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Single Nucleotide Polymorphisms from CSF2, FLT1, TFPI and TLR9 Genes Are Associated with Prelabor Rupture of Membranes

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1. Introduction

A prelabor rupture of membranes (PROM) concerns the membranes with amniotic fluid leakage that occurs before the onset of labor or regular uterine activity and is observed in approximately 40% of preterm deliveries [1–3]. Based on the gestational age at the time of membrane rupture, the PROM is divided into a preterm PROM (pPROM), if the disturbance occurs before the 37th week, and the term PROM (tPROM), observed from the 37th week [1]. TPROM is diagnosed in roughly 8% of full-term pregnancies, followed by adverse maternal and perinatal outcomes, including placental abruption, cord compression,
cord prolapse, a risk of caesarean delivery and maternal and neonatal infection [1,2,4]. In
turn, pPROM affects about 3–4% of all births, causing one-third of all preterm labors and
being one of the most serious complications of pregnancy [5–7]. The occurrence of pPROM
is accompanied by increased maternal and offspring morbidity, as well as fetal and neonatal
mortality [8–10]. PPROM is associated with an increased risk of intra-amniotic infection,
acute and chronic histological chorioamnionitis, clinical chorioamnionitis, cord prolapse,
placental abruption and postpartum endometritis [8–11]. The complications in neonates
include pulmonary hypoplasia, respiratory distress syndrome (RDS), bronchopulmonary
dysplasia (BPD), adverse neurodevelopmental outcomes, intraventricular hemorrhage
(IVH), retinopathy of prematurity (ROP), cardiovascular diseases, necrotizing enterocolitis
(NEC), sepsis and death [9,10].

Disorders in the hemostatic system and angiogenesis have been reported among the
factors, which determine the occurrence of PROM [12–15]. In vitro studies have shown
thrombin participation in fetal-membrane-weakening processes, accompanied by their
remodeling and apoptosis, similar to the changes that occur physiologically fetal mem-
branes in term pregnancies [16,17]. Thrombin can compromise the membranes, both
directly, affecting the extracellular matrix by converting matrix metalloproteinase (MMP)
proteins from their precursor forms to the active form of the enzyme, and indirectly, via
protease-activated receptors (PAR1, 3 and 4) [18–22]. In the case of tissue factor (TF), which
initiates the activation of blood clotting through thrombin synthesis, it has been found
that an increased expression is associated with pPROM [13,23]. In turn, the tissue factor
pathway inhibitor (TFPI) that prevents the TF-dependent coagulation pathway has been
found to be reduced in women with pPROM, compared to full-term pregnancies [13]. The
granulocyte-macrophage colony-stimulating factor (GM-CSF, CSF2) has also been reported
to be involved in fetal membrane weakening and is characterized by an increased activity
in the fetal membranes with PROM, compared to labor on time [15,24].

The most common infections associated with pPROM are caused by genital mycoplas-
mas: Ureaplasma and Mycoplasma hominis [25–28]. The toll-like receptors (TLRs) are the
key molecules in the immune response against a variety of microorganisms, including
bacteria [29–31]. Increased levels of amniotic-fluid-soluble forms of TLR2 (sTLR2) and
TLR4 (sTLR4) have been found in women with pPROM in the case of microbial invasion
of the amniotic cavity (MIAC), determined by the presence of PCR products for the gen-
ital mycoplasmas (Ureaplasma spp. and M. hominis), Chlamydia trachomatis and/or
the growth of any bacteria except coagulase-negative Staphylococcus epidermidis [12,32].
For TLR2, TLR4, TLR6 and TLR9 receptors, their participation in angiogenesis, one of
the key processes involved in the development and function of the placenta, has also
been confirmed [14,33–35]. TLR2 has been demonstrated to induce the expression of the
angiogenic factor angiopoietin 2 (ANGPT2), as well as the MMP2 and MMP9 proteins,
involved in destabilization and development of blood vessels [36]. The macrophage-
activating lipopeptide-2 (MALP2), isolated from Mycoplasma spp., was found to interact
with TLR2/6 receptors to induce GM-CSF-mediated angiogenesis [14,37]. TLR9 has been
shown to correlate with the inhibition of angiogenesis by downregulating the vascular
endothelial growth factor (VEGF) and upregulating the soluble VEGF receptor 1 (sVEGFR1,
sFLT1) at the fetomaternal interface with preeclampsia [33]. In addition, TLR9 has also
been reported to be associated with increased mRNA and protein levels of TF but with a de-
creased transcription, secretion and activity of TFPI in human coronary artery endothelial
cells and with activation of blood clotting in mice [38].

Given the genetic alterations in angiogenic factors, CSF2 rs25881, rs25882 and rs27438
were associated with an increased risk of preterm birth in European-American women,
while rs721121, rs4705916, rs743564 and rs6898270 were correlated with a reduced risk
of the disease in both European and African-American pregnant women [39]. For FLT1,
rs748252 was reported to be involved in an approximately two-fold increased risk of
spontaneous preterm labor in women with bacterial vaginosis prior to the 37th week of
gestation [40]. Among African-American women, a minor A allele in FLT1 rs12428494
was associated with spontaneous preterm delivery, determined before the 34th week of pregnancy [41]. In turn, it was found that the FLT1 rs722503 polymorphism correlated with an increased susceptibility to preeclampsia in a dominant model, designed with regards to Iranian women [42]. The T allele in rs722503 was significantly more prevalent among women with preeclampsia, compared to healthy controls [42]. A study conducted on placentas with intrauterine growth restriction (IUGR) demonstrated an involvement of TFPI in alternative splicing and angiogenesis-related processes [43]. However, it was found that the frequency of genotypes and alleles for TFPI rs8176592 was similar, both in women with recurrent miscarriages and in healthy controls [44]. The genotype prevalence rates for TFPI T-33C and C-399T polymorphisms were also similar, both in cases with recurrent pregnancy loss and in the control group, while the TFPI -287C allele (TC + CC genotypes) was considered to protect against the disease [45]. In the case of the TLR9 gene, it was reported that the CT and TT genotypes within rs352140 were significantly more common in neonates with placental inflammation compared to the CC genotype [46]. So far, it has been shown that chorioamnionitis is associated with preterm labor and related postpartum diseases [46]. In Ukrainian pregnant women, TLR9 rs352140 has been reported to play a key role in the development of spontaneous abortion [47]. The AA and GA genotypes, as well as the A allele in rs352140, significantly increased the risk of miscarriage [47]. Among Ghanaian primiparas infected with Plasmodium falciparum, TLR9 rs187084 was associated with a six-fold increased risk of low birth weight in full-term infants but was not involved in preterm delivery [48].

Since there is a lack of data on the contribution of genetic changes associated with hemostasis and angiogenesis to the occurrence of PROM, the current research aimed to demonstrate the role of the four SNPs, located in CSF2 (rs25881), FLT1 (rs722503), TFPI (C-399T) and TLR9 (rs352140) genes, in the presented disease.

2. Materials and Methods

2.1. Women with PROM and Healthy Controls

The study was conducted prospectively in 360 women with singleton pregnancy, treated at the Department of Obstetrics, Perinatology and Gynecology of the Polish Mother’s Memorial Hospital-Research Institute (PMMH-RI), in Lodz, Poland, during the period between August 2016 and December 2020 (see Table 1). The population consisted of 180 women with PROM, observed between 14 and 41 weeks of gestation, and 180 healthy full-term women without PROM as controls. In the studied cohort, 126 (70.0%) women were diagnosed with pPROM before the 37th week of pregnancy, while 54 (30.0%) women with tPROM from 37 weeks of gestation (see Table 1 and Table S1). The presence of PROM was confirmed by the AMNIOQUICK® test (BIOSYNEX SA, Illkirch-Graffenstaden, France). The pregnant women included in the study were aged between 18 and 44 years, the PROMs were aged between 18 and 43 years, and the controls were aged between 18 and 44 years. The women with pPROM ranged in age from 18 to 43 years, while those with tPROM ranged from 19 to 37 years in age. Detailed clinical characteristics of the women in terms of asthma and respiratory system infections, bleeding, diabetes mellitus (DM), hypertension, hypothyroidism, serological conflict, threatened miscarriage and genitourinary infections, are presented in Table 1 and Table S1. Women were excluded from the study in the following cases: multiple pregnancy, congenital disorder, genetic syndrome, structural uterine defect, endometriosis, myoma uterus, placenta previa, cervical insufficiency, condition after amniocentesis, fetal abnormality and growth disorders, including fetal growth restriction (FGR), small for gestational age (SGA) and large for gestational age (LGA). The study was approved by the Research Ethics Committee at the PMMH-RI (approval numbers 14/2019 and 15/2019). Clinical samples were obtained for diagnostic purposes, then anonymized for research. Informed consent forms were signed by all the study participants, as recommended by the Research Ethics Committee.
Table 1. Characteristics of women with prelabor rupture of membranes and healthy controls.

|                                    | Controls | PROM a Cases | \( p \)-Value b | pPROM c Cases | \( p \)-Value |
|------------------------------------|----------|--------------|------------------|---------------|--------------|
| Number of women                    | 180      | 180          | 0.057            | 126           | \( \leq 0.001 \) |
| Age (years)                        | 28 (18–44) | 30 (18–43) |                  | 31 (18–43)    |              |
| No. d of pregnancy, \( n \) (%)    |          |              |                  |               |              |
| 1                                  | 113 (62.8%) | 87 (48.3%)  | 0.013            | 47 (37.3%)    | \( \leq 0.001 \) |
| 2                                  | 51 (28.3%) | 55 (30.6%)  |                  | 45 (35.7%)    |              |
| 3                                  | 12 (6.7%) | 26 (14.4%)  |                  | 22 (17.5%)    | \( \leq 0.001 \) |
| 4                                  | 4 (2.2%)  | 7 (3.9%)     |                  | 7 (5.5%)      |              |
| 5                                  | 0 (0.0%)  | 3 (1.7%)     |                  | 3 (2.4%)      |              |
| 6                                  | 0 (0.0%)  | 2 (1.1%)     |                  | 2 (1.6%)      |              |
| Asthma and respiratory system infections | 4 (2.2%) | 10 (5.6%) | 0.085            | 6 (4.8%)      | 0.183        |
| Diabetes mellitus                  | 3 (1.7%) | 7 (3.9%)     | 0.168            | 5 (4.0%)      | 0.189        |
| Hypertension                       | 22 (12.2%) | 19 (10.6%) | 0.619            | 13 (10.3%)    | 0.606        |
| Hypothyroidism                     | 27 (15.0%) | 36 (20.0%) | 0.212            | 25 (19.8%)    | 0.267        |
| Serological conflict               | 11 (6.1%) | 4 (2.2%)    | 0.055            | 2 (1.6%)      | 0.045        |
| Threatened miscarriage             | 0 (0.0%)  | 15 (8.3%) | \( \leq 0.001 \) | 12 (9.5%) | \( \leq 0.001 \) |
| Urogenital infections              | 20 (11.1%) | 20 (11.1%) | 1.000            | 11 (8.7%)     | 0.497        |
| APTT (s) e                          | 28.3 (23.6–34.8) | 27.7 (21.7–44.5) | 0.103 | 28.1 (21.7–44.5) | 0.322 |
| No. [\( \times 10^{9}/L \)]        | 211.5 (150–398) | 214 (59–457) | 0.220 | 220.5 (59–457) | 0.900 |
| PDW [fL] f                          | 13.7 (9.0–23.7) | 13.3 (8.8–24.9) | 0.597 | 12.9 (8.8–24.9) | 0.010 |
| MPV [fL] g                          | 11.1 (9.0–14.1) | 11.1 (8.8–14.6) | 0.809 | 10.9 (8.8–14.6) | 0.024 |
| PCT [%] h                           | 0.24 (0.17–0.39) | 0.23 (0.06–0.50) | 0.086 | 0.23 (0.06–0.50) | 0.257 |
| Delivery, \( n \) (%)              |          |              |                  |               |              |
| Weeks of pregnancy                 | 39 (37–41) | 35 (17–41) | \( \leq 0.001 \) | 33 (17–40)    | \( \leq 0.001 \) |
| Natural                            | 82 (45.6%) | 78 (44.8%) | 0.891            | 49 (40.8%)    | 0.419        |
| C-section i                        | 98 (54.4%) | 96 (55.2%) |                  | 71 (59.2%)    |              |
| Fetal sex, \( n \) (%)             |          |              |                  |               |              |
| Female                             | 96 (53.3%) | 72 (42.6%) | 0.045            | 50 (43.5%)    | 0.099        |
| Male                               | 84 (46.7%) | 97 (57.4%) |                  | 65 (56.5%)    |              |
| Neonatal data                      |          |              |                  |               |              |
| Weight (percentiles)               | 73 (10–100) | 63.5 (0–100) | 0.004 | 56.5 (0–100) | 0.002 |
| Apgar in 1 min                     | 10 (7–10) | 9 (0–10) | \( \leq 0.001 \) | 7 (0–10) | \( \leq 0.001 \) |
| Apgar in 5 min                     | 10 (7–10) | 9 (0–10) | \( \leq 0.001 \) | 8 (0–10) | \( \leq 0.001 \) |

a PROM, prelabor rupture of membranes; b \( p \)-value, statistically significant results are marked in bold; c pPROM, preterm PROM; d No., number; e APTT (s), activated partial thromboplastin time (second); f PDW, platelet distribution width; g MPV, mean platelet volume; h PCT, plateletcrit; i C-section, caesarean section.
2.2. Collection and Analysis of Blood Samples

Two S-Monovette tubes (Sarstedt, Numbrecht, Germany) were filled with peripheral venous blood, collected by puncture from each woman on the day of admission for research purposes. EDTA KE/1.2 mL tubes were used for complete blood count (CBC) and DNA extraction, while 9 NC/1.4 mL coagulation tubes were used to determine the activated partial thromboplastin time (APTT). Platelet (PLT) parameters, including PLT count, platelet distribution width (PDW), the mean platelet volume (MPV) and plateletcrit (PCT), as part of the CBC, were determined using Fluorocell PLT reagent on a Sysmex XN-2000 Automated Hematology System (Sysmex, Kobe, Japan). The PLT count is normal between $150 \times 10^9 / L$ and $400 \times 10^9 / L$, and the MPV reference range is 8.0 to 10.0 fl, according to the manufacturer (Sysmex, Kobe, Japan). APTT was assessed using the HemosIL APTT-SP reagent on an ACL TOP 550 CTS automated system (Instrumentation Laboratory, Werfen Company, Bedford, MA, USA). The normal range of APTT is 23 to 36.9 s, as reported by the manufacturer. Total DNA was extracted from 200 µL of whole-blood samples using the Syngen Blood/Cell DNA Mini Kit (Syngen Biotech, Wroclaw, Poland). Purified DNA was eluted from a mini spin column in 100 µL of buffer DE and stored at $-20^\circ C$ until further analysis.

2.3. Genotyping of Single Nucleotide Polymorphisms (SNPs)

Four single nucleotide polymorphisms (SNPs) from CSF2 (rs25881), FLT1 (rs722503), TPPI (C-399T) and TLR9 (rs352140) genes were genotyped by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. SNPs were selected for the study, taking into account (1) their localization in the genes, associated with angiogenesis and PROM, and (2) their possible influence on protein function. For the three genes, i.e., CSF2, FLT1 and TLR9, SNPs were found from the National Center for Biotechnology Information (NCBI) SNP database (dbSNP) (https://www.ncbi.nlm.nih.gov/snp/ accessed on 22 September 2021) [49]. The minor allele frequency (MAF) of rs25881, rs722503 and rs352140 was $>10\%$, as reported by the NCBI Allele Frequency Aggregator (ALFA) project. Primer sequences, restriction enzymes and RFLP profiles are shown in Table S2, as previously described [42,49–51]. The PCR mixes, with a final volume of 25 µL, contained up to 0.5 µg of total DNA, 0.2 mM dNTP mix, 0.4 µM of each primer, specific for the polymorphism to be evaluated, 1x polymerase B buffer and 0.5 U of Perpetual Taq DNA Polymerase (EURx, Gdańsk, Poland). The PCR program included initial denaturation at 95 $^\circ C$ for 3 min, 40 cycles of denaturation at 95 $^\circ C$ for 30 s, annealing at 55–58 $^\circ C$, depending on SNP for 40 s, extension at 72 $^\circ C$ for 1 min and final extension at 72 $^\circ C$ for 7 min. The amplicons were digested with 10 U of the appropriate enzymes at defined temperatures for 16 h. PCR and restriction digestions were performed on a T100 Thermal Cycler (Bio-Rad, Singapore). The PCR and RFLP products were separated in 1–3.0% agarose gels (see Figure 1), prepared in 1× TAE buffer, depending on the length of the tested DNA fragments, and visualized in the ChemiDoc XR+ imaging system (Bio-Rad, Hercules, CA, USA).

2.4. Statistical Analysis

The study groups of the women with PROM and healthy controls and their offspring were characterized by descriptive statistics and then compared using Pearson’s chi-square and Mann–Whitney U tests. The female clinical data and the APTT and PLT parameters were described and compared among groups using the NCSS 2004 software. The Hardy–Weinberg equilibrium and the prevalence rates of alleles, genotypes and multiple-SNP variants for CSF2, FLT1, TLR9 and TPPI polymorphisms were determined using the SNPStats software [52]. The allele distribution among the examined groups of women was estimated using Pearson’s chi-square test. The relationships between genetic changes and the prevalence of PROM were estimated using logistic regression analyses to define inheritance models. Multiple-SNP analyses were performed using the expectation–maximization (EM) algorithm to estimate the frequency of complex variants in the PROM and healthy control groups. Adjustment analyses of the results for the APTT, PLT parameters and preg-
nancy disorders, including asthma and respiratory infections, bleeding, DM, hypertension, hypothyroidism, serological conflict, threatened miscarriage and urogenital infections, were also performed using logistic regression models. The results were considered statistically significant at the significance level of $p \leq 0.050$.

3. Results

3.1. Characteristics of the Pregnant Women

The women with PROM and the healthy controls were of similar age ($p = 0.057$, see Table 1). The women with pPROM were significantly older compared to both the control group and the women with tPROM ($p \leq 0.001$, see Table 1 and Table S1). Many pregnancies occurred significantly more frequently in PROM cases than in the control group ($p \leq 0.050$, see Table 1). Women in more than one pregnancy demonstrated pPROM more often than tPROM ($p \leq 0.001$, see Table S1). The methods of delivery, including natural labor and caesarean section, were similarly distributed in the study groups ($p = 0.891$ and $p = 0.419$, see Table 1). The gestational age at delivery was significantly shorter in women with PROM than in those in the control group ($p \leq 0.001$). As for the offspring of the women, male births were more common in the women with PROM, compared to healthy controls ($p = 0.045$). In addition, the fetal weight and Apgar at 1 and 5 min were significantly lower in the PROM cases than in the control group ($p \leq 0.050$). A comparison between pPROM and tPROM showed similar fetal weight ($p = 0.240$, see Table S1), but significantly lower Apgar score (after 1 and 5 min) was found in the women with pPROM ($p \leq 0.001$).

Figure 1. Products of PCR-RFLP assays for the CSF2 rs25881 (A), FLT1 rs722503 (B), TFPI C-399T (C) and TLR9 rs352140 (D) polymorphisms. Digestions were performed with the endonucleases Bpl (A), AvaII (B), HinfI (C) and BstUI (D), followed by separation in 2.5–3.0% ethidium bromide stained agarose gels. The numbers to the right of the electropherograms show the lengths of the separated DNA fragments. M: 50 bp DNA marker; Ud: undigested PCR product; CC, CT, TT: genotypes in the tested SNPs.
3.2. Parameters of Hemostasis

APTT and PCT achieved similar values in the studied groups of pregnant women ($p > 0.050$, see Table 1 and Table S1). The PLT count was comparable between PROMs and controls; however, the count was significantly higher in women with pPROM than in those with tPROM ($p = 0.028$). In turn, PDW and MPV were similar in cases of PROM and healthy controls, but they reached lower values in the pPROM women when compared to the control group and the women with tPROM ($p \leq 0.050$).

3.3. Hardy–Weinberg Equilibrium

For all tested groups of pregnant women, the Hardy–Weinberg (H–W) equilibrium was maintained for the genotypes in FLT1 rs722503 and TFPI C-399T SNPs ($p > 0.050$). In the case of CSF2 rs25881, the H–W equilibrium was found in the women with pPROM and in the healthy control group, but it was not confirmed in the tPROM cases ($p = 0.027$). Regarding TLR9 rs352140, genotypes were found in the H–W equilibrium in the women with tPROM and in the healthy controls, while the deviation was significant in the pPROM women ($p \leq 0.001$).

3.4. Genetic Alterations in CSF2, FLT1, TFPI and TLR9 Polymorphisms

A single-SNP analysis provided similar prevalence rates of genotypes in CSF2 rs25881, FLT1 rs722503, TFPI C-399T and TLR9 rs352140 SNPs among the women with PROM or pPROM and the healthy controls (see Table S3a,b). Similar prevalence rates of genotypes for the studied polymorphisms were also found in the pPROM and tPROM cases (see Tables S4–S7). Alleles in the tested SNPs had a similar distribution pattern among the studied groups of pregnant women (see Tables S5 and S6). However, the CT heterozygotes in the CSF2 polymorphism were significantly more frequent among the women with pPROM when compared to those with tPROM after correction by DM (OR 2.28 95% CI 1.04–5.01, $p = 0.032$, see Table 2).

A multiple-SNP analysis showed that the TT variants for CSF2 and FLT1 polymorphisms correlated with an approximately two-fold increase in the PROM risk when corrected for APTT, PLT parameters and pregnancy disorders ($p \leq 0.050$, see Table 3 and Table S7). CC double-SNP variants for TLR9 and TFPI polymorphisms were also associated with an almost two-fold higher risk of PROM, corrected by APTT (OR 1.94 95% CI 1.08–3.50, $p = 0.028$, see Table 3). Triple-SNP variants of TTC for the CSF2, FLT1 and TLR9 polymorphisms were associated with an increased risk of PROM when adjusted for APTT (OR 19.54 95% CI 1.22–311.80, $p = 0.037$). In turn, TCT variants for those three SNPs correlated with a reduced risk of PROM after adjusting for PLT (OR 0.13 95% CI 0.13–0.14, $p \leq 0.001$). Similarly, CCTC variants for CSF2, FLT1, TLR9 and TFPI SNPs were significantly less frequent among the PROMs than the healthy controls, corrected by APTT (OR 0.04 95% CI 0.01–0.28, $p = 0.002$). It was found that TTT triple-SNP variants for FLT1, TLR9 and TFPI polymorphisms, as well as CCCC and TTTC complex variants for all the tested SNPs, were correlated with an increased risk of PROM ($p \leq 0.050$).
Table 2. Distribution of genotypes in CSF2, FLT1, TFPI and TLR9 SNPs between women with term and preterm PROM, adjusted for diabetes mellitus.

| Polymorphism | Genetic Model | Genotype | Genotype Prevalence, n (%) | OR (95% CI) | p-Value |
|--------------|---------------|----------|---------------------------|-------------|---------|
|              |               |          | tPROM | pPROM |               |           |
| **CSF2**     | Codominant    | CC       | 40 (74.1%) | 82 (65.1%) | 1.00 | 0.055 |
|              |               | CT       | 10 (18.5%) | 41 (32.5%) | 2.16 (0.97–4.78) | 0.160 |
|              |               | TT       | 4 (7.4%) | 3 (2.4%) | 0.42 (0.09–1.96) | 0.160 |
|              | Dominant      | CC       | 40 (74.1%) | 82 (65.1%) | 1.00 | 0.055 |
|              |               | CT-TT    | 14 (25.9%) | 44 (34.9%) | 1.66 (0.81–3.41) | 0.160 |
|              | Recessive     | CC-CT    | 50 (92.6%) | 123 (97.6%) | 1.00 | 0.055 |
|              |               | TT       | 4 (7.4%) | 3 (2.4%) | 0.34 (0.07–1.56) | 0.160 |
|              | Overdominant  | CC-TT    | 44 (81.5%) | 85 (67.5%) | 1.00 | 0.055 |
|              |               | CT       | 10 (18.5%) | 41 (32.5%) | 2.28 (1.04–5.01) | 0.032 |
| **FLT1**     | Codominant    | TT       | 36 (66.7%) | 71 (56.4%) | 1.00 | 0.370 |
|              |               | CT       | 16 (29.6%) | 48 (38.1%) | 1.59 (0.79–3.20) | 0.370 |
|              |               | CC       | 2 (3.7%) | 7 (5.6%) | 1.80 (0.35–9.23) | 0.160 |
|              | Dominant      | TT       | 36 (66.7%) | 71 (56.4%) | 1.00 | 0.370 |
|              |               | CT-CC    | 18 (33.3%) | 55 (43.6%) | 1.61 (0.82–3.16) | 0.160 |
|              | Recessive     | TT-CT    | 52 (96.3%) | 119 (94.4%) | 1.00 | 0.600 |
|              |               | CC       | 2 (3.7%) | 7 (5.6%) | 1.52 (0.30–7.68) | 0.230 |
|              | Overdominant  | TT-CC    | 38 (70.4%) | 78 (61.9%) | 1.00 | 0.370 |
|              |               | CT       | 16 (29.6%) | 48 (38.1%) | 1.52 (0.76–3.05) | 0.370 |
| **TFPI**     | Codominant    | CC       | 43 (79.6%) | 103 (81.8%) | 1.00 | 0.530 |
|              |               | CT       | 11 (20.4%) | 22 (17.5%) | 0.74 (0.33–1.70) | 0.530 |
|              |               | TT       | 0 (0%) | 1 (0.8%) | NA (0.00–NA) | 0.530 |
|              | Dominant      | CC       | 43 (79.6%) | 103 (81.8%) | 1.00 | 0.570 |
|              |               | CT-TT    | 11 (20.4%) | 23 (18.2%) | 0.79 (0.35–1.78) | 0.570 |
|              | Recessive     | CC-CT    | 54 (100%) | 125 (99.2%) | 1.00 | 0.380 |
|              |               | TT       | 0 (0%) | 1 (0.8%) | NA (0.00–NA) | 0.380 |
|              | Overdominant  | CC-TT    | 43 (79.6%) | 104 (82.5%) | 1.00 | 0.470 |
|              |               | CT       | 11 (20.4%) | 22 (17.5%) | 0.74 (0.32–1.69) | 0.470 |
| **TLR9**     | Codominant    | TT       | 13 (24.1%) | 33 (26.2%) | 1.00 | 0.510 |
|              |               | CT       | 32 (59.3%) | 81 (64.3%) | 0.88 (0.41–1.90) | 0.510 |
|              |               | CC       | 9 (16.7%) | 12 (9.5%) | 0.54 (0.18–1.57) | 0.510 |
|              | Dominant      | TT       | 13 (24.1%) | 33 (26.2%) | 1.00 | 0.560 |
|              |               | CT-CC    | 41 (75.9%) | 93 (73.8%) | 0.80 (0.38–1.69) | 0.560 |
|              | Recessive     | TT-CT    | 45 (83.3%) | 114 (90.5%) | 1.00 | 0.270 |
|              |               | CC       | 9 (16.7%) | 12 (9.5%) | 0.59 (0.23–1.49) | 0.270 |
|              | Overdominant  | TT-CC    | 22 (40.7%) | 45 (35.7%) | 1.00 | 0.810 |
|              |               | CT       | 32 (59.3%) | 81 (64.3%) | 1.08 (0.56–2.11) | 0.810 |

* n, number; tPROM, term prelabor rupture of membranes; pPROM, preterm PROM; OR, odds ratio; 95% CI, confidence interval; p-value, statistically significant result is marked in bold; NA, not analyzed.
Table 3. Association of multiple-SNP variants for CSF2, FLT1, TLR9 and TFPI polymorphisms with PROM after correction by APTT and PLT parameters.

| Categorical Covariate | Polymorphisms/Alleles | Controls | PROM b Cases | OR c (95% CI d) | p-Value e |
|-----------------------|-----------------------|----------|-------------|----------------|-----------|
|                       | rs25881               | rs722503 | rs352140    | C-399T         |           |
| APTT f                | C                     | T        | -           | -              | 0.629     | 0.592 | 1.00 | — | 0.990 |
|                       | C                     | C        | -           | -              | 0.199     | 0.228 | 0.99 (0.53–1.86) | 0.990 |
|                       | T                     | T        | -           | -              | 0.118     | 0.181 | 2.39 (1.23–4.64) | 0.011 |
|                       | T                     | C        | -           | -              | 0.054     | 0.000 | 0.00 (−Inf–Inf) | 1.000 |
|                       | C                     | T        | T           | C              | 0.356     | 0.318 | 1.00 | — | 0.011 |
|                       | C                     | T        | T           | C              | 0.275     | 0.273 | 1.46 (0.67–3.16) | 0.340 |
|                       | C                     | C        | T           | C              | 0.134     | 0.122 | 0.74 (0.23–2.40) | 0.610 |
|                       | C                     | T        | T           | T              | 0.079     | 0.129 | 1.96 (0.80–4.83) | 0.150 |
|                       | T                     | T        | C           | C              | 0.064     | 0.106 | 3.17 (0.63–15.81) | 0.160 |
|                       | T                     | T        | C           | C              | 0.038     | 0.051 | 19.54 (1.22–311.80) | 0.037 |
|                       | T                     | C        | C           | -              | 0.026     | 0.000 | 0.00 (−Inf–Inf) | 1.000 |
|                       | -                     | -        | T           | C              | 0.555     | 0.503 | 1.00 | — | 0.028 |
|                       | -                     | -        | C           | C              | 0.364     | 0.400 | 1.94 (1.08–3.50) | 0.028 |
|                       | -                     | -        | T           | T              | 0.039     | 0.066 | 2.88 (0.50–16.61) | 0.240 |
|                       | -                     | -        | C           | T              | 0.041     | 0.031 | 0.80 (0.08–8.42) | 0.860 |
|                       | C                     | T        | T           | C              | 0.326     | 0.269 | 1.00 | — | 0.940 |
|                       | C                     | T        | C           | C              | 0.244     | 0.255 | 1.03 (0.44–2.46) | 0.002 |
|                       | C                     | C        | T           | C              | 0.125     | 0.115 | 0.04 (0.01–0.28) | 0.210 |
|                       | T                     | T        | T           | C              | 0.080     | 0.118 | 0.41 (0.10–1.67) | 0.009 |
|                       | T                     | T        | C           | C              | 0.053     | 0.094 | 13.87 (1.96–98.21) | 0.004 |
|                       | T                     | T        | C           | T              | 0.036     | 0.052 | 35.00 (3.14–390.81) | 0.004 |
|                       | C                     | T        | T           | T              | 0.029     | 0.056 | 1.60 (0.27–9.60) | 0.610 |
|                       | C                     | C        | C           | C              | 0.032     | 0.011 | 0.09 (0.00–2.29) | 0.140 |
|                       | T                     | C        | T           | C              | 0.026     | NA i     | 1.17 (0.20–8.86) | 0.860 |
|                       | C                     | T        | C           | T              | 0.030     | NA i     | 0.01 (0.00–1.30) | 0.066 |
|                       | C                     | C        | C           | T              | 0.010     | 0.019 | 2.67 (0.11–66.45) | 0.550 |
Table 3. Cont.

| Categorical Covariate | Polymorphisms/Alleles | Multiple-SNP Variant Frequency | OR \(^c\) (95% CI \(^d\)) | \(p\)-Value \(^e\) |
|------------------------|-----------------------|--------------------------------|--------------------------|------------------|
|                        | CSF2                  | FLT1                           | TLR9                     | TFPI             |
|                        | rs25881               | rs722503                        | rs352140                 | C-399T           |
| PLT \(^b\)             |                       |                                 |                          |                  |
| C                      | T                     | -                              | -                        | Controls         | 0.629 (0.592)   | 1.00               | —                  |
| C                      | T                     | -                              | -                        | Controls         | 0.199 (0.228)   | 1.15 (0.79–1.69)  | 0.460              |
| T                      | T                     | -                              | -                        | Controls         | 0.118 (0.181)   | 1.57 (1.01–2.43) | 0.045              |
| T                      | C                     | -                              | -                        | Controls         | 0.054 (0.000)   | 0.00 (−Inf–Inf)  | 1.000              |
| C                      | T                     | T                              | -                        | Controls         | 0.356 (0.318)   | 1.00               | —                  |
| C                      | T                     | C                              | -                        | Controls         | 0.275 (0.273)   | 1.17 (0.70–1.96) | 0.540              |
| C                      | T                     | T                              | -                        | Controls         | 0.134 (0.122)   | 1.01 (0.52–1.96) | 0.980              |
| T                      | T                     | T                              | -                        | Controls         | 0.079 (0.129)   | 1.67 (0.88–3.16) | 0.120              |
| C                      | C                     | C                              | -                        | Controls         | 0.064 (0.106)   | 1.61 (0.79–3.30) | 0.190              |
| T                      | T                     | C                              | -                        | Controls         | 0.038 (0.051)   | 1.66 (0.61–4.49) | 0.320              |
| T                      | C                     | T                              | -                        | Controls         | 0.026 (0.000)   | 0.13 (0.13–0.14) | 0.001              |
| T                      | C                     | C                              | -                        | Controls         | 0.030 (0.000)   | 0.00 (−Inf–Inf)  | 1.000              |
| -                      | T                     | T                              | C                        | Controls         | 0.405 (0.385)   | 1.00               | —                  |
| -                      | T                     | C                              | C                        | Controls         | 0.280 (0.309)   | 1.24 (0.78–1.98) | 0.370              |
| -                      | C                     | T                              | C                        | Controls         | 0.150 (0.119)   | 0.84 (0.46–1.54) | 0.570              |
| -                      | C                     | C                              | C                        | Controls         | 0.084 (0.090)   | 1.11 (0.57–2.17) | 0.770              |
| -                      | T                     | T                              | T                        | Controls         | 0.029 (0.066)   | 2.75 (1.05–7.19) | 0.040              |
| -                      | T                     | C                              | T                        | Controls         | 0.033 (0.012)   | 0.00 (−Inf–Inf)  | 1.000              |
| -                      | C                     | C                              | T                        | Controls         | 0.008 (0.019)   | 2.42 (0.29–20.09) | 0.410              |
| C                      | T                     | T                              | C                        | Controls         | 0.326 (0.269)   | 1.00               | —                  |
| C                      | T                     | C                              | C                        | Controls         | 0.244 (0.255)   | 1.50 (0.86–2.60) | 0.150              |
| C                      | T                     | C                              | C                        | Controls         | 0.125 (0.115)   | 1.10 (0.53–2.30) | 0.800              |
| T                      | T                     | C                              | C                        | Controls         | 0.080 (0.118)   | 1.81 (0.91–3.60) | 0.094              |
| C                      | C                     | C                              | C                        | Controls         | 0.053 (0.094)   | 1.92 (0.87–4.27) | 0.110              |
| C                      | T                     | T                              | T                        | Controls         | 0.029 (0.056)   | 2.70 (0.98–7.40) | 0.055              |
| T                      | T                     | C                              | C                        | Controls         | 0.036 (0.052)   | 1.91 (0.67–5.40) | 0.230              |
| T                      | C                     | C                              | C                        | Controls         | 0.026 (NA)      | 0.00 (−Inf–Inf)  | 1.000              |
| C                      | T                     | C                              | T                        | Controls         | 0.030 (NA)      | 0.00 (−Inf–Inf)  | 1.000              |
| T                      | C                     | T                              | C                        | Controls         | 0.032 (0.011)   | 0.31 (0.01–12.37) | 0.530              |
| C                      | T                     | C                              | T                        | Controls         | 0.010 (0.019)   | 1.88 (0.29–12.08) | 0.510              |
| Categorical Covariate | Polymorphisms/Alleles | Multiple-SNP a Variant Frequency | OR c (95% CI d) | p-Value e |
|-----------------------|-----------------------|---------------------------------|-----------------|-----------|
|                       | CSF2                  | FLTI                            | TLR9            | TFPI      |
|                       | rs25881               | rs722503                        | rs352140        | C-399T    |
| PDW h                 |                       |                                 |                 |           |
|                       | C                     | T                               | -               | -         |
|                       | C                     | T                               | -               | -         |
|                       | T                     | C                               | -               | -         |
|                       | T                     | C                               | -               | -         |
| MPV i                 |                       |                                 |                 |           |
|                       | C                     | T                               | -               | -         |
|                       | C                     | T                               | -               | -         |
|                       | T                     | C                               | -               | -         |
|                       | T                     | C                               | -               | -         |
| PCT i                 |                       |                                 |                 |           |
|                       | C                     | T                               | -               | -         |
|                       | C                     | T                               | -               | -         |
|                       | T                     | C                               | -               | -         |
|                       | T                     | C                               | -               | -         |
| PLT + MPV             |                       |                                 |                 |           |
|                       | C                     | T                               | -               | -         |
|                       | C                     | T                               | -               | -         |
|                       | T                     | C                               | -               | -         |
|                       | T                     | C                               | -               | -         |
| PDW + MPV             |                       |                                 |                 |           |
|                       | C                     | T                               | -               | -         |
|                       | C                     | T                               | -               | -         |
|                       | T                     | C                               | -               | -         |
|                       | T                     | C                               | -               | -         |
| MPV + PCT             |                       |                                 |                 |           |
|                       | C                     | T                               | -               | -         |
|                       | C                     | T                               | -               | -         |
|                       | T                     | C                               | -               | -         |
|                       | T                     | C                               | -               | -         |
| PLT + PDW + MPV       |                       |                                 |                 |           |
|                       | C                     | T                               | -               | -         |
|                       | C                     | T                               | -               | -         |
|                       | T                     | C                               | -               | -         |
|                       | T                     | C                               | -               | -         |
**Table 3. Cont.**

| Categorical Covariate | Polymorphisms/Alleles | Multiple-SNP Variant Frequency | OR (95% CI) | p-Value |
|-----------------------|-----------------------|--------------------------------|-------------|---------|
|                       | CSF2                  | FLT1                           | TLR9        | TFPI    | Controls | PROM b Cases |
|                       | rs25881               | rs722503                       | rs352140    | C-399T  |           |              |
| PLT + PDW + PCT       |                       |                                |             |         |           |              |
| C                     | T                     | -                              | -           |         | 0.629    | 0.592       | 1.00        | —        |
| C                     | C                     | -                              | -           |         | 0.199    | 0.228       | 1.24 (0.84–1.83) | 0.280    |
| T                     | T                     | -                              | -           |         | 0.118    | 0.181       | 1.70 (1.09–2.66) | **0.019**|
| T                     | C                     | -                              | -           |         | 0.054    | 0.000       | 0.00 (–Inf–Inf)  | 1.000    |
| PLT + MPV + PCT       |                       |                                |             |         |           |              |
| C                     | T                     | -                              | -           |         | 0.629    | 0.592       | 1.00        | —        |
| C                     | C                     | -                              | -           |         | 0.199    | 0.228       | 1.24 (0.84–1.83) | 0.280    |
| T                     | T                     | -                              | -           |         | 0.118    | 0.181       | 1.71 (1.09–2.66) | **0.019**|
| T                     | C                     | -                              | -           |         | 0.054    | 0.000       | 0.00 (–Inf–Inf)  | 1.000    |
| C                     | T                     | T                              | C           |         | 0.326    | 0.269       | 1.45 (0.82–2.56) | 0.200    |
| C                     | T                     | C                              | C           |         | 0.244    | 0.255       | 2.08 (1.02–4.23) | **0.045**|
| C                     | T                     | T                              | C           |         | 0.125    | 0.115       | 1.83 (0.65–5.15) | 0.260    |
| T                     | T                     | T                              | C           |         | 0.080    | 0.118       | 2.00 (0.89–4.48) | 0.092    |
| T                     | C                     | T                              | C           |         | 0.053    | 0.094       | 1.66 (1.06–2.60) | **0.026**|
| T                     | T                     | T                              | T           |         | 0.029    | 0.056       | 1.00        | —        |
| PDW + MPV + PCT       |                       |                                |             |         |           |              |
| C                     | T                     | -                              | -           |         | 0.629    | 0.592       | 1.00        | —        |
| C                     | C                     | -                              | -           |         | 0.199    | 0.228       | 1.22 (0.83–1.81) | 0.360    |
| T                     | T                     | -                              | -           |         | 0.118    | 0.181       | 1.69 (1.08–2.64) | **0.022**|
| T                     | C                     | -                              | -           |         | 0.054    | 0.000       | 1.00        | —        |

a SNP, single nucleotide polymorphism; b PROM, prelabor rupture of membranes; c OR, odds ratio; d 95% CI, confidence interval; e p-value, statistically significant results are marked in bold; f APTT, activated partial thromboplastin time; g PLT, platelet; h PDW, platelet distribution width; i MPV, mean platelet volume; j PCT, plateletcrit; k Inf, infinity; l NA, not analyzed.
A comparison between the women with pPROM and the healthy controls showed that TT double-SNP variants for \textit{CSF2} and \textit{FLT1} polymorphisms and TTT triple-SNP variants for \textit{CSF2}, \textit{FLT1} and \textit{TLR9} polymorphisms correlated with an increased risk of disease when corrected by APTT and PLT parameters ($p \leq 0.050$, see Tables S8–S10). Adjusting the results for pregnancy disorders also revealed that the TT variants for \textit{CSF2} and \textit{FLI} T SNPs were associated with an approximately two-fold increase in the pPROM risk ($p \leq 0.050$, see Table 4), while the TCT complex variants for the \textit{CSF2}, \textit{FLT1} and \textit{TLR9} SNPs correlated with a reduced risk of disease, corrected by a serological conflict (OR 0.01 95% CI 0.00–0.05, $p \leq 0.001$, see Table 4). The complex TCC and TTT variants for the \textit{FLT1}, \textit{TLR9} and \textit{TFPI} SNPs were correlated with a significantly higher risk of pPROM when considering the APTT and PLT parameters ($p \leq 0.050$). In turn, triple-SNP CCC variants for those three polymorphisms were associated with a significantly lower risk of pPROM after adjusting to APTT (OR 0.04 95% CI 0.00–0.66, $p = 0.026$). The CCC variants for \textit{CSF2}, \textit{FLT1} and \textit{TFPI} polymorphisms were also associated with an increased risk of pPROM after adjusting for PLT parameters (OR 1.65 95% CI 1.03–2.64, $p = 0.036$).

Moreover, quadruple-SNP variants CTTT and TTTC for \textit{CSF2}, \textit{FLT1}, \textit{TLR9} and \textit{TFPI} polymorphisms were also found to correlate with a higher risk of pPROM when adjusted for PLT parameters.

A further analysis showed that different double-, triple- and quadruple-SNP variants for the studied polymorphisms with C alleles in \textit{CSF2} rs25881, \textit{FLT1} rs722503, \textit{TFPI} C-399T SNPs and the T allele in \textit{TLR9} rs352140 polymorphism were significantly more frequent in the women with pPROM compared to the PROM subjects, after adjusting for APTT, PLT parameters and pregnancy disorders ($p \leq 0.050$, see Tables S9 and S10). The complex TTTT variants for all the tested SNPs were more common in the pPROM women than in those with tPROM, corrected by PLT and PDW (OR 6.05 95% CI 4.71–7.78, $p \leq 0.001$).
Table 4. Relationship between multiple-SNP variants for CSF2, FLT1 and TLR9 polymorphisms and occurrence of preterm PROM after correction for pregnancy disorders.

| Pregnancy Disorders                  | Polymorphisms/Alleles | Multiple-SNP a Variant Frequency | OR c (95% CI d) | p-Value e |
|--------------------------------------|------------------------|----------------------------------|-----------------|-----------|
|                                      | CSF2 rs25881           | FLT1 rs722503                     | TLR9 rs352140   | Controls  | pPROM b Cases |                   |
| Asthma and respiratory system infections |                        |                                  |                 | 0.629     | 0.568      | 1.00  | 1.39 (0.92–2.09) | 0.110 |
|                                       | C                     | T                                |                 | 0.199     | 0.246      | 1.30 (1.06–2.78) | 0.028 |
|                                       | T                     | T                                |                 | 0.118     | 0.187      | 1.00  | —                 |
|                                       | T                     | C                                |                 | 0.054     | 0.000      | 0.00 (−Inf–Inf) | 1.000 |
|                                       | C                     | T                                |                 | 0.356     | 0.299      | 1.00  | —                 |
|                                       | C                     | T                                |                 | 0.275     | 0.276      | 1.36 (0.76–2.43) | 0.300 |
|                                       | C                     | C                                |                 | 0.134     | 0.150      | 1.59 (0.77–3.28) | 0.210 |
|                                       | T                     | T                                |                 | 0.079     | 0.134      | 1.98 (0.97–4.04) | 0.065 |
|                                       | C                     | C                                |                 | 0.064     | 0.089      | 1.69 (0.73–3.93) | 0.220 |
|                                       | T                     | C                                |                 | 0.038     | 0.045      | 2.09 (0.65–6.76) | 0.220 |
|                                       | T                     | C                                |                 | 0.030     | 0.008      | 0.00 (−Inf–Inf) | 1.000 |
|                                       | T                     | T                                |                 | 0.026     | 0.000      | 0.00 (−Inf–Inf) | 1.000 |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | C         | T          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
|                                        |                        |                                  |                 | T         | C          |                   |
|                                        |                        |                                  |                 | T         | T          |                   |
| Pregnancy Disorders | Polymorphisms/Alleles | Multiple-SNP a Variant Frequency | OR c (95% CI d) | p-Value e |
|---------------------|-----------------------|----------------------------------|-----------------|----------|
|                     | CSF2 rs25881 FLT1 rs722503 TLR9 rs352140 | Controls | pPROM b Cases |                      |
| **Diabetes mellitus** | C T - | 0.629 | 0.568 | 1.00 | — |
|                     | C C - | 0.199 | 0.246 | 1.35 (0.90–2.02) | 0.150 |
|                     | T T - | 0.118 | 0.187 | 1.72 (1.06–2.77) | 0.027 |
|                     | T C - | 0.054 | 0.000 | 0.00 (–Inf–Inf) | 1.000 |
|                     | C T T | 0.356 | 0.299 | 1.00 | — |
|                     | C T C | 0.275 | 0.276 | 1.29 (0.61–2.72) | 0.510 |
|                     | C C T | 0.134 | 0.150 | 1.41 (0.52–3.77) | 0.500 |
|                     | T T T | 0.079 | 0.134 | 1.94 (0.93–4.05) | 0.080 |
|                     | C C C | 0.064 | 0.089 | 1.63 (0.50–5.34) | 0.420 |
|                     | T T C | 0.038 | 0.045 | 1.73 (0.03–95.93) | 0.790 |
|                     | T C C | 0.030 | 0.008 | 0.11 (0.00–8.01 × 10^{15}) | 0.910 |
|                     | T C T | 0.026 | 0.000 | 0.11 (0.00–7.18 × 10^{11}) | 0.880 |
| **Hypertension** | C T - | 0.629 | 0.568 | 1.00 | — |
|                     | C C - | 0.199 | 0.246 | 1.35 (0.90–2.03) | 0.140 |
|                     | T T - | 0.118 | 0.187 | 1.71 (1.06–2.75) | 0.029 |
|                     | T C - | 0.054 | 0.000 | 0.00 (–Inf–Inf) | 1.000 |
|                     | C T T | 0.356 | 0.299 | 1.00 | — |
|                     | C T C | 0.275 | 0.276 | 1.26 (0.71–2.23) | 0.440 |
|                     | C C T | 0.134 | 0.150 | 1.46 (0.71–2.99) | 0.300 |
|                     | T T T | 0.079 | 0.134 | 1.92 (0.94–3.92) | 0.074 |
|                     | C C C | 0.064 | 0.089 | 1.62 (0.70–3.75) | 0.260 |
|                     | T T C | 0.038 | 0.045 | 1.90 (0.59–6.12) | 0.280 |
|                     | T C C | 0.030 | 0.008 | 0.00 (–Inf–Inf) | 1.000 |
|                     | T C T | 0.026 | 0.000 | 0.00 (–Inf–Inf) | 1.000 |
| Pregnancy Disorders | Polymorphisms/Alleles | Multiple-SNP<sup>a</sup> Variant Frequency | OR<sup>c</sup> (95% CI<sup>d</sup>) | p-Value<sup>e</sup> |
|---------------------|-----------------------|---------------------------------|---------------------------------|-----------------|
| CSF2 rs25881 | FLT1 rs722503 | TLR9 rs352140 | Controls | pPROM<sup>b</sup> | Cases |
| Hypothyroidism | | | C | T | - | 0.629 | 0.568 | 1.00 | — |
| | | | C | C | - | 0.199 | 0.246 | 1.36 (0.90–2.04) | 0.140 |
| | | | T | T | - | 0.118 | 0.187 | 1.71 (1.06–2.77) | 0.029 |
| | | | T | C | - | 0.054 | 0.000 | 0.00 (~Inf–Inf) | 1.000 |
| | | | C | T | T | 0.356 | 0.299 | 1.00 | — |
| | | | C | T | C | 0.275 | 0.276 | 1.28 (0.72–2.26) | 0.400 |
| | | | C | C | T | 0.134 | 0.150 | 1.47 (0.72–2.98) | 0.290 |
| | | | T | T | T | 0.079 | 0.134 | 2.00 (0.98–4.10) | 0.058 |
| | | | T | C | C | 0.064 | 0.089 | 1.65 (0.72–3.80) | 0.240 |
| | | | T | T | C | 0.038 | 0.045 | 1.76 (0.53–5.79) | 0.360 |
| | | | T | C | C | 0.030 | 0.008 | 0.01 (~Inf–Inf) | 1.000 |
| | | | T | C | T | 0.026 | 0.000 | 0.00 (~Inf–Inf) | 1.000 |
| Serological conflict | | | C | T | - | 0.629 | 0.568 | 1.00 | — |
| | | | C | C | - | 0.199 | 0.246 | 1.32 (0.88–1.98) | 0.180 |
| | | | T | T | - | 0.118 | 0.187 | 1.66 (1.03–2.69) | 0.039 |
| | | | T | C | - | 0.054 | 0.000 | 0.00 (~Inf–Inf) | 1.000 |
| | | | C | T | T | 0.356 | 0.299 | 1.00 | — |
| | | | C | T | C | 0.275 | 0.276 | 1.27 (0.68–2.39) | 0.450 |
| | | | C | C | T | 0.134 | 0.150 | 1.37 (0.65–2.86) | 0.410 |
| | | | T | T | T | 0.079 | 0.134 | 1.93 (0.94–3.96) | 0.076 |
| | | | T | C | C | 0.064 | 0.089 | 1.63 (0.64–4.14) | 0.310 |
| | | | T | T | C | 0.038 | 0.045 | 1.53 (0.18–12.75) | 0.690 |
| | | | T | C | C | 0.030 | 0.008 | 0.14 (0.00–1.44 × 10<sup>6</sup>) | 0.810 |
| | | | T | C | T | 0.026 | 0.000 | 0.01 (0.00–0.05) | <0.001 |
Table 4. Cont.

| Pregnancy Disorders                   | Polymorphisms/Alleles | Multiple-SNP $^a$ Variant Frequency | OR $^c$ (95% CI $^d$) | p-Value $^e$ |
|---------------------------------------|-----------------------|-------------------------------------|------------------------|-------------|
|                                       | CSF2 rs25881          | FLT1 rs722503                       | TL9 rs352140           | Controls    | pPROM $^b$ Cases |                                      |
| Threatened miscarriage                | C                     | T                                  | -                      | 0.629       | 0.568        | 1.00                        | —                                      |
|                                       | C                     | C                                  | -                      | 0.199       | 0.246        | 1.34 (0.89–2.02) | 0.170                   |
|                                       | T                     | T                                  | -                      | 0.118       | 0.187        | 1.69 (1.03–2.77) | 0.037                   |
|                                       | T                     | C                                  | -                      | 0.054       | 0.000        | 0.00 (−Inf–Inf)  | 1.000                   |
|                                       | C                     | T                                  | T                      | 0.356       | 0.299        | 1.00                        | —                                      |
|                                       | C                     | T                                  | C                      | 0.275       | 0.276        | 1.25 (0.70–2.23) | 0.460                   |
|                                       | C                     | C                                  | T                      | 0.134       | 0.150        | 1.54 (0.71–3.36) | 0.280                   |
|                                       | T                     | T                                  | T                      | 0.079       | 0.134        | 1.94 (0.90–4.21) | 0.094                   |
|                                       | C                     | C                                  | C                      | 0.064       | 0.089        | 1.34 (0.55–3.28) | 0.530                   |
|                                       | T                     | T                                  | C                      | 0.038       | 0.045        | 1.66 (0.44–6.24) | 0.450                   |
|                                       | T                     | C                                  | C                      | 0.030       | 0.008        | 0.00 (−Inf–Inf)  | 1.000                   |
|                                       | T                     | C                                  | T                      | 0.026       | 0.000        | 0.22 (0.00–1.71 × 10$^3$) | 0.740 |
| Urogenital infections                 | C                     | T                                  | -                      | 0.629       | 0.568        | 1.00                        | —                                      |
|                                       | C                     | C                                  | -                      | 0.199       | 0.246        | 1.35 (0.90–2.02) | 0.150                   |
|                                       | T                     | T                                  | -                      | 0.118       | 0.187        | 1.70 (1.03–2.75) | 0.031                   |
|                                       | T                     | C                                  | -                      | 0.054       | 0.000        | 0.00 (−Inf–Inf)  | 1.000                   |
|                                       | C                     | T                                  | T                      | 0.356       | 0.299        | 1.00                        | —                                      |
|                                       | C                     | T                                  | C                      | 0.275       | 0.276        | 1.30 (0.71–2.39) | 0.390                   |
|                                       | C                     | C                                  | T                      | 0.134       | 0.150        | 1.41 (0.63–3.14) | 0.410                   |
|                                       | T                     | T                                  | T                      | 0.079       | 0.134        | 1.91 (0.91–4.00) | 0.089                   |
|                                       | C                     | C                                  | C                      | 0.064       | 0.089        | 1.57 (0.62–3.99) | 0.350                   |
|                                       | T                     | T                                  | C                      | 0.038       | 0.045        | 1.58 (0.21–12.03) | 0.660                   |
|                                       | T                     | C                                  | C                      | 0.030       | 0.008        | 0.23 (0.00–1.33 × 10$^3$) | 0.740 |
|                                       | T                     | C                                  | T                      | 0.026       | 0.000        | 0.14 (0.00–4.16 × 10$^5$) | 0.790 |

$^a$ SNP, single nucleotide polymorphism; $^b$ pPROM, preterm prelabor rupture of membranes; $^c$ OR, odds ratio; $^d$ 95% CI, confidence interval; $^e$ p-value, statistically significant results are marked in bold; $^f$ Inf, infinity.
4. Discussion

We found in our study that genetic changes, localized in SNPs from *CSF2*, *FLT1*, *TFPI* and *TLR9* genes, were associated with PROM, when the results had been adjusted for APTT, PLT parameters or pregnancy disorders. Among the double-SNP variants, the TT complex genotypes for *CSF2* rs25881 and *FLT1* rs722503 polymorphisms correlated with an increased risk of PROM and pPROM, after adjusting for APTT, PLT parameters or pregnancy disorders. In women of European and American origin, *CSF2* rs25881 was found to be associated with preterm birth; however, no predelivery events were defined, such as preterm labor or spontaneous rupture of membranes [39]. Regarding *FLT1* rs722503, the T allele was previously reported to be associated with an increased risk of preeclampsia in populations of Iranian and white pregnant women [42,53]. Similarly, AA homozygotes in rs722503 were estimated to be susceptible to preeclampsia in women from the Philippines [54]. We also found that the CC double-SNP variants for the *TLR9* rs352140 and *TFPI* C-399T SNPs were associated with an approximately two-fold higher risk of PROM when the results were corrected by APTT. In patients with stable coronary heart disease, the T allele in the *TFPI* C-399T polymorphism was correlated with an increased thrombin generation in vivo, and TT homozygotes were associated with an extended ex vivo thrombin generation delay time [55]. Moreover, in cultured human coronary artery endothelial cells, TLR9 was found to shift the TF and TFPI balance towards a procoagulant phenotype when induced by bacterial DNA [38]. In the case of TF, increased mRNA and protein levels and activity were determined, while *TFPI* had lower transcription, secretion and activity [38]. Considering our results, a combined participation of the tested SNPs from *TLR9* and *TFPI* genes, involved in hemostasis in PROM, seems likely.

Regarding the coagulation parameters, it was previously shown that prothrombin time (PT) and APTT were significantly shorter in preterm labor [56]. In the case of PLT parameters, the PLT count, as well as MPV and PCT, were also shown as correlating with PROM [57]. Among women with pPROM in the first trimester, a significantly higher PLT count and reduced MPV values were determined compared to healthy controls [57]. In our cohort of women, we also found significantly lower MPV values in the pPROM cases than in the healthy controls. Therefore, it seems important to adjust the genetic results of this study to the APTT and PLT parameters. To date, MPV reduction has been associated with chronic inflammatory disorders, including inflammatory bowel disease, rheumatoid arthritis, acute rheumatic fever and ankylosing spondylitis [58].

Taking into account triple-SNP variants for *CSF2*, *FLT1* and *TLR9* polymorphisms, we found that TTC variants correlated with an approximately 20-fold increased risk of PROM when adjusted by APTT, while TCT variants were associated with a decreased risk of the disease after correction by the PLT count. Among the pPROM cases, the TTT complex variants were significantly more prevalent compared to the healthy controls when the results were adjusted for PLT parameters. Conversely, TCT variants were correlated with a reduced risk of pPROM after adjusting for serological conflict. Regarding *TLR9* rs352140, CT and/or TT genotypes were previously found to be involved in placental inflammation and maternal pattern of inflammation [46]. Another study, performed in the Polish population, showed *TLR9* rs352140 as a possible genetic risk factor for cervical cancer [59]. In terms of our results, it also seems possible that *CSF2*, *FLT1* and *TLR9* SNPs collaborate in the development of PROM. It is noteworthy that TLR9 has been reported to inhibit angiogenesis by downregulating VEGFA and upregulating sFLT1 in placentas from an animal model of preeclampsia and in trophoblasts [33]. Similarly, the TLR9 ligand, oligodeoxynucleotide (ODN) 1826, has been found to induce sFLT1 secretion from macrophages and decrease the number of aortic ring vessel sprouts [60]. In the suture-induced corneal angiogenesis model, ODN 1826 has also been determined to reduce the length and volume of hemangiogenesis and lymphangiogenesis [60]. We found in our study that TTT triple-SNP variants for *FLT1*, *TLR9* and *TFPI* polymorphisms were associated with an increased risk of PROM and pPROM when adjusted by APTT or PLT parameters. Similarly, TCC variants were more common in the women with pPROM than
in the healthy controls, while CCC complex variants correlated with a decreased risk of the disease when corrected by APTT. Taking into account the results, obtained also by the quadruple-SNP analysis, the participation of the four studied polymorphisms in PROM seems justified. An additional comparison between the pPROM and tPROM cases showed that the C alleles for the CSF2, FLT1 and TFPI polymorphisms, and the T allele for the TLR9 SNP in different complex variants, were more common in the women with pPROM after adjusting for APTT, PLT parameters and pregnancy disorders.

In this study, we corrected the results for the following complications of pregnancy: asthma and respiratory infections, bleeding, DM, hypertension, hypothyroidism, serological conflict, threatened miscarriage and urogenital infections. So far, many studies have described the relationship between these diseases and the occurrence of PROM [61–65]. DM-adjusted analyses showed that CSF2 rs25881 CT heterozygotes are significantly more common in pPROM compared to tPROM cases. Similarly, the TT double-SNP variants for the CSF2 and FLT1 polymorphisms had an approximately two-fold increased risk of PROM when the results were adjusted for DM and other pregnancy disorders. Previously, pre-pregnancy DM, as well as nulliparity, maternal age and body mass index (BMI), were termed the predictors of pPROM [66]. In another study, gestational DM (GDM) was associated with an increased incidence of vulvovaginal candidiasis, PROM, preterm labor, chorioamnionitis/puerperal infection, as well as macrosomia, and neonatal hypoglycemia [67]. Asthma was found to be positively associated with PROM, both at preterm and term pregnancies, while chronic bronchitis correlated with a reduced risk of the disease [68]. In turn, vaginal bleeding has been shown to correlate with a shorter gestational age at membrane rupture and delivery, as well as lower birth weight, more frequent placental abruption, RDS, IVH and perinatal death [69]. Considering hypertensive disorders, gestational hypertension was found to be associated with an approximately four-fold increased risk of PROM, while preeclampsia was correlated with about two-fold higher risk [70]. Moreover, hypertension was associated with an increased risk of term rather than preterm PROM [70]. In turn, hypothyroidism in pregnancy was correlated with a slight FGR, a higher risk of PROM and the development of hypertension and GDM [62]. Similarly, threatened miscarriage was associated with much more frequent PROM, preterm labor and low-birth-weight newborns [71]. PPROM was found more prevalent in women with threatened miscarriage and in a high-risk group with the risk factor for spontaneous abortion compared to healthy controls [72]. Another significant risk factor for subsequent pPROM turned out to be the composition of the vaginal microbiota, and vaginal dysbiosis was correlated with unfavorable short-term outcomes of mothers and newborns [73,74]. Therefore, it is important to correct the obtained genetic results for the presented study population of pregnant women for possible risk factors of PROM.

5. Conclusions

Polymorphisms of the genes involved in hemostasis and angiogenesis, including CSF2 rs25881, FLT1 rs722503, TFPI C-399T and TLR9 rs352140, contribute to PROM.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/genes12111725/s1, Table S1: Characteristics of women with prelabor rupture of membranes, Table S2: PCR-RFLP assays and genotype profiles for CSF2, FLT1, TL9, and TFPI polymorphisms, Table S3a: Distribution of genotypes of CSF2, FLT1, TFPI, and TLR9 polymorphisms between women with PROM and healthy controls, Table S3b: Relationship between genotypes of CSF2, FLT1, TFPI, and TLR9 SNPs and the incidence of preterm prelabor rupture of membranes, Table S4: Distribution of genotypes of CSF2, FLT1, TFPI, and TLR9 SNPs between women with term and preterm PROM, Table S5: Distribution of alleles from CSF2, FLT1, TFPI, and TLR9 polymorphisms in women with PROM and healthy controls, Table S6: Incidence of alleles for CSF2, FLT1, TFPI, and TLR9 polymorphisms in women with PROM and healthy controls, Table S7: Inclusion of allelic factors for CSF2 and FLT1 polymorphisms and the incidence of PROM, adjusted to the pregnancy disorders, Table S8: Multiple-SNP variants for CSF2, FLT1, TLR9, and TFPI polymorphisms and the incidence of preterm PROM, after adjusting for APTT and PLT parameters, Table S9: Differences in the prevalence
of multiple-SNP variants for CSF2, FLT1, TLR9, and TFPI polymorphisms between women with term and preterm PROM, corrected for APTT and PLT parameters, Table S10: Distribution of multiple-SNP variants for CSF2, FLT1, TLR9, and TFPI polymorphisms between women with term and preterm PROM, corrected for pregnancy disorders.

Author Contributions: Conceptualization, W.I.W., M.K. (Marian Kacerovsky) and M.G.; data curation, W.I.W.; formal analysis, W.I.W., M.K. (Marian Kacerovsky), M.K. (Michał Krekora), P.K. and M.G.; funding acquisition, W.I.W. and M.G.; investigation, W.I.W., M.K. (Michał Krekora), P.K. and M.G.; methodology, W.I.W., M.K. (Michał Krekora), P.K. and M.G.; project administration, W.I.W.; resources, W.I.W., M.K. (Michał Krekora), P.K. and M.G.; supervision, M.G.; visualization, W.I.W.; writing—original draft, W.I.W.; writing—review and editing, W.I.W., M.K. (Marian Kacerovsky), M.K. (Michał Krekora), P.K. and M.G. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the Polish Ministry of Science and Higher Education, Polish Mother’s Memorial Hospital-Research Institute (grant supporting statutory research).

Institutional Review Board Statement: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Research Ethics Committee at the Polish Mother’s Memorial Hospital-Research Institute (approval numbers: 14/2019 and 15/2019).

Informed Consent Statement: Informed consent was obtained from all individual participants included in the study.

Data Availability Statement: All data and materials, as well as software application, support the published claims and comply with field standards.

Conflicts of Interest: The authors have no conflicts of interest to declare that are relevant to the content of this article.

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