Prevalence of F5 1691G>A, F2 20210G>A, and MTHFR 677C>T polymorphisms in Bosnian women with pregnancy loss

Emir Mahmutbegović1, Damir Marjanović2,3, Edin Medjedović4, Nevena Mahmutbegović5, Serkan Dogan2, Amina Valjevac6, Ewa Czerska7, Anna Pawińska-Matecka7, Agnieszka Madlani6, Grażyna Adler*8

1Institution of Health Protection of Women and Motherhood Canton Sarajevo, Sarajevo, Bosnia and Herzegovina, 2International Burch University, Sarajevo, Bosnia and Herzegovina, 3Institute for Anthropological Research, Zagreb, Croatia, 4Gynecology Clinic, Clinical Center of University of Sarajevo, Sarajevo, Bosnia and Herzegovina, 5Neurology Clinic, Clinical Center of University of Sarajevo, Sarajevo, Bosnia and Herzegovina, 6Laboratory for Molecular Medicine, Center for Genetics, Medical Faculty, University of Sarajevo, Sarajevo, Bosnia and Herzegovina, 7Central Laboratory, Regional Hospital, Szczecin, Poland, 8Department of Gerontobiology, Pomeranian Medical University, Szczecin, Poland

ABSTRACT

The relationship between genetic risk factors of thrombophilia and pregnancy loss (PL) is being discussed. The focus has been on F5 1691G>A, F2 20210G>A, and MTHFR 677C>T polymorphisms that may predispose women to microthrombosis during the stages of embryo implantation and placentation. Although, the frequencies of these polymorphisms were reported in different populations, such studies have not yet been performed in Bosnian population. In this study, we determined the prevalence of F5 G>A (rs6025), F2 G>A (rs1799963) and MTHFR C>T (rs1801133) polymorphisms in Bosnian women. A total of 154 women with PL, mean age 33 (±5.4) years, were enrolled in the study. As a control group, 154 mothers [mean age 31.4 (±6.7) years] with at least one live-born child were included. We used real-time polymerase chain reaction (PCR) to determine the frequencies of F5 G>A and F2 G>A genotypes, and PCR-restriction fragment length polymorphism (RFLP) for analyzing MTHFR C>T genotypes. The frequency of heterozygotes for F5 and F2 was significantly higher in women with venous thrombosis (VT) compared to women without VT (p = 0.047 and p = 0.001, respectively). There was no significant difference in the distribution of MTHFR genotypes and alleles between these two groups. In addition, we observed no significant differences in the genotype and allele frequencies between the group with PL and control group, for all investigated polymorphisms. The allele frequencies for 1691A (F5), 20210A (F2), and 677T (MTHFR) reported in this study are consistent with the data obtained for other European countries, however, we were not able to confirm the association between the three polymorphisms and PL in Bosnian women.

KEY WORDS: Pregnancy loss; risk factors; polymorphisms; thrombophilia; F5; F2; MTHFR

INTRODUCTION

There is a growing evidence that women with thrombophilia are at increased risk, not only for pregnancy-related venous thromboembolism (VTE), but also for other vascular complications in pregnancy, such as fetal loss, pre-eclampsia (PE) and intrauterine growth restriction (IUGR) [1].

According to the definition, pregnancy loss (PL) is the spontaneous abortion of an embryo or fetus before the 20th week of pregnancy or when the fetus weighs <500 g or measures <25 cm [2].

The F5 gene mutation 1691G>A (rs6025) results in an altered variant of factor V, namely Factor V Leiden, which cannot be easily cleaved by activated protein C (aPC). The 1691G>A mutation increases the risk of venous thrombosis (VT) up to 50-100 fold in adult homozygous [3]. In European populations, the frequency of F5 1691A allele is higher in Bosnians (6.0%) than in the Czechs (2.6%), Ukrainians (1.5%), and Croats (1.4%) [4]. Numerous studies indicated that the F5 1691G>A is responsible for 3-42% of PL [5].

The F2 20210G>A (rs1799963) mutation, results in increased prothrombin levels and is considered as another risk factor for VT [6]. The heterozygotes have 2-5-fold increased risk of thrombosis which may also increase the risk for PL. The
The prevalence of F2 20210G>A in European Caucasians is from 1% to 8%, and from 1% to 12% in Mediterranean populations. Furthermore, in European Caucasians with VTE, the prevalence of 20210G>A is from 3% to 17%, and in Mediterranean populations with VTE this figure is from 3% to 24% [7-9]. The F2 20210A allele frequency in Bosnians is 6.0%, and is higher than in Poles and Ukrainians, 2.5% and 1.7%, respectively [4].

The 677C>T mutation (rs1801133) of the MTHFR gene leads to a defective methylene tetrahydrofolate reductase (MTHFR) enzyme that has only 50% of the normal activity and is a common cause of elevated levels of homocysteine, previously identified as a risk factor for VTE. The frequency of MTHFR 677T allele in Bosnians is 37.5% and is higher than those reported for the Czechs, Ukrainians, Norwegians, and Swedes (25.0%, 29.1%, 29.0%, and 25.0%, respectively), as well as for Austrians and the Dutch (each 29.3%)[4]. The American College of Obstetricians and Gynecologists recommends against screening for MTHFR variants to determine the presence of thrombophilia in pregnant women [10]. On the other hand, the co-inheritance of the alleles linked to thrombophilia, such as the F5 1691A, F2 20210A, and MTHFR 677T, may increase the risk for PL.

The prevalence of the genetic risk factors for thrombophilia is higher in Bosnian population compared to several European populations [4]. Therefore, the aim of our study was to investigate the prevalence of F5 G>A (rs6025), F2 G>A (rs1799963) and MTHFR C>T (rs1801133) polymorphisms in Bosnian women with miscarriages and to determine the correlation with the risk of PL.

MATERIALS AND METHODS

The medical history and buccal swabs were collected at the Institution of Health Protection of Women and Motherhood (Sarajevo, Bosnia and Herzegovina) from 2013 to 2015. The study was conducted according to the standards of the Declaration of Helsinki (1975, revised 2000), and the protocol was approved by the local Bioethical Committee (decision reference numbers 10-1285-03-14, KB-0012/46/15, and KB-0012/133/13). A total of 154 women with PL were enrolled in the study, and 121 (78.6%) of these women had at least one live born child. In this group, all pregnancies were anembryonic, and miscarriage took place between 6 and 28 weeks gestation. As a control group, 154 mothers without PL and with at least one live-born child were included. All 308 women were of Bosnian origin and came from the general population of Sarajevo (an estimated population of 1,691,534 inhabitants [http://fzs.ba/index.php/popis-stanovnistva/popis-stanovnistva-2013/konacni-rezultati-popisa-2013/]). In our study ethnicity refers to linguistic, cultural, religious, and political aspects. Clinical and lab data were recorded for each women in the StatView computer software version 5.0 (SAS Institute Inc. Cary, NC, USA).

Data on obstetric history, body weight, number of successful pregnancies, and history of VT are shown in Table 1.

DNA extraction and amplification

Genomic DNA from buccal swabs was extracted using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The extraction was performed according to the manufacturer’s instructions. DNA samples were stored at +4°C for further analyses.

Following DNA extraction from buccal swabs, polymerase chain reaction (PCR) was performed. The F5 G>A and F2 G>A genotypes were determined by real-time PCR on a Roche LightCycler® 2.0 (Roche, Bazylea, Switzerland) and using Gene Proof PCR kits (Imogena, Brno, Czech Republic). The MTHFR C>T genotypes were analyzed by PCR-restriction fragment length polymorphism (PCR-RFLP) using the following primers: forward 5’-CAGGCGCAGCCCCGCAAGC-3’ and reverse 5’-AGGACCGTGCGGTTAGAGTG-3’ (TIB MolBiol, Poznan, Poland). The PCR reactions were performed on a Hightech Thermocycler Cycler-Technology for Life (SensoQuest, Gottingen, Germany) according to previously described techniques by Schmitz et al. [11]. After digestion, the genotypes were determined by electrophoresis in 2-3% agarose gels, stained with DNA-star dye (Lonza, Inc., Rockland, ME, USA).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 23.2. (IBM Corp, Armonk, NY). Deviation of Hardy–Weinberg equilibrium (HWE) was assessed by Student's t-test for independent samples. Difference in genotype distribution between women with and without PL was determined using Fisher’s exact test. Odds ratio (OR) and 95% confidence interval (CI) were calculated. A value of p < 0.05 was considered statistically significant.

RESULTS

In our study group, 9.7% (n = 15) women with PL and 7.8% (n = 12) without PL had VT, but there was no statistically significant difference between the two groups (χ² = 0.688; p = 0.44).

A positive family history of VT was more frequent in the group of women with PL: 24.7% (n = 38) compared to the control group, 16.2% (n = 25), and this difference was statistically significant (χ² = 3.372; p = 0.045). Both groups were in HWE, and we observed no significant differences in the genotype and allele frequencies between the group with PL and control group, for all investigated polymorphisms (p > 0.05).
The distribution of genotypes and alleles in women with PL and control group is presented in Table 2. The distributions of genotypes and alleles in women with PL and control group in relation to the number of PL and the presence of VT are shown in Tables 3 and 4, respectively.

The F5 GA genotype was more frequent in women with multiple miscarriages (the borderline statistical significance, \(p = 0.051, \chi^2 = 7.791\)). No statistically significant correlation between the F2 and MTHFR genotype and allele distribution and the number of PL was observed.

Both F5 and F2 heterozygotes were significantly more frequent in women with VT compared to women without VT (\(p = 0.047\) and \(0.001\), respectively). There was no statistically significant difference in the distribution of MTHFR genotypes and alleles between women with VT and those without VT.

**DISCUSSION**

Pregnancy is a physiological state linked to increased clotting, decreased anticoagulant activity, and decreased fibrinolysis [12].

Although the allele A of the F5 gene (1691A) is a risk factor for thrombosis, the results on the association between F5 A allele and PL are contradictory. In our study, the frequency of 1691A allele was 7.8% in both groups (i.e., women with and without PL), and the differences in the genotype and allele distributions between the two groups were not significant (\(p > 0.05\)). Similarly, Polish, German, and Turkish studies showed no significant differences in the frequency of F5 1691A allele between women with PL and those without PL (\(p > 0.05\)). In the above-mentioned studies, the following frequencies of the 1691A allele were reported in women with PL versus women without PL: 3.2% versus 3.0% in the Polish study, 10.9% versus 7.4% in the German study, and 7.9% versus 7.0% in the Turkish group [13-15]. In Serbian women with and without PL, the frequency of the 1691A allele was 4.0% versus 1.5%, respectively (\(p = 0.05\)) [16]. Similarly, studies on Lebanese and Palestinian women with PL confirmed that the 1691A allele was significantly more frequent in PL group compared to controls (\(p < 0.001\) and \(p < 0.0001\), respectively) [6,17]. Surprisingly, in an Israeli study, the frequency of

### TABLE 1. Characteristics of the study group

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Women with PL (mean±SD)</th>
<th>Women without PL (mean±SD)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>32.9±5.1</td>
<td>31.7±6.6</td>
<td>0.069</td>
</tr>
<tr>
<td>Weight</td>
<td>73.9±9.3</td>
<td>74.7±9.2</td>
<td>0.484</td>
</tr>
<tr>
<td>Number of pregnancies</td>
<td>1.4±1.1</td>
<td>1.2±0.8</td>
<td>0.066</td>
</tr>
<tr>
<td>VT, n (%)</td>
<td>15 (9.7)</td>
<td>12 (7.8)</td>
<td>0.344</td>
</tr>
<tr>
<td>VT in the family, n (%)</td>
<td>38 (24.7)</td>
<td>25 (16.2)</td>
<td>0.045</td>
</tr>
</tbody>
</table>

PL: Pregnancy loss; VT: Venous thrombosis; SD: Standard deviation

### TABLE 2. Distribution of genotypes and alleles of F5, F2, and MTHFR polymorphisms in women with and without PL

<table>
<thead>
<tr>
<th>Gene polymorphism</th>
<th>Women with PL n=154</th>
<th>Women without PL n=154</th>
<th>Compared genotypes</th>
<th>(p) value</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>F5 1691G&gt;A</strong></td>
<td></td>
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<tr>
<td>Genotypes, n (%)</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>142 (92.2)</td>
<td>142 (92.2)</td>
<td>GA versus GG</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>12 (7.8)</td>
<td>12 (7.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele frequency, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>296 (96.1)</td>
<td>296 (96.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>12 (3.9)</td>
<td>12 (3.9)</td>
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<tr>
<td><strong>F2 20210G&gt;A</strong></td>
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<tr>
<td>Genotypes, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>148 (96.1)</td>
<td>149 (96.8)</td>
<td>GA versus GG</td>
<td>0.759</td>
<td>1.208 (0.36-4.06)</td>
</tr>
<tr>
<td>GA</td>
<td>6 (3.9)</td>
<td>5 (3.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>Allele frequency, n (%)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>G</td>
<td>302 (98.1)</td>
<td>303 (98.4)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>6 (1.9)</td>
<td>5 (1.6)</td>
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<tr>
<td><strong>MTHFR 677C&gt;T</strong></td>
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<tr>
<td>Genotypes, n (%)</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CC</td>
<td>61 (39.6)</td>
<td>71 (46.1)</td>
<td>CC versus CT+TT</td>
<td>0.215</td>
<td>0.767 (0.49-1.21)</td>
</tr>
<tr>
<td>CT</td>
<td>76 (49.4)</td>
<td>74 (48.1)</td>
<td>CT versus CC+TT</td>
<td>0.819</td>
<td>1.053 (0.67-1.65)</td>
</tr>
<tr>
<td>TT</td>
<td>17 (11.0)</td>
<td>9 (5.8)</td>
<td>TT versus CC+CT</td>
<td>0.106</td>
<td>1.999 (0.86-4.64)</td>
</tr>
<tr>
<td>Allele frequency, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>198 (64.3)</td>
<td>216 (70.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>110 (35.7)</td>
<td>92 (29.9)</td>
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</tr>
</tbody>
</table>

PL: Pregnancy loss; OR: Odds ratio; CI: Confidence interval
the mutated F5 allele was higher in women without PL compared to women with PL (p < 0.05) [18].

Rodger et al. [19] performed a meta-analysis on the relationship between mothers, carriers of the F5 1691A and F2 20210A alleles, and complications of pregnancy. They demonstrated no significant association between the mutated alleles and pregnancy complications, including PL [19]. In the present study, we also showed no significant differences in the frequency of F2 20210A allele and obstetric complications between women with and without PL (p > 0.05); the frequency of the mutated allele was 1.9% and 1.6%, respectively.

Although Sehirali et al. [20] found the association between the F2 20210A allele and PL in Turkish women (p < 0.05) [20], these results should be interpreted with caution due to the small sample size; they included 55 women with PL and 49 controls.

Rey et al. [21] conducted a meta-analysis of 31 studies, published between 1975 and 2002. Their conclusion was that the F5 1691A allele is linked to early (OR: 2.01, 95% CI: 1.13-3.58), late recurrent fetal loss (OR: 7.83, 95% CI: 2.83-21.67), and late non-recurrent fetal loss (OR: 3.26, 95% CI: 1.82-5.83). The F2 20210A allele was linked to PL in the first trimester (OR: 2.32, 95% CI: 1.12-4.79), repeated miscarriages (OR: 2.56, 95% CI: 1.04-6.29), and late non-recurrent fetal loss (OR: 2.3, 95% CI: 1.09-4.87) [21].

In Spanish women with three or more unexplained PL, significant differences between the 677T allele of the MTHFR gene and PL were not found [22].

In a Greek study, Sotiriadis et al. [23] reported that the F5 1691G>A, F2 20210G>A, and MTHFR 677C>T polymorphisms were not significantly associated with increased risk for PL (p = 1.00, p = 0.652, and p = 0.869, respectively) [23]. In another Greek study, the association between PL in the second trimester and primary PL with the F5 1691G>A and F2 20210G>A was found (p = 0.03, p = 0.038, respectively), but not with the MTHFR 677C>T (p = 0.134) [24].

In the present study, the frequency of F5 1691A and F2 20210A alleles was significantly higher in women with VT compared to women without VT, 9.3% versus 3.4% (χ² = 4.739; p = 0.047) and 9.3% versus 1.1% (χ² = 19.199; p = 0.001), respectively. In 2006, Robertson et al. [25] reported a relationship between the risk of VTE and adverse pregnancy outcomes and thrombophilia in pregnancy. The authors concluded that the risk of VTE for F5 1691A homozygotes was 34.4 (95% CI: 9.86-120.05), while for the heterozygotes the risk was 8.32 (95% CI: 5.44-12.70) [25].

In a meta-analysis of 63 studies, the authors found that the F5 1691G-A mutation was associated with a 4.5- and 8.6-fold increased risk of the first VTE during pregnancy (95% CI: 4.0-8.7).
CONCLUSION

In summary, we were not able to confirm the association between the F5 1691G>A, F2 20210G>A, and MTHFR 677C>T polymorphisms and PT in Bosnian women. The significant differences in the frequency of mutated alleles were found only between women with VT and those without VT.

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DECLARATION OF INTERESTS

The authors declare no conflict of interests.

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