers.

Genotyping of the Leu-Met54 polymorphism was done by polymerase chain reaction (PCR) amplification of a 170-bp-long fragment using the primers described by Humbert et al.[9] The PCR products are cleaved by *Nla*III in the presence of BSA at 37°C for 3 hours. The digested products are analyzed on a 15% polyacrylamide gel, stained with ethidium bromide, and examined under UV transillumination. The L allele corresponded to the nondigested 170-bp-long fragment, while the M allele corresponded to a 126-bp and a 44-bp fragment. A similar protocol was used for genotyping the Gln-Arg-191 polymorphism using the primers described by Humbert et al.[9]

Carotid Duplex Scanning

Color-coded equipment (Diasonics, VingMed CFM 750) was used to determine atherosclerotic vessel wall abnormalities of the carotid arteries. All B-mode and Doppler data were transferred to a Macintosh personal computer for postprocessing and storage on optical disks. The imaging protocol involved scanning of both common and internal carotid arteries in multiple longitudinal and transverse planes and has been previously described.[20,21] The examinations were done by one experienced physician. Image quality was assessed and graded into good (common and internal carotid arteries clearly visible and internal carotid arteries detectable over a distance of >2 cm), fair (common and internal carotid arteries sufficiently visible and internal carotid arteries detectable over a distance of at least 2 cm), and poor (common and internal carotid arteries insufficiently visible or internal carotid arteries detectable over a distance of <2 cm). The image quality was good in 308 (97%) and fair in 8 (3%) individuals. It was never poor. Measurements of maximal plaque diameter were done in longitudinal planes, and the extent of atherosclerosis was graded according to the most severe visible changes in the common and internal carotid arteries as 0=normal, 1=vessel wall thickening (>1 mm), 2=minimal plaque (<2 mm), 3=moderate plaque (2 to 3 mm), 4=severe plaque (>3 mm), and 5=lumen completely obstructed. Assessment of the intrarater reliability of this score was done in 50 randomly selected subjects and yielded a κ value of 0.83.

Statistical Analysis

We used the Statistical Package for Social Sciences (SPSS/PC+) for data analysis. Categorical variables among the paraoxonase genotypes were compared by χ^2 test. Assumption of normal distribution for continuous variables was tested by Lilliefors statistics. Normally distributed continuous variables were compared by 1-way ANOVA, whereas the Kruskal-Wallis test was used for comparison of non-normally distributed variables. ANCOVA and logistic multivariate regression analysis were used to adjust for possible confounding in the comparison of risk factors among paraoxonase genotypes. Allele frequencies were calculated by the gene counting method, and Hardy-Weinberg equilibrium was assessed by χ^2 test. To test the differences in sonographic score among the 3 genotypes at both

TABLE 1. Distribution of Paraoxonase Genotypes Defined by Amino Acid Substitution at Positions 54 and 191

	AA	AB	BB	Total
LL	47	71	19	137
LM	81	50	1	132
MM	44	3	0	47
Total	172	124	20	316

A indicates glutamine; B, arginine at position 191; L, leucine; and M, methionine at position 54.

polymorphic sites, Kruskal-Wallis 1-way ANOVA was used. To assess the relative contribution of the paraoxonase genotypes on the presence of carotid atherosclerosis, we used multiple logistic regression analysis. The sonographic score was dichotomized into normal (grade 0) or abnormal (grade 1 through 5). Vessel wall thickening (grade 1) was considered to be abnormal because it has been shown to represent an early stage of atherosclerosis and to be associated with an increased risk for future stroke.[22,23] Forward selection stepwise regression analysis with age and sex forced into the model assessed independent predictors of carotid disease. At each step, each variable not in the model was assessed as to its contribution to the model, and the most significant variable was added to the model. This process continued until no variable not in the model made a significant ($P<0.05$) contribution. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated from the β coefficients and their standard errors.

Results

The genotypes LL, LM, and MM were noted in 137 (43.4%), 132 (41.8%), and 47 (14.9%) study participants. The AA, AB, and BB genotypes occurred in 172 (54.4%), 124 (39.2%), and 20 (6.3%) study participants, respectively. The genotypes of both polymorphisms were in Hardy-Weinberg equilibrium. As shown in Table 1, there was a moderate association between the 2 polymorphisms, with arginine at position 191 being with 1 exception always concurrent with leucine at position 54.

Table 2 compares demographic variables and risk factors among the LL, LM, and MM genotype subsets. There was no significant difference between groups, with the exception of lower glucose levels and less frequent cardiac disease in those with the LM genotype. The significant difference in blood glucose level remained unchanged after adjustment for age, sex, and cardiac disease ($P=0.03$) but was no longer present after correction for use of antidiabetic treatment ($P=0.26$). Correction for use of lipid-lowering treatment did not materially change the results of comparisons of blood lipids between the 3 investigational subsets. The between-group difference for cardiac disease remained significant after adjustment for age, sex, and glucose level ($P=0.04$). There was no significant difference among the AA, AB, and BB genotype subsets when the demographic variables and risk factors listed in Table 2 were compared (data not shown).

Sonographic scores among the 3 genotypes for both polymorphisms are shown in Table 3. Overall, there were 63 (47.7%) subjects with the LM genotype and 23 (48.9%) subjects with the MM genotype, but 86 (62.7%) subjects with the LL genotype showed an abnormal sonographic score. Subjects homozygous for the L allele had higher grades of carotid abnormalities than subjects with either the LM or MM genotype ($P=0.022$). Logistic regression analysis yielded an unadjusted OR of 1.86 (95% CI, 1.18 to 2.94; $P=0.007$) for abnormal sonographic findings in the LL genotype relative to the other 2 genotypes. The OR after adjustment for age and

TABLE 2. Demographics and Risk Factors Among Paraoxonase Leu-Met54 Genotypes

	Genotypes			
Variable	LL (n=137)	LM (n=132)	MM (n=47)	*P*
Age, y	59.8±5.9	60.0±6.4	60.0±6.1	0.61*
Sex, no. male (%)	64 (46.7)	68 (51.5)	26 (55.3)	0.54†
Hypertension, n (%)	41 (29.9)	34 (25.8)	18 (38.3)	0.27†
Diabetes mellitus, n (%)	10 (7.3)	3 (2.3)	3 (6.4)	0.16†
Fasting glucose, mg/dL	95.5±23.0	89.9±13.6	97.7±25.8	0.045*
Cardiac disease, n (%)	57 (41.6)	37 (28.8)	21 (44.7)	0.03†
Smoking status, n (%)				
Never smoker	67 (48.9)	74 (56.1)	30 (63.8)	
Ex-smoker	50 (36.5)	37 (28.0)	11 (23.4)	
Current smoker	20 (14.6)	21 (15.9)	6 (12.8)	0.35†
Body mass index, kg/m^2	26.8±3.7	26.4±3.8	26.5±3.5	0.60*
Hematocrit, %	36.5±14.5	35.8±15.2	34.6±16.6	0.18*
Triglycerides, mg/dL	157.2±100.8	134.0±75.3	149.3±75.5	0.46*
Cholesterol, mg/dL	228.4±39.1	227.0±39.4	227.3±37.9	0.65‡
LDL cholesterol, mg/dL	148.4±36.6	149.2±34.0	149.3±32.8	0.64‡
HDL cholesterol, mg/dL	49.0±16.0	50.65±14.5	48.2±15.5	0.71*
Lipoprotein(a), mg/dL	26.3±29.0	19.6±23.1	48.2±15.5	0.47*
Fibrinogen, mg/dL	308.4±75.1	303.1±72.4	299.0±66.4	0.84*

*Kruskal-Wallis test, †χ^2 test; ‡1-way ANOVA.

TABLE 3. Paraoxonase Genotypes and Duplex Score

Duplex Score	Genotype Leu-Met54				Genotype Gln-191-Arg			
	LL (n=137)	LM (n=132)	MM (n=47)	*P**	AA (n=172)	AB (n=124)	BB (n=20)	*P**
0	51 (37.2%)	69 (52.3%)	24 (51.1%)		80 (46.5%)	57 (46.0%)	7 (35.0%)	
1	5 (3.6%)	7 (5.3%)	1 (2.1%)		6 (3.5%)	6 (4.8%)	1 (5.0%)	
2	52 (38.0%)	38 (28.8%)	15 (31.9%)		58 (33.7%)	41 (33.1%)	6 (30.0%)	
3	22 (16.1%)	10 (7.6%)	6 (12.8%)		21 (12.2%)	11 (8.9%)	6 (30.0%)	
4	7 (5.1%)	8 (6.1%)	1 (2.1%)	0.022	7 (4.1%)	9 (7.3%)	0	0.481

*Kruskal-Wallis test.

sex was 1.98 (95% CI, 1.23 to 3.20; $P=0.005$) and 1.88 (95% CI, 1.16 to 3.05; $P=0.01$) when adjusting for age, sex, fasting glucose level, and cardiac disease. Evaluation of the effect of the Gln-Arg-191 polymorphism on carotid atherosclerosis demonstrated that subjects with the BB genotype had the highest prevalence of carotid abnormalities. This difference between the genotypes did not reach statistical significance ($P=0.481$).

The Gln-Arg-191 polymorphism did not modulate the effect of the LL genotype on carotid disease because atherosclerotic changes were present in almost identical frequency in 74 (62.7%) subjects in the LL/AA group and in 12 (63.2%) individuals in the LL/BB group.

The relative contribution of the paraoxonase LL genotype to the presence of carotid atherosclerosis was determined by stepwise forward logistic regression analysis (Table 4). Age and sex was forced in the model. This analysis demonstrated the LL genotype to be significantly and independently associated with carotid atherosclerosis ($P=0.014$). Plasma fibrinogen entered first (OR, 1.005 per mg/dL), LDL cholesterol second (OR, 1.012 per mg/dL), the LL genotype third (OR, 1.907), and cardiac disease fourth (OR, 1.748). No other variables such as total cholesterol, HDL cholesterol, triglycerides, hypertension, diabetes mellitus, smoking, hematocrit, or body mass index were entered into the model. The ORs for the associations with age and sex were 1.09 ($P=0.0003$) and 1.66 ($P=0.05$) per year, respectively.

Discussion

Our data suggest that the paraoxonase LL genotype at position 54 is a significant and independent predictor of carotid atherosclerosis in a middle-aged and elderly population. Homozygosity for the L allele is associated with higher frequency and severity of carotid abnormalities, whereas heterozygosity for this allele results in no risk increase.

We failed to detect a significant association between the Gln-Arg-191 polymorphism and carotid disease, although atherosclerotic lesions were more common in BB than in AB or AA carriers. We found that individuals with the combination of the LL/BB genotypes had frequency of atherosclerosis virtually identical to that of those with combined LL/AA genotypes. This indicates that the Gln-Arg-191 polymorphism has no effect on carotid atherosclerosis per se and does not modulate the effect of the L allele on atherosclerosis. The Gln-Arg-191 polymorphism was defined as the molecular basis for the difference of paraoxonase activity observed against the artificial substrate, paraoxon.[9] It was frequently reported to be associated with coronary heart disease.[12–14] Blatter-Garin et al[24] reported for the first time that in whites the Leu-Met54 polymorphism was significantly and independently of the polymorphism at position 191 associated with the concentration and activity of paraoxonase. These authors also found that the LL genotype predicted coronary heart disease.[24] Similarly to our results, the effect was present in LL homozygotes only, indicating a recessive effect of the L allele. Sanghera et al[25] investigated the effect of the Gln-Arg-191 polymorphism on coronary heart disease in the genetically distinct populations of Chinese and Asian Indians and found a race-specific association with coronary heart disease with the B allele only in Indian cohort. The inconsistency of associations in different populations strongly indicates that the polymorphism at position 191 is not causally related to atherosclerosis but is rather a marker for a functional sequence variant in its vicinity. Whether the Leu-Met54 polymorphism represents this functional variant is unclear and cannot be elucidated by association studies.

TABLE 4. Final Logistic Regression Analysis: Predictors of Carotid Atherosclerosis

Variable	β	SE	*df*	*P*	OR	95% CI
Age, y*	0.082	0.022	1	0.0003	1.086/y	1.04–1.135
Sex*	0.507	0.261	1	0.0519	1.660	0.996–2.768
Fibrinogen, mg/dL	0.005	0.002	1	0.0057	1.005/mg/dL	1.001–1.009
LDL cholesterol, mg/dL	0.012	0.004	1	0.0028	1.012/mg/dL	1.004–1.019
LL paraoxonase genotype	0.646	0.264	1	0.0144	1.907	1.137–3.198
Cardiac disease	0.558	0.275	1	0.0423	1.748	1.020–2.997

*Forced into the model.

Several recent publications support the role of paraoxonase in atherosclerosis. Paraoxonase is tightly associated with antiatherogenic HDL. According to recent studies, only certain subfractions of HDL are able to reduce the risk of atherosclerosis. Results from apoA-I and apoA-II transgenic (tg) mice underline this assumption.[26–28] ApoA-II tg mice are prone to atherosclerosis while apoA-I tg mice were found to be protected against it, even though both had significantly increased HDL levels compared with control mice.[29] HDL isolated from the apoA-I tg mice had been shown to protect against the accumulation of lipid peroxides on LDL, whereas HDL from apoA-II tg mice had no similar effect. The loss of ability of the apoA-II HDL to protect against LDL oxidation was associated with a decreased level of paraoxonase. Substitution of apoA-II HDL with paraoxonase restored its antioxidative ability.[29] Paraoxonase is associated with a certain subfraction of HDL also containing apoA-I and apoJ.[7] This HDL subfraction seems to play a central role in the antioxidative effect of HDL.[30] Navab et al[31] have found that in HepG2 cell culture, minimally oxidized LDL induces an increase in the apoJ/paraoxonase ratio due to altered transcriptional rates. They also reported an increase in the apoJ/paraoxonase ratio in different animal models of atherosclerosis on atherogenic diet, such as in mice prone to atherosclerosis, in apoE knockout mice, and in LDL receptor knockout mice. Interestingly, normolipidemic patients with coronary artery disease also had a significantly higher apoJ/paraoxonase ratio than normolipidemic controls.[30]

Moreover, paraoxonase immunoreactivity was found in atherosclerotic lesions, and the intensity of immunoreactivity in the arterial walls increased with the progression of atherosclerosis.[32] Recently, it was described by the same authors that paraoxonase is present in the interstitial fluid in an enzymatically active form.[33] This is in line with the hypothesis that paraoxonase prevents the accumulation of lipid peroxides on LDL, a process that has to take place in the subendothelial space. If paraoxonase is involved in atherogenesis by its ability to prevent accumulation of lipid peroxides on LDL, and if the association between the L allele and carotid atherosclerosis is causal, then one would expect that the L isoform is less effective in preventing the oxidative modification of LDL than the M isoform. A recent report from Mackness et al[34] supports this hypothesis. These authors investigated the antioxidative effect of HDL isolated from individuals carrying the AA, AB, or BB genotype on Cu^{2+}-induced oxidation of LDL. They found that HDL from BB subjects completely lost its ability to prevent LDL oxidation within 6 hours, whereas HDL from AB or AA individuals still kept 23% and 40% of its original protective activity, respectively. Given the strong linkage disequilibrium between the B and the L allele, it is most likely that the L isoform has similar effects. In line with this suggestion, another recent work from Mackness et al[35] showed that HDL from subjects with the MM/AA genotype most effectively protects oxidative modification, whereas HDL from subjects with the LL/BB genotype has the lowest antioxidative potential.

Several groups reported that the genotype at the paraoxonase locus influences the concentration and/or activity of serum paraoxonase, which partly maybe due to an altered expression of the paraoxonase gene.[36] A weakness of our study is that due to the lack of frozen serum from our participants, we were not able to measure paraoxonase concentration and activity to test the effect of genotype on these parameters in our collective.

Our study is an allelic association study and cannot provide an explanation for the mechanism(s) of the paraoxonase genotypes leading to carotid disease. It might be that there exists a true causal relationship between leucine at position 54 in the paraoxonase enzyme and atherosclerosis; however, the possibility that the association is due to a linkage disequilibrium between the L allele and another functional allele in its neighborhood cannot be excluded. The PON1 gene is a member of a multigene family including PON2 and PON3 located at the same locus on chromosome 7.[37] Recently, Hegele et al[38] and Sanghera et al[39] described 2 polymorphisms present in the PON2 gene with possible clinical relevance. However, function of the PON2 gene product is still unknown, making the estimation of the importance of these findings difficult.

In summary, this is the first report on a positive association between the paraoxonase LL genotype and carotid atherosclerosis. Independent of other vascular risk factors, homozygosity for the L allele was associated with a 1.91-fold increased risk for carotid disease. If our results can be confirmed in other ethnic groups, the Leu-Met54 variant, which can be easily determined by conventional DNA technology, may be considered to be included in the early risk assessment for stroke.

Acknowledgments

This project was partly supported by the Austrian Research Foundation Project P11691, SFB702 (Dr Kostner) and by the Franz Lanyar Stiftung of the Karl-Franz University (Dr H. Schmidt), Graz, Austria. The excellent technical assistance of Johann Semmler is appreciated.

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Angiotensinogen Polymorphism M235T, Carotid Atherosclerosis and Small-Vessel Diseae-Related Cerebral Abnormalities

4.2

Angiotensinogen Polymorphism M235T, Carotid Atherosclerosis, and Small-Vessel Disease–Related Cerebral Abnormalities

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Abstract—The angiotensinogen M235T polymorphism has been linked to hypertension and cardiovascular disease. We studied the role of this polymorphism as a risk factor for carotid atherosclerosis and small-vessel disease–related brain abnormalities. A total of 431 randomly selected community-dwelling subjects without clinical evidence for strokes underwent angiotensinogen genotyping and carotid Duplex scanning; 1.5-T brain magnetic resonance imaging (MRI) was done in 396 individuals. At 3-year follow-up, we reexamined 343 and 267 study participants by ultrasound and brain MRI, respectively. Carotid atherosclerosis was graded on a 5-point scale. Small-vessel disease–related brain abnormalities were deep or subcortical white matter lesions or lacunes. Progression of carotid atherosclerosis and MRI findings was rated by direct imaging comparison by 3 independent raters. The M/M, M/T, and T/T genotypes were seen in 20.9%, 52.9%, and 18.1% of subjects, respectively. The M235T polymorphism was neither associated with baseline carotid findings nor with progression of carotid atherosclerosis. There was a trend toward more frequent small-vessel disease–related MRI abnormalities in the T/T than in the other genotypes at the baseline examination. Progression of brain lesions occurred significantly more commonly in T/T than in M/M and M/T carriers ($P<0.001$). Logistic regression analysis identified the T/T genotype (odds ratio, 3.19; $P=0.002$) and arterial hypertension (odds ratio, 3.06; $P=0.03$) as significant independent predictors of lesion progression. These data suggest that the angiotensinogen T/T genotype at position 235 is a genetic marker for brain lesions from and progression of small vessel disease but not for extracranial carotid atherosclerosis. **(*Hypertension*. 2001;38:110-115.)**

Key Words: angiotensinogen ■ genetics ■ carotid arteries ■ atherosclerosis ■ vessels

Angiotensinogen is a liver protein that interacts with renin to produce angiotensin I, the prohormone of angiotensin II, which increases vascular tone and promotes sodium retention. The plasma concentrations of angiotensinogen have been directly related to arterial blood pressure and were found to be modified by variations in the angiotensinogen gene.[1] A missense mutation in exon 2 of the angiotensinogen gene (T704-C) encoding threonine instead of methionine at position 235 (M235T) of the amino acid sequence commonly occurs among various ethnic groups. The frequency of the T235 allele ranges from 0.35 in whites to approximately 0.80 in blacks.[2] A recent meta-analysis with an overall sample size of 27 906 subjects attributed an increased risk for arterial hypertension to the presence of the T-allele. In comparison with the MM reference group, the excess risk was 31% in TT homozygotes and 11% in MT heterozygotes.[3] Moreover, several studies found this genetic variant to be associated with coronary artery disease and myocardial infarction.[4–7]

There is little information on a possible link between this genetic variant and cerebrovascular disease. Two studies showed no relation with carotid intima-media-thickness,[8,9] but there have been no investigations assessing the relation of the M→T interchange with atheromatous carotid disease and intracranial small vessel disease, for which arterial hypertension is the most important risk factor.[10] It is also undetermined as to whether the M235T polymorphism relates to progression of extracranial or intracranial atherosclerosis. We therefore extended previous work by investigating the association between the M235T polymorphism with carotid atherosclerosis and with small-vessel disease–related cerebral damage in the setting of a longitudinal study in community-dwelling middle-aged and elderly persons. We used Doppler sonography and brain magnetic resonance imaging (MRI) to monitor the study participants over a time period of 3 years.

Received October 13, 2000; first decision November 16, 2000; revision accepted January 10, 2001.

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Methods

Subjects and Design

The study population consisted of participants of the Austrian Stroke Prevention Study, a single-center, prospective follow-up 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 institutional review committee of the Department of Neurology and by the ethics committee of the Karl-Franzens University Graz, Austria. The procedure of recruitment and diagnostic workup of study participants has been described previously.[11] The baseline cohort consisted of 2000 persons without a history or signs of neuropsychiatric disease. Every fourth study participant or, in case of refusal, the next, was invited to enter phase II of the study, which included carotid Doppler sonography and brain MRI. From a total of 496 phase II participants, all underwent Duplex scanning of the carotid arteries, and 461 volunteered to undergo an MRI study. Since 1993, blood sampling for DNA analysis was done in a total of 431 individuals. There were 207 men and 224 women. The mean age was 60.2±6.1years. The sample consisted exclusively of whites of central European origin. Doppler sonography was available in all of them and MRI in 396 individuals. At the second study panel 3 years after baseline, we were able to reexamine 341 individuals of the 431 participants who underwent neuroimaging and DNA sampling, following the same protocol. From the 90 individuals who could not be reexamined, 7 had died and another 7 had had a stroke, which is an end point in our study. Sixty-five subjects were contacted by telephone but refused to undergo the extensive diagnostic workup a second time. Eleven individuals could not be reinvited because we were unable to reach them after 3 phone calls and a letter. All attendees who were reexamined after 3 years underwent a second duplex scanning, but 74 refused to undergo a second MRI study because of claustrophobic sensations at the initial evaluation. The individuals who participated in the duplex scan and MRI follow-up studies did not differ from those who dropped out in age, gender, and risk factors for stroke. They were also comparable in terms of genotype frequency and baseline sonographic and MRI findings.

Historic information and laboratory findings at baseline were considered for risk factor diagnosis. The definitions for risk factor diagnosis have been previously described.[11]

DNA Isolation and Genotyping

Genomic DNA was isolated from whole blood by Qiagen genomic tips. The M235T polymorphism was identified by a nonisotropic method involving restriction typing after polymerase chain reaction, as described elsewhere.[12] The DNA was visualized directly by ethidium bromide staining. After digestion, the M235 allele that lacked the restriction site was a 163-bp fragment and the T235 allele that had the restriction site was a 140-bp fragment.

Carotid Duplex Scanning

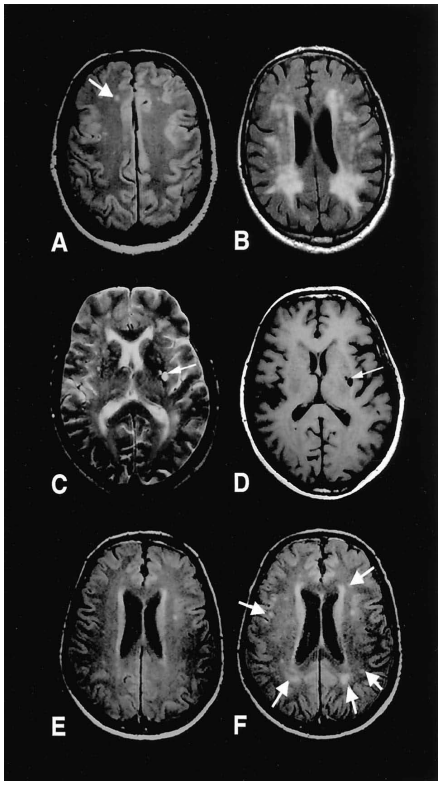
Color-coded equipment (Diasonics, VingMed CFM 750) was used to determine atherosclerotic vessel wall abnormalities of the carotid arteries at baseline and 3-year follow-up examination. All B-mode and Doppler data were transferred to a Macintosh personal computer for processing and storage on optical disk. The imaging protocol involved scanning of both common carotid arteries (CCA) and internal carotid arteries (ICA) in multiple longitudinal and transverse planes and has been previously described.[13] The examinations were performed by 3 readers without knowledge of the clinical data of the individuals. Image quality was assessed and graded as good (CCA and ICA clearly visible and ICA detectable over a distance of >2 cm), fair (CCA and ICA sufficiently visible and ICA detectable over a distance of at least 2 cm), and poor (CCA or ICA insufficiently visible or ICA detectable over a distance of <2 cm). There was no poor quality study at the baseline and follow-up examination. At baseline and follow-up, the extent of atherosclerosis was graded according to the most severe visible changes in the CCA and ICA as 0, normal; 1,vessel wall thickening >1 mm; 2, minimal plaque (<2 mm); 3, moderate plaque (2 to 3 mm); 4, severe plaque (>3 mm), and 5, lumen completely obstructed. The interrater variability for grading the extent of sonographic changes was independently assessed in 200 vessels in 50 subjects. The κ values for interrater agreement for the sonographic score between the 3 sonographers ranged from 0.89 to 0.95. The most severe change in any vessel and the sum of scores of the 4 vessels has been recorded. The difference of the sum of scores of both CCA and ICA between baseline and follow-up was used to define regression or progression of carotid atherosclerosis.

Magnetic Resonance Imaging

MRI was performed on 1.5-T superconducting magnets (Gyroscan S 15 and ACS, Philips) with proton-density and T2-weighted (TR/TE 2000 to 2500/30 to 90 ms) sequences in the transverse orientation. T1-weighted images (TR/TE 600/30 ms) were generated in the sagittal plane. Slice thickness was 5 mm, and the matrix size used was 128×256 pixels. MRI protocols at baseline and 3-year follow-up were identical. The scanning plane was always determined by a sagittal and coronal pilot to ensure consistency in image angulation throughout the study. The baseline and follow-up scans of each study participant were read independently by 3 investigators blinded to the clinical data of study participants. Blinding of the readers for the date of the examinations was impossible because the format of hard copies changed from baseline to follow-up. The scans were evaluated for small-vessel disease–related abnormalities. According to numerous histopathological correlations, these changes consisted of white matter hyperintensities (WMH) and lacunar lesions.[14,15] WMH were graded according to our scheme as absent (grade 0), punctate (grade 1), early confluent (grade 2), and confluent (grade 3).[16] The κ values for interrater agreement regarding WMH grade ranged from 0.63 to 0.70. The number of WMH was also recorded. Caps and periventricular lining were disregarded because these changes probably represent normal anatomical variants.[15] Lacunes were focal cerebrospinal fluid–containing lesions that involved the basal ganglia, the internal capsule, the thalamus, or brain stem not exceeding a maximum diameter of 10 mm. Progression of small-vessel disease–related brain lesion was defined to be present if WMH increased in grade or number or if new lacunar lesions occurred at the follow-up examination. Rating of lesion progression was determined by direct scan comparison and relied on majority judgment of the 3 assessors. In the case of complete disagreement, consensus was found in a joint reading session. The κ values for interrater agreement for progression of small-vessel disease–related brain abnormalities ranged from 0.61 to 0.69. We also looked at different grades of progression. We considered a change from baseline by 1 to 4 punctate WMH to represent minor progression. Progression was rated to be marked if there was a difference of more than 4 WMH or a transition to early confluent or confluent WMH or if new lacunar lesions were seen. The Figure displays examples for punctate and confluent WMH representing the range of WMH extent seen in our study as well as examples for lacunes and for WMH progression.

Statistical Analysis

We used the Statistical Package for Social Sciences (SPSS 8.0) for data analysis. The degree of agreement for sonographic and MRI rating was expressed by the means of κ statistics. According to Fleiss,[17] a κ value <0.40 reflects poor agreement; between 0.40 and 0.75, fair to good agreement; and >0.75, excellent agreement. Categoric variables among the M235T genotypes were compared by means of the χ^2 test. Assumption of normal distribution for continuous variables was assessed by Lilliefors statistics. Normally distributed continuous variables were compared by 1-way ANOVA; the Kruskal-Wallis test was used for comparison of nonnormally distributed variables. Allele frequencies were calculated by the gene-counting method, and Hardy-Weinberg equilibrium was assessed by means of the χ^2 test. Logistic regression analysis assessed the relative contribution of the M235T genotypes on carotid ultrasound and brain MRI findings. Analyses with ultrasound findings as the dependent variable were adjusted for age and gender. In the selection of covariates for analyses on small-vessel disease–related brain damage, we followed a recent review on risk factors for these MRI



Composite shows WMH at top, single left basal ganglionic lacune in middle, and progression of WMH at bottom. A, Punctate WMH (arrow); B, widespread confluent WMH. A and B represent observed extremes of WMH. C and D, Lacunar lesion is demonstrated on corresponding T2-weighted (C) and T1-weighted (D) images. Lacunes are isointense to cerebrospinal fluid in each scan sequence. E, Baseline scan in 67-year-old study participant showing few punctate WMH in centrum semiovale. F, Follow-up study in same subject after 3 years demonstrates multiple, new, and partly confluent WMH (arrows).

lesions.[18] Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated from the β coefficients and their standard errors.

Results

The frequencies of the M and T alleles were 0.55 and 0.45. The MM, MT, and TT genotypes were noted in 125 (29.0%), 228 (52.9%), and 78 (18.1%) study participants. The genotypes were in Hardy-Weinberg equilibrium. Table 1 compares demographic variables and vascular risk factors among the genotype subsets. As can be seen from this table, TT carriers were older and had a higher frequency of heart disease and arterial hypertension. There was also a nonsignificant trend toward higher diastolic blood pressure values at examination in participants with the TT genotype. The association with hypertension was more pronounced in women than in men. A total of 16 (26.2%) and 29 (24.4%) women with the MM and MT genotype were hypertensive as opposed to 21 (47.7%) female TT carriers (P=0.03). The figures in men were 22 (34.4%), 37 (33.9%), and 14 (41.2%), respectively (P=0.57). The systolic and diastolic blood pressure values were also highest in the TT genotype subset in both genders. Yet, when ANCOVA was used to correct for the between-group differences in age and antihypertensive medication, the association remained only significant for diastolic blood pressure in women (P=0.04).

A total of 231 (53.6%) study participants showed atherosclerotic changes of the carotid arteries. They occurred in 74 (59.2%) subjects with the MM genotype, 117(51.3%) with the MT genotype, and 40 (51.3%) with the TT genotype (P=0.33). A breakdown of the severity of carotid atherosclerosis by genotype is given in Table 2. There were no significant differences among the 3 investigational subsets. At the 3-year follow-up examination, regression of carotid lesions occurred in 2 (0.6%) individuals, 259 (75.5%) subjects had stable findings, and 82 (23.9%) participants showed plaque progression. Plaque progression occurred at similar frequencies among the 3 genotypes. It was seen in 27 (28.1%) MM carriers, 40 (21.9%) MT carriers, and 15 (23.4%) TT carriers (P=0.57). There existed no gender difference for the associations between the M235T variant and baseline frequency or progression of carotid atherosclerosis. The age- and gender-adjusted odds ratios of the MT and TT genotypes relative to the MM genotype for the presence of carotid atherosclerosis were 0.73 (95% CI, 0.45 to 1.16; P=0.18) and 0.61 (95% CI, 0.33 to 1.11; P=0.11). Those for progression of sonographic findings were 0.69 (95% CI, 0.28 to 1.23; P=0.21) and 0.72 (95% CI, 0.39 to 1.54; P=0.40).

At baseline, MRI detected WMH in 183 (46.2%) study participants. Lacunar lesions were seen in 32 (8.1%) subjects. Homozygotes for the T allele had higher grades of WMH and tended to show lacunes more frequently than their counterparts with either the MM or MT genotypes (Table 3). The number of WMH was also highest in the TT subset, but the differences to the other genotypes were not significant. The difference for WMH grade between the 3 genotypes seen in the univariate analysis was no longer significant when multinominal logistic regression analysis was used to correct for possible confounding by age, gender, hypertension, heart disease, and systolic and diastolic blood pressure. After 3 years, we noted progression of small-vessel disease–related cerebral abnormalities, including both WMH or lacunes in 52 (19.5%) participants. As shown in Table 3, lesion progression was more than twice as common in subjects with the TT genotype than in the comparative groups (P<0.001). The most pronounced difference between the genotype subsets was seen for marked progression. The association between the TT genotype and progression of brain abnormalities was significant in women (P=0.03) and in men (P=0.01). Logistic regression analysis yielded an unadjusted OR of 3.78 (95% CI, 1.89 to 7.56; P<0.0001) for lesion progression in the TT genotype relative to the two other genotypes. Adjustment for age and gender altered the OR only marginally to 3.42 (95% CI, 1.70 to 6.66; P=0.001) and 3.75 (95% CI, 1.87 to 7.52; P<0.0001). We used multivariable logistic regression to assess the relative contribution of the TT relative to the other two genotypes combined on the progression of

TABLE 1. Demographics and Risk Factors Among Angiotensinogen M235T Genotypes

Variable	MM (n=125)	MT (n=228)	TT (n=78)	*P*
Age, y	60.0±6.1	59.8±6.1	61.9±6.2	0.3*
Gender, male	64 (51.2%)	109 (47.8%)	34 (43.6%)	0.57†
Hypertension	39 (31.4%)	69 (29.6%)	36 (45.6%)	0.03†
Systolic blood pressure, mm Hg	140.1±21.7	136.3±18.5	142.8±22.6	0.10*
Diastolic blood pressure, mm Hg	85.6±9.0	84.8±9.1	87.9±10.5	0.05*
Heart disease	42 (33.6%)	78 (34.2%)	39 (50%)	0.03†
Diabetes mellitus	6 (4.8%)	13 (5.7%)	5 (6.4%)	0.88†
Fasting glucose, mmol/L	5.1±0.06	5.3±1.3	5.3±1.4	0.80*
Smoking status				
Never	73 (58.4%)	121 (53.1%)	52 (66.7%)	
Ex-smoker	15 (12.0%)	30 (13.2%)	8 (10.3%)	
Current	37 (29.6%)	77 (33.8%)	18 (23.1%)	0.33*
Triglycerides, mmol/L	1.6±1.0	1.6±0.9	1.7±1.1	0.98*
Total cholesterol, mmol/L	5.9±1.0	5.9±1.0	6.1±1.1	0.37‡
LDL cholesterol, mmol/L	3.8±0.9	3.9±0.9	4.1±1.0	0.12‡
HDL cholesterol, mmol/L	1.3±0.4	1.3.0±0.4	1.2±0.4	0.58*
Apolipoprotein B, μmol/L	2.4±0.5	2.4±0.5	2.4±0.7	0.63*
Apolipoprotein A-I, μmol/L	64.3±10.7	60.7±10.7	60.7±14.3	0.75*
Fibrinogen, μmol/L	8.8±2.3	8.8±2.3	9.1±2.3	0.75*

*Kruskal-Wallis test, †χ^2 test, ‡1-way ANOVA.

small-vessel disease–related brain abnormalities with adjustment for the putative confounders age, gender, arterial hypertension, hypertensive treatment, diabetes mellitus, heart disease, and plasma fibrinogen. Table 4 displays this risk factor model. As can be seen from this table, the TT genotype and arterial hypertension were the only variables found to be significantly associated with the progression of small-vessel disease–related brain abnormalities. The interaction term arterial hypertension×TT genotype was not associated with lesion progression (OR, 0.67; $P=0.58$).

Discussion

WMHs and lacunes represent small-vessel disease–related brain damage, which, in early stages, is often clinically silent.[18] Our study in an elderly population has shown that the T235 variant of the angiotensinogen gene is associated with the presence and progression of these brain abnormalities. The relation between the M235T polymorphism and lesion progression occurred independent of arterial hypertension, which per se was moderately linked to both the genomic variant and lesion progression. Clinically, progression of small-vessel disease–related cerebral abnormalities is thought to be associated with cognitive dysfunction and gait disturbances, symptoms known to be a major source of disability in our aging societies.[18] Identification of factors relating to the progression of this type of brain damage is thus of relevance regarding the prediction of possible clinical consequences and the development and initiation of preventive measures.

TABLE 2. Carotid Doppler Sonography Findings at Baseline by Genotype

Maximum Doppler Score*, n (%)	MM (n=125)	MT (n=228)	TT (n=78)	*P*
0	51 (40.8%)	111 (48.7%)	38 (48.7%)	
1	9 (7.2%)	8 (3.5%)	2 (2.6%)	
2	46 (36.8%)	70 (30.7%)	21 (26.9%)	
3	15 (12.0%)	27 (11.8%)	12 (15.4%)	
4	4 (3.2%)	12 (5.3%)	5 (6.4%)	0.45†
Sum of scores‡	2.4±2.5	2.4±2.9	2.6±2.9	0.85§

*Doppler score 5 (complete obstruction) was not seen; †χ^2 test; ‡sum of maximum score in left and right common and internal carotid arteries; §1-way ANOVA.

In line with the population-based ARIC study and the NHLBI family heart study,[9] which measured intima-media thickness of the common carotid arteries, and a single case-control study[8] assessing also the degree of stenosis, we failed to show a relation between the M235T polymorphism and carotid atherosclerosis. The lack of association with carotid atherosclerosis in the presence of an association with brain lesions linked to arteriolosclerosis appears contradictory at first glance. Yet, large- and small-vessel disease represent distinct vascular pathologies, which may also be reflected by differences in the profiles of genetic susceptibility factors.

The frequency of M and T homozygotes in our sample paralleled that of population samples from France and North America,[1] New Zealand,[4] and Germany,[7] indicating that there

TABLE 3. Angiotensinogen M235T Polymorphism and Small-Vessel Disease–Related Cerebral Abnormalities on MRI: Baseline Findings and 3-Year Progression

Baseline	MM (n=118)	MT (n=201)	TT (n=77)	P*
WMH grade, n (%)				
0	60 (50.8)	115 (57.2)	38 (49.4)	
1	39 (33.1)	56 (27.9)	17 (22.1)	
2	14 (11.9)	22 (10.9)	20 (26.0)	
3	5 (4.2)	8 (4.0)	2 (2.6)	0.04
WMH No. (mean±SD)	4.6±11.2	4.0±7.9	5.2±9.2	0.57
Lacunes, n (%)				
Absent	110 (93.2)	188 (93.5)	66 (85.7)	
Present	8 (6.8)	13 (6.5)	11 (14.3)	0.08
3-y Follow-up, n	82	135	50	
Progression, n (%)†				
Absent	70 (85.4)	115 (85.2)	30 (60.0)	
Present	12 (14.6)	20 (14.8)	20 (40.0)	0.001
Progression extent, n (%)‡				
Minor	8 (9.8)	11 (8.1)	10 (20.0)	
Marked	4 (4.9)	9 (6.7)	10 (20.0)	0.002

*χ^2 test; †progression of either type of abnormality; ‡minor represents change of 1 to 4 punctate WMH, marked represents change of >4 WMH or transition to early confluent or confluent WMH or new lacunar lesions.

was no sample bias in this community-dwelling cohort. The modest overall association between the T235 polymorphism with arterial hypertension is in keeping with a previous meta-analysis of 69 case-control studies.[3] Blood pressure directly relates to plasma concentration of angiotensinogen, with the angiotensinogen level being elevated in subjects carrying the T235 variant.[1] We observed a much closer relation between the T235 variant and blood pressure among women. Angiotensinogen gene expression is known to be estrogen-dependent,[19] but all female participants in our study were postmenopausal. This implies other estrogen-independent yet undetermined differential gene-gene or gene-environment interactions across genders to contribute to the gender-specific difference in the blood pressure of homozygotes for the T allele, at least in higher age groups. Notably, the NHLBI Family Heart Study, which had an age distribution similar to the current cohort, also reported a greater association between the T235 polymorphism and hypertension in women than in men.[20]

TABLE 4. Logistic Regression Model of Risk Factors for Progression of Small-Vessel Disease–Related Cerebral Abnormalities on MRI

Variable	OR	95% CI
TT genotype	3.19	1.54, 6.63
Hypertension	3.06	1.15, 8.20
Antihypertensive therapy	0.60	0.21, 1.75
Age, y	1.04	0.98, 1.10
Gender (male)	0.87	0.45, 1.68
Diabetes	0.69	0.17, 2.82
Heart disease	1.14	0.56, 2.29
Fibrinogen, mg/dL	1.002	0.99, 1.01

*Each OR is adjusted for other factors listed in the table.

Several histopathologic correlations substantiate that lacunes and WMH are linked to small-vessel disease of the brain. Lacunes represent small cavities caused by infarctions, which are often located in areas irrigated by the deep and superficial cerebral arterioles,[14] whereas the histological correlates of WMH show much greater diversity.[15] Punctate lesions frequently correspond to a perivascular reduction in myelin content with atrophy of the neuropil and thus constitute only minor tissue damage, probably from low permeability through thickened arteriolar walls. Early confluent and confluent WMH indicate more extensive tissue damage including myelin pallor, loss of fibers, reactive gliosis, and sometimes even small lacunar cavities consistent with more advanced microangiopathy. Arterial hypertension is the most important risk factor for small-vessel disease–related brain changes besides age.[11,18]

The mechanism(s) responsible for the effect(s) of the angiotensinogen T235 variant on progression of small-vessel disease–related cerebral abnormalities remains speculative at this time. It is clear from our data that elevated blood pressure can only partly explain this association because homozygosity for the T allele predicted lesion progression independent of arterial hypertension. One explanation for the relation is that the T235 variant represents merely a marker in linkage disequilibrium, with a close-by etiologically important polymorphism. Conceivably, this could be a recently identified mutation in the promoter region of the angiotensinogen gene (A-6), which was seen to be in tight linkage disequilibrium with T235 and caused elevated angiotensinogen expression.[21] Angiotensinogen is the precursor peptide of the vasoactive hormone angiotensin II, which has multiple proatherogenic effects, including induction of smooth muscle cell hypertrophy, stimulation of vascular fibrosis, plasminogen activator inhibitor-1 stimulation, free radical formation, and increased endothelin secretion.[22] Most importantly in the context of our results, there exists an independent renin-angiotensin system in the brain that might contribute to or amplify cerebral small-vessel disease, an effect that might not be reflected in the systemic circulation.[23] The fact that the M235T angiotensinogen polymorphism is at some distance from the angiotensin cleavage site supports that this genomic variant is rather a marker for another functionally important mutation in the vicinity. However, one cannot exclude with certainty that angiotensinogen has other yet unknown functions unrelated to its role as a prohormone. These functions could then be altered by mutations distant from the site of cleavage, such as T235. The large ratio of the size of the precursor (452 to 453 amino acids) to product (10 amino acids) encourages a teleological argument regarding alternate functions of angiotensinogen.[24] By contrast, a relatively high degree of sequence divergence (>35%) between rodents and human angiotensinogens argues against such functions because it shows that there is little pressure to conserve much of the amino acid sequence of this protein.[24] Whatever mechanism is responsible for the association between the angioten-

sinogen M235T polymorphism and progression of brain abnormalities caused by small-vessel disease, our data suggest components of the renin-angiotensin system to play a role in the pathogenesis of arteriolosclerosis independent of their effects on blood pressure. Consequently, intervention in the renin-angiotensin system could exert beneficial effects on the evolution of small-vessel disease–related brain damage and its clinical consequences beyond what can be expected from the lowering of blood pressure alone.

Acknowledgments

The study was funded by the Austrian Science Fund, project P13180-MED. The authors are grateful to Birgit Reinhart for administrative work and to Johann Semmler and Irmgard Pölzl for excellent technical assistance.

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Beta-Fibrinogen Gene Polymorphism (C148-->T) is Associated with Carotid Atherosclerosis

4.3

β-Fibrinogen Gene Polymorphism (C_{148}→T) Is Associated With Carotid Atherosclerosis

Results of the Austrian Stroke Prevention Study

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Abstract—Polymorphisms at the β-fibrinogen locus have been shown to be associated with plasma concentration of fibrinogen and coronary heart disease. The effect of the genetic heterogeneity of fibrinogen on carotid atherosclerosis has not been determined so far. We examined the influence of the C_{148}→T polymorphism on carotid disease in a large cohort of middle-aged to elderly subjects without evidence of neuropsychiatric disease. This polymorphism is located close to the consensus sequence of the interleukin-6 element and may represent a functional sequence variant. The genotype of 399 randomly selected, neurologically asymptomatic individuals, aged 45 to 75 years, was determined by denaturing gradient gel electrophoresis. Carotid atherosclerosis was assessed by color-coded duplex scanning and was graded on a five-point scale ranging from 0 (=normal) to 5 (=complete luminal obstruction). The *C/C*, *C/T,* and *T/T* genotypes were noted in 226 (56.6%), 148 (37.1%), and 25 (6.3%) individuals, respectively. The *T/T* genotype group demonstrated higher grades of carotid atherosclerosis than did the *C/C* and *C/T* genotypes (*P*=.003). Logistic regression analysis created a model of independent predictors of carotid atherosclerosis that included apolipoprotein B (odds ratio [OR], 1.17/10 mg/dL), age (OR, 2.46/10 years), lifetime tobacco consumption (OR, 1.03/1000 g), presence of the β-fibrinogen promoter *T/T* genotype (OR, 6.17), plasma fibrinogen concentration (OR, 1.05/10 mg/dL), and cardiac disease (OR, 1.80). These data suggest that the β-fibrinogen promoter *T/T*148 genotype represents a genetic risk factor for carotid atherosclerosis in the middle-aged to elderly. **(*Arterioscler Thromb Vasc Biol*. 1998;18:487-492.)**

Key Words: fibrinogen ■ genetics ■ atherosclerosis ■ carotid arteries

There are numerous studies describing an association between plasma fibrinogen levels and coronary heart disease,[1–6] stroke,[5–7] and carotid atherosclerosis.[8–18] However, it is unclear whether the elevations in plasma fibrinogen level are a causal factor in the development of atherosclerosis or only an epiphenomenon of the atherogenic process.

Fibrinogen concentration is controlled by genetic and environmental factors, including smoking, obesity, use of contraceptives, trauma, and lack of exercise, which have been reported to elevate fibrinogen concentrations.[19–21] Fibrinogen level also increases with age and in the presence of diabetes mellitus, hypertension, or lipid abnormalities.[5,6]

The estimate of heritability for fibrinogen is in the range of 30% to 50%, depending on the study design.[22,23] Theoretically, any gene coding for proteins involved in fibrinogen metabolism may have an impact on the genetic regulation of the plasma fibrinogen level. The synthesis of the Bβ chain has been shown to be the rate-limiting step in the formation of fibrinogen.[24] The 5′ region of the β gene contains binding sites for several *trans*-acting factors, which largely control expression of the gene.[25–27] Several polymorphisms have been identified within this region.[28–30] One of them, the C_{148}→T polymorphism, is located close to an interleukin-6–responsive element and may affect fibrinogen gene expression, mainly in response to the acute-phase reaction.[28] There are some population-based studies that have investigated the effect of polymorphisms in the promoter region of the β-fibrinogen gene on fibrinogen level and the risk of coronary atherosclerosis.[30–32] Their results are controversial. To our knowledge, this is the first investigation on the effect of the C_{148}→T polymorphism on carotid atherosclerosis.

Methods

Study Population

Individuals aged 45 to 75 years and stratified by gender and 5-year age groups were randomly selected from the official register of residents of the city of Graz, Austria. They were all white and of central European origin. They received a written invitation to participate in the Austrian Stroke Prevention Study, a single-center, prospective, follow-up study in our community. The study had been approved by the Medical Ethics Committee of the Karl-Franzens University of Graz. Written, informed consent was obtained from all study participants. The rationale and design of the Austrian Stroke Prevention Study have been previously described.[33] In brief, the objective of the study was to

Received October 13, 1997; revision accepted November 26, 1997.

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examine the frequency of cerebrovascular risk factors and their effects on carotid atherosclerosis as well as on cerebral morphology and function in the elderly. The inclusion criteria for the study were no history of neuropsychiatric disease and a normal neurological examination. From a total of 8193 individuals invited between September 1991 and March 1994, a sample of 2794 subjects agreed to participate, of whom 1998 individuals fulfilled the inclusion criteria. All study participants underwent a structured clinical interview, a physical and neurological examination, three blood pressure readings, electrocardiography, and echocardiography as well as laboratory investigations including blood cell count and a complete blood chemistry panel. Every fourth study participant was then invited to enter phase II of the Austrian Stroke Prevention Study, which included Doppler sonography, magnetic resonance imaging, single-photon emission computed tomography, and neuropsychological testing. Since 1993 we also performed β-fibrinogen promoter genotyping in all phase II attendees. The current study cohort consists of those 399 individuals who underwent both carotid duplex scanning and assessment of the β-fibrinogen polymorphism. There were 204 women and 195 men. The mean±SD age of this cohort was 60.1±6.0 years.

Vascular Risk Factors

The diagnosis of major risk factors for stroke, including arterial hypertension, diabetes mellitus, and cardiovascular disease, relied on the individual's history and appropriate laboratory findings. A detailed description of the definition of these risk factors is given elsewhere.[34,35]

Study participants were defined as current smokers, ex-smokers, or never-smokers. For current smokers and ex-smokers, information was obtained about the daily number of items smoked and the smoking duration in years. The data on the amount of tobacco were converted to grams of tobacco consumed during their lifetime by using the following conversion factors: 1 cigarette=1 g, 1 cheroot=3 g, and 1 cigar=5 g. Body mass index was calculated as weight in kilograms divided by the square of height in meters squared. The regular use of estrogen replacement therapy was recorded among all female study participants.

For measurements of hematocrit, blood was obtained from a large antecubital vein without stasis. Lipid status, including the level of triglycerides, total cholesterol, LDL cholesterol, HDL cholesterol, lipoprotein(a), apolipoprotein B, and apolipoprotein A-I, was determined for each study participant. Triglycerides and total cholesterol were enzymatically determined by using commercially available kits (Uni-Kit III "Roche" and MA-Kit 100 "Roche," Hoffman –La Roche). HDL cholesterol was measured by the use of the TDx REA cholesterol assay (Abbott). LDL cholesterol was calculated by the equation of Friedewald. The lipoprotein(a) concentration was determined by the electroimmunodiffusion method using a reagent kit containing monospecific anti-lipoprotein(a) antiserum and the Rapidophor M3 equipment (Immuno AG). The levels of apolipoprotein B and A-I were assessed by an immunoturbidimetric method utilizing polyclonal antibodies and a laser nephelometer (Behringwerke AG). The plasma fibrinogen concentration was measured according to the Clauss method[35] by using the recommendations and reagents of Behringwerke AG.

Isolation of DNA and Genotype Analysis

High-molecular-weight DNA was extracted from peripheral whole blood by using Qiagen genomic tips (Qiagen Inc) according to the protocol of the manufacturer. Genotyping was performed by denaturing gradient gel electrophoresis (DGGE). This procedure is routinely used in our laboratory and is preferred over restriction enzyme digestion because it detects point mutations within the amplified DNA fragment with high sensitivity and does not require further processing of polymerase chain reaction (PCR) products.[36,37] A 417-bp-long fragment containing part of the promoter region and exon 1 (from −263 to +114 nucleotides) of the β-fibrinogen gene and a 40-bp-long GC clamp serving as an artificial high-melting-point domain for DGGE was amplified by using two oligonucleotides (5′-GC clamp CTC TTT GAG GAG TGC CCT AAC TTC C-3′ and 5′-TGT CGT TGA CAC CTT GGG ACT TAA C-3′). PCR was performed on 1 μg of genomic DNA in a buffer containing 10 mmol/L Tris (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 0.5 μmol/L of each primer, and 1 U of *Taq* DNA polymerase in a final volume of 50 μL. After 5 minutes at 94°C, amplification was carried out in 40 cycles consisting of 1 minute at 94°C, 1 minute at 52°C, and 2 minutes at 72°C on a Mastercycler (Eppendorf). A final elongation step was performed for 10 minutes at 72°C. Amplification was assessed by electrophoresing 5 μL of the PCR product on a 1.5% agarose gel stained with ethidium bromide.

The melting domain map for the DGGE analysis was calculated with the MELT87 computer algorithm.[38] PCR products were analyzed on 8% polyacrylamide gels containing a 20% to 50% linearly increasing denaturing gradient (100% denaturant is equivalent to 7 mol/L urea and 40% [vol/vol] deionized formamide). Electrophoresis was performed at 100 V at 60°C for 16 hours in TAE buffer (40 mmol/L Tris acetate, 1 mmol/L EDTA, pH 7.5). Gels were stained with ethidium bromide and examined under UV illumination. DNA samples with known genotypes were used initially to determine the position of the bands of the different genotypes. The *C/C* genotype corresponded to the lower band; the *T/T* genotype, the higher band; and the *C/T* genotype, a four-band pattern resulting from the two homoduplexes and two heteroduplexes.

Sequencing of the control DNA samples used as standards for the DGGE analysis was performed on a model 373A automated DNA sequencer (Perkin Elmer/Applied Biosystems Inc) and applying the dye terminator cycle sequencing ready reaction kit (model No. 402079, Perkin Elmer/Applied Biosystems).

Carotid Artery Duplex Scanning

A color-coded device (Diasonics, VingMed CFM 750) was used to determine atherosclerotic vessel wall abnormalities of the carotid arteries. All B-mode and Doppler data were transferred to a Macintosh personal computer for postprocessing and storage on optical disks. The imaging protocol involved scanning of both common and internal carotid arteries in multiple longitudinal and transverse planes and has been previously described.[33,34] The examinations were performed by one experienced physician (S.H.). Image quality was assessed and graded into good (common and internal carotid arteries clearly visible and internal carotid arteries detectable for a distance >2 cm), fair (common and internal carotid arteries sufficiently visible and internal carotid arteries detectable for a distance of ≥2 cm), and poor (common and internal carotid arteries insufficiently visible or internal carotid arteries detectable for a distance <2 cm). Examinations of poor quality were excluded from further analysis. Measurements of maximal plaque diameter were done in longitudinal planes, and the extent of atherosclerosis was graded according to the most severe visible changes in the common and internal carotid arteries as follows: 0=normal, 1=vessel wall thickening (<1 mm), 2=minimal plaque (one ≤2 mm), 3=moderate plaque (two ≤3 mm), 4=severe plaque (>3 mm), and 5=lumen completely obstructed. Assessment of the intrarater reliability of this score was done in 50 randomly selected subjects and yielded a kappa value of 0.83.

Statistical Analysis

We used the Statistical Package for the Social Sciences (SPSS/PC+) for data analysis. Categorical variables among the three β-fibrinogen genotypes were compared by the χ^2 test. Assumption of a normal distribution for continuous variables was tested by Lilliefors statistics. Normally distributed continuous variables were compared by one-way ANOVA, whereas the Kruskal–Wallis test was used for comparison of nonnormally distributed variables. To assess the relative contribution of the three β-fibrinogen genotypes on the presence of carotid atherosclerosis, we used multiple logistic regression analysis. The sonographic score was dichotomized as normal (grade 0) or abnormal (grades 1 to 5). Odds ratios (ORs) and 95% confidence intervals (CIs) with and without adjustment for age were calculated from the β coefficients and their SEs. We used first-order interaction terms to evaluate whether or not the association between β-fibrinogen genotype and the presence of carotid atherosclerosis was modified by plasma fibrinogen level, lifetime tobacco consumption, or use of hormone replacement therapy.[19–21] These factors were considered because previous studies demonstrated that polymorphisms at the

TABLE 1. Demographics and Risk Factors Among β-Fibrinogen Genotypes

Variable	Genotypes			P
	C/C (n=226)	*C/T* (n=148)	*T/T* (n=25)	
Age, y	60.1±5.8	59.7±6.0	61.9±6.6	.29*
Sex, male	110 (58.7)	71 (48.0)	14 (56.0)	.76†
Hypertension, n (%)	76 (33.6)	47 (31.8)	7 (28.0)	.82†
Diabetes mellitus, n (%)	9 (4.0)	10 (6.8)	2 (8.0)	.41†
Fasting glucose, mg/dL	92.7±19.2	94.9±21.8	99.2±30.1	.31‡
Cardiac disease, n (%)	82 (36.3)	55 (37.2)	13 (52.0)	.30†
Ex-smokers, n (%)	24 (10.6)	25 (16.9)	3 (12.0)	
Current smokers, n (%)	78 (34.5)	46 (31.1)	8 (32.0)	.52†
Lifetime tobacco consumption $\times 10^3$ g	85.6±147.6	100.8±156.7	138.0±278.4	.57*
Estrogen replacement, n (%)	33 (28.7)	19 (24.7)	3 (27.3)	.83†
Body mass index, kg/m^2	26.7±3.6	26.4±3.9	26.6±3.5	.70*
Hematocrit, %	34.4±16.2	36.7±14.4	37.2±14.8	.33*
Triglycerides, mg/dL	147.4±86.6	141.6±84.1	154.3±103.0	.54*
Cholesterol, mg/dL	229.5±38.1	229.8±42.2	233.1±41.5	.91‡
LDL cholesterol, mg/dL	149.7±34.7	150.9±37.6	158.1±28.9	.54‡
HDL cholesterol, mg/dL	50.4±15.6	50.6±15.3	44.3±12.4	.10*
Lipoprotein(a), mg/dL	22.5±26.5	27.9±34.7	18.7±17.9	.60*
Apolipoprotein B, mg/dL	142.3±31.4	144.2±37.1	147.8±33.3	.84*
Apolipoprotein A-I, mg/dL	174.9±31.9	174.0±30.6	167.4±23.6	.49*
Fibrinogen, mg/dL	304.7±76.8	306.2±68.9	300.8±69.7	.87*

*Kruskall-Wallis test.
†χ^2 test.
‡One-way ANOVA.

β-fibrinogen locus may influence the plasma concentration of fibrinogen and that this may in turn be modified by smoking and hormone therapy. We used forward stepwise regression to create a model of independent predictors of carotid disease. At each step, each variable not in the model was assessed as to its contribution to the model, and the most significant variable was added to the model. This process continued until no variable not in the model made a significant ($P<.05$) contribution. ORs and 95% CIs were calculated from the β coefficients and their SEs.

Results

Using DGGE between nucleotides −263 and +114 in the β-fibrinogen gene, we were able to detect only one sequence variation. This sequence alteration was identical to the known $C_{148}\rightarrow T$ polymorphism. The overall allele frequency of the *T*148 allele was found to be .248, similar to what has been observed in other countries in Europe.[34,35] The *C/C*, *C/T*, and *T/T* β-fibrinogen promoter genotypes were noted in 226 (56.6%), 148 (37.1%), and 25 (6.3%) study participants, respectively, and these values were in Hardy-Weinberg equilibrium.

Table 1 compares demographic variables and risk factors among the three genotypes. As shown in this table, there were no statistically significant differences among the three groups. The plasma fibrinogen concentration was almost equal in the three subsets. There were also no differences in fibrinogen level among the *C/C*, *C/T*, and *T/T* β-fibrinogen promoter genotypes in current smokers (306.4±85.8 mg/dL, 300.6±62.5 mg/dL, and 299.7±95.0 mg/dL, respectively; *P*=.92) and in the subset of 55 women who were on estrogen replacement therapy (282.3±69.6 mg/dL, 277.4±56.0 mg/dL, and 288.0±46.0 mg/dL, respectively; *P*87).

The quality of carotid duplex examinations was good in 389 (97.5%) and fair in 8 (2.0%) subjects. Only 2 (0.2%) studies were of poor quality and were thus excluded from further analysis. As shown in Table 2, subjects carrying the *T/T* β-fibrinogen promoter genotype had atherosclerotic carotid abnormalities more commonly than did their counterparts with either the *C/C* or *C/T* genotype with very similar sonographic findings. Table 2 demonstrates that the most striking differences between the *T/T* genotype and the two other genotypes were seen with respect to the extremes of the duplex score. Normal findings occurred in only 12.0% of *T/T* carriers but in 46.4% of *C/C* and 45.9% of *C/T* carriers. By contrast, grade 4 atherosclerotic changes were noted in 20.0% of subjects with the *T/T* genotype but in only 4.0% and 4.7% of those with the *C/C* and *C/T* genotypes, respectively. There

TABLE 2. β-Fibrinogen Promoter Genotypes and Duplex Score

Duplex Score Grade, n (%)	Genotype			P
	C/C (n=224)	*C/T* (n=148)	*T/T* (n=25)	
0	104 (46.4%)	68 (45.9%)	3 (12.0%)	
1	5 (2.2%)	9 (6.1%)	2 (8.0%)	
2	76 (33.9%)	45 (30.4%)	11 (44.0%)	
3	30 (13.4%)	19 (12.8%)	4 (16.0%)	
4	9 (4.0%)	7 (4.7%)	5 (20.0%)	.003

*χ^2 test.

TABLE 3. Final Logistic Regression Model of Carotid Atherosclerosis

Variable	β	SE	df	P	OR	95% CI
Apolipoprotein B, mg/dL	0.016	0.004	1	<.00001	1.17/10 mg/dL	1.08; 1.26
Age, y	0.090	0.021	1	<.00001	2.46/10 years	1.65; 3.67
Tobacco consumption, g	3.2×10^{-6}	9.3×10^{-7}	1	.0004	1.03/1000 g	1.01; 1.05
T/T β-fibrinogen genotype	1.820	0.674	1	.007	6.17	1.70; 22.36
Fibrinogen, mg/dL	0.005	0.002	1	.006	1.05/10 mg/dL	1.01; 1.09
Cardiac disease	0.588	0.259	1	.023	1.80	1.09; 2.95

were no individuals with grade 5 changes. Overall, atherosclerotic carotid abnormalities were recorded in 53.6% of the *C/C* and 54.1% of the *C/T* carriers but in 88.0% of the *T/T* carriers, and this difference was statistically significant (*P*=.003). The unadjusted and age-adjusted ORs for abnormal sonographic findings in the *T/T* genotype relative to the other two genotypes was 6.29 (1.91 to 20.71 95% CI; *P*=.003) and 5.97 (1.77 to 20.15 95% CI; *P*=.005), respectively. On the basis of our finding that the *T/T* genotype subset was, on average, slightly older than those with the *C/C* and *C/T* genotypes, we repeated our analyses after matching the investigational groups for age to avoid overreliance on statistical adjustment. For 22 individuals of the *T/T* group, we were able to randomly select 3 individuals of the *C/C* and *C/T* groups each of whose ages were ±2 years of that of a given *T/T* carrier. The ages of the matched *C/C*, *C/T*, and *T/T* subgroups were 62.6±6.1, 62.9±6.4, and 62.8±6.5 years, respectively, (*P*=.97), and there were no significant between-group differences in demographics and vascular risk factors. As in the entire cohort, *T/T* carriers demonstrated a higher frequency and severity of carotid atherosclerosis (*P*=.01). In this age-matched subset of study participants, the ORs for the presence of carotid artery disease associated with the *T/T* genotype was 4.38 (1.28 to 15.04 95% CI; *P*=.02). The interaction terms *T/T* genotype×plasma fibrinogen, *T/T* genotype×lifetime tobacco consumption, and *T/T* genotype×use of oral contraceptives were not associated with evidence of carotid atherosclerosis in the total study group. The respective ORs were 1.001 (0.98 to 1.02 95% CI; *P*=.92), 1.0002 (0.99 to 1.001; *P*=.65), and 0.50 (0.42 to 0.67; *P*=.64). When we used forward stepwise regression analysis to create a model of predictors of atherosclerotic changes in the carotid arteries, the *T/T* genotype remained significantly and independently associated with evidence of abnormal sonographic findings. Apolipoprotein B entered the model first, age second, lifetime tobacco consumption third, the *T/T* β-fibrinogen promoter genotype fourth, plasma fibrinogen fifth, and cardiac disease sixth (Table 3). All other variables, including male sex, hypertension, diabetes mellitus, fasting blood glucose level, current and former smoking status, body mass index, other lipid fractions, and hematocrit did not enter the model.

Discussion

In the current study, we have demonstrated an association between the *T/T*148 genotype at the β-fibrinogen locus and the presence of carotid atherosclerosis in a neurologically asymptomatic, randomly selected population aged 45 to 75 years. *T/T* carriers tended to be older than their counterparts with the *C/C* or *C/T* genotype, but the β-fibrinogen promoter polymorphism remained significantly related with vessel wall status even after adjustment for age by either multivariate logistic regression analysis or analysis of subgroups of study participants closely matched for age. We found that not only the *T/T* genotype but also plasma fibrinogen concentration are independent predictors of carotid atherosclerosis in addition to the well-established risk factors age, apolipoprotein B, lifetime tobacco consumption, and cardiac disease. The $C_{148}\rightarrow$T polymorphism was not correlated with plasma fibrinogen concentrations in our study participants.

There are at least 10 polymorphisms present in the β-fibrinogen gene.[29] Of particular interest are sequence alterations in the 5′-flanking region of the gene, because this region contains several regulatory elements that control gene expression under different conditions.[25–27] The increased fibrinogen synthesis during the acute-phase reaction is due to a higher transcriptional rate and is mainly mediated by interleukin-6.[39] Anderson et al[26] characterized two distinct sequence elements required for maximal induction of transcription by interleukin-6. One of them is similar to the interleukin-6 responsive-element core sequence of the rat α_2-macroglobulin gene promoter and lies between nucleotides −137 and −143. The other is a CAAT-enhancer binding protein (C/EBP) binding site between nucleotides −124 and −133. The $C_{148}\rightarrow$T polymorphism lies in the direct vicinity of these regions. It is thought to modulate acute-phase fibrinogen response by altering the binding of hepatic nuclear proteins to this part of the DNA.[40] Thus, this polymorphism may represent a functional sequence variant. Some evidence for such a mechanism has been given in a study by Montgomery et al,[41] who reported that the acute rise in fibrinogen concentration after physical activity in young men is influenced by the $G_{455}\rightarrow$A (β *Hae*III) polymorphism, which is tightly linked with the $C_{148}\rightarrow$T polymorphism.[28,29] We have found that subjects homozygous for the *T*148 allele have a 6.17-fold increased risk for carotid atherosclerotic abnormalities when compared with subjects with the *C/C* or *C/T* genotype after adjustment for age. Because this trend was seen to occur independently of fibrinogen concentration, we speculate that a transient, fibrinogen promoter genotype–dependent rise of fibrinogen levels in response to repeated extrinsic and intrinsic stimuli might play a role in the etiology of carotid atherosclerosis, irrespectively of the possible atherogenic effects of baseline fibrinogen concentrations. We most likely measured baseline fibrinogen values, as our study participants were clinically normal at the time of the examination. We found that tobacco consumption in all subjects and use of oral contraceptives in women did not modify the association of the

β-fibrinogen promoter genotype and carotid atherosclerosis. It is therefore unlikely that these two factors were extrinsic stimuli in the present study cohort.

There have been several studies that have addressed the question of the extent of variation in the β-fibrinogen gene and its influence on the baseline plasma content of fibrinogen. In the ECTIM Study, the $G_{455}\rightarrow A$ (β *Hae*III) polymorphism was shown to be significantly associated with the level of fibrinogen.[31] In the EARS Study, the association between the $G_{455}\rightarrow A$ (β *Hae*III) polymorphism and plasma fibrinogen was present, but this relation was affected by sex, hormonal status, and smoking habits.[32] In a recent report from the ECTIM Study, the presence of the *Bcl*I polymorphism in the 3′-region, which is also tightly linked with the $C_{148}\rightarrow T$ polymorphism,[29] was shown to be highly correlated with the severity of coronary atherosclerosis in myocardial infarction patients. The *Bcl*I polymorphism has also been implicated by Fowkes et al[42] in occlusive peripheral artery disease. Similar to our results, they found that the polymorphism was associated with the presence of atherosclerosis without influencing plasma fibrinogen levels. A recent study by Carter et al[43] has investigated the effect of the arginine-to-lysine substitution at position 448 on fibrinogen levels and the risk for stroke. They observed an association between the Bβ448 polymorphism and baseline fibrinogen levels in male patients only, but similar to our results, not in male control subjects or females. Regarding the effect of genotype on stroke, they found that the polymorphism was associated with a lower risk in females. The authors therefore speculated that genetic variations at the β-fibrinogen locus might modulate the risk for stroke through different mechanisms in males and females. The participants of the EARS Study were young men and women (aged 18 to 26 years) and those of the ECTIM Study, young to middle-aged men (25 to 64 years). The study population investigated by Fowkes et al[42] and Carter et al[43] consisted of men and women with an age range of 55 to 74 years and 68 to 82 years, respectively, close to the age range of our study population. It is conceivable that the effect of the polymorphism on fibrinogen level and its dependence on environmental factors and sex disappears with advancing age and therefore was not detectable in older study populations like those examined by Fowkes, Carter, and us.

Another explanation for the controversial results on the association between genotype and fibrinogen level in the different studies may be the high intraindividual variation in fibrinogen level[44–46] as measured by the Clauss method.[35] This method has been used not only by us but also by all major previous investigations. The Clauss method is a functional assay measuring the level of clottable fibrinogen with a reported batch error of 5% to 7%.[44–46] Using this method, Rosenson et al[44] examined the intraindividual variation in fibrinogen concentration over a 6-week period. They found a coefficient of variation of 17.8%, comprising both biological fluctuations as well as methodological variations. On the basis of their calculations, at least four measurements are required for an accurate fibrinogen concentration assessment in a given individual, which is not practicable for risk assessments in population studies. In population-based studies, the ability of the investigation to detect fibrinogen concentration–associated effects is mainly dependent on sample size. According to Rosenson et al,[44] our sample was large enough to detect a possible association between fibrinogen concentration and carotid atherosclerosis in general. Nevertheless, the limited number of subjects with the *T/T* genotype might have led to an underestimation of the effect of genotype on fibrinogen level in this subset. However, also on the basis of our data, we cannot exclude the possibility that the $C_{148}\rightarrow T$ polymorphism per se is not a functional sequence variant but is in linkage disequilibrium with another important, yet-undefined sequence alteration in the β-fibrinogen gene or in another gene in its neighborhood.

In summary, our study demonstrates the first evidence of a significant association between the *T/T*148 genotype at the β-fibrinogen gene and carotid atherosclerosis. The results should be interpreted with caution, given the small number of *T/T* homozygotes. A larger cohort will ultimately be required to confirm whether or not this association is real.

Acknowledgments

This project was partly supported by the Austrian Research Foundation Project P11691 MED (to G.M.K.) and by the Franz Lanyar Stiftung of the Karl-Franzens University (to H.S.), Graz, Austria. The excellent technical assistance of Johann Semmler and Anita Gradert is appreciated.

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General Discussion

5

In this thesis we studied the course of cerebral small vessel disease and its predictors. The studies presented here were conducted as part of the Austrian Stroke Prevention Study (ASPS), a single-center, longitudinal cohort study on the cerebral effects of vascular risk factors in the normal elderly population of the city of Graz, Austria. The ASPS was started in 1991 and enrolled 1998 men and women between the age of 50 and 75 years of whom 498 underwent extensive baseline and follow-up evaluations, including blood pressure readings, ECG, echocardiography, blood cell count and complete blood chemistry as well as brain MRI, Doppler sonography, SPECT and neuropsychiatric testing. The cohort is followed up in 3-year intervals. Currently the third follow-up panel of the ASPS is underway. A detailed description of the study design, definition of risk factors and the MRI protocol of the ASPS is given in Chapter 2.1.

In this chapter I will give a summary of main findings (Chapter 5.1) and discuss methodological issues of our studies (Chapter 5.2). I will address, the clinical relevance of these results (Chapter 5.3) and will extent on future objectives of research on cerebral small vessel disease (Chapter 5.4).

5.1. Main findings

Progression studies

In 1999 we reported the first large-scale longitudinal study on the progression of white matter lesions (WML) (Chapter 2.1). The follow-up time was 3 years and 273 of the ASPS participants having baseline and follow-up MRI scans were included in the study. The rate of overall progression was 18%. Lesion progression was marked in 8% of study participants. Importantly, no regression of lesions was observed. Major predictors of progression were diastolic blood pressure and the grade of WML at baseline. A neuropsychological test battery assessing memory and learning abilities, conceptual reasoning, attention, and speed as well as visuopractical skills was administered at baseline and at follow-up. We observed no decrease of cognitive functions in individuals with lesion progression during the three year of follow-up.

Results of the 6-year follow-up study, largely confirmed the previous findings (Chapter 2.2). Remarkably, although we used a different measure of lesion progression as in the previous manuscript, rate of progression was very similar in the two analyses. It was 9% after 3 years and 17% after 6-years follow-up. A novel finding of this study was that the grade of white matter lesions at baseline was the only significant predictor of progression explaining 28.5% of the variance in volume change. All participants with confluent and 58% of those with early-confluent WML at baseline showed progression over the 6-year follow-up, compared with 15% in participants with punctate lesions and 2% with normal MRI at baseline. The median volume change in subjects with confluent lesions at baseline was 4.8 cm^3 after 3 years and 9.3cm^3 after 6-years follow-up.

The median volume change in subjects with early confluent lesions at baseline was 0.7 cm^3 after 3 years and 2.7cm^3 after 6-years follow-up. By contrast subjects with punctuate lesions at baseline showed minimal increase in lesion load (median change 0.1 cm^3 at 3-years; 0.2 cm^3 at 6-years), while there was no volume increase in participants without WML at baseline. Again we did not observe regression of lesions during follow-up. Data on the relationship between lesion progression and cognitive performance for the 6-year follow-up is still under study.

Heritability of cSVD

The genetic aspects of cerebral small vessel disease are reviewed in Chapter 3.1. So far three studies have reported heritability estimates for WML volume. The first study was conducted in normal elderly male twins, the second was a family based approach including middle-aged and elderly men and women and the third studied hypertensive siblings (1-3). The latter two studies were published recently and are therefore not discussed in Chapter 3.1. Although the study design and populations were different in these studies, all three studies found that WML are highly heritable with h^2 ranging between 0.55 and 0.73. These high heritability estimates underline the relevance of searching for genes involved in the etiology of WML. Identification of genes probably will provide a better understanding of the pathogenesis of this common pathology of the elderly and ultimately may lead to the development of novel preventive and therapeutic measures. This is of particular interest, given the fact that there exists no established therapy for this entity.

Different strategies can be followed for finding genes in complex traits. Most commonly used are non-parametric linkage studies, such as sib-pair studies or association studies. Candidate gene studies are most powerful if one has substantial knowledge of the pathways involved in a trait as is the case in cSVD.

We hypothesize that early confluent and confluent WMLs as well as lacunar lesions represent ischemic damage of the brain parenchyma due to arteriolosclerosis. Based on this hypothesis we developed a model for the selection of candidate genes for these phenotypes, which we summarize in Figure 1. In our earlier work we also used the term microangiopathy-related cerebral damage (MARCD), which described exactly the same phenotype.

The genes selected to initiate association studies included the apolipoprotein E gene (APOE), the paraoxonase 1 gene (PON1), angiotensinogen gene (AGT) and the beta fibrinogen gene (FGB). The APOE gene was selected based on its association with aging, serum lipid concentrations (vascular systemic factors), as well as brain parenchymal damage and repair (4). The PON1 gene was selected based on its protective role against oxidative damage through which it may modulate brain vulnerability to ischemic damage (5). The AGT gene was

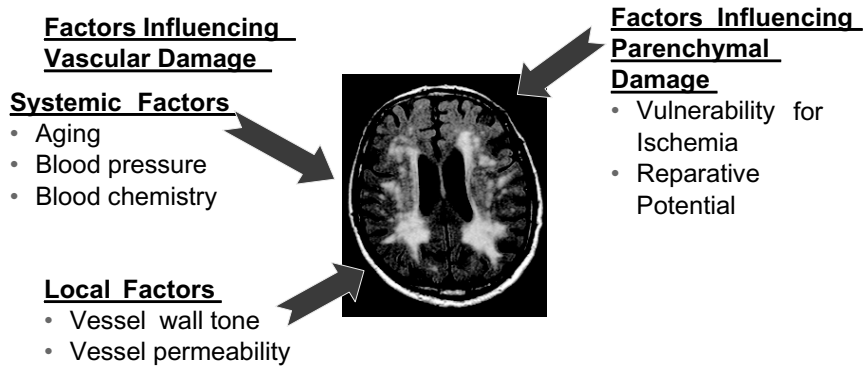

Figure 1 Working model for selection of candidate genes for cerebral small vessel disease

selected due to its central role in the regulation of the renin-angiotensin system and blood pressure (6). Hypertension is besides age the major risk factor for cSVD. The FGB gene was selected because syntheses of the beta chain is rate limiting in fibrinogen assembly (7) and positive associations between plasma fibrinogen and cSVD have been reported previously (8).

For each candidate gene, only polymorphisms with suspected functional relevance were studied. Functional relevance was evaluated based on location (promoter region, conserved sequence, functional domain) as well as based on available in vitro (biochemical activity, cell expression) and in vivo data (correlation with intermediate phenotype, eg. serum concentration, blood pressure) (6).

In this thesis polymorphisms investigated in cSVD were also tested for association with carotid atherosclerosis. We use carotid atherosclerosis as a paradigm for large vessel disease. Common and differential associations in small and large vessel disease may shed light to shared and distinct features of these two vascular processes.

Candidate genes involved only in cSVD

In Chapter 3.2. we investigated the association between cSVD and the apolipoprotein E isoforms 2, 3 and 4. We found, that carriers of the 2/3 genotype had a 3 fold higher risk for cSVD compared to carriers of the 3/3 genotype after adjustment for possible confounders. The association was highly significant ($p=0.01$) and was present inspite of a favourable effect of the 2/3 genotype on lipid levels. We did not find an association between the 3/4 genotype and cSVD.

The manuscript presented in Chapter 4.2 investigates the association between the frequent AGT gene variation, M235T and both prevalence and progression of cerebral small and large vessel disease. The M235T was neither

associated with baseline carotid pathology nor with progression of carotid plaques. The important finding of this study was, that the TT genotype enhanced the risk both for the severity of cSVD at baseline (statistical trend) and for the progression of cSVD (adjusted OR 3.2; 95% CI 1.5 to 6.6). Contrary to our expectation, the effect of the M235T polymorphism on cSVD was independent of hypertension. Based on the fact that the M235T polymorphism is located outside of the renin cleavage site, we hypothesized that this polymorphism is a marker, and the enhanced risk associated with it is due to linkage disequilibrium with a functional variant possibly in the AGT promoter.

In Chapter 3.4 I report a series of three studies evaluating the role of angiotensinogen (AGT) gene promoter haplotypes in cSVD. In the first study presented in Chapter 3.4.1, we described the presence of 4 polymorphic nucleotides at position –6(g/a), -20(a/c), -152(g/a) and –217(g/a) in the AGT promoter. These single nucleotide polymorphic sites (SNPs) constructed five novel promoter haplotypes (coded as A,B,C,D and E). We found that subjects homozygous for the –20:c allele had a significantly higher frequency of cSVD than carriers of the –20:c/a or –20:a/a genotypes (50% vs. 22% and 20%, p=0.02). There was also a trend for higher cSVD frequency in carriers of the –6: a/a genotype compared to carriers of the –6:g/a genotype and –6:g/g genotype (31% vs. 18% and 21% respectively; p=0.054). The major finding of this study was that homozygosity for the B-haplotype (-6:a; -20:c; -152:g and –217:g) enhanced the risk for cSVD 8-fold (95% CI 2.1 to 31.1; p=0.003) compared to the wild-type A-haplotype (-6:g; -20:a; -152:g and –217:g). The effect was much stronger than that of the SNPs by themselves. The association between the B-haplotype and cSVD was not mediated by hypertension. This suggests that the effect of the B-haplotype is probably mediated through an altered cerebral expression of AGT resulting in enhanced renin-angiotensin system (RAS) activity in the brain.

In Chapter 3.4.2 we reviewed the literature on the molecular biology of the AGT gene and on the relevance of its genetic variations in vascular diseases and hypertension. We studied various pathways linking the AGT promoter B-haplotype to cSVD. Based on this review our working hypothesis has focused on the effect of RAS on vascular smooth muscle cells in cerebral arterioles.

The first step in our working hypotheses was that the B-haplotype alters the promoter activity of the AGT gene. To test the hypothesis that it is rather the local brain RAS than the systemic that links the B-haplotype to cSVD, we studied transcriptional activity of the AGT gene in astrocytes, which are the major source of cerebral AGT, as well as in hepatocytes, which are the major source of systemic AGT (6).

In the following Chapter 3.4.3 we showed that the B-haplotype significantly enhances basal promoter activity compared to the A-haplotype in

both cell-types. Its effect was stronger in astrocytes than in hepatocytes (2.4±0.09 fold vs. 1.6±0.06fold, respectively). Importantly the effect in astrocytes was dependent on the presence of both SNPs (-6:a and –20:c) distinguishing the A- and B-haplotypes, while in hepatocytes the –20:c on its own was sufficient to enhance promoter activity. This is in line with our epidemiological data showing, that the haplotype but not the single SNPs is relevant for cSVD.

In this chapter we also tested linkage disequilibrium between the promoter haplotypes and 5 newly genotyped SNPs scattered over the AGT gene. We inferred 9 haplotypes through this analysis. By investigating the evolutionary relation of these haplotypes also including the published chimpanzee sequence, we found, that the B-haplotype probably represents an ancient AGT promoter variant. This is a further, though indirect support for the functional relevance of this haplotype.

Candidate genes involved only in carotid atherosclerosis

In Chapter 4.3 we investigated the association between the beta-fibrinogen promoter polymorphism, C-148T and carotid atherosclerosis. Homozygosity for the T allele significantly and strongly enhanced the risk for carotid atherosclerosis (adjusted OR 6.2; 95%CI 1.7 to 22.4; p=0.007). The effect was independent of the measured fibrinogen level, which by itself was also associated with the phenotype. There was no relationship between the beta-fibrinogen genotype and fibrinogen concentration in our cohort. We hypothesized that the lack of association is due to the fact that the polymorphism, which is located in the vicinity of two interleukin-6 responsive elements, modulates fibrinogen levels in the acute phase response, but not at baseline. However, a low statistical power due to the small number of TT homozygotes and high intraindividual variability of fibrinogen concentration may also explain the lack of association. We found no indication that the C-148T polymorphism modulates the risk for cSVD in our cohort (unpublished data).

Candidate genes involved in both cSVD and in carotid atherosclerosis

In Chapter 3.3. we present a study on the association between the PON1 polymorphisms at position 54 (L/M) and 191(A/B) and prevalence and progression of cSVD. Homozygotes for the L allele at position 54 tended to have higher grades of cSVD at baseline (p=0.08). The main finding of this study was the significant association between the LL genotype and progression of cSVD (adjusted OR=2.65; p=0.004). The association was not modulated by the A/B polymorphism at position 191, as the frequency of lesion progression in subjects with the LL/AA and LL/BB haplotypes was almost identical (23.9% vs. 26.3%). Although the LL genotype also significantly increased the risk for carotid atherosclerosis (adjusted OR 1.9; 95% CI 1.14 to 3.2; p=0.01) (Chapter 4.1), its

effect on cSVD was not mediated by carotid atherosclerosis.

5.2. Methodological Issues

Definition of phenotypes

cSVD extend and progression

For the rating of WML the modified Fazekas rating scale was used (9). This scale differentiates deep white matter lesions from periventricular lesions and separates them into punctuate, early-confluent and confluent lesions. The scale is validated by histopathological correlations which showed that early-confluent and confluent lesions are associated with cerebral microangiopathy, while punctuate lesions frequently represent non-vascular changes (10). In our genetic epidemiological studies the phenotype cSVD was defined as the presence of early-confluent or confluent WML or lacunar lesions (d<10mm). This definition does not quantify the extent of abnormalities and therefore semi-automated algorithms to measure white lesion volume have been also incorporated in the MRI protocol of the ASPS. In our first study with a 3-years follow-up period volumetric measurements were not yet available. Here we graded progression of WML according to the change in the number of lesions and lesion grade. A change was considered to be marked if it exceeded 4 punctate abnormalities or if there was a transition in WML grade to early-confluent or confluent WML. A problem of this method is that enlarging confluent lesions will not be considered to represent progression.

By contrast, a quantitative assessment of lesion volume was performed in the 6-year follow-up study. Further strength of this analysis was the long follow-up period with 3 MRI scans obtained in each individual separated by equal intervals. This allows to study not only progression but also the course of this process over time. Lesion progression was assessed as a continuous variable but it was also dichotomised. Progression was considered present if lesion volume change exceeded the possible measurement error of 1.81cm^3, which was defined as 95% confidence interval of the most pronounced difference between repeated measurements on 50 randomly selected scans.

Carotid atherosclerosis

Ultrasonographic measures of the carotid arteries are frequently used as surrogate markers for generalised atherosclerosis (11). In the ASPS, carotid atherosclerosis is defined based on the most severe visible atherosclerotic plaque in the common and internal carotid arteries on either side. A detailed description of the protocol is given in Chapter 4.2. Briefly, severity of carotid atherosclerosis is graded as 0=normal vessel; 1=vessel wall thickening (<1mm), 2= minimal plaque (1mm<d<2mm), 3=moderate plaque (2mm<d<3mm), 4=severe plaque

(d>3mm), and 5=lumen completely obstructed.

Carotid artery plaque (CAP) as surrogate marker for atherosclerosis is not as frequently used as carotid intima media thickness (IMT) (11). Its heritability is estimated to be lower than that of IMT (23-28% vs. 20-40%, respectively) (12,13). IMT and CAP describe different aspects of carotid atherosclerosis. IMT primarily assesses media thickening with the intima contributing only 2 to 20% to the IMT value. IMT is obtained as a mean value of several measurements at predefined sites of the carotid arteries. Although increased IMT may reflect the presence of a large single plaque forcing mean IMT to higher values, it more likely reflects the presence of vascular smooth muscle cell hyperplasia associated with early stages of atherosclerosis or with adaptation of the vessel to flow, shear or tensile stress. Yet, increased carotid IMT is a strong predictor of hypertensive end-organ disease and stroke, while CAP is a better predictor of coronary artery disease (11,12). CAP describes focal thickening of the vessel wall, which probably represents a later stage in atherosclerosis than the diffuse thickening of the media. Common vascular risk factors like cholesterol levels, smoking, hypertension, diabetes, age and gender explain about 15% of the variation seen in IMT and over 50% of the variation seen in CAP (11). Based on these differences between CAP and IMT we expect that the proportion of shared genetic background with cSVD is less for CAP than for IMT.

Association studies

Genetic dissection of a complex trait such as cSVD can be targeted through linkage and association studies (14). Association studies offer opportunities beyond linkage studies as they can evaluate the contribution of genes with low penetrance to the distribution of a trait in the population further they allow to estimate interactions between genetic and environmental factors.

There is a still an ongoing debate on how to perform and analyze such studies correctly. Phenotypic complexity, genetic heterogeneity and the presence of gene-gene and gene-environment interactions are inherent to the nature of complex traits and represent challenges for both linkage and association studies. Other issues like level of statistical significance and interpretation of positive findings deserve particular attention in relation to association studies.

Phenotypic complexity

In order to maximize the probability of finding an association to a complex trait, it is crucial to define the phenotype with care. It has been recommended to use intermediate phenotypes, which are also commonly termed as endophenotypes instead of the manifest disease (15). An intermediate phenotype is a preclinical stage of a disease and represents a biological pathway leading to higher susceptibility to clinical disease. The advantage of using

intermediate phenotypes is that they are more directly related to the action of a gene and show less complexity than manifest diseases. In this context MRI detected early confluent and confluent WML and lacunes as well as Doppler sonography detected atherosclerosis in the carotid arteries can be regarded as intermediate phenotypes for cerebral small and large vessel disease and for their clinical manifestations stroke or subcortical vascular dementia. Optimally endophenotypes are measured on a quantitative scale and show considerable variation within the population. It has been shown that genetic an analysis of a quantitative trait is more powerful than analyses of the same trait dichotomized into discrete variables (16). It is therefore our major interest to measure the volume of WML and to use the extent of WML as a quantitative trait in our future genetic studies. A weakness of using endophenotypes instead of manifest disease is that genetic factors involved in outbreak of a disease can not be investigated. Also a significant proportion of people presenting the endophenotype will never manifest the disease. Therefore the interpolation of the results to the clinical practice has to be more cautious.

Another important issue in the genetic study of a complex trait is the precision of phenotypic information. The availability of such data is a prerequisite to detect the presence of phenotypic variability but also of genetic heterogeneity within a trait. Phenotypic variability refers to differences such as disease severity, age at onset or clinical symptoms in carriers of the same genotype. By definition subjects manifesting the disease within this variability are expected to be genetically homogenous and all of them should be defined as cases in order to enhance the power of an association study. On the other hand the observed phenotypic differences may define clinical subtypes and may potentially indicate the presence of genetic heterogeneity within the trait (17). The presence of genetic heterogeneity introduces noise into the data as the expected small effect of the gene under study will be masked by the effect of other genes and will reduce the power of the study to detect association. While in linkage studies only locus heterogeneity is of concern, in association studies also the presence of allelic heterogeneity represents a problem. Allelic heterogeneity is frequently observed in Mendelian diseases and is also expected to be present in complex traits (17).

The most straightforward approach to handle heterogeneity is sample stratification. The rationale of stratification is that different disease models operate within different strata and the pathological role of a gene is conditional on the presence or absence of the stratifying variable. The main disadvantage of sample stratification is the reduced sample size within the strata, and in turn a reduction in statistical power. Other methods aiming to handle heterogeneity in association studies include cluster analyses, latent class analyses and factor analyses. All of these methods like sample stratification are dependent on the availability of relevant covariate data and their usefulness for association studies

still has to be evaluated (17).

Finally, the comparability of diagnostic criteria across studies has to be mentioned. It is desirable to use international guidelines and standardized protocols for phenotyping. A major drawback for genetic studies in cSVD is the lack of such guidelines. Each major study in the field uses its own scale to describe WMLs. Recently efforts were undertaken to correlate these different scales and evaluate their usefulness for cross-sectional and longitudinal studies. Based on these studies the Fazekas rating scale has an excellent inter-rater agreement and correlates well with other WML scoring systems, yet it is rather a typological than a quantitative scale (18).

Statistical significance

A major concern regarding association studies is the level of statistical significance of an observed positive association. Presently most association studies investigate only a small number of polymorphisms in a small number of candidate genes. A correction for multiple testing is generally not considered. Recently, it has been proposed that analogous to linkage studies more stringent significance levels should be used in association studies especially in genome wide studies. The point is that the prior probability that a gene variant will be associated with a trait of interest is very low (1 to 30.000 if one considers only the number of genes in the human genome). This would translate to a p-value as low as $2.6x10^{-7}$, which is hardly ever seen in association studies (19). Careful selection of candidate genes and polymorphisms based on previous evidence such as linkage and association data or functional data can increase prior probability. It has been suggested that journals should require explicit, standardized description of previous evidence and authors should propose estimates of prior probability based on this evidence (19). However it is difficult to envision how estimates for prior probability for an association should be calculated. Firstly, it can be expected that a complex trait will be associated with more than one gene and there will be interactions with other genes and with environmental factors and there will be a variation of these factors among populations. Secondly, a review of published data in order to search for previous evidence will probably overestimate prior probability due to publication bias. A search of the newly developed Genetic Association Database, which deposits association data in a standardized manner, may provide a less biased overview of data (20).

With the advent of high-throughput genotyping a genome-wide scan in association studies become technically feasible. Depending on the linkage disequilibrium structure in a population the estimated number of markers needed to cover the genome is 50.000 to 60.000 (20). Contrary to linkage studies, where we expect, that one gene will be linked to a Mendelian disease, we expect true associations between several genes and a complex trait in association studies. The

question of statistical significance for genome-wide association studies is still not clarified, although different approaches, like the usage of the false discovery rate, have been proposed (21).

Interpretation of a positive association

An observed positive association should be interpreted cautiously (22). The association might be true and causal, meaning that the mutation itself by altering function or availability of the gene product leads to the development of the disease. Proving the causality of a mutation requires supplementary studies including histopathology, cell expression or animal studies. Also replication of a positive association in an unrelated population is considered to be a strong indicator for a true and causal relationship between mutation and phenotype. However, it is uncommon that positive associations are replicated by follow-up studies. Partly the difficulties for replicating results may have biological explanations like dependence of the gene effect on the presence of other genetic or environmental factors differentially present in the initial and in the follow-up population (22). Another explanation might be the use of non-standardized criteria for phenotype definitions across different studies. A recent call for a "Human Phenome Project" in Nature Genetics to systematically collect and describe human phenotypic information illustrates the magnitude of this problem (23). As discussed in the previous section there is a need for an internationally accepted uniform protocol to define and type cSVD based on MRI findings and develop a uniformly used nomenclature for these lesions.

Beside a causal relationship a true positive association can also be due the presence of LD. There exist about 10 million SNPs in the human genome and most of them are neutral regarding human diseases. However, a portion of them will represent genetic factors enhancing the liability to common diseases. The demonstration of LD between disease and a marker SNP indicates the presence of a causal mutation within the neighborhood of the marker. Based on recent publications significant LD in humans is expected in a swept radius of over 100kb (24).

Nevertheless, there is a substantially high likelihood that the observed association is a false positive finding due to population stratification or due to chance (25). If a subgroup within the population has a higher susceptibility to disease and also has a higher frequency of the investigated allele, then a spurious positive association between disease and allele may be found. Awareness of this problem termed population admixture led to the use of ethnically more homogeneous study populations and more careful selection of cases and controls originating from the same source population. However even small stratification can introduce considerable bias, if sample sizes are large. The method of genomic control can adjust for bias due to hidden population substructure by using genotype information from additional unlinked markers (26). Other methods

like the transmission disequilibrium test (TDT), uses family-based controls, in order to prevent bias due to admixture (27). Recruiting relatives is however more difficult than recruiting unrelated controls and in some cases relatives are not available (26).

Expression study

As discussed above dissecting the causality of an observed association requires supplementary studies. In this thesis we investigated the biological effect of AGT promoter variants using a transient transfection assay. As a model system we used cultured astrocytoma cells transfected with a reporter plasmid containing the luciferase gene driven by the inserted promoter variants. Our results showing an enhanced transcriptional activity of the risk B-haplotype suggest a causal relationship between AGT promoter B-haplotype and cSVD. There are however some methodological issues, which have to be discussed in order to interpret the biological relevance of these findings.

Complexity of gene regulation in eukaryotes

Transient transfection assays measure the rate of transcription initiation, which is generally considered as the major controlling mechanism of gene expression. Other mechanisms such as RNA processing and post-translational modification however may influence the amount of mature protein synthesized. Also recent studies indicate that gene expression in eukaryotes is regulated in a hierarchical manner including regulation at the DNA sequence level, at the chromatin level and at the nuclear level (28-29). Due to the lack of experimental methods to manipulate nuclear organization or chromatin structure there is little knowledge on how these different regulatory mechanisms interact with each other. The importance of interactions between different levels is, however, demonstrated by the observation of transvection, where regulatory elements located on one allele can interact with the promoter of the other allele on the homologous chromosome by probably coming physically close to each other due to nuclear compartmentalization. Hierarchically higher order regulatory mechanisms may even compensate the enhanced transcriptional activity of a promoter. In transient transfection assays the promoter is cloned into a reporter plasmid lacking its natural chromatin and nuclear context. Also the large copy number the plasmids present in the transfected cell may lead to aberrant function (39). Thus one should be cautious about transferring results from transient transfection assays to intact organisms.

It is also important to note that transcriptional initiation is a highly dynamic process fluctuating by orders of magnitude within minutes. Adjacent cells within a tissue may show very different transcriptional activity depending on the availability of transcription factors and on environmental or developmental

conditions of the cell. In our experiments promoter activity was studied under constant basal conditions. The AGT promoter contains several putative binding sites for transcription factors such as estrogen and glucocorticoid responsive elements or acute phase response elements. Their effect might be mediated differentially by the B-haplotype. Thus investigation of the promoter activity under induced conditions such as hormonal treatment or hypoxia is of interest.

In or experiments we used a 1.3 kb long fragment of the AGT promoter spanning nucleotides from +44 to –1222 relative to the transcriptional start site. Previous studies have shown that this fragment is functionally active in hepatocytes and astrocytes. Our results showing a differential transcriptional activity of the haplotypes indicate that haplotype dependent regulatory mechanisms can be investigated by using this fragment (31-34). Clearly, cis-acting elements interacting with the B-haplotype may be present further upstream, in introns or downstream of the AGT gene. Recent studies showed that the physical distance between a core promoter and its cis-acting elements ranges between some few hundreds up to >100kb in humans (28). Looping or bending of the DNA allows proteins associated with these distant elements to interact with the core promoter. Distant control regions including enhancers, silencers or locus control regions, probably represent a higher position in the regulatory hierarchy and may influence the accessibility of the whole locus (28,30). Therefore some researchers argue that before initiating promoter studies efforts should be undertaken to find distant control regions (30). However, these are difficult to find and frequently do not function in transient assays, which makes such studies more time-consuming. Due to limited resources we decided to perform our experiments using the published 1.3kb promoter sequence. It is important to be aware of the probable presence of distant regions controlling AGT expression and to adapt the experimental design accordingly, if such sequences become known.

Selection of cell model

Cells being studied in a transient transfection assay must fulfill three criteria (30). The cells must express the gene of interest endogenously. They must survive in culture for the time of the experiment and a protocol to transfect them with the reporter plasmid must be available. In general immortal cell lines represent the best choice for such studies, while primary cell are more difficult to isolate and maintain and are rarely available in sufficient quantity. Primary cells are also more resistant to transfection procedures than transformed cells. In our experiments we used the A172, a grade III astrocytoma cell line as a cellular model for cerebral AGT expression and HepG2 cells, a hepatoma cell line as cellular model for hepatic AGT expression. Both cell lines produce AGT endogenously and have been used by other groups to study the AGT promoter (34).

A disadvantage of using transformed cell lines compared to primary cells

is that due to their malignant phenotype these cells will probably not express all the transcription factors of normal astrocytes and, and contrary, they probably also express transcription factors, such as oncogens, which are normally not present in astrocytes. Recently, primary human astrocytes became commercially available but their usefulness for studying AGT promoter activity still has to be evaluated. While primary astrocytes represent a more physiological model than astrocytoma cells, one should bear in mind that gene expression is also dependent on the area surrounding a cell located in the brain (35). Therefore immunohistochemistry or in situ hybridization to visualize AGT expression in post mortem brain tissue from subjects carrying different promoter haplotypes may deliver more information on the effect of the haplotypes in their natural context. Beside astrocytes also other cell types like vascular smooth muscle cells, endothel cells and neurons have been shown to synthesize AGT and may contribute to AGT synthesis at the site of cSVD (36). The effect of the promoter haplotypes in these cells has not yet been investigated.

5.3. What did we learn from these studies about cSVD?

Natural course of cSVD

We have shown that the type of WMLs determines the rate by what these lesions progress, with early-confluent and confluent lesions progressing at the highest speed. Importantly subjects with confluent WML have a "malignant" course as lesion volume almost doubles over a relatively short time period of 6 years. We never observed regression of lesions. Even though the use of different scales to estimate extent and progression of lesions hampers the comparability of results of different studies, all studies published so far confirm that these lesions progress over time (37). Studies are however less coherent regarding predictors of progression. Diastolic and systolic blood pressure, age, diabetes, cigarette smoking and infarcts on the initial scan have been indicated to be related to lesion progression (38). In our cohort baseline WML grade was the strongest predictor of progression and if this variable was included in a multivariable model all other associations including age and blood pressure, became non-significant. One may debate if defining WML grade at baseline as a predictor of WML progression is meaningful as both baseline WML as well as progression of WML is the manifestation of the same process observed at different time points and their strong correlation is self-evident. Nonetheless, this observation is important in daily routine in order to estimate a subject's prognosis based on the baseline MR finding.

There are only a few studies with longitudinal MRI and clinical assessments. So far only the Cardiovascular Health Study found a positive association between WML progression and cognitive decline, the other studies

were negative (38). Probably, the follow-up time of these studies is still too short to measure cognitive decline due to lesion progression with acceptable power. Increasing gait and balance dysfunctions due to WML progression have also been reported (39). The clinical consequences of progression still need to be evaluated in our cohort.

Etiology of cSVD

We have determined novel genetic risk factors for cSVD. In the first place our results on the AGT gene deserve special attention. Our epidemiological and molecular biological data on this gene strongly suggest that overactivity of the cerebral RAS is involved in the development of cSVD. The vascular effects of AT-II, which represent the biologically active end-product of RAS in the plasma, are well documented. It is a potent vasoconstrictor and promotes vSMC hyperplasia and hypertrophy. It modulates NADH/NADPH oxidase and extracellular superoxide dismutase activity and extracellular matrix protein syntheses (40-43). Systemic RAS activity is regulated at the synthesis of hepatic AGT, which is in turn controlled by the transcriptional rate of the AGT gene (6, 36). Regulation of cerebral RAS activity is less well understood. If cerebral RAS is regulated similarly as systemic RAS, then increased promoter activity of the AGT gene may lead to RAS overactivity in the brain. This in turn may result in an imbalance of physiological processes like brain perfusion, autoregulation of cerebral blood flow, oxidative state of the vessel wall or blood brain barrier function. Each of these processes are likely to be involved in cSVD (44).

The findings that the PON1 LL genotype is independently associated with both small and large vessel disease, suggest that it acts through different mechanisms in the two vascular pathologies. The association with carotid atherosclerosis probably depends on a reduced protection of LDL against oxidation, which is a crucial and early event during atherosclerosis (45). The capacity of paraoxonase to protect LDL from oxidation is indeed lowest in individuals with the LL genotype (5). The effect of the LL genotype on cSVD may depend on its cerebral action. Paraoxonase is located on a subfraction of HDL together with clusterin. This subfraction of HDL is highly abundant in interstitial fluid and it is the only lipoprotein found in the brain. Its suggested function is to protect cellular membranes and possibly also myelin from oxidation. The association between cSVD and PON1 polymorphism suggests that oxidative damage plays a role in these lesions and that PON1 polymorphism modulates vulnerability of the brain to radicals. Oxidative damage is part of the aging process and may therefore partly explain the strong association of cSVD with age (46).

Our findings on the apoE gene and cSVD are controversial. Our study is the only study showing an association between the apoE 2 allele and cSVD, while

other studies found association with the apoE 4 allele or showed no association with either apoE variant (47). Based on diverse functions apoE has in the central nervous and in the vascular system, the hypothesis that apoE is involved in cSVD is well supported (4). ApoE may enhance arteriolosclerosis, as it can modulate vascular smooth muscle cell proliferation, or may modulate repair mechanisms, remyelinisation in the brain upon ischemia by delivering lipids. Yet, most of the pathologic functions are assigned to the apoE 4 allele, while the apoE 2 allele is rather considered to be protective. Nonetheless, some epidemiological studies reported deleterious effects for the apoE 2 allele (48). Further studies on the role of apoE variants in cSVD are needed.

The lack of association between the beta-fibrinogen polymorphism and cSVD and its strong association with carotid atherosclerosis may suggest that the thrombosis-haemostasis pathway does not play a significant role in cSVD while it is important in atherosclerosis. We also did not observe an association between plasma fibrinogen level and cSVD in our cohort, although some other studies reported such association. It is important to keep in mind that the C-148T polymorphism probably modulates the increase of fibrinogen upon iterleukin-6 response, which occurs during acute phase or in smokers while it probably does not influence baseline fibrinogen level (49). Therefore an analysis of the association between the C-148T polymorphism and cSVD stratified on smoking is of interest, but due to the low frequency of the T allele for such an analyses a larger sample than in our study is needed.

In summary the results presented in this thesis suggest that cSVD manifested as early confluent and confluent WML or lacunes on MRI represents a chronic and progressive brain pathology. We identified genetic factors, which significantly alter the risk for this entity. Although we observed an association between AGT haplotype and blood pressure, PON1 polymorphism and paraoxonase concentration and apoE isoforms and serum lipid levels, the increased risk for cSVD was not mediated by these associations. We hypothesize that these genetic variations most probably act through local cerebral effects rather then through systemic effects. In order to test this hypothesis it will be important to study the localization of the candidate gene products at the mRNA and protein level in areas of brain damage. Our finding indicating that overactivity of the cerebral RAS is involved in the etiology of cSVD suggests this pathway is a possible treatment target and that drugs acting on the RAS exerts effects beyond those expected by lowering blood pressure.

5.4. Future research

Refined assessment of WML features (threshold, location, atrophy) and new imaging techniques (magnetization transfer, diffusion tensor imaging)

Although a moderate association between leuko-araiosis and cognitive as well as affective disturbances has been repeatedly described in cross-sectional studies, this could not yet be shown in longitudinal investigations (50,51). Also, clinicians are commonly confronted with cases that have extensive white-matter lesions and/or lacunes but virtually normal cognitive and affective functions. In contrast, other persons with the same extent of lesions may exhibit pronounced deficits. The advent of new MRI techniques including diffusion tensor imaging, magnetisation transfer imaging, functional MRI, but also the functional imaging methods such as SPECT or PET promise to characterise brain lesions beyond what can be expected from conventional MRI sequences (52). These methods have the potential to demonstrate interruptions of white-matter fibre tracks, to determine the severity of tissue destruction and axonal damage in the area of a given lesion but also in normal appearing white matter (52,53). Probably, these methods will also allow to study remote effects of lesions in different brain areas and to evaluate the plasticity of the brain in order to compensate for lesion-related detrimental effects.

The natural course of small vessel disease-related brain damage and its clinical consequences

The most important finding of studies on the natural course of microangiopathy-associated brain damage was that the lesion type substantially predicts the rate of future progression (37). While punctate white-matter lesions show negligible progression over a six-year observational period, it was shown that the volume of confluent abnormalities almost doubles within the same time interval (37). This indicates a malignant course even though the clinical consequences of this process are yet unclear. Validation of confluent white-matter lesions as surrogate markers in dementia trials is an important research topic for the coming years.

Future Genetic Research

Identification of risk factors and factors that modify the course of the disease has high priority in this field as this may ultimately allow developing effective treatment strategies. Genetic linkage and association studies will probably contribute to establish such factors. First genetic association studies provided promising results but these findings have to be replicated in independent populations. Association studies investigating new candidate genes such as other members of the RAS, and its antagonist, the nitric oxide system, as well as genes

involved in free radical production and antioxidative mechanisms or in brain repair should be performed in order to extend the results of our previous studies on the relevance of these pathways in cSVD pathology. It will be important to establish international collaborations with the aim to replicate results in independent samples as well as to investigate to gene-gene and gene-environment interactions in large samples.

Linkage studies using affected pedigree members or isolated populations will allow genome-wide screening for risk factors and disease-modifying factors, and very likely will extent and supplement the results of genetic association studies. We are currently estimate the feasibility of such a study by contacting relatives of the ASPS participants.

Future molecular biological research

Concomitant histopathological correlation and investigations on the expression of genes at tissue and cellular level will be mandatory to determine the functional importance of gene variants identified by genetic studies. A brain bank of cSVD cases has been initiated in our laboratory. Samples embedded in paraffin are currently used for immunohistochemistry and for in situ hybridization experiments to investigate expression pattern of individual candidate genes in such lesions. We also collect snap frozen tissue samples, which should allow studying gene expression pattern using a global approach such as microarray experiments (54). The advantage of global approaches is the possibility to identify novel pathways leading to cSVD indicated by altered expression of gene clusters.

Success of future research on cSVD will depend on the international collaboration of large epidemiological studies and on the interdisciplinary efforts of experts to dissect molecular pathways leading to this pathology.

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Summary-Samenvatting

Summary

Cerebral small vessel disease (cSVD) is a common finding in the elderly. It is manifested as early-confluent and confluent white matter lesions as well as lacunar lesions on magnetic resonance imaging of the brain. These lesions may lead to cognitive impairment, gait disturbances and depression as they progress. Risk factors for cSVD are age and hypertension. The heritability is estimated to range from 55 to 75%. The aim of this thesis was to better understand the pathogenesis of cSVD by investigating the development of these lesions over time and by the identification of genetic risk factors for cSVD. These results may ultimately lead to the development of preventive and therapeutic measures of cSVD and its clinical consequences.

All studies presented in this thesis were conducted as part of the Austrian Stroke Prevention Study, a single-center, longitudinal cohort study on the cerebral effects of vascular risk factors in the normal elderly population of the city of Graz, Austria. The ASPS was started in 1991 and enrolled 1998 participants aged between of 50 and 75 years of whom 498 underwent extensive baseline and follow-up evaluations, including blood pressure readings, ECG, echocardiography, blood cell count and complete blood chemistry as well as brain MRI, Doppler sonography, SPECT and neuropsychiatric testing and DNA banking. The cohort is followed up in 3-year intervals.

this study was that the only significant predictor of lesion progression is the grade of white matter lesions at the baseline MRI. All participants with confluent and 58% of those with early-confluent WML at baseline showed progression over the 6-year follow-up, compared with 15% in participants with punctate lesions and 2% with normal MRI at baseline. We never observed regression of lesions. Thus early-confluent and confluent lesions represent progressive and probably malignant cerebrovascular pathology.

In Chapter 3 we give a review on the genetic aspects of cSVD and present a series of 5 studies investigating the association between cSVD and genetic variants in candidate genes. We found that the APOE 2/2 genotype, the PON1 54 L/L genotype and the AGT promoter B/B genotype increase the risk for cSVD. In case of the AGT promoter B-haplotype, we showed that its effect might be due to an increased transcriptional activity of the AGT promoter in astrocytes, which represent the major source of AGT in the brain. By using an evolution-based approach, we showed that the B-haplotype represent an ancient promoter variant in humans.

In chapter 4 we present three studies investigating the association of genetic variants in candidate genes and CAP. We argue that simultaneous investigation of genetic factors in both cSVD and CAP may shed light to overlapping and distinguishing features of cerebral small and large vessel pathology. Our

result showed that the PON1 L/L genotype enhances the risk for CAP, but its effect on cSVD is not mediated by the association with CAP. We found no association between the AGT gene variant M235T and CAP, a variant which enhanced the risk both for the severity of cSVD at baseline and for the progression of cSVD. On the contrary, the FGB T/T –148 genotype showed a strong association with CAP, but had no effect on cSVD risk.

Chapter 5 provides a general discussion on our main findings, methodological issues of our studies and the clinical relevance of the results. Finally future objectives of research on cerebral small vessel disease in the context of this thesis are discussed.

Samenvatting

Cerebrale micro-angiopathie, de zgn. 'cerebral small vessel disease'(cSVD), komt vaak voor bij ouderen. De afwijkingen hiervan manifesteren zich op MRI (magnetic resonance imaging) afbeeldingen, zowel als (vroeg-) confluerende witte stof laesies, als ook in de vorm van lacunaire laesies. Bij progressie kunnen deze laesies symptomen veroorzaken als cognitieve achteruitgang, stoornissen in de gang en depressie. Risicofactoren voor cSVD zijn leeftijd en hypertensie. De erfelijkheid (heritability) wordt geschat op 55% tot 75%. Het doel van dit proefschrift was een beter begrip van de pathogenese van cSVD, door het onderzoeken van de ontwikkeling van dit soort laesies door de tijd en door het identificeren van genetische risicofactoren voor cSVD. Deze resultaten zouden uiteindelijk kunnen leiden tot de ontwikkeling van preventieve en therapeutische mogelijkheden voor cSVD en de klinische sequelae ervan.

Alle studies die in dit proefschrift worden beschreven, zijn uitgevoerd in het kader van de 'ASPS' (Austrian Stroke Prevention Study, een Oostenrijkse studie naar de preventie van cerebrovasculaire accidenten), een longitudinale cohort studie, uitgevoerd in en door één centrum, naar de cerebrale effecten van vasculaire risicofactoren onder ouderen (binnen de algemene bevolking) van de stad Graz in Oostenrijk. De ASPS is gestart in 1991 en er zijn 1998 deelnemers zijn geïncludeerd, in leeftijd variërend van 50 tot 75 jaar, van wie er 498 uitgebreid onderzoek ondergingen bij de start van de studie en tijdens vervolgonderzoeken. Deze hielden o.a. in: bloeddruk metingen, ECG, bloedonderzoek en MRI cerebrum, doppler onderzoek, SPECT, neuropsychiatrische testen en DNA opslag. De vervolgonderzoeken vonden plaats met een interval van 3 jaar.

Hoofdstuk 1 is een algemene inleiding betreffende de epidemiologie, pathophysiologie en genetica van cSVD. In dit hoofdstuk worden de definiëring beschreven van de phenotypes, cSVD en plaques in de arteria carotis (CAP), welke

gebruikt werden in de genetische associatie studies. Voorts worden ook het doel van de studie en de onderzoeksopzet beschreven.

In hoofdstuk 2 worden de snelheid en determinanten van progressie van witte stof laesies beschreven na 3 en 6 jaar vervolgonderzoek. Een nieuwe bevinding van deze studie was, dat de enige significante voorspeller van progressie de gradatie van de witte stof laesies is op de MRI scan bij de start van het onderzoek. Bij alle deelnemers met confluerende witte stof laesies en 58% van hen met vroeg-confluerende laesies was er sprake van progressie gedurende 6 jaar vervolgonderzoek. Dit in vergelijk met 15% progressie bij deelnemers met punt laesies, en 2% bij deelnemers met een normale MRI bij de start van het onderzoek. Dus zijn vroeg-confluerende en confluerende laesies een indicator voor progressieve en waarschijnlijk kwaadaardige cerebrovasculaire pathologie.

In hoofdstuk 3 geven wij een overzicht van de genetische aspecten van cSVD en presenteren wij een serie van 5 studies, waarin de associatie tussen cSVD en genetische varianten van kandidaat-genen werd onderzocht. Het APOE 2/2 genotype, het PON1 54 L/L genotype en het AGT promoter B/B genotype bleken geassocieerd met een verhoogd risico op cSVD. Bij het AGT promoter B-haplotype werd aangetoond, dat het effect mogelijk kon worden toegeschreven aan een toename in transcriptie van de AGT promoter in astrocyten, die de grootste bron van AGT zijn in de hersenen. Door gebruik te maken van een vergelijk van de sequentie, konden wij aantonen dat het B-haplotype een zeer oude promotor variant in mensen vertegenwoordigt.

In hoofdstuk 4 presenteren wij drie studies naar de associatie tussen genetische varianten in kandidaat-genen en CAP. Wij bespreken, dat gelijktijdig onderzoek naar genetische factoren en zowel cSVD als CAP, mogelijk meer duidelijkheid zou kunnen geven over overlappende maar ook onderscheidende kenmerken van cerebrale micro- en macro-angiopathie. Onze resultaten toonden aan dat PON1 L/L genotype het risico op CAP vergroot, maar dat het effect op cSVD niet gemedieerd wordt door de associatie met CAP. Wij vonden geen associatie tussen de AGT genvariant M235T en CAP, een variant die wel het risico op zowel op de ernst van cSVD bij het begin van het onderzoek, als op progressie van cSVD vergroot. In tegendeel toonde het FGB TT –148 genotype een sterke associatie met CAP, maar had het geen effect op het risico op cSVD.

Hoofdstuk 5 biedt een algemene discussie van onze belangrijkste bevindingen, methodologie van onze studies en de klinische relevantie van de resultaten. Tot slot worden toekomstige onderzoeksdoelen, in de context van deze promotie, voor onderzoek rond cerebrale micro-angiopathie besproken.

About the Author

Helena Schmidt was born as Helena Becsagh, on December 21, 1963 in Budapest, Hungary. She studied medicine at the Semmelweis Medical University, Budapest, Hungary and received her degree Doctor of Medicine in 1989. After passing the examinations of the Educational Commissions for Foreign Medical Graduates (ECFMG) in 1990, she became a research fellow at the at the Department for Biomathematics and at the Laboratory for Flow Cytometry, at Roswell Park Cancer Institute, Buffalo, New York, where she participated in a research project on chronic fatigue syndrome. In 1991 she returned to Austria, where she passed the nostrification examinations for foreign medical graduates at the Karl-Franzens University, Graz. In 1992 she started her carrier as a research assistant at the Institute for Medical Biochemistry, at the Karl-Franzens University, Graz. She coordinated the genetic analyses of familial hypercholesterolemia, as part of the international MEDPED project, in Austria. Since 1993 she is responsible for the genetic studies conducted in course of the Austrian Stroke Prevention Study. In 2001, she received the venia legendi for medical biochemistry at the Medical Faculty of the Karl-Franzens University, Graz. She currently holds the position of an associate professor for medical biochemistry with special attention on molecular biology at the Medical University Graz. Presently, she is head of the Laboratory for DNA analyses at the Institute for Molecular Biology and Biochemistry, Medical University Graz and Vice-chairmen of the Institute for Molecular Biology and Biochemistry. In 1998 she started her study in genetic epidemiology at the Erasmus University, Rotterdam, the Netherlands. She obtained her Master of Science degree in 2001. In the same year she spent one month as guest researcher at the National Institute for Aging, NIH Bethesda, USA. In 2003 she finished her 2-year trainee in "Didactics with Professionalism and Success for University Teachers" at the University of Graz. She also obtained courses on project management, neurolinguistic programming, conflict management, time management, and presentation techniques provided by the University of Graz.

She is married to Reinhold Schmidt since 1987 and has one daughter, Veronika, who was born in 1990. Her hobbies are jogging, swimming, biking, gardening, reading and dancing.

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Acknowledgements

It´s seven years now since I first entered the Erasmus University in 1998. Meanwhile it is like coming home when I get off the elevator at 22nd floor and walk over to the Genetic Epidemiology Unit. It´s nice to feel integrated and I would like to thank all those colleges and friends, whom I got known during these years and whose support made this work possible.
Above all I would like to thank my promoter prof.dr.Cornelia van Duijn for her guidance, advices and the friendly way of supporting me in my study, never getting exhausted by answering all my questions. Thank you, dear Cornelia, for giving me the feeling of being part of your group despite being in Austria most of the time. I am also most indebted to my co-promoter, dr.Yurii Aulchenko for his support in statistical analyses and study design and for always being available to take some more questions. I would also like to thank Lodewijk Sandkuiyl, for all I learned from him in those five years he was teaching me and for all the helpful discussions on my work. I felt very privileged to learn from the most distinguished teachers, prof.dr.A. Hofman, prof.dr.BA. Oostra, prof.dr T.Stijnen, dr. J.Houwing-Duistermaat, dr.J Wittman, prof.P.Heutink, prof.dr.K.Rothman, prof.dr.D.Clayton, prof.dr.M.Hills, prof.dr.Kleinbaum, prof.dr.S.Lemeshow, prof. dr.J.Ott, dr.SM.Leal, prof.dr.E.Thompson during my study at Erasmus University. I am grateful for the excellent courses they provided and for their enthusiasm in teaching students. I would also like to thank prof.dr.L.Launer for giving me the opportunity to spend time at the Institute of Aging in Bethesda and for sharing her research experience with me. Dear Lenore, it was a wonderful time which I and my family enjoyed very much. Thank you so much for offering this possibility to me.

My co-students Fernando Rivadeneira and Fakhredin Ali Sayed Tatabaei, deserve to be particularly acknowledged. We spent a lot of time together discussing but we also enjoyed student life at the Erasmus University. Dear Fernando and Fakhredin, many thanks for your friendship and for your instant support as my paranymphs during the preparation of this thesis. I also feel particularly bound to say thank you to Mrs Kolff who was my landlady since my first visit in Rotterdam. She gave me the feeling of being at home at her house, always cared for me and supported me whenever I had a problem during all these years. I also would like to thank Mark Sea for his help in translating the summary into Dutch.

In Austria I would like to thank in first place prof.dr.GM Kostner, who is my chief at the Institute for Molecular Biology and Biochemistry. The constant support and trust, I received from him during my work was most important. Dear Gerhard, thank you for giving me the freedom to set up a new field in our institute and for your advice in planning my carrier and in persuing my scientific interest. I very much appreciate what you have done for me during these years. To have Ing.Johann Semmler on my side when getting the opportunity to establish

the DNA-laboratory at our Institute was an invaluable support. Dear Hans, I know that without your expertise and committment this thesis would not have been possible, and I would like to thank you so much for your long-term collaboration and loyalty.

All my appreciations go to my collegues at the Department of Neurology and at the Center for Neuroimaging, at the Medical University Graz. Dr.Reinhold Schmidt, prof.dr.Franz Fazekas, dr.Peter Kapeller, dr.Christian Enzinger, and Birgit Reinhart have set up the excellent data bank which provides the basis for my research. I thank you all for your friendly and kind way taking up me into your group. I especially would like to thank to my husband, Reinhold with whom we spent endless hours discussing the data and planning future projects during these years. Dear Reinhold, I am glad to have you as partner on my side and share my interest also on research with you.

I would also like to thank the co-authors of the manuscripts presented in this thesis for their contributions, especially to Natascha Schweighofer, prof.dr.S Frank and Irmgard Pölzl for their work on the angiotensinogen promoter.

Last but not least my loving thanks go to my family, to my parents, to my husband, Reinhold and to my beautiful daughter, Veronika. Thank you for all the love, care and support I got from you.

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