Markers of Endothelial and Hemostatic Activation and Progression of Cerebral White Matter Hyperintensities
Longitudinal Results of the Austrian Stroke Prevention Study

Hugh S. Markus, FRCP; Beverley Hunt, FRCPath; Kiran Palmer, BSc; Christian Enzinger, MD; Helena Schmidt, PhD; Reinhold Schmidt, MD

Background and Purpose—The pathogenesis of cerebral small vessel disease (SVD) is poorly understood, but endothelial activation and dysfunction may play a causal role. Cross-sectional studies have found increased circulating markers of endothelial activation, but this study design cannot exclude causality from secondary elevations. Confluent white matter hyperintensities (WMHs) on magnetic resonance imaging (MRI) appear to represent asymptomatic cerebral SVD. In a prospective study, we determined whether circulating markers of endothelial activation predicted progression of WMH.

Methods—In the community-based Austrian Stroke Prevention Study, MRI was performed at baseline in 296 subjects and repeated at 3 and 6 years. The following were measured on baseline plasma samples: intercellular adhesion molecule (ICAM), thrombomodulin, tissue factor plasma inhibitor, prothrombin fragments 1 and 2, and D-dimers.

Results—ICAM was associated with age- and gender-adjusted WMH lesion progression at both 3 and 6 years, respectively; (odds ratio [OR], 1.007; 95% confidence interval [CI], 1.002 to 1.012; \( P = 0.004 \)) and 1.004; 95% CI, 1.000 to 1.009 per ng/mL; \( P = 0.057 \)). After multivariate analysis controlling for other cardiovascular risk factors and C-reactive protein, 3-year OR was 1.010 (95% CI, 1.004 to 1.017; \( P = 0.001 \)) and 6-year OR was 1.008 (1.002 to 1.014 per ng/mL; \( P = 0.006 \)). Baseline log lesion volume was a strong independent predictor of progression but associations remained after controlling for this (3-year OR, 1.011; 95% CI, 1.002 to 1.020; \( P = 0.039 \)) and 6-year OR, 1.009; 95% CI, 1.000 to 1.017; \( P = 0.004 \)). There was no association between WMH progression and other markers.

Conclusions—ICAM levels are related to progression of WMH on MRI. The prospective study design increases the likelihood that this association is causal and supports a role of endothelial cell activation in disease pathogenesis. In contrast, we found no evidence for coagulation activation being important. (Stroke. 2005;36:1410-1414.)

Key Words: cerebrovascular disorders • lacunar infarction • magnetic resonance imaging • risk factors • white matter hyperintensities

Cerebral small vessel disease (SVD) causes one quarter of all ischemic strokes, a large proportion of cases of vascular dementia, and contributes to age-related cognitive decline. It results in lacunar infarction, resulting from ischemia in the territory of a single perforating artery, as well as in more diffuse ischemic changes referred to as leukoaraisosis. Leukoaraisosis, which is seen as low signal on computed tomography imaging or as high signal on T2 or FLAIR magnetic resonance imaging (MRI), is most prominent in the periventricular and deep white matter regions. At autopsy in patients with symptomatic cerebral SVD, thickening and hyaline deposition of the small perforating end arterioles supplying the white matter can be seen. In some cases of larger symptomatic lacunar infarction, localized microatheroma was found at the origin of the deep perforating arterioles. The neuropathological appearances corresponding to leukoaraisosis are neuronal loss, ischemic demyelination, and gliosis.

White matter high-signal changes can seen on MRI of normal subjects, particularly with increasing age. Such white matter hyperintensities (WMHs) may be punctate or confluent. Correlations of MRI appearances with neuropathology have shown that punctate WMHs often represent widened perivascular spaces without substantial ischemic damage. In contrast, confluent lesions appear to be ischemic and result from similar vascular changes to those seen in patients with symptomatic SVD. Imaging a community population to identify the presence of confluent WMH may therefore provide a surrogate marker to investigate the pathogenesis of SVD. A particularly powerful approach may be to study relationships between risk factors and progression of WMH; recent longitudinal studies have shown that confluent lesions progress relatively rapidly.
The pathogenesis of SVD is not fully understood. Hypertension is the major risk factor but fails to account for much of the risk. Several lines of evidence suggest that chronic endothelial dysfunction plays a pivotal role. This may be impaired cerebral reactivity and autoregulation, both of which have been observed in SVD. Endothelial dysfunction can be assessed in vivo by measuring soluble plasma markers. These are released into the circulation in response to endothelial perturbation by a number of different stimuli. Expression of intercellular adhesion molecule 1 (ICAM) is a precondition for the adhesion and transendothelial migration of lymphocytes; increased blood levels of soluble ICAM reflect endothelial cell activation. Thrombomodulin (TM) is normally expressed on the endothelial cell surface where, once activated by thrombin, it regulates the activity of protein C. Increased plasma levels are thought to reflect endothelial damage. Levels of both ICAM and TM are elevated in patients with symptomatic SVD. However, such changes could be merely secondary to systemic vascular or cerebral parenchymal damage. Prospective studies offer a more robust method to determine whether such associations are causal or secondary. Therefore, we determined whether plasma markers of endothelial function predicted disease progression, determined by WMH on MRI, in a community population. Previous work has also suggested a prothrombotic state may be important in symptomatic SVD. Therefore, we also determined levels of 2 markers of hemostatic turnover, D-dimer, and prothrombin fragments 1 and 2.

Subjects and Methods

Study Population

The Austrian Stroke Prevention Study is a single-center prospective follow-up study on the cerebral effects of vascular risk factors in the normal elderly population of Graz, Austria. Individuals aged 50 to 75 years, stratified by gender and 5-year age groups, were randomly selected from the official community register. Between September 1991 and March 1994, 8193 were invited to participate and 2794 agreed; the first 2007 eligible subjects were enrolled. All were examined by a trained neurologist. Exclusion criteria included neuropsychiatric disease, including stroke and dementia, with the latter screened for by the Mini Mental State Examination. Every fourth study participant or, in case of refusal, the next participant was invited for an imaging and neuropsychology substudy, including MRI; 509 subjects were included in this part. MRI was repeated at 3 or 6 years or both. The current article uses data before data analysis, leaving 267 samples. Baseline characteristics are shown in Table 1. Baseline lesion volume was highly correlated. The intraclass correlation coefficients ranged from 0.96 at baseline to 0.98 at 6-year follow-up.

Laboratory Methods

Blood was obtained in sodium citrate, centrifuged at 3000 g for 10 minutes, and plasma separated and stored at −70°C. Levels of circulating markers were measured using sandwich enzyme-linked immunosorbent assay-based assays for the following: ICAM (R&D Systems, UK); TM and tissue factor pathway inhibitor (TFPI), (Diagnostica Stago and American Diagnostica, Axis Shield, Scotland); prothrombin fragments 1 and 2 (Dade Behring, Sysmex UK Ltd); and D-Dimers (Biopool, Alpha Laboratories UK). Intra-assay (inter-assay) coefficients of variation for ICAM1, TM, TFPI, pro-thrombin fragments 1 and 2, and D-Dimers were 3.6% (7.4%), 4.8% (4.0%), 7.2% (7.4%), 6.0% (9.0%), and 3.6% (4.8%), respectively. High-sensitivity C-reactive protein was assayed with an enzyme-linked immunosorbent assay.

MRI

MRI was performed on 1.5-T systems (Gyrosan S 15 and ACS; Philips Medical Systems) using proton density and T2-weighted (TR/TE 2000 to 2500/50 to 90 ms) sequences in the transverse orientation. Additionally, sagittal T1-weighted images (TR/TE 580 to 620/15 to 20 ms) were acquired. Baseline and follow-up MRI protocols were identical. All slice thicknesses were 5 mm and acquisition matrices were 205 × 256.

Image analysis was performed in a 2-stage process. First, white matter lesions were identified on the hard copies by an experienced investigator. Lesion volume was then calculated semi-automatically on the original data. This allowed the same lesions to be identified on repeat scans and lesion volume quantification to be performed blind to patient identity and time of scan. White matter lesions were defined as focal areas hyperintense on proton density and T2-weighted images, and graded into punctate, early confluent, or confluent lesions, as previously described. Caps and pencil-thin lining were disregarded because these changes represent normal anatomical variants. The reader marked and roughly outlined on an overlaid transparency all white matter lesions on the baseline MRI initially, and then on the follow-up scans from the same individual. The identified lesions then had their volumes measured on T2 images on an UltraSPARC workstation (Sun Microsystems) using DISPunc (David Plummer, UCL, London, UK). The hard copy with lesions outlined on the overlaid transparency was placed alongside the corresponding images displayed on the workstation. Computer-based volumetric measurements were performed blinded to subject identity and order of scans. Total lesion volume (cm³) was calculated by multiplying the total lesion area by slice thickness. The operator had a proven maximum median intrarater coefficient of variation of 6.4%.

Scans of 50 randomly selected participants were analyzed on 2 occasions, separated by a few months, to establish the error in volumetric assessments. We set the error range as the 95% confidence interval (CI) of the most pronounced difference between repeated measurements (~1.59 to 1.81 cm³). Lesion progression was defined as an increase in WMH volume that exceeded measurement error (1.81 cm³) at 3- and 6-year follow-up. All decreases in lesion volume were within the range of measurement error. The WMH lesion load results from the first and second repeatability studies were highly correlated. The intraclass correlation coefficients ranged from 0.96 at baseline to 0.98 at 6-year follow-up.

Statistical Analysis

Distributions of thrombomodulin, TFPI, and D-dimer were skewed and required logarithmic transformation to obtain normal distributions. We determined associations between markers and lesion progression, already defined, using logistic regression. Because of strong associations between age and lesion volume and progression, we initially determined age- and gender-adjusted relationships and then for significant associations, adjusted for other cardiovascular risk factors (hypertension, diabetes, smoking (current, ex-smoker, nonsmoker), body mass index, and serum cholesterol, triglyceride, and fibrinogen. We also adjusted for C-reactive protein. We also determined associations with absolute change in lesion volume at 3 and 6 years, using multivariate regression.

Because progression was related to baseline WMH lesion load, we controlled for this in multivariate analysis. Baseline values of zero were replaced with 0.1 to allow logarithmic transformation that best fit the distribution of lesion volume.

Results

Seventeen samples were visually lipidemic and excluded before data analysis, leaving 267 samples. Baseline characteristics are shown in Table 1. Baseline lesion volume was...
median 0.3, range 0 to 35.4, mean (standard deviation [SD]) 1.32 (3.49) cm³, and grading of WMHs was none in 82 (30.7%), punctate in 148 (55.4%), early confluent in 26 (9.7%), and confluent in 11 (4.1%).

In 259 subjects rescanned at 3 years, WMH load was median (range) 0.3 (0 to 42.6), mean (SD) 1.88 (4.83) cm³, and in 196 rescanned at 6 years WMH load was median 0.45 (0 to 65.9) and mean (SD) 2.74 (7.35) cm³. At 3 years, 24 (9.3%) showed disease progression, whereas at 6 years 34 (17.3%) showed disease progression.

Relationships between conventional cardiovascular risk factors and WMH progression are shown in Table 2. Progression at both time points was associated with age and hypertension, but not with other risk factors. Baseline log lesion volume was a strong independent predictor of progression at 3-year (odds ratio [OR], 24.466 log cm³; 95% CI, 6.939 to 86.263; \( P < 0.0001 \)) and 6-year (OR, 24.264 log cm³; 95% CI 7.023 to 83.836; \( P < 0.0001 \)) values after adjustment for age, gender, and conventional risk factors.

ICAM was associated with age- and gender-adjusted WMH lesion progression at both 3 and 6 years; 3-years OR was 1.007 (95% CI, 1.002 to 1.012) per ng/mL (\( P = 0.004 \)) and 6-year OR was 1.004 (95% CI, 1.000 to 1.009) per ng/mL (\( P = 0.057 \)). Unadjusted mean [SD] ICAM levels were higher in subjects who showed lesion progression at both 3 (372.60 [86.49] versus 312.75 [88.42] ng/mL; \( P = 0.003 \)) and 6 years (347.44 [83.29] versus 309.01 [92.55] ng/mL). After multivariate analysis controlling for conventional risk factors and also C-reactive protein, ICAM remained associated with WMH lesion progression at both 3 and 6 years (3-year OR, 1.007 (95% CI, 1.002 to 1.012) per ng/mL (\( P = 0.004 \)) and 6-year OR was 1.004 (95% CI, 1.000 to 1.009) per ng/mL (\( P = 0.057 \)).

### Table 1. Baseline Characteristics in 267 Subjects in Whom Plasma Samples Were Analyzed and Divided According to Whether Lesion Progression Was Present at 3 and 6 Years

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All (n=267)</th>
<th>Progression at 3 Years</th>
<th>Progression at 6 Years</th>
<th>( P ) Univariate</th>
<th>Progression at 3 Years</th>
<th>Progression at 6 Years</th>
<th>( P ) Univariate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>60.0 (6.0)</td>
<td>63.1 (5.0)</td>
<td>59.6 (6.0)</td>
<td>0.004</td>
<td>58.6 (5.5)</td>
<td>63.8 (6.4)</td>
<td>0.000</td>
</tr>
<tr>
<td>Male</td>
<td>135 (50.6%)</td>
<td>111 (50.7%)</td>
<td>108 (49.3%)</td>
<td>0.794</td>
<td>77 (50.7%)</td>
<td>18 (56.3%)</td>
<td>0.698</td>
</tr>
<tr>
<td>Hypertension</td>
<td>79 (29.6%)</td>
<td>56 (25.6%)</td>
<td>14 (60.9%)</td>
<td>0.0001</td>
<td>38 (25.0%)</td>
<td>17 (53.1%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>230.2 (39.5)</td>
<td>232.0 (40.3)</td>
<td>219.9 (40.0)</td>
<td>0.170</td>
<td>229.2 (40.0)</td>
<td>220.6 (42.7)</td>
<td>0.302</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.7 (3.7)</td>
<td>26.6 (3.8)</td>
<td>26.8 (2.6)</td>
<td>0.707</td>
<td>26.7 (3.9)</td>
<td>26.0 (2.5)</td>
<td>0.341</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>12 (4.5%)</td>
<td>12 (5.5%)</td>
<td>0 (0%)</td>
<td>0.250</td>
<td>5 (3.3%)</td>
<td>0 (0%)</td>
<td>0.589</td>
</tr>
<tr>
<td>Current smoker</td>
<td>28 (10.5%)</td>
<td>21 (9.6%)</td>
<td>3 (13.0%)</td>
<td>0.461</td>
<td>13 (8.6%)</td>
<td>3 (9.4%)</td>
<td>0.336</td>
</tr>
<tr>
<td>Exsmoker</td>
<td>83 (31.1%)</td>
<td>64 (29.2%)</td>
<td>9 (39.1%)</td>
<td>0.005</td>
<td>47 (30.9%)</td>
<td>14 (43.8%)</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>306.0 (77.1)</td>
<td>307.0 (76.7)</td>
<td>304.8 (83.3)</td>
<td>0.908</td>
<td>307.6 (77.6)</td>
<td>316.3 (80.4)</td>
<td>0.579</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>145.5 (68.4)</td>
<td>144.1 (84.3)</td>
<td>141.7 (66.9)</td>
<td>0.870</td>
<td>148.1 (91.4)</td>
<td>130.3 (71.6)</td>
<td>0.231</td>
</tr>
<tr>
<td>ICAM, ng/mL</td>
<td>319.3 (89.3)</td>
<td>312.8 (88.4)</td>
<td>372.6 (86.5)</td>
<td>0.005</td>
<td>309.0 (92.6)</td>
<td>347.4 (83.3)</td>
<td>0.031</td>
</tr>
<tr>
<td>Thrombomodulin, ng/mL</td>
<td>43.0 (23.9)</td>
<td>43.2 (24.9)</td>
<td>44.0 (26.1)</td>
<td>0.764</td>
<td>44.1 (28.7)</td>
<td>43.9 (22.3)</td>
<td>0.943</td>
</tr>
<tr>
<td>TFPI, ng/mL</td>
<td>375.9 (446.5)</td>
<td>395.8 (470.4)</td>
<td>305.8 (206.2)</td>
<td>0.956</td>
<td>351.2 (451.7)</td>
<td>358.4 (251.2)</td>
<td>0.151</td>
</tr>
<tr>
<td>D-dimers, ng/mL</td>
<td>206.6 (118.8)</td>
<td>207.1 (116.8)</td>
<td>229.8 (169.0)</td>
<td>0.676</td>
<td>201.7 (112.0)</td>
<td>234.6 (195.0)</td>
<td>0.555</td>
</tr>
<tr>
<td>PF1+2, nmol/mL</td>
<td>2.50 (1.51)</td>
<td>2.45 (1.47)</td>
<td>2.61 (1.30)</td>
<td>0.595</td>
<td>2.46 (1.67)</td>
<td>2.68 (1.27)</td>
<td>0.434</td>
</tr>
</tbody>
</table>

Values indicate mean (SD) or proportion (%). For thrombomodulin, D-dimers, and PF1+2, untransformed values are shown but statistics are calculated on log-transformed values (see Subjects and Methods).

### Table 2. Relationship (OR, 95% CI, and \( P \)) Between Conventional Cardiovascular Risk Factors and Progression at 3 and 6 Years on Multivariate Analysis

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>3-Year Progression</th>
<th>6-Year Progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>1.105 (1.017–1.201)</td>
<td>1.160 (1.069–1.259)</td>
</tr>
<tr>
<td>Male</td>
<td>0.447 (0.151–1.321)</td>
<td>0.783 (0.293–2.092)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>6.509 (1.924–12.972)</td>
<td>3.714 (1.510–9.136)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.000 (4.8±19)</td>
<td>0.801 (2.7±19)</td>
</tr>
<tr>
<td>Smoking status</td>
<td>1.504 (0.871–2.599)</td>
<td>1.365 (0.828–2.252)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>0.970 (0.844–1.115)</td>
<td>0.903 (0.779–1.046)</td>
</tr>
<tr>
<td>Cholesterol, mg/dL</td>
<td>0.990 (0.976–1.003)</td>
<td>0.998 (0.986–1.010)</td>
</tr>
<tr>
<td>Triglyceride, mg/dL</td>
<td>1.002 (0.995–1.009)</td>
<td>0.999 (0.993–1.006)</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>0.998 (0.991–1.004)</td>
<td>0.999 (0.993–1.005)</td>
</tr>
</tbody>
</table>

Smoking is categorized as never, ex-smoker and current smoker. Hypertension is defined as history of hypertension with repeated blood pressure > 160/95, treatment for hypertension, or 2 readings at the examination > 160/95.
endothelial dys-
release plays a crucial role in regulation of cerebral blood
and blood–brain barrier function. Endothelial nitric oxide

adhesion of circulating leukocytes to endothelial cells and


elevated because of, rather than caused by, the


correlated with age- and gender-
adjusted change in absolute lesion volume at 3 years


cerebral SVD,6,9 although a prospective study found no


cerebral SVD.21 Expression of ICAM on brain microvascular endo-
thelium may allow activated leukocytes to enter the central
nervous system via binding to leukocyte ligand on ICAM.22

Therefore, although our data provide evidence that endo-
thelial cell activation plays a role in WMH progression,


process is mediated by cellular adhesion molecules, including
ICAM, expressed on the endothelial membrane. Pathological
studies have shown increased ICAM expression in several
components of the atherosclerotic plaque.17 Prospective
studies have also shown associations between soluble ICAM and
risk of future myocardial infarction,18,19 supporting a role in
atherosclerosis, although this may be partly caused by con-
founding by conventional risk factors and socioeconomic
status.20 Increased blood–brain barrier permeability has been
suggested as playing a role in the pathogenesis of cerebral
SVD.21 Expression of ICAM on brain microvascular endo-
thelium may allow activated leukocytes to enter the central
nervous system via binding to leukocyte ligand on ICAM.22

Some potential limitations of the data merit consideration.
First, because they are based on the storage of samples at
−70°C for up to 7 years, the possibility of protein degrada-
tion cannot be excluded. However, this seems unlikely
because concentrations of ICAM were similar to those
reported in fresh plasma. Second, previous studies have found
ICAM is elevated in patients with acute and chronic inflam-
matory disorders and unstable coronary symptoms. However,
participants in this study were healthy when blood samples
were obtained.

In contrast to the associations with ICAM, we found no
associations with other plasma markers of endothelial and
hemostatic activation. Previous studies have found an asso-
ciation between plasma TM and advanced symptomatic
cerebral SVD,6,9 although a prospective study found no
association with 87 cases of all subtypes of ischemic stroke.24

Our results failed to show an association with progression of
WMH, not supporting a role for TM in the pathogenesis of
early cerebral SVD. TM expression in cerebral small vessels
is low to absent in many areas;25 this may be relevant to the
lack of association. It is possible that our sample size is too
small to detect associations with these markers. We per-
formed power calculations, based on the means and SDs of
measurements in those who progressed and did not at 3 years;
if such differences were significant, sample sizes required for
detection would be: thrombomodulin, 37492; TFPI, 1034;
D-dimers, 1152; and prothrombin fragments 1 and 2, 3742.
Therefore, such differences would only be detected by a
much larger study and would be an order of magnitude lower
than those with ICAM.

TFPI is the major physiological inhibitor of the effect of
tissue factor. It binds deactivated factor Xa within the tissue
factor VIIa/Xa complex, preventing thrombin formation. The
relationship between TFPI and stroke has been little studied. Decreased plasma levels have been found in adult patients with ischemic stroke, including atherothrombotic and lacunar infarction. In a cross-sectional study of advanced symptomatic cerebral SVD, patients with widespread leukoaraiosis had lower TFPI levels and a higher TF/TFPI ratio than those with isolated lacunar infarction, but no prospective studies have investigated these relationships. Ours is the first prospective study to our knowledge, and it failed to show a relationship.

It has been suggested that a prothrombotic state plays a role in cerebral SVD. Prothrombin fragments 1 and 2 are markers of thrombin generation, which arises from conversion of prothrombin to thrombin. D-dimer is a marker of fibrinolytic turnover and has been elevated in studies assessing those at risk for future atherothrombotic events. We found no relationship between prothrombin fragments 1 and 2 or D-dimer and progression of WMH. This does not support a role of activation of coagulation in progression of early cerebral SVD. However, it could be important in pathogenesis of acute ischemic events resulting in lacunar stroke in patients with established SVD; this would not have been detected in our study of asymptomatic individuals.

In summary, this study shows ICAM levels are significantly related to WMH progression. This provides support for endothelial activation being involved in early pathogenesis. In contrast, our results do not support a role for hemostatic changes in early disease, and this would be consistent with local thrombosis playing a secondary role of endothelial activation. Our results, together with previous studies implicating a role of endothelial activation and dysfunction in the disease, would suggest that therapies that stabilize the endothelium may have a role in preventing disease progression.

Acknowledgments

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References

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