High von Willebrand Factor Levels Increase the Risk of First Ischemic Stroke

Influence of ADAMTS13, Inflammation, and Genetic Variability

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Background and Purpose—Elevated von Willebrand factor (vWF) concentrations are associated with an increased risk of ischemic heart disease. Several factors influence vWF antigen levels and activity, including blood group, genetic variability, acute-phase response, and proteolysis by ADAMTS13 and Metalloprotease with Thrombospondin motif (ADAMTS13), a determinant of proteolytic cleavage of vWF. We assessed how these factors affect the relation between vWF and the occurrence of stroke to understand the underlying mechanism.

Methods—In a case-control study of 124 first-ever ischemic stroke patients and 125 age- and sex-matched controls, we studied vWF antigen (vWF:Ag), vWF ristocetin cofactor activity (vWF:RCo), ADAMTS13 activity, the −1793C/G polymorphism in the vWF gene, and C-reactive protein.

Results—vWF antigen and activity levels were significantly higher in cases than in controls. The relative risk of ischemic stroke was highest in individuals in the upper quartile of vWF:Ag (odds ratio, 3.2; 95% CI, 1.4 to 7.5) and vWF:RCo (odds ratio, 2.1; 95% CI, 0.9 to 4.8) compared with individuals in the lowest quartiles. In individuals with ADAMTS13 in the lowest quartile, the relative risk of stroke was 1.7 (95% CI, 0.7 to 3.9) compared with the highest quartile. C-reactive protein, ADAMTS13, and genetic variation did not affect the association between vWF and the relative risk of stroke, whereas blood group did affect the association.

Conclusions—vWF antigen and activity are associated with the occurrence of acute ischemic stroke. This relation is unaffected by the severity of the acute-phase response or by genetic variation or degradation. (Stroke. 2006;37:2672–2677.)

Key Words: ADAMTS13 ■ hemostasis ■ ischemic stroke ■ von Willebrand factor

Von Willebrand factor (vWF), a plasma glycoprotein that is a mediator of platelet adhesion, becomes available when the endothelium is damaged. vWF is released as ultralarge (UL) multimers, which form spontaneous high-strength bonds with the platelet glycoprotein Ib-IX-V complex, resulting in platelet aggregation and thrombus formation.1 The concentration and activity of vWF are influenced by several factors, including blood group, inflammation, and proteolysis by ADAMTS13 and Metalloprotease with Thrombospondin motif (ADAMTS13). Genetic variations in the vWF gene are associated with vWF levels, eg, the −1793C/G polymorphism in the promotor region of the vWF gene. The G allele is associated with higher levels of vWF antigen than is the C allele.2

Proteolysis by ADAMTS13 also determines vWF activity. ADAMTS13 is a recently discovered metalloprotease that specifically cleaves the bond between tyrosine-842 and methionine-843 in the A2 domain of vWF multimers, resulting in 2 fragments of 176 and 140 kDa.3-4 These fragments are less active in platelet aggregation compared with the uncleaved ULvWF multimers. A deficiency of ADAMTS13 is seen in patients with thrombotic thrombocytopenic purpura, which is characterized by cerebral ischemia caused by platelet thrombosis in the cerebral microcirculation.5-6

Several studies have shown that high vWF antigen levels are a risk factor for arterial thrombosis.7-9 However, information on vWF and cerebrovascular disease is limited and inconsistent.8,10-12 We hypothesized that ADAMTS13 plays a role in arterial thrombosis because reduced ADAMTS13 activity will result in less degradation of ULvWF multimers and thereby in increased vWF activity. The aim of our study was to further investigate the role of vWF in ischemic stroke and to assess how the factors aforementioned can affect the relation between vWF and the occurrence of ischemic stroke.
Subjects and Methods

Study Design
We performed a case-control study in which cases were consecutively recruited patients with a first-ever acute ischemic stroke who were admitted to the Department of Neurology at Erasmus University Medical Center. Population controls were partners or friends of the patients. They were matched by age and sex, white, without a history of stroke, and unrelated to the patient. The study design has previously been described in more detail elsewhere.13

Blood Sampling and Procedures
Venous blood samples were taken between 7 and 14 days after the stroke under strictly standardized conditions. From a random group of 64 patients, blood was also collected 3 months after the stroke.

Blood was collected into citrate (0.105 mol/L) with use of the Vacutainer (Becton-Dickinson, Plymouth, UK) system. Blood was centrifuged (2000g for 30 minutes at 4°C), and plasma was stored in aliquots at −80°C until use. Genomic DNA was isolated according to standard salting-out procedures.

Plasma Level Measurements
vWF antigen (vWF:Ag) was determined with an in-house ELISA with polyclonal rabbit anti-human vWF antibodies (DakoCytomation, Glostrop, Denmark). vWF ristocetin cofactor activity (vWF:RCo) was measured by an aggregometric method with the use of formalin-fixed platelets and ristocetin (Diagnostica Stago, Asnières, France). FVIII:C was measured by a 1-stage clotting assay with platelet (Organon Teknika, Durham, NC) and FVIII-deficient plasma (Biopool, Umeå, Sweden). The intra-assay coefficients of variation for vWF:Ag, vWF:RCo, and FVIII:C were 6.3%, 7.9%, and 5.2%, respectively.

ADAMTS13 activity was measured with a rapid functional assay that determines the digestion of vWF by ADAMTS13, based on the method described by Gerritsen with minor modifications, as described before.14 Normal pooled plasma obtained from 40 healthy volunteers was used for calibration. The intra-assay coefficient of variation was 11%.

The concentration of C-reactive protein (CRP) was determined with a sensitive in-house enzyme immunoassay with rabbit antibodies against human CRP as catching antibodies and goat antibodies against human CRP as tagging antibodies (Dako). The intra-assay coefficient of variation for CRP was 3.5%.

−1793C/G Polymorphism in the vWF Gene
The DNA fragment containing the polymorphism was amplified by polymerase chain reaction (PCR) with forward primer 5′AGCCACGGGACGGTGGCAG-3′ and reverse primer 5′TAC AAAAATGGGAGTGAG-3′. The PCR comprised 4 minutes at 95°C of initial denaturation, followed by 32 cycles of 1 minute at 94°C, 1 minute at 65°C, and 2 minutes at 72°C. Subsequently, the PCR product of 264 bp was digested by AcI, which cleaves the C allele into 2 fragments of 180 and 84 bp, whereas the common G allele remains uncleaved. The fragments were separated on a 2% agarose gel and visualized under UV light.15

Statistical Analysis
The levels of vWF:Ag, vWF:RCo, FVIII, and ADAMTS13 were compared in cases and controls and in subgroups according to ABO blood group with Student t test. vWF:Ag, vWF:RCo, FVIII, and ADAMTS13 activity were divided into quartiles based on the distribution in the control group. The relation between the levels of these variables and ischemic stroke was estimated by logistic regression, with the lowest quartile as the reference, except for the ADAMTS13 activity, for which the highest quartile was the reference group. We adjusted for several well-known risk factors for vascular diseases, including smoking, hypertension, diabetes mellitus, and hyperlipidemia. A P value <0.05 was considered to indicate statistical significance. In the subgroup analysis by blood group, logistic regression was performed with quartiles of vWF, which were
data collected for each subgroup separately. Correlations were estimated with the Spearman correlation coefficient.

The relation between the levels of vWF and the −1793C/G polymorphism was studied by ANOVA, after adjustment for age, sex, and blood group. The association of the −1793C/G polymorphism with the occurrence of ischemic stroke was estimated by logistic regression and is presented with its 95% CIs. The statistical analysis was performed with SPSS software, version 11.

Results
The study included 124 patients and 125 sex- and age-matched controls, and their characteristics are presented in Table 1. Plasma levels were available for all individuals. Genetic analysis could be performed for 109 patients and 119 controls.

vWF:Ag levels were significantly higher in cases (mean±SD, 1.47±0.66 U/mL) compared with controls (1.23±0.50 U/mL; P=0.002). Levels of vWF:RCo were also higher in cases than in controls (1.37±0.74 versus 1.13±0.47 U/mL; P=0.002).

The respective levels for FVIII in cases and controls were 1.40±0.46 versus 1.17±0.70 U/mL (P=0.003). The relative risk of stroke (odds ratio [OR]) for vWF:Ag as a continuous variable was 2.20 (95% CI, 1.3 to 3.7; Table 2). Individuals with the highest levels of vWF:Ag (highest quartile) had a significantly increased relative risk 3.21 (95% CI, 1.4 to 7.5) compared with individuals in the lowest quartiles (Table 3).

In the highest quartiles of vWF:RCo, the relative risk of

### Table 1. Baseline Characteristics

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Cases (n=124)</th>
<th>Controls (n=125)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>56±12</td>
<td>56±12</td>
<td>NS</td>
</tr>
<tr>
<td>Female sex</td>
<td>58 (47%)</td>
<td>59 (47%)</td>
<td>NS</td>
</tr>
<tr>
<td>Blood group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>54 (44%)</td>
<td>63 (50%)</td>
<td>0.37</td>
</tr>
<tr>
<td>Non-0</td>
<td>68 (56%)</td>
<td>62 (50%)</td>
<td></td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>61 (49%)</td>
<td>37 (30%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Hypertension</td>
<td>60 (48%)</td>
<td>24 (19%)</td>
<td>0.000</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>18 (14%)</td>
<td>5 (4%)</td>
<td>0.004</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>78 (61%)</td>
<td>84 (67%)</td>
<td>0.70</td>
</tr>
<tr>
<td>Positive family history</td>
<td>75 (61%)</td>
<td>56 (45%)</td>
<td>0.013</td>
</tr>
<tr>
<td>CRP</td>
<td>2.01</td>
<td>1.42</td>
<td>0.008</td>
</tr>
</tbody>
</table>

Data for age are presented as mean±SD. Data for CRP are presented as median (25% to 75% range). Other data are counts and (percentages).

### Table 2. Levels of vWF and ADAMTS13 in Cases and Controls

<table>
<thead>
<tr>
<th>vWF/ADAMTS13</th>
<th>Cases</th>
<th>Controls</th>
<th>P</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF:Ag</td>
<td>1.47±0.66</td>
<td>1.23±0.50</td>
<td>0.002</td>
<td>2.2 (1.3–3.7)</td>
</tr>
<tr>
<td>vWF:RCo</td>
<td>1.37±0.74</td>
<td>1.13±0.47</td>
<td>0.002</td>
<td>1.9 (1.2–3.0)</td>
</tr>
<tr>
<td>ADAMTS13</td>
<td>0.96±0.41</td>
<td>1.03±0.44</td>
<td>0.23</td>
<td>0.7 (0.4–1.4)</td>
</tr>
<tr>
<td>FVIII:C</td>
<td>1.40±0.70</td>
<td>1.17±0.46</td>
<td>0.003</td>
<td>1.2 (0.9–1.5)</td>
</tr>
</tbody>
</table>

All values are in U/mL and indicate mean±SD. ORs (95% CI) per U/mL were adjusted for blood group. P values indicate the significance of the t test for the differences between cases and controls.
stroke was 2.06 (95% CI, 0.9 to 4.8), The risk in the highest quartile for VWF was 1.5 (95% CI, 0.7 to 3.4). Additional adjustment for previous cardiovascular disease (atrial fibrilation, myocardial infarction, intermittent claudication, peripheral arterial diseases, and vascular surgery) did not affect the relation with risk. Analysis of VWF levels in the blood samples collected 3 months after the stroke showed slightly increased vWF:Ag in cases compared with controls (1.32 versus 1.13). vWF:RCo values (1.13 versus 0.41) and similar vWF:RCo values (1.13 versus 0.41 U/mL).

As expected, lower levels of VWF:Ag were found in all individuals with blood group O (1.14 ± 0.53 U/mL) compared with individuals with blood group non-O (1.53 ± 0.59 U/mL). The levels of VWF:RCo were also lower in individuals with blood group O than in individuals with blood group non-O. Within both cases and controls, higher levels were found in those with blood group non-O (Table 4). The relative risk of stroke in individuals increased with increasing VWF levels (P for trend=0.002), and in the highest quartile of VWF:Ag with blood group O, the relative risk was 5.9 (95% CI, 1.6 to 21.5), whereas in subjects with blood group non-O, the relative risk was 1.6 (95% CI, 0.5 to 5.2; P for trend=0.69; the Figure).

Levels of ADAMTS13 were slightly but not significantly lower in cases (0.96 ± 0.41 U/mL) than in controls (1.03 ± 0.44 U/mL; P=0.23). The OR for ADAMTS13 in the lowest quartile was 1.7 (95% CI, 0.7 to 3.9), with the highest quartile as the reference group. A weak but significant correlation was found between ADAMTS13 and VWF:Ag (r=-0.14, P=0.05) and VWF:RCo (r=-0.18, P=0.01). Adjustment for ADAMTS13 had only a minor effect on the correlation between VWF parameters and stroke risk. Because it had been previously reported that individuals with blood group O had significantly higher levels of ADAMTS13, we performed a subgroup analysis by blood group. The levels of ADAMTS13 in the controls with blood group non-O were somewhat lower than in the controls with blood group O (P=0.06). ADAMTS13 levels were similar in the various blood groups in the cases (Table 4).

The median level of CRP was 2.07 mg/L in cases and 1.42 mg/L in controls (P=0.11). Although CRP was significantly correlated with the levels of VWF:Ag (r=0.19) and VWF:RCo (r=0.24), adjustment for CRP did not influence the relation between these VWF parameters and stroke risk.

The allele distribution of the -1793C/G polymorphism was in Hardy-Weinberg equilibrium. The frequency of the minor allele of the -1793C/G polymorphism was 0.33 (95% CI, 0.3 to 0.4) in cases and 0.30 (95% CI, 0.24 to 0.36) in controls (P=NS). The levels of VWF:Ag were slightly higher in the group with the GG genotype in cases (1.65 ± 0.56 U/mL) than in the other genotypes (1.52 ± 0.61 U/mL for the CC genotype and 1.39 ± 0.60 for the CG genotype; P=0.41).

In the patients with ischemic stroke and the GG genotype, we also observed higher levels of VWF:RCo (1.64 ± 0.56 U/mL) than in patients with other genotypes (VWF:RCo in the CC genotype was 1.47 ± 0.86 U/mL [P=0.11] and 1.23 ± 0.57 U/mL in the CG genotype). Similar but weaker trends were seen in controls (Table 5). The ORs for stroke were 0.9 (95% CI, 0.5 to 1.5) in the CG genotype and 0.7 (95% CI, 0.3 to 1.6) in the GG genotype, with the CC genotype as the reference.

**Discussion**

The present study shows a significant relation between increased VWF:Ag levels and the occurrence of ischemic stroke. A similar relation was observed for VWF:RCo activity. In this study, VWF levels were associated with genetic variation, ADAMTS13 activity, inflammation, and blood

| TABLE 3. Relation Between VWF Levels, ADAMTS13, FVIII:C, and Stroke by Quartiles (Q1–Q4) |
|-----------------------------------|----------------|----------------|----------------|----------------|
|                                  | Q1     | Q2     | Q3     | Q4     |
| VWF:Ag, U/mL                     | <0.84  | 0.84–1.17 | 1.17–1.52 | >1.52  |
| OR (95% CI)                      | 1 (reference) | 1.8 (0.8–4.0) | 2.4 (1.0–4.8) | 3.2 (1.4–7.5) |
| VWF:RCo, U/mL                    | <0.78  | 0.78–1.06 | 1.06–1.46 | >1.46  |
| OR (95% CI)                      | 1 (reference) | 1.6 (0.7–3.4) | 1.4 (0.7–3.2) | 2.1 (0.9–4.8) |
| ADAMTS13, U/mL                   | <0.70  | 0.70–0.95 | 0.95–1.34 | >1.34  |
| OR (95% CI)                      | 1.7 (0.7–3.9) | 1.9 (0.9–4.3) | 2.0 (0.9–4.5) | 1 (reference) |
| FVIII:C, U/mL                    | <0.90  | 0.90–1.13 | 1.13–1.41 | >1.41  |
| OR (95% CI)                      | 0.70 (0.3–1.5) | 1.0 (0.4–2.1) | 1.5 (0.7–3.4) |  |

Values presented are mean ± SD for VWF antigen and activity and ADAMTS13 in U/mL.

| TABLE 4. Levels of VWF and ADAMTS13 in Individuals With Blood Group O vs Non-O |
|-----------------------------------|----------------|----------------|----------------|
|                                  | Blood Group O | Blood Group Non-O | P    |
| VWF:Ag                           | 1.30 ± 0.62  | 1.59 ± 0.66      | 0.017 |
| VWF:RCo                          | 1.15 ± 0.69  | 1.54 ± 0.74      | 0.004 |
| ADAMTS13                         | 0.96 ± 0.41  | 0.97 ± 0.41      | 0.89  |
|                                  | 1.00 ± 0.39  | 1.47 ± 0.50      | 0.000 |
|                                  | 0.93 ± 0.38  | 1.33 ± 0.47      | 0.000 |
|                                  | 1.11 ± 0.44  | 0.95 ± 0.43      | 0.06  |

Values presented are mean ± SD for VWF antigen and activity and ADAMTS13 in U/mL.
group. However, after adjustment for these factors, the
relation between vWF and stroke remained similar.
It is well known that vWF levels are dependent on ABO
blood groups. Previously, it has been reported that individuals
with blood group non-O have higher levels of vWF.\textsuperscript{17} It has
been reported that individuals with blood group non-O also
have a higher risk of arterial thrombosis.\textsuperscript{18,19}

We also observed higher vWF levels in blood group non-O
individuals and therefore performed a subgroup analysis by
blood group. Interestingly, we found a higher relative risk of
stroke in individuals in the highest quartile of vWF levels
with blood group O (OR, 5.9; 95% CI, 1.6 to 21.5) compared
with individuals in the highest quartile of vWF level with
blood group non-O (OR, 1.6; 95% CI, 0.5 to 5.2). The
underlying mechanism for this finding is unclear. Some
mechanisms have been proposed through which blood group
affects vWF, such as the clearance of vWF, which differs in
the various blood groups owing to the varying carbohydrate
structure of plasma glycoproteins.\textsuperscript{20} Furthermore, gene loci
were suggested to have a quantitative effect on plasma levels
of vWF. ABO blood group locus is one of these, located on
chromosome 9q34. Because the gene is located very close to
that for ADAMTS13, we investigated with HAPMAP (www.
hapmap.org) whether there was any linkage disequilibrium,
but the genes were in different linkage disequilibrium
blocks.

vWF:Ag and vWF:RCo in the samples collected 3 months
after stroke were similar to the levels in controls. Possible

\begin{table}
\centering
\begin{tabular}{lccc}
\hline
 & CC & CG & GG \\
Cases & (n=52) & (n=47) & (n=10) \\
\hline
vWF:Ag & 1.52±0.61 & 1.39±0.60 & 1.65±0.56 & 1.23±0.45 & 1.24±0.51 & 1.25±0.71 & P=0.41 \\
vWF:RCo & 1.47±0.86 & 1.23±0.57 & 1.64±0.56 & 1.08±0.40 & 1.22±0.52 & 0.99±0.53 & P=0.11 \\
\hline
\end{tabular}
\caption{Relation Between the vWF \textsuperscript{−}1793C/G Polymorphism and vWF Levels in Cases and Controls}
\end{table}
explanations for this difference between vWF and risk between the acute and the convalescent phase are that the increase in vWF levels that we observed shortly after the event might have been due to an acute-phase response, but adjustment for the sensitive, early acute-phase marker CRP and the late acute-phase marker fibrinogen had only a minimal effect on the OR. This indicates that inflammation may play a role in determining levels of vWF, but it will not be the only important regulatory mechanism, because vWF levels are independent of the inflammation that is also associated with the relative risk of ischemic stroke. Another plausible explanation is that the use of drugs (such as statins) and a healthier lifestyle (such as smoking cessation) may have decreased the vWF levels in the patients.

The −1793CG polymorphism in the promoter region of the vWF gene is associated with vWF levels in plasma. We have previously shown that carriers of the G allele have a significantly increased risk of acute myocardial infarction. This indicates that genetic variation in the vWF gene, resulting in higher vWF levels, may be causally related to the relative risk of arterial thrombosis. In the present study, the highest levels of vWF (vWF:Ag and vWF:RCo) were also found in individuals with the GG genotype (P=NS). However, there was no indication that carriers of the G allele have a higher relative risk of ischemic stroke.

Proteolytic cleavage of ULvWF by ADAMTS13 into less-active, smaller multimers determines the activity levels of vWF in plasma. We therefore hypothesized that ADAMTS13 activity might be associated with the risk of stroke. This is the first study to determine the level of ADAMTS13 in patients with ischemic stroke, and indeed, we observed that levels of ADAMTS13 activity were somewhat lower in ischemic stroke patients compared with controls. In individuals with the lowest levels of ADAMTS13, the risk of ischemic stroke was almost doubled (OR, 1.6; 95% CI, 0.7 to 3.8) compared with individuals in the highest quartile of ADAMTS13 level. However, these results were not statistically significant, and these findings should be confirmed in studies with a larger patient population. Our study has shown that the levels of vWF and ADAMTS13 are negatively associated, similar to what has been found in a variety of conditions in previous studies.

Therefore, we can only speculate about the underlying mechanism: low levels of ADAMTS13 resulting in higher vWF levels or an increase of vWF levels resulting in lower ADAMTS13 levels. In addition, ADAMTS13 levels in our control group were also dependent on blood group, with individuals with blood group O having higher levels than non-O, as has been reported earlier. This was not seen in our patient group, which might indicate that the increase in vWF levels found in ischemic stroke patients overrules the difference in vWF due to blood group.

In conclusion, this study has shown clearly that high levels of vWF:Ag and vWF activity are associated with an increased risk of stroke. Our results indicate that inflammation, genetic variation, and degradation by ADAMTS13 partly determine vWF levels; however, we did not demonstrate an interaction with the effect of vWF on ischemic stroke risk.

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Disclosures
None.

References
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