Is factor V Leiden a risk factor for thrombotic microangiopathies without severe ADAMTS13 deficiency?

Soraya Krieg, Jan-Dirk Studt, Irmela Sulzer, Bernhard Lämmle, Johanna A. Kremer Hovinga
Department of Hematology and Central Hematology Laboratory, Inselspital, University of Bern, Bern, Switzerland

Summary
About 60% of patients diagnosed with acute thrombotic thrombocytopenic purpura (TTP) display a severe ADAMTS13 deficiency. Recently, Raife et al. concluded from a small case series, that factor V Leiden (FVL) might constitute a risk factor for acute thrombotic microangiopathy (TMA) without severe ADAMTS13 deficiency. Therefore, we determined ADAMTS13 activity and FVL carrier-ship in 256 consecutive patients presenting with various forms of acute TMA, including patients diagnosed with TTP or hemolytic-uremic syndrome (HUS). The overall prevalence of FVL was 8.2% (6.25% among patients diagnosed with TTP, and 9% among those with HUS) concordant with the FVL prevalence reported in Europe. FVL was present in 9.9% of patients with ADAMTS13 activity <10% and in 9.7% of those with normal ADAMTS13 activity (>50%). We conclude that FVL is not more prevalent in TMA patients without as compared to those with severe ADAMTS13 deficiency. The prevalence of FVL carriers in certain HUS subgroups (HUS with ADAMTS13 activity >50%) reaching 12.3% suggests that a contributory role of FVL in the pathogenesis of defined forms of HUS needs further study.

Keywords
ADAMTS13 activity, factor V Leiden, HUS, thrombotic microangiopathy, TTP

Introduction
Thrombotic microangiopathies (TMAs) are rare disorders characterized by microangiopathic hemolytic anemia with schistocytes, microvascular platelet clumping leading to thrombocytopenia and often ischemic organ dysfunctions. Based on prevailing clinical symptoms and underlying disorders, TMA are generally divided into three principal groups, hemolytic-uremic syndrome (HUS) when renal involvement prevails, thrombotic thrombocytopenic purpura (TTP) in the presence of neurological symptoms, and secondary TMAs in association with various clinical conditions including autoimmune disorders, hematopoietic stem cell transplantation, neoplasia, certain drugs and other conditions (1). The epidemic form of HUS (D’HUS) results from infection with verotoxin-producing bacteria and is predominantly, but not exclusively, diagnosed in children. The pathophysiology of the atypical form of HUS is poorly understood, although lately some familial cases have been linked to mutations and/or deficiencies of the complement regulatory proteins, factor H and membrane cofactor protein (MCP) (2, 3).

Acute idiopathic TTP is characterized by the failure to process unusually large, extremely adhesive VWF multimers resulting in systemic platelet aggregation. Processing of VWF involves the von Willebrand factor-cleaving protease, ADAMTS13. Its severe deficiency (<5% of normal), either constitutional due to compound heterozygous or homozygous ADAMTS13 gene mutations or acquired as a result of circulating ADAMTS13 inhibitory autoantibodies, is a specific finding of acute TTP (4). The sensitivity of severe ADAMTS13 deficiency for the clinical diagnosis of TTP remains equivocal, with a reported prevalence of about 60% [range 33–100% (5–12)]. Apparently, other pathogenetic factors may lead to a condition clinically indistinguishable from that seen in severe ADAMTS13 deficiency.

Recently, Raife and coworkers (13), based on a small case series, hypothesized that factor V Leiden (FVL) constituted a pathophysiological risk factor in a subset of patients suffering from acute TMA. They determined ADAMTS13 activity and FVL carrier-ship in Caucasians with acute TMA. In the subset of 11 patients with normal ADAMTS13 activity the prevalence of...
FVL was 36%, while none of the 16 patients with severe ADAMTS13 deficiency carried this mutation. The FVL mutation, which results in resistance to activated protein C (APC) is the most prevalent hereditary abnormality associated with venous thrombosis in Caucasians (14–16). A possible connection of FVL with acute TMA might necessitate a more individualized treatment regimen including anticoagulant drugs complementing plasma exchange therapy. Our aim was, therefore, to estimate the prevalence of FVL carriers in a large group of patients with acute TMA, either with or without severe ADAMTS13 deficiency.

Materials and methods

Patients
Between January 2001 and July 2003, ADAMTS13 activity was determined in plasma samples of 396 consecutive patients referred to our laboratory for diagnostic purposes (10). A diagnosis of acute TMA was based on the concomitant presence of thrombocytopenia (platelet count <130x10^9/L) and microangiopathic hemolytic anemia with fragmented erythrocytes (schistocytes) on the peripheral blood smear, and elevated lactate dehydrogenase values. Adhering to these criteria we initially excluded 101 patients from further study as they did not suffer from an acute TMA (patients in remission or not fulfilling the diagnostic criteria of acute TMA, n=35; or investigation for another hematological disorder, e.g. immune thrombocytopenia, Evans’ syndrome, chronic hemolysis or thrombocytopenia of unknown origin and other conditions, n=21) or because of missing clinical information (n=45).

The remaining 295 patients were assigned to one of eight predefined clinical categories adopting the diagnosis of the referring clinicians: 1) acute idiopathic TMA without further allocation into TTP or HUS; 2) neoplasia- or chemotherapy-associated TMA; 3) hematopoietic stem cell transplantation-associated TMA; 4) presence of an additional or alternative disease that might have caused the presenting disorder; 5) idiopathic TTP either at initial presentation or at relapse; 6) HUS not further specified by the referring clinician; 7) typical HUS with (bloody) diarrhea prodrome (D’HUS); and 8) atypical HUS, i.e. acute HUS with atypical presentation (no enterocolitis prodrome) and/or exclusion of infection with verotoxin-producing bacteria. A total of 3 patients were re-classified by the investigators (2 patients diagnosed by the referring clinicians with HUS within two months of hematopoietic stem cell transplantation were allocated to the subcategory “hematopoietic stem cell transplantation-associated TMA” and one patient with a clinical diagnosis of acute TTP who suffered from disseminated cancer was reclassified to have “neoplasia- or chemotherapy-associated TMA”).

From the study cohort of 295 patients, another 39 had to be excluded for the following reasons: There was no plasma left from a sample drawn before initiation of plasma therapy (n=21); referral occurred twice (n=8); non-Caucasians (n=3); APC-sensitivity ratio in plasma was not conclusive and genotyping failed (n=2); or a factor V phenotype-genotype mismatch was conceivable (n=4; one patient after liver transplantation, 3 after hematopoietic stem cell transplantation); and because of missing safety installations for handling plasma of a patient suffering from Hanta virus infection (n=1).

Eight of the remaining 256 Caucasian patients were referred from local hospitals while all others were treated at tertiary care centers. Patients’ countries of origin were Austria (n=4), Belgium (1), Croatia (7), Czech Republic (4), Denmark (3), France (1), Germany (168), Hungary (1), the Netherlands (1), Norway (5), Portugal (1), Slovakia (2), Sweden (2), Switzerland (53), Turkey (1) and the United States (2). Plasma samples were withdrawn at presentation before initiation of any form of plasma therapy and shipped frozen to our laboratory. After ADAMTS13 activity determination, samples were refrozen and stored at -80°C until further use.

The study was approved by the responsible ethics committee (Kantonale Ethische Kommission, Bern, Switzerland).

Assay of ADAMTS13 activity
ADAMTS13 activity was determined using a quantitative immunoblotting assay as previously described (4, 5). ADAMTS13 activity was read from the immunoblots by two independent investigators without knowledge of the clinical diagnosis. Five arbitrary categories of ADAMTS13 activity were made: <5%, severe deficiency: 5–9%, borderline severe deficiency: 10–25%, moderate deficiency: 26–50%, mild or minimal deficiency; and >50%, normal (8, 10).

Factor V clotting activity, modified APC-resistance and factor V genotype analysis
Factor V clotting activity (FV:C) was measured by a prothrombin time-based assay using Innovin® and factor V deficient substrate plasma and expressed as percentage of a standard human plasma pool (all Dade Behring, Marburg, Germany).

The FVL status was determined by the modified APC resistance test (Coastest® APC Resistance V, Chromogenix, Mölndal, Sweden) having a specificity and sensitivity of 100% for the FVL genotype, which is achieved by the predilution (1:5) of the patient’s plasma in factor V deficient plasma (16). It should be kept in mind, that acquired APC resistance, e.g. due to increased FVIII clotting activity, is not assessed by this test. FVII status was established based on in-house APC sensitivity ratio cut-off values: non-carriers ≥ 2.2 and heterozygous carriers between ≥ 1.50 and ≤ 1.90, provided the base-line aPTT (aPTT of patient’s plasma prediluted in factor V deficient plasma without addition of APC) was ≤ 42s and FV:C in patient’s plasma was ≥ 50%.

The FVL status was considered doubtful when an equivocal APC sensitivity ratio <1.5, or between > 1.9 and < 2.2 was present and whenever the sample failed the quality control criteria (base-line aPTT >42s and FV:C <50%). Prior to this study, these cut-off values had been validated by the investigation of the FVL status in 485 consecutive samples of thrombosis patients, as well as in 89 healthy controls (17), confirming a sensitivity and specificity of the modified APC resistance test of 100% each as was evidenced by concurrent genotyping.

In patients with a doubtful FVL status (65/256 patients) DNA was extracted from patient’s plasma using QIAamp Blood Mini Kit (Qiagen, Basel, Switzerland) according to manufacturer’s instructions. Whenever possible 400 µl of plasma were processed.
Genotyping was performed using the LightCycler Factor V Leiden Mutation Detection Kit (Roche, Basel, Switzerland).

**Statistical analysis**

Allele frequencies were calculated by gene counting and compared by χ² analysis or Fisher's exact test whenever an expected value was < 5. The statistical analysis was performed using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL). A p value of < 0.05 was considered statistically significant.

**Results**

Allocation to one of the eight clinical categories of acute TMA was based on the diagnosis established by the referring clinicians (Table 1). Severe ADAMTS13 deficiency was found in 23% (59/256) of patients investigated, among them 49 patients diagnosed with idiopathic TTP (40 at first presentation and 9 at relapse) while none of the 122 patients diagnosed with HUS had an ADAMTS13 activity <5%.

Table 2: Prevalence of the factor V Leiden mutation in relation to ADAMTS13 activity and clinical category of thrombotic microangiopathy (TMA).

<table>
<thead>
<tr>
<th>Clinical Category</th>
<th>ADAMTS13 activity</th>
<th>ADAMTS13 activity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5%</td>
<td>≥5%</td>
</tr>
<tr>
<td>All TMA†</td>
<td>6.8%</td>
<td>8.6%</td>
</tr>
<tr>
<td>Idiopathic TTP</td>
<td>6.1%</td>
<td>6.3%</td>
</tr>
<tr>
<td>HUS‡</td>
<td>0%</td>
<td>0%</td>
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† Including all TMA categories (categories 1–8 from Table 1).
‡ Including categories of hemorrhagic uremic syndrome (HUS) not specified, HUS with diarrhea proctodeum and atypical HUS (categories 6, 7 and 8 from Table 1).

The FVL status was determined by the modified APC resistance test alone in 191/256 (74.6%) of patients. The remaining 65 (25.4%) patients were genotyped because of an equivocal modified APC sensitivity ratio (n = 11 patients), base-line aPTT > 42s (n = 22), FV:C < 50% (n = 6) or combinations thereof (n = 26). Twenty-one (8.2%) patients were identified to be FVL carriers, a prevalence that is in line with that reported for the FVL mutation throughout Europe (range 2.8–14% (18)), especially, however, with that for Germany (4–9% (18–20)) and Switzerland (4–7% (17, 21)), these two countries referring 86.3% of all TMA patients (168 [65.6%] and 53 [20.7%] patients, respectively). All patients were heterozygous except for one homozygous FVL carrier with neoplasia-associated TMA and severe ADAMTS13 deficiency. The genotype distribution was in Hardy-Weinberg equilibrium for the whole group of patients as well as for the subcategories TTP and HUS.

**Discussion**

We studied ADAMTS13 activity and the presence of the FV Leiden mutation in a large group of patients suffering from various forms of acute TMA. Among all patients with an ADAMTS13 activity <5%, 6.8% carried the FVL mutation and among those without severe ADAMTS13 deficiency 8.6%, a difference which was not statistically significant (Table 2). A slightly higher FVL prevalence of 9.9% was observed among patients with ADAMTS13 activity <10% (severe and borderline severe deficiency taken together) compared to 7.6% in patients with ADAMTS13 activity of ≥10% (not significant). Exclusion of patients with intermediate ADAMTS13 activity (between 5–50% or 10–50%, respectively) that is, comparison of patients with severe (and borderline severe) ADAMTS13 deficiency and those with normal ADAMTS13 activity, as was done by Raife et al. (13) did not reveal a significantly different FVL prevalence.
Thus, FVL is not more prevalent in TMA patients without as compared to those with severe ADAMTS13 deficiency. To avoid small sample sizes leading to statistical instability of the results, we restricted sub-group analysis to the clinical categories of idiopathic TTP and HUS, the latter comprising all three HUS sub-categories. While 62.5% of patients with idiopathic TTP were carriers of the FVL mutation, the prevalence of FVL among all HUS patients was slightly higher (9%), a difference that was, however, not statistically significant.

There are limitations to this study. Many centers and physicians have been involved in diagnosing acute TMA and therefore uniformity in clinical assessment was not granted despite given diagnostic criteria. Only a quarter of the patients were genotyped for diagnosing or excluding FV Leiden carrier-ship. Given the sensitivity and specificity of 100% of the modified APC resistance test for the FV Leiden genotype it is unlikely that the study results were affected by this diagnostic strategy. Although the study was set out to compare the number of FVL carriers among patients suffering from acute TMA with and without severe ADAMTS13 deficiency, the main limitation of our study is the absence of a control group of healthy individuals. Given the fact that the 256 patients investigated stem from different countries and areas of Europe with a wide range of reported prevalences of FVL, such a control group should have consisted of healthy individuals recruited at the place of origin of each patient. These controls were not available and a comparison with our own laboratory control group recruited in Switzerland (6.7% of FVL carriers (17)) was considered inappropriate. Therefore, we can not exclude that the FVL prevalence in certain diagnostic groups and/or ADAMTS13 activity categories is slightly higher than in the general local population. In this context it is noteworthy that 3 out of 28 (10.7%) adults with a diagnosis of HUS and 10 out of 81 (12.3%) HUS patients with normal ADAMTS13 activity (> 50%) were carriers of the FVL mutation. This is of special interest since FVL is associated with a procoagulant state and increased thrombin formation (22), which was recently reported to be present in verotoxin-induced HUS (23, 24). Furthermore, a possible role of FVL would also be in line with the pathological anatomical finding of fibrin rich and platelet poor thrombi reported in HUS (25).

To conclude, our data suggest that FVL is not a major risk factor for acute TMA, even though we cannot exclude a contributory role in certain subgroups of patients and further studies on the role of FVL in well defined groups of patients with HUS are warranted.

References