Prospective Evaluation of the Risk Conferred by Factor V Leiden and Thermolabile Methyleneetrahydrofolate Reductase Polymorphisms in Pregnancy

Ronan P. Murphy, Catherine Donoghue, Ruth J. Nallen, Melwyn D’Mello, Carmen Regan, Alexander S. Whitehead, Desmond J. Fitzgerald

Abstract—Factor V (FV) Leiden and thermolabile methylenetetrahydrofolate reductase (MTHFR) are 2 common polymorphisms that have been implicated in vascular thrombosis. We determined whether these mutations predicted an adverse outcome in pregnancy. Second, we looked for an interaction between these 2 mutations in patients with recurrent fetal loss or thrombosis in pregnancy. Primigravid subjects at their booking visit to the National Maternity Hospital (Holles Street, Dublin, Ireland) were screened for the polymorphisms. Thermolabile MTHFR and FV Leiden genotypes were detected by either restriction fragment length polymorphism or heteroduplex capillary chromatography. The carrier frequency of FV Leiden in the screened primigravid population was 2.7% (allele frequency 1.36%), all being heterozygous for the mutation. This value was lower than expected from previous studies in European populations. Forty-nine percent of the screened population (289 of 584) were heterozygous for thermolabile MTHFR, and 10.6% were homozygous (62 of 584). The frequency of the 2 polymorphisms was no higher in those who subsequently developed preeclampsia (n = 12) or intrauterine growth retardation (n = 9), and none of the screened population developed thrombosis. However, the frequency of FV Leiden was higher in patients who subsequently miscarried after the first trimester of pregnancy (allele frequency of 5.5%, P = 0.0356). Among those positive for FV Leiden, 3 of 27 miscarried, compared with 24 of 572 of FV Leiden–negative patients (11% versus 4.2%). No interaction was found between the 2 mutations in the control or patient populations. In patients with a prior history of venous thrombosis, the carrier rate of FV Leiden was increased (4 of 33, allele frequency of 7.6%, P = 0.0115). In contrast, the carrier frequency for thermolabile MTHFR was no higher, and there was no interaction between the 2 mutations. Neither mutation occurred at a significantly higher frequency in patients with a prior history of recurrent fetal loss. In conclusion, FV Leiden is a risk factor for thrombosis in pregnancy and possibly for second-trimester miscarriage independent of thermolabile MTHFR. However, prospective analysis suggests that the risk conferred by FV Leiden is low in a primigravid population. The thermolabile MTHFR genotype was not implicated in any adverse outcome. (Arterioscler Thromb Vasc Biol. 2000;20:266-270.)

Key Words: factor V Leiden polymorphism ■ thermolabile methylenetetrahydrofolate reductase polymorphism ■ pregnancy ■ venous thrombosis ■ recurrent fetal loss ■ genetic risk factors

Functional polymorphisms in genes controlling hemostasis contribute to an increased risk of thrombotic events. However, the penetrance of any single mutation is highly variable, even in families with a history of thrombosis. One explanation for the variable penetrance is that combinations of polymorphisms in several genes may act synergistically to increase the risk of thrombosis.

Factor V (FV) Leiden and the thermolabile (T) methyleneetrahydrofolate reductase (MTHFR) polymorphisms have been implicated as risk factors for thrombosis.1-3 The FV Leiden variant arises as a result of a point mutation at nucleotide position 1691, resulting in an arginine to a glutamine substitution at position 5064 that reduces its sensitivity to inactivation by activated protein C. FV Leiden has been associated with familial thrombophilia5 and indeed, is the commonest inherited risk factor for venous thrombosis. Compared with those without the mutation, heterozygous carriers have a 7-fold increased risk of venous thrombosis,6,7 and homozygous individuals have a risk that is increased up to 100-fold.8

MTHFR is critical in the metabolism of homocysteine, as it effects the NADPH-linked reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. A C-to-T missense mutation at nucleotide 677 results in an enzyme that is
thermolabile and exhibits reduced activity compared with the wild type. TT MTHFR homozygotes are predisposed to increased plasma homocysteine levels, particularly in individuals with low folate.8,10 Hyperhomocysteinemia has been implicated in premature vascular disease11 and more recently, in venous thrombosis12 and unexplained early pregnancy loss.13

Although it has been suggested that FV Leiden increases the risk of thrombotic events in pregnancy, the carrier frequency of this mutation is 4% to 15%,6,14 far in excess of the risk of thrombosis (1 to 2 per 1000). Even in a family with the mutation and a history of thrombosis, the penetrance is highly variable, suggesting that other factors are involved.15 Because both of these mutations are common, we asked whether the presence of T MTHFR increased the risk of FV Leiden. An interaction has been shown between homocysteine and FV Leiden mutation was performed as follows. Genomic DNA (500 ng) and 25 pmol of sense and antisense primers (sense primer FV1-TGC GGA AAT GCC CCA TTA, to prime for fragment 1 [F1]; or FV2B-AAG GAC AAA AGT ACC TGT ATT CCA, to prime for F2) were used in a reaction containing 2 U of Taq polymerase enzyme (Promega) and MgCl₂ at a concentration of 1.5 mmol/L in a final volume of 50 μL. The PCR program was as follows: initial denaturation of 95°C for 5 minutes, followed by 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute in a 35-cycle reaction. Dimethyl sulfoxide was added to a concentration of 3.5% in the PCR for F2 to optimize amplification. PCR amplification for the region containing the MTHFR mutation was as follows: 20 pmol of both sense primer (5'-TGA AGG AGA AGG TGT CTG CGG GA-3'; exonic sequence) and antisense primer (5'-AGG ACG ATG CCG TGA GAG AGT G-3'; intronic sequence) were used in a reaction volume of 50 μL containing 200 μmol/L dNTPs, 1.5 mmol/L MgCl₂, 50 mmol/L Tris-HCl (pH 9), 50 mmol/L KCl, 2 U of Taq polymerase (Promega). 200 ng of genomic DNA, and 1% Triton X-100 (for MTHFR PCR only). The PCR cycling conditions were 94°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute for 40 cycles. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

The restriction fragment length polymorphism analysis for the FV Leiden mutation was carried out as described previously by using both restriction enzymes MnlI and NlaIII (New England Biolabs).14,18 A similar protocol was used to analyze the MTHFR T mutant by using the restriction enzyme HinfI (New England Biolabs).19 The FV Leiden mutation is associated with the loss of an MnlI site in F1. This results in a band shift from 118 to 155 bp on visualization of the electrophoresed product. Conversely, the mutation combined with the sequence of primer 2B used to amplify product F2 results in the acquisition of a novel NlaIII site, resulting in a shift of 91 to 67 bp. Amplification with the MTHFR-specific primers yields a 198-bp product. On restriction analysis with HinfI enzyme and in the presence of the mutant T allele, the PCR product is cleaved, giving rise to a 175- and a 23-bp fragment. In the absence of the mutation, no cleavage is observed.

FV analysis was also carried out using heteroduplex analysis and capillary electrophoresis on a Perkin-Elmer ABI PRISM 310 automated sequencer.20 The primers were as follows:21 sense 5'-CAT GAG AGA CAT CCG CTC TG-3' and antisense 5'-GAC CTA ACA TGT TCT AGC CAG AAG-3' (labeled with a HEX fluor). The heteroduplex generator probe was a kind gift from Dr Derrick Bowen, Hemostasis Research Laboratory, The Arthur Bloom Center, Cardiff, Wales. A nondenaturing matrix (native 3% polymer) and a 37-cm capillary were used for electrophoresis. Analysis was carried out using GENESCAN software. All reagents and software were supplied by Perkin-Elmer.

### Statistical Analysis

The Pearson χ² test probability was computed by using STATEXACT 3 software for exact nonparametric inference.21

### Results

Of the primigravid subjects asked to participate in the study, 94% agreed. The mean age (±SEM) at the time of presentation was 25±0.2 years, and the mean gestational age was 14.2±0.26 weeks. The allele frequencies of the 2 polymorphisms in the 3 study groups are shown in Table 1. The allelic frequency of FV Leiden was lower than expected, at 1.36% (a carrier rate of 2.7%), all of the carriers being heterozygotes. The allelic frequency for T MTHFR was similar to that previously reported in Western populations.

### DNA Analysis

Total genomic DNA was extracted from whole human blood by a salting-out procedure.17 Care was taken to avoid contamination, and a sterile-water blank was taken through each batch of isolations and used as a polymerase chain reaction (PCR) control. PCR amplification of human genomic DNA for the region containing the FV Leiden mutation was performed as follows: Genomic DNA (500 ng) and 25 pmol of sense and antisense primers (sense primer FV1-TGC CCA GTG CTT AAC AGA CCA; antisense primer FV2A-TCT CTT

### Methods

#### Study Groups

The research protocol was approved by the Ethics Committee at the National Maternity Hospital, Holles Street, Dublin, Ireland, and all participants gave written, informed consent. All consecutive primigravid subjects attending 2 specific booking clinics over a 4-month period were asked to participate. Patients with a history of hypertension or thrombosis were excluded. A venous blood sample was drawn into EDTA for genetic analysis on their first clinic visit. All parameters tested in the primigravid group.

In addition, blood was obtained from patients with a prior history of venous thrombosis and/or pulmonary embolism during pregnancy, the diagnosis of which was based on peripheral Doppler studies and/or a ventilation/perfusion lung scan. All of the patients underwent a thrombophilia screen, and all parameters tested were normal.

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### Table 1. Allele Frequencies of FV Leiden and T MTHFR in the Groups Studied

<table>
<thead>
<tr>
<th>Group</th>
<th>FV Leiden</th>
<th>RFL (%)</th>
<th>RT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primigravida</td>
<td>35.36%</td>
<td>31.25%</td>
<td>23.33%</td>
</tr>
<tr>
<td>RFL</td>
<td>1.36%</td>
<td>2.44%</td>
<td>7.58%</td>
</tr>
</tbody>
</table>

RFL indicates recurrent fetal loss; RT, recurrent thrombosis.

GAA GGA AAT GCC CCA TTA, to prime for fragment 1 [F1]; or FV2B-AAG GAC AAA AGT ACC TGT ATT CCA, to prime for F2 were used in a reaction containing 2 U of Taq polymerase enzyme (Promega) and MgCl₂ at a concentration of 1.5 mmol/L in a final volume of 50 μL. The PCR program was as follows: initial denaturation of 95°C for 5 minutes, followed by 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute in a 35-cycle reaction. Dimethyl sulfoxide was added to a concentration of 3.5% in the PCR for F2 to optimize amplification. PCR amplification for the region containing the MTHFR mutation was as follows: 20 pmol of both sense primer (5'-TGA AGG AGA AGG TGT CTG CGG GA-3'; exonic sequence) and antisense primer (5'-AGG ACG ATG CCG TGA GAG AGT G-3'; intronic sequence) were used in a reaction volume of 50 μL containing 200 μmol/L dNTPs, 1.5 mmol/L MgCl₂, 50 mmol/L Tris-HCl (pH 9), 50 mmol/L KCl, 2 U of Taq polymerase (Promega). 200 ng of genomic DNA, and 1% Triton X-100 (for MTHFR PCR only). The PCR cycling conditions were 94°C for 1 minute, 63°C for 1 minute, and 72°C for 1 minute for 40 cycles. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

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The relationship between mutations and adverse outcomes in this prospective analysis of 593 primigravidas is shown in Tables 2 and 3. There was no correlation between MTHFR thermolabile homozygosity and the various outcomes ($\chi^2=4.347$, $P=0.0356$). However, the presence of FV Leiden was shown to be significantly more frequent in those who subsequently miscarried ($\chi^2=7.104$, $P=0.0356$). Among those positive for FV Leiden, 3 of 27 miscarried compared with 24 of 572 of FV Leiden–negative patients (11% versus 4.2%). We used the same statistical model to determine whether there was any association between TT MTHFR and multiple pregnancy-related conditions, including thrombosis, fetal loss, preeclampsia, and retinal arterial occlusion.30

We also retrospectively studied patients with recurrent fetal loss (n=41, 32±0.74 years) or a history of thrombosis in pregnancy (n=33, 29±0.97 years). The genotype distribution was compared with the prospectively studied patients. The Pearson $\chi^2$ analysis of the genotypes in these groups is shown in Tables 4 and 5. There was a statistically significant association between FV Leiden and recurrent thrombosis (Table 4, $\chi^2=12.04$, $P=0.0115$) but not recurrent fetal loss. There was no significant difference in the distribution of the T MTHFR allele between normal primigravid subjects and the groups characterized by thrombosis in pregnancy and recurrent fetal loss. Moreover, there was no interaction between the 2 polymorphisms in these groups.

**TABLE 2. Distribution of FV Leiden Genotypes and Outcomes for the Control Group of Primigravid Subjects (n=588)**

<table>
<thead>
<tr>
<th>FV</th>
<th>Normal</th>
<th>PET</th>
<th>Miscarriage</th>
<th>FGR</th>
<th>Thrombosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/−</td>
<td>13</td>
<td>…</td>
<td>3</td>
<td>…</td>
<td>…</td>
<td>16</td>
</tr>
<tr>
<td>+/+</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>…</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>−/−</td>
<td>527</td>
<td>12</td>
<td>24</td>
<td>9</td>
<td>…</td>
<td>572</td>
</tr>
<tr>
<td>Total</td>
<td>540</td>
<td>12</td>
<td>27</td>
<td>9</td>
<td>…</td>
<td>588</td>
</tr>
</tbody>
</table>

$\chi^2$ 0.2959 7.104 0.2219 …

$P$ 1.000 0.0356 1.000 …

PET indicates preeclamptic toxemia; FGR, fetal growth retardation; and +/−, +/+ indicate heterozygous and homozygous for FV Leiden, respectively. Five cases were excluded owing to incomplete data.

**TABLE 3. Distribution of MTHFR Genotypes and Outcomes for the Control Group of Primigravidas (n=584)**

<table>
<thead>
<tr>
<th>MTHFR</th>
<th>Normal</th>
<th>PET</th>
<th>Miscarriage</th>
<th>FGR</th>
<th>Thrombosis</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/−</td>
<td>270</td>
<td>3</td>
<td>11</td>
<td>5</td>
<td>…</td>
<td>289</td>
</tr>
<tr>
<td>+/+</td>
<td>56</td>
<td>3</td>
<td>4</td>
<td>…</td>
<td>…</td>
<td>62</td>
</tr>
<tr>
<td>−/−</td>
<td>214</td>
<td>5</td>
<td>9</td>
<td>4</td>
<td>…</td>
<td>233</td>
</tr>
<tr>
<td>Total</td>
<td>540</td>
<td>11</td>
<td>24</td>
<td>9</td>
<td>…</td>
<td>584</td>
</tr>
</tbody>
</table>

$\chi^2$ 4.092 0.9627 1.040 …

$P$ 0.1334 0.6877 0.7947 …

PET indicates preeclamptic toxemia; FGR, fetal growth retardation; and +/−, +/+ indicate heterozygous and homozygous for T MTHFR, respectively. Nine cases were excluded owing to incomplete data.

**TABLE 4. Genotype Frequencies of FV Leiden Among Both the Thrombotic Group and the Recurrent Fetal Loss Group Compared With the Normal Outcomes of the Primigravid Control Group**

<table>
<thead>
<tr>
<th>Factor V</th>
<th>Thrombosis</th>
<th>Recurrent Fetal Loss</th>
<th>Control Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/−</td>
<td>3</td>
<td>2</td>
<td>13 (2.4%)</td>
</tr>
<tr>
<td>+/+</td>
<td>1</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>−/−</td>
<td>29</td>
<td>39</td>
<td>527 (97.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>41</td>
<td>540</td>
</tr>
</tbody>
</table>

$\chi^2$ 12.04 0.8334 …

$P$ 0.0115 0.6452 …

+/−, +/+ indicate heterozygous and homozygous for FV Leiden, respectively.

**TABLE 5. Genotype Frequencies of T MTHFR Among Both the Thrombosis Group and the Recurrent Fetal Loss Group Compared With the Normal Outcomes of the Primigravid Control Group (n=540)**

<table>
<thead>
<tr>
<th>MTHFR</th>
<th>Thrombosis</th>
<th>Recurrent Fetal Loss</th>
<th>Control Population</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/−</td>
<td>5</td>
<td>19</td>
<td>270 (50%)</td>
</tr>
<tr>
<td>+/+</td>
<td>1</td>
<td>3</td>
<td>56 (10.4%)</td>
</tr>
<tr>
<td>−/−</td>
<td>9</td>
<td>18</td>
<td>214 (39.6%)</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>40</td>
<td>540</td>
</tr>
</tbody>
</table>

$\chi^2$ 2.52 0.6169 …

$P$ 0.2771 0.7014 …

+/−, +/+ indicate heterozygous and homozygous for T MTHFR, respectively.
other case-control and cohort studies that have implicated FV Leiden in second-trimester but not in first-trimester miscarriages. A possible explanation for this fact is that recurrent, first-trimester miscarriage reflects a failure in implantation, and second-trimester miscarriage reflects a thrombotic event in the placenta. There were no thrombotic events in the screened population, although this was not unexpected, as the frequency of thrombosis is 1 to 2 in 1000 pregnancies.

The MTHFR TT genotype did not influence the rate of late miscarriage, and importantly, no interaction was found between these 2 mutations in determining the risk of events. However, homocysteine levels were not measured in our study groups, and because folate supplementation during pregnancy is common, the underlying effect of the MTHFR TT genotype may have been masked. Our findings suggest that widespread screening for these mutations in unselected populations is unlikely to be clinically useful.

We also examined the allelic frequency of these mutations in 2 groups of patients, 1 with a history of thromboembolic disease in pregnancy and a second with recurrent fetal loss. The cause of recurrent fetal loss is largely unknown. However, in some cases, there is an underlying prothrombotic disorder, such as the presence of lupus anticoagulant, and recently, mutations in the thrombin-binding domain of thrombomodulin result in recurrent fetal loss in mice. The frequency of FV Leiden in patients with a history of thrombosis was higher than expected, consistent with previous reports. However, the frequency of FV Leiden was only marginally higher in patients with recurrent fetal loss. Moreover, there was no association between T MTHFR and these 2 disorders, and, as in the control population, no linkage was found between the 2 mutations in these patient groups. Therefore, the presence of T MTHFR did not increase the risk attributable to FV Leiden.

It is difficult to explain why there was an association between FV Leiden and late miscarriage, yet we could not show a significant association with recurrent fetal loss. The numbers of subjects were relatively small, and this may be a factor. It is also possible that FV Leiden alone is insufficient to cause fetal loss. During the course of the study, we identified 2 sisters who were heterozygous for FV Leiden and who had experienced recurrent fetal loss, with 4 losses in 1 case and 6 in the second. Both also were positive for lupus anticoagulant and antiphospholipid antibodies. These findings were in agreement with recent findings that FV Leiden may contribute to the hypercoagulability of some subjects with antiphospholipid antibodies. In the population reported, we screened all cases for other prothrombotic factors, and in all cases these factors were absent. However, differences in genetic background may explain variations in risk of FV Leiden between studies.

In conclusion, FV Leiden occurred more frequently in patients with prior thromboembolic disease but not in patients with recurrent fetal loss. No association between these events and T MTHFR was found, and there was no interaction between the mutations in these patient populations. Prospective identification of FV Leiden and T MTHFR did not predict adverse outcomes, other than a weak association between miscarriage and FV Leiden. Thus, widespread screening of the pregnant population for either of these mutations is unlikely to be fruitful in identifying patients at risk of adverse pregnancy outcomes.

References

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