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Abstract

Objective. There is growing evidence that hypertensive pregnancy complications and other adverse pregnancy outcomes are associated with the presence of inherited or acquired thrombophilias. As hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome is one of the most severe forms of pre-eclampsia we aimed to assess the prevalence of the factor V Leiden, the prothrombin 20210G >A mutation and the methylenetetrahydrofolate reductase (MTHFR) 677C >T polymorphism in women with HELLP syndrome and in their fetuses from the same index pregnancy. Design. The study was performed retrospectively in a case-control design. Sample. Seventy-one mother-child pairs with HELLP syndrome and 79 control mother-child pairs with uncomplicated pregnancies were included in the study. Methods. Genotyping of the three thrombophilic mutations was performed using the LightCycler technology. The chi-squared test was used for statistical analysis. Main outcome measures were maternal and fetal genotypes and their correlation with clinical parameters. Results. Maternal heterozygosity for factor V Leiden was significantly more prevalent in the HELLP group than in controls (OR 4.45, 95% CI 1.31-15.31). No significant association was observed for maternal prothrombin mutation or MTHFR polymorphism (p=0.894, p=0.189, respectively). The fetal genotype was not associated with HELLP syndrome for any of the three mutations investigated. Analysis of gene-gene interactions and genotype-phenotype correlation with respect to clinical parameters and perinatal outcome revealed no further differences. Conclusions. Our study confirms that women heterozygous for factor V Leiden have an increased risk of developing HELLP syndrome, while the most frequent mutations of the prothrombin and MTHFR gene do not play a major role in the pathogenesis of HELLP syndrome.
Maternal factor V Leiden mutation is associated with HELLP syndrome in Caucasian women

Running title: Factor V Leiden is associated with HELLP

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Abstract

Objective: There is growing evidence that hypertensive pregnancy complications and other adverse pregnancy outcomes are associated with the presence of inherited or acquired thrombophilias. As HELLP syndrome is one of the most severe forms of pre-eclampsia we aimed to assess the prevalence of the factor V Leiden, the prothrombin 20210G>A mutation and the MTHFR 677C>T polymorphism in women with HELLP syndrome and in their fetuses from the same index pregnancy.

Design: The study was performed retrospectively in a cas-control design. Sample: 71 mother-child pairs with HELLP syndrome and 79 control mother-child pairs with uncomplicated pregnancies were included in the study. Methods: Genotyping of the three thrombophilic mutations was performed using the LightCycler technology. The chi-squared test was used for statistical analysis. Main outcome measures: Maternal and fetal genotypes and their correlation with clinical parameters.

Results: Maternal heterozygosity for factor V Leiden was significantly more prevalent in the HELLP group than in controls (OR 4.45, 95%CI 1.31-15.31). No significant association was observed for maternal prothrombin mutation or MTHFR polymorphism ($p=0.894$, $p=0.189$ resp.). The fetal genotype was not associated with HELLP syndrome for any of the three mutations investigated. Analysis of gene-gene interactions and genotype-phenotype correlation with respect to clinical parameters and perinatal outcome revealed no further differences.

Conclusions: Our study confirms that women heterozygous for factor V Leiden have an increased risk of developing HELLP syndrome, while the most frequent mutations of the prothrombin and MTHFR gene do not play a major role in the pathogenesis of HELLP syndrome.
Key words

Factor V Leiden; HELLP syndrome; MTHFR; Prothrombin; Thrombophilia
Introduction

HELLP (hemolysis, elevated liver enzymes, low platelets) syndrome is a life-threatening form of pre-eclampsia, complicating about 0.17 - 0.8% of all live births (1). Diagnosis of HELLP is based on the evidence of intravascular hemolysis, impaired liver function and thrombocytopenia. Approximately 15% of women with pre-eclampsia/eclampsia show the complete spectrum of HELLP syndrome and thus have an increased risk for significant perinatal morbidity and mortality, both maternal and fetal (2).

There is growing evidence that hypertensive pregnancy complications and other adverse pregnancy outcomes are associated with the presence of inherited or acquired thrombophilias (3;4). However, the results of single studies remain controversial. Most of the inherited thrombophilias are found in the protein C system and affect the physiological balance between pro- and anticoagulant factors (5). Inherited resistance to activated protein C (APC) is known as risk factor for thrombosis. A single point mutation in the gene for factor V (G to A transition at nucleotide position 1691, factor V Leiden mutation), which predicts replacement of Arg(R)506 in the APC-cleavage site with a Gln(Q) and results in an impaired degradation of factor V, accounts for more than 90% of the cases of APC-resistance (6). The prevalence of factor V Leiden mutation in the normal population ranges from 3 to 7% in Europe and the USA (7).

The prothrombin gene mutation (20210G>A) is a second common genetic risk factor for thrombosis, leading to an increased plasma concentration of prothrombin with a subsequently higher rate of fibrin formation (8). In Europe the carrier frequency of the prothrombin mutation is reported to be 1-4% with a nearly twofold higher prevalence in Southern than in Northern Europe (9).

Homozygosity for the polymorphism 677C>T in the methylenetetrahydrofolate reductase (MTHFR) gene is associated with hyperhomocysteinemia and may thus increase the risk for vascular disorders (10). The MTHFR 677C>T polymorphism can be found in homozygous
state in about 14% of the general Caucasian population with higher carrier rates in Southern compared to Northern Europe (11).

Over the last decade more than 70 studies including several meta-analyses have investigated the prevalence of thrombophilic gene mutations in pre-eclampsia, yielding contradictory results [for review see (4;12)]. Only 14 studies have addressed the association between these mutations and the development of HELLP syndrome, also reporting conflicting data (13-26). Most of these studies have included a small number of HELLP patients and have not assessed the fetal genotype. Furthermore, data from HELLP patients are often included in the results for pre-eclampsia patients.

In this study we aimed to assess the prevalence of the most common inherited thrombophilias factor V Leiden, prothrombin 20210G>A and MTHFR 677C>T in a well-defined cohort of Caucasian women with a history of HELLP syndrome compared to healthy female controls. As vascular features of the placenta are thought to be involved in the pathogenesis of pre-eclampsia, and as the placenta is of fetal origin (27;28), fetuses from the index pregnancies were also genotyped for the three common thrombophilic mutations in order to determine whether a paternal contribution is of central importance.

**Materials and Methods**

**Study cohort**

71 women with HELLP syndrome and their children from the index pregnancy were included in our study. Patients were recruited at the Department of Obstetrics and Gynecology, Aachen University Hospital, and nationwide by the German pre-eclampsia society. 79 healthy mother-child-pairs with uncomplicated pregnancies who delivered at Aachen Univeristy Hospital or obstetric departments in the surrounding area were enrolled as controls. All subjects were of white Caucasian origin. The diagnosis of HELLP syndrome was based on the presence of hemolytic anemia (serum haptoglobin levels < 0.3 g/L or serum lactate dehydrogenase (LDH)
> 300 IU/L), elevated liver enzymes (elevation of serum aspartate aminotransferase (AST) or serum alanine aminotransferase (ALT) over norm), and evidence of thrombocytopenia, defined as a platelet count of < 100.000/µl. The study was approved by the Institutional Ethics Committee and informed consent was obtained from all subjects.

**DNA studies**

Genomic DNA was isolated from blood leukocytes as described by Miller et al. (29). Analysis of the defined point mutations of factor V, prothrombin and MTHFR was performed using the LightCycler technology (Roche Diagnostics, Mannheim, Germany). For analysis of the corresponding sites in human factor V (1691G>A) and human prothrombin (20210G>A) commercially available kits (LightCycler-Factor V Leiden mutation detection kit, LightCycler-Prothrombin (20210G>A) mutation detection kit/ Roche, Mannheim, Germany) were used. MTHFR 677C>T genotyping was performed as described by von Ahsen et al. (30) with slight modifications. In brief, a 198 bp fragment containing the mutation site was amplified with 0.1 µM primer MTHFR-rev [5´-d(AGG ACG GTG CGG TGA GAG TG)-3´] and 0.5 µM primer MTHFR-for [5´-d(TGA AGG AGA AGG TGT CT*G CGG GA)-3´] carrying a LC-Red640 group at T*. PCR reaction was performed in a 1x “LC™-DNA Master Hybridization Probes” including additional 1.5 mM MgCl₂ and 0.1 µM primer MTHFR-probe [5´-d(AGC TGC GTG ATG ATG AAA TCG GCT CC-Fluorescein)-3´]. Samples were initially denaturated for 30 sec at 95°C (ramp rate 20°C/sec) and amplified for 55 cycles of 0 sec at 94°C (ramp rate 20°C/sec), 5 sec at 50°C (ramp rate 20°C/sec), and 5 sec at 72°C (ramp rate 20°C/sec). The melting curve analysis was performed in 1 cycle of 95°C for 0 sec and 40°C for 1 min, each with a temperature transition rate of 20°C/sec, and then ramping to 80°C with a transition rate of 0.1°C/sec and continuous fluorescence measurement. Each run included DNA controls of all genotypes (verified by sequencing) and a water control. All
primers were obtained from Metabion (Martinsried, Germany) or TipMOL (Berlin, Germany).

Statistical analysis

Results for the two groups were compared by using the $\chi^2$-test. $p$-values $< 0.05$ were regarded as statistically significant. Statistical analyses were performed with SPSS for Windows®.

Results

Clinical characteristics of the study groups are shown in Table 1. 89% of HELLP patients presented with signs of pre-eclampsia according to ISSHP criteria (blood pressure $\geq 140/90$ mmHg after 20th week of gestation on at least two independent occasions accompanied by proteinuria $\geq 0.3$ g/24 h or $\geq 1$ g/l ($\geq 2+$ on urinary dipstick)). The number of primiparous women was higher in the HELLP group (88.7% vs. 65.8%). Furthermore, cases and controls differed significantly with respect to blood pressure, gestational age at delivery and birth weight of the infants.

Maternal genotype distribution for the mutations in the prothrombin and factor V gene and the polymorphism in the MTHFR gene is presented in Table 2. Heterozygosity for factor V 1691G>A mutation was more prevalent in HELLP patients than in controls (16.9% vs. 3.8%, OR 4.45, 95%CI 1.31-15.13, $p$=0.006). None of the patients or controls had a homozygous mutation of factor V Leiden.

No differences were observed in maternal genotype frequencies of prothrombin 20210G>A mutation ($p$=0.894) or MTHFR 677C>T polymorphism ($p$=0.189).

Fetal blood samples were obtained from all of the 71 HELLP pregnancies and from 78 of the control pregnancies. For technical reasons genotyping was not possible in all samples. Fetal genotypes were not associated with maternal HELLP syndrome for any of the three mutations
investigated (Table 3), thus excluding a relevant paternal contribution to the development of the disease.

In order to evaluate the influence of thrombophilic mutations on disease severity and perinatal outcomes, genotype-phenotype correlation was performed for all three mutations separately in the HELLP subjects following the items in table 1. No significant association was found for any of the clinical variables investigated: gestational age at delivery, infant’s birth weight, systolic and diastolic blood pressure, platelet count, liver enzymes (AST, ALT) and hemolysis (LDH).

Furthermore, analysis of gene-gene interactions in the mother, in the fetus and between mother and fetus was performed. In the HELLP group, neither mothers nor children were compound heterozygous for factor V Leiden or the prothrombin gene mutation whereas among the controls, one child and one mother were compound heterozygous. Only one child in the HELLP group carried the MTHFR-677-TT genotype and was heterozygous for the prothrombin gene mutation. The combination of heterozygous factor V Leiden and homozygosity for MTHFR – 677-T was not found at all in our study population. Regarding the gene-gene interactions between mother and fetus, the frequency of mothers who had inherited the mutant allele to their offspring was the same in patients and controls for the three mutations investigated.

**Discussion**

To our knowledge this is the largest study published so far on the relation between HELLP syndrome and the thrombophilic gene mutations factor V Leiden, prothrombin 20210G>A and MTHFR in mothers and fetuses. The post hoc statistical power calculation revealed that given 17% heterozygosity in the HELLP group and 4% in the control population and accepting an alpha error level of 10%, the statistical power would be as high as 91%.
Previous association studies on thrombophilic genotypes and HELLP syndrome are summarized in Table 4 (13-26). Most of them were limited to a small sample size and genotyping was not done simultaneously for all of the three most common inherited thrombophilias. However, in line with our results, an increased risk for HELLP syndrome in factor V Leiden carriers was reported in two Caucasian samples (22;25). In contrast to most of the other studies, Nagy et al. (20) reported a significantly increased prevalence of the MTHFR 677TT genotype in Caucasian HELLP patients from Hungary. Demir et al. (15) also found a positive association for the MTHFR 677C>T polymorphism in Turkish women with hypertensive pregnancy disorders including 20 patients with HELLP syndrome, but as the latter subgroup has not been analyzed separately, a comparison with other studies on HELLP syndrome and thrombophilia is rather difficult. In two other studies cited in Table 4, HELLP subgroup analysis has not been performed either (16;24). Furthermore, some studies have included patients from heterogeneous ethnic backgrounds (19;24), a critical point considering the great differences in allelic frequencies across populations (31).

In contrast to the HELLP syndrome, the relation between pre-eclampsia in general and inherited thrombophilia has been more extensively investigated. In a recent review and meta-analysis including studies published in English between 1980 and June 2003, Robertson et al. (4) described an increased risk of pre-eclampsia for heterozygous factor V Leiden (OR 2.19, 95%CI 1.46-3.27), heterozygous prothrombin 20210G>A (OR 2.54, 95%CI 1.52-4.23) and homozygous MTHFR 677C>T (OR 1.37, 95%CI 1.07-1.76). In 2005 Lin et al. performed another meta-analysis including studies published up to November 2002 (32). A two-fold increase in pre-eclampsia risk was demonstrated for the factor V Leiden mutation (OR 1.81, 95%CI 1.14-2.87) while no effects were detected for prothrombin G mutation or homozygosity for MTHFR 677C>T. However, when the more recent studies published up to the end of 2007 are additionally considered, there is no evidence for a major effect of these
common thrombophilias on pre-eclampsia risk as most studies failed to demonstrate a significant association (31).

Fetal genes related to vascular conditions are also supposed to influence the risk of pre-eclampsia and HELLP because the placenta is of fetal origin and vascular features of the placenta are thought to be involved in the pathogenesis of these disorders (27;28). Therefore we assessed the fetal genotype for factor V Leiden, prothrombin 20210G>A and MTHFR 677C>T. No influence on maternal risk of HELLP syndrome was observed.

Several studies have addressed the role of fetal inherited thrombophilias in pre-eclampsia, only three of them have included a small number of HELLP pregnancies (Table 5)(19, 23,28,33-38). On the whole, there was no evidence for a strong association. Nevertheless, Dizon-Townson et al.(35) found a trend towards an increase of maternal pre-eclampsia among fetal factor V Leiden carriers \((p=0.06)\) which became significant when the cases were separated according to ethnicity. In African-American and Hispanic mothers of fetal factor V Leiden carriers, pre-eclampsia revealed to be significantly more frequent than in whites \((p=0.04)\). Furthermore, Anteby et al. (33) showed that fetal factor V Leiden and prothrombin mutations may lead to a more severe course of pre-eclampsia, intrauterine growth-restriction and placental abruption: Fetuses with factor V Leiden or prothrombin mutation were born earlier \((p=0.04)\) and weighed less \((p=0.02)\) than fetuses without these thrombophilic mutations. Thus a certain fetal impact on maternal pre-eclampsia and other severe obstetric complications may not be completely ruled out. Our results clearly show that there is no paternal effect in addition to maternal inheritance of factor V Leiden mutation which should be found with an increased frequency in the fetuses since the mutation is more prevalent in mothers with HELLP syndrome.

In summary, our data confirm that Caucasian women carrying a heterozygous factor V Leiden mutation have an increased risk of developing HELLP syndrome (OR 4.45). In contrast, no significant association was found for prothrombin 20210G>A mutation and MTHFR 677C>T
polymorphism. No further differences were detected when thrombophilic genotypes were related to the clinical features of HELLP syndrome and perinatal outcome. Moreover, there was no evidence for a fetal genetic influence on the mother’s risk of developing HELLP syndrome. We further may conclude from our study that gene-gene interactions do not have a major impact on the role of thrombophilic mutations in the etiology of HELLP syndrome.

References


Table legends

Table 1. Clinical characteristics of the study population.

Table 2. Maternal genotypes for the 3 mutations/ polymorphisms investigated. Statistical analysis was performed by use of chi-squared test, d.f. 1.

Table 3. Fetal genotypes for the 3 mutations/ polymorphisms investigated. Statistical analysis was performed by use of chi-squared test, d.f. 1.

Table 4. Studies on genetic thrombophilias and risk of HELLP

Table 5. Association studies of fetal genetic thrombophilias and risk of PE
Table 1. Clinical characteristics of the study population.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HELLP (n=71)</th>
<th>Controls (n=79)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30.0 ± 3.9</td>
<td>30.9 ± 5.0</td>
<td>0.245</td>
</tr>
<tr>
<td>Primiparity (%)</td>
<td>n=63 (88.7 %)</td>
<td>n=52 (65.8 %)</td>
<td>0.229</td>
</tr>
<tr>
<td>Week of gestation at delivery</td>
<td>33.0 ± 4.5</td>
<td>39.7 ± 1.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Infant’s birth weight (g)</td>
<td>1714.7 ± 836.7</td>
<td>3416.5 ± 396.4</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>168.7 ± 30.0</td>
<td>118.5 ± 6.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>105.1 ± 17.7</td>
<td>72.4 ± 5.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Minimal platelet count (G/l)</td>
<td>48 (11 - 97)</td>
<td>249 (150 - 349)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>AST max (U/l)</td>
<td>104 (23 – 1190)</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>ALT max (U/l)</td>
<td>117 (31 – 1140)</td>
<td>n.d.</td>
<td>-</td>
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<tr>
<td>LDH max (U/l)</td>
<td>579 (227 – 3460)</td>
<td>n.d</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.D., laboratory parameters as median (range). Normal range for AST was 5-15 U/l, for ALT 5-19 U/l.
Table 2. Maternal genotypes for the 3 mutations/polymorphisms investigated.

<table>
<thead>
<tr>
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<th></th>
<th>P</th>
<th>OR</th>
<th>(95%CI)</th>
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</thead>
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<tr>
<td></td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Prothrombin (20210G&gt;A)</strong></td>
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<td></td>
<td></td>
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<tr>
<td>HELLP (n=71)</td>
<td>68</td>
<td>95.8</td>
<td>3</td>
<td>4.2</td>
<td>0</td>
<td>0.894</td>
<td>1.11</td>
<td>(0.23-5.34)</td>
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<tr>
<td>Controls (n=79)</td>
<td>76</td>
<td>96.2</td>
<td>3</td>
<td>3.8</td>
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<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
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<tr>
<td><strong>Factor V (1691G&gt;A)</strong></td>
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<tr>
<td>HELLP (n=71)</td>
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<td>83.1</td>
<td>12</td>
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<td>(1.31-15.13)</td>
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<tr>
<td>Controls (n=79)</td>
<td>76</td>
<td>96.2</td>
<td>3</td>
<td>3.8</td>
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<td>n</td>
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<td>%</td>
<td>n</td>
<td>%</td>
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<td></td>
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<tr>
<td><strong>MTHFR (677C&gt;T)</strong></td>
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<td>HELLP (n=71)</td>
<td>30</td>
<td>42.3</td>
<td>34</td>
<td>47.9</td>
<td>7</td>
<td>9.9</td>
<td>0.189</td>
<td>0.52 (0.22-1.20)</td>
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<tr>
<td>Controls (n=79)</td>
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<td>44.3</td>
<td>29</td>
<td>36.7</td>
<td>15</td>
<td>19</td>
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</table>

CI confidence interval, OR odds ratio
**Table 3.** Fetal genotypes for the 3 mutations/ polymorphisms investigated. Frequencies are given in parantheses.

<table>
<thead>
<tr>
<th></th>
<th>Prothrombin (20210G&gt;A)</th>
<th>Factor V (1691G&gt;A)</th>
<th>MTHFR (677C&gt;T)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GA</td>
<td>AA</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>HELLP children (n=71)</td>
<td>68</td>
<td>95.8</td>
<td>3</td>
</tr>
<tr>
<td>Control children (n=78)</td>
<td>74</td>
<td>94.9</td>
<td>4</td>
</tr>
<tr>
<td>HELLP children (n=69)</td>
<td>64</td>
<td>92.8</td>
<td>5</td>
</tr>
<tr>
<td>Control children (n=77)</td>
<td>72</td>
<td>93.5</td>
<td>5</td>
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</table>

CI confidence interval, OR odds ratio