Inherited thrombophilia and recurrent pregnancy loss: a single-center case-control study in North-Western Algeria

Ilhem Nassour-Mokhtari 1, Bouchra Loukidi 1, Abdellatif Moussouni 2,3*, Reda Bettioui 3, Riad Benhabib 4, Hafida Merzouk 1, Amaria Aouar 3 and Katia Allal-Taouli 1,5

Abstract

Background: Recurrent pregnancy loss (RPL) is a common disorder that affects around 3 to 5% of pregnant women. It has different causes, and in about 50%, it is of unknown etiology. Thrombophilia might increase the risk of RPL by adversely affecting the normal placental vascular function. Our study aimed to determine the frequency of factor V Leiden (FVL) and prothrombin G20210A gene mutations in Algerian women with RPL and to correlate their presence with the occurrence of such health's problem. A total of 80 women with previous fetal losses and 100 age-matched women with no history of fetal loss were recorded. Participants were tested for activated protein C resistance (APCR), protein C (PC), protein S (PS), and antithrombin (AT) deficiencies. The screening of FVL and prothrombin G20210A mutations was also done using a duplex polymerase chain reaction.

Results: APCR was detected in 6.25% of cases and was absent in controls (p = 0.011). PC and PS deficiencies were documented in 7.5% of patients. FVL was detected in 8.33% of patients and was absent in controls (p = 0.047). Prothrombin G20210A mutation was found in 8.33% of patients compared to 11.11% of controls (p = 0.631). A significant association of FVL mutation with the abortion which occurred in the second trimester was found (p = 0.001).

Conclusion: There is a significant association between FVL mutation and RPL especially the loss occurring during the second trimester. No correlation was found regarding prothrombin G20210A mutation.

Keywords: Recurrent pregnancy loss, Thrombophilia, FVL, APCR

Background

Recurrent pregnancy loss (RPL) represents a significant health problem with a rate of 5% among women in the reproductive age [1]. RPL or miscarriage can be defined as the loss of three or more successive pregnancies before viability and includes all pregnancy losses from the time of conception until 24 weeks of gestation [2]. Miscarriages can be subdivided into early pregnancy losses (which are most common) that occur before the 12th week of gestation and late pregnancy losses that occur in the 12th week to 21st week of gestation [3, 4]. The cause of recurrent miscarriage remains unknown (idiopathic) in more than 50% of cases [5]; however, various genetic, anatomical, endocrine, and infective factors as well as thrombophilic states have been implicated [6].

Thrombophilia is defined as a predisposition to arterial or venous thrombotic complications as a result of hemostatic system defects [7]. It may be acquired, like the antiphospholipid syndrome, or inherited [8]. Thrombotic disorders are common cause of RPL and may be seen in 40–50% of cases [9]. In fact, pregnancy is a hypercoagulable state, and if the pregnancy is affected by
thrombophilia, the hypercoagulable state becomes worse and may impair blood flow through the maternal veins, leading to deep vein thrombosis, and clots in the placental blood vessels, leading to fetal growth restriction and/or fetal demise [10]. Factors associated with thrombophilia include factor V Leiden (FVL) mutation, activated protein C resistance (APCR), prothrombin G20210A gene mutation, protein C (PC) deficiency, protein S (PS) deficiency, antithrombin III (AT III) deficiency, and endothelial cell dysfunction [11].

The polymorphisms G20210A of prothrombin gene and G1691A of factor V gene are the most extensively studied thrombophilic mutations in association to recurrent miscarriage [12]. Previous studies have reported prevalence of FVL mutation among women with recurrent miscarriage ranging from 3 to 42% [13], while its prevalence in Caucasian population is 4 to 7% [14]. FVL mutation is autosomal dominant disorder in which the glutamine to arginine missense mutation occurs at nucleotide 1691 of the factor V gene [15]. The resulting arginine (Arg) at amino acid 506 is substituted with glutamine (Gln), and this factor V mutation induces the APCR and contributes to increased risk of thrombosis [15].

The prothrombin G20210A mutation results from G to A substitution at position 20,210 in the 3’-untranslated region of the prothrombin gene; it induces high plasma levels of thrombin [16] and facilitates clot formation in heterozygous individuals, who have a twofold higher risk of clotting in comparison to non-carriers [17, 18].

Many studies done worldwide have shown a significant correlation between FVL and RPL [19]. This approach may be helpful to solve this major health problem by an appropriate antithrombotic treatment [20]. In Algeria, there is a lack of data regarding FVL and thrombophilia implication in general in recurrent abortions. For this reason, a case-control study was planned.

The main goal of this study was to determine the frequency of prothrombin G20210A and FVL polymorphisms and to correlate their presence with RPL occurring in local population. The frequencies of APCR, PC, and PS deficiencies were also determined.

Methods

Patients and controls

The case-control study was conducted at the laboratory of Hematology, Hospital of Tlemcen, Algeria, from January 2016 to July 2017, after getting approval from the institutional ethics committee. Informed consent was obtained from all the cases and the controls.

Eighty women of reproductive age (19–45 years) with history of at least 3 unexplained miscarriages before 10 weeks of amenorrhoea (WA) or at least 2 late miscarriages between 10 and 16 WA or at least one intrauterine fetal demise (IUFD) beyond 16 weeks were included as cases, while 100 age-matched females with at least one live-born children and no personal history of pregnancy loss were taken as controls. The definition of IUFD in terms of gestational age varies across geographical settings [21]. In our study, we defined it as the death of an unborn baby at 16 weeks’ gestation or more.

Those excluded from among the cases were patients with chronic pathologies (arterial hypertension, diabetes), pregnant ones, and women taking anticoagulant medications.

A uniform questionnaire was used to collect information about age, residency, parity, medical and obstetric history, consanguineous marriage, and familial history of miscarriage. Data were collected by direct interview between the researcher and each participant.

Laboratory evaluation

Venous blood was collected on 0.109 M tri-sodium citrate and was centrifuged twice at 2500 g for 15 min at room temperature in order to obtain plasma with relatively few remaining platelets. Plasma was then frozen and stored in small aliquots at –20 °C until tested. Ethylene diamine tetraacetic acid (EDTA) anticoagulant samples were used for deoxyribonucleic acid (DNA) analysis. EDTA blood was immediately stored at – 40 °C.

Women in both the control and study groups underwent the below-mentioned tests: prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen, APCR, and prothrombin. Women in the study group additionally underwent the following tests: PC, PS, and AT.

Phenotypic tests

The analysis was carried out at the Thrombosis Unit. Before examining the blood sample, the plasma was thawed in a water bath for 15 min at a temperature of 37 °C. All tests were performed by the fully automatic clinical laboratory analyzer “STA COMPACT” using “Diagnostica Stago” reagents. The laboratory investigation was initiated with global coagulation tests, including PT, APTT, and fibrinogen to assess blood clotting function.

The principle of the APCR study is a disproportionate prolongation of the clotting time, in the presence of APC and calcium. Before the test plasma is mixed with factor V deficient plasma, to ensure the normal starting concentration of the other factors. Clotting is initiated with Crotalus viridis helleri poison, which activates factor X [22]. Patients presenting a clotting time of 120 s or more were considered as APCR negative, whereas those with a clotting time less than 120 s were considered as APCR positive. For the prothrombin, the plasma level was determined.
Functional assays for PC are either coagulometric or chromogenic methods. Both types of assays are based on the activation of PC in patient plasma using snake venom. The coagulometric assays are based on the ability of a patient’s activated PC to degrade activated factors V and VIII, thereby prolonging the APTT-based clotting time. Laboratory investigation of PS deficiency is a clot-based method that measures the ability of PS to serve as a cofactor for activated PC, augmenting degradation of activated factors V and VIII and thereby prolonging clotting time. About the AT, the functional assays are chromogenic methods that measure AT activity related to its ability to inhibit thrombin (activated prothrombin) or activated factor X [23].

**Genotypic tests**

Due to financial considerations, the number of subjects was reduced to 60 in the study group and to 45 in the control group. Genomic DNA was extracted from blood samples using Wizard® Genomic DNA Purification Kit from Promega.

FVL and prothrombin G20210A analysis was performed using a duplex PCR followed by restriction using a single endonuclease, HindIII. Primers for the factor V gene G1691A determination and those for the prothrombin gene G20210A determination are mentioned in Table 1. Thermocycling conditions are 94°C (1 min), 58°C (1 min), and 72°C (2 min) for 40 cycles. The PCR products were digested with 15 U of HindIII restriction enzyme. The restricted products were separated by electrophoresis through a 2% agarose gel stained with ethidium bromide and directly visualized under UV light. Figure 1 shows the different migration patterns observed. For both factor V and prothrombin alleles, the normal genotypes produce undigested PCR products (241 and 345 base pair (bp), respectively), whereas mutated homozygous lead to restricted fragments (209 + 32 and 322 + 23 bp, respectively). The heterozygous patterns are characterized by the presence of undigested and digested amplified fragments [24].

**Statistical analysis**

The statistical analysis was carried out using MINITAB (18.1). Continuous variables were expressed as mean, whereas categorical data were expressed in the form of frequency. The comparison between patient and control groups and the association between patients’ clinical features and laboratory analysis were assessed using the chi-square (χ²) test. A p value < 0.05 was considered statistically significant.

**Results**

A total of 180 subjects were included: 80 (44.44%) cases and 100 controls. The demographic data of all participants are represented in Table 2.

Among the cases, the percentage of patients presenting a PC, PS, or AT deficiency is represented in Table 3. By comparing the results of the screening tests between case and control groups, no significant difference has been found in the following parameters: APTT (p = 0.051), fibrinogen (p = 0.275), and prothrombin (p = 0.434); however, the difference was statistically significant concerning PT (p = 0.024) and APCR (p = 0.011) (Table 4). Five cases of APCR were found in the patient group, whereas APCR was absent in controls.

FVL mutation was present in 5 (8.33%) cases with the heterozygous form, while it was absent in all the controls (Table 5). FVL mutation was significantly associated with RPL (p = 0.047).

Prothrombin G20210A polymorphism was present in 5 (8.33%) cases and in 5 (11.11%) controls; consequently, no association was found between this polymorphism and the occurrence of RPL (p = 0.631) (Table 5).

Regarding the clinical features of patients, a significant correlation was found between the age and type of pregnancy loss (p = 0.01) and between age and number of pregnancy loss (p = 0.009). In fact, women older than 35 years presented more IUFD and have a high number of pregnancy loss (> 3).

A significant correlation was also found between FVL and type of pregnancy loss (p = 0.001); the IUFD was more frequent than miscarriage in heterozygous patients.

### Table 1: Specific primer sequences, restriction enzymes, and restriction digestion products’ sizes for FVL and prothrombin G20210A.

| Gene   | Length (bp) | Primer’s sequence | Restriction enzyme | Restriction digestion product size (bp) |
|--------|-------------|-------------------|-------------------|----------------------------------------|
|        |             |                   |                   | Normal | Heterozygous | Homozygous |
| FVL    | 241         | Forward: 5’TCAAGGCAGGAACACACCACC-3’ Reverse: 5’GGTTACTTCAAGGACAAGAAATCCTGTAAGCT-3’ | HindIII | 241 | 241 + 209 + 32 | 209 + 32 |
| Prothrombin | 345    | Forward: 5’TCTAGAAACAGTGGCTTGCC-3’ Reverse: 5’ATAGCAGTGGAGCATTGAAG-3’ | HindIII | 345 | 345 + 322 + 23 | 322 + 23 |
Discussion
Though the role of the two common thrombophilic mutations (FVL, prothrombin G20210A) in fetal loss has been well-studied in different populations, their real impact is still under debate. Therefore, it is of great importance to explore the association between these mutations and RPL in Algerian women. According to the results of our study, a significant correlation was found between FVL and RPL occurrence, while no association was observed regarding prothrombin G20210A mutation.

As there is regional and ethnic variation in the distribution of mutations, we compared our rates firstly with reports from the same region. In Tunisia, Mahjoub et al. reported that the frequency of the mutant FV (0.1400 vs. 0.0276; \( p < 0.001 \)) but not prothrombin 20210 (0.0100 vs. 0.0225; \( p = 0.159 \)) allele was higher in patients than controls, respectively. APCR with FVL was seen in 27% of patients compared to 11.5% of controls [16]. In agreement with our results, they found that FVL was a significant predictor for recurrent abortions.

In Saudi Arabia, a study showed that the frequencies of FVL and prothrombin mutations among recurrent miscarriages patients were relatively high compared to general incidence supporting the hypothesis of considering them as RPL genetic factors [25].

A recent Turkish study reported a statistically meaningful data (\( p < 0.01 \)) related to the relationship between RPL and thrombophilia-associated gene polymorphisms such as heterozygous FVL and heterozygous prothrombin G20210A [26].

Unlike the results of the two previous studies, we found that prothrombin G20210A mutation was present in both case and control groups with heterozygous form and was not associated with the RPL. The same finding was observed by Silver et al. who tried to ascertain whether women carrying mutation of the prothrombin gene G20210A were at higher risk of RPL. They

| Sizes (bp)  | Prothrombin genotypes. | FV genotypes. |
|------------|------------------------|---------------|
| 345        |                        |               |
| 322        |                        |               |
| 241        |                        |               |
| 209        |                        |               |

Fig. 1 Electrophoretic patterns for duplex FV and prothrombin PCR. Lane 1, size marker (1-kb ladder); lane 2, homozygous prothrombin (digested PCR products)/normal FV (undigested PCR products); lane 3, normal prothrombin/homozygous FV; lanes 4 through 9, normal prothrombin/normal FV; lane 10, normal prothrombin/heterozygous FV. The smallest restricted fragments (32 and 23 bp) are not visible on the gel. N, normal allele; m, mutated allele.

Table 2 Demographic data of patient and control groups

|                                | Study group | Control group | \( p \) value |
|--------------------------------|-------------|---------------|--------------|
| Number                         | 80          | 100           |              |
| Age (years), mean (range)      | 33.28 (19–45) | 32.84 (21–45) | 0.94         |
| Number of previous pregnancy loss, mean (range) | 3 (2–13) | – |              |
| Type of pregnancy loss          | Miscarriage | 60            |              |
|                                | IUFD        | 7             |              |
|                                | Miscarriage + IUFD | 13         |              |
| Number of participant with a previous live birth | 46          | 100           |              |
| Consanguinity                  | 18          | 10            | 0.021        |
| Number of participant with a familial history of pregnancy loss | 15          | 8             | 0.031        |
recruited 5188 women, and 4167 blood samples were taken in the first trimester and analyzed for the gene mutation G20210A. Only 3.8% of the women tested had a mutation of prothrombin G20210A, and their pregnancy loss rates were similar to those of women without the mutation. The authors thus concluded that the prothrombin gene mutation G20210A was not associated with pregnancy loss [27].

What was remarkable in our study is the high frequency of prothrombin G20210A mutation: 9.52% in all the studied population. In discordance with our finding, Liatsikos et al. [28] reported that the prevalence of this mutation varies from 0.2 to 3%, being lowest in Africa (0–0.3%) and highest in Southern Europe (3%). The mean value in Northern Europe is 2%. For FVL, its prevalence varies from 0.6 to 7.0%, with the lowest frequency observed in Africa (0–0.6%) and the highest in Southern Europe (7%). The mean prevalence in Northern Europe is 4% [29].

The 5 cases of APCR found by us were congenital as confirmed by the genotypic tests.

No homozygous case of FVL was found in the study group, and all cases of FVL mutation were found in second trimester. Kashif et al’s [30] cases for FVL mutation were also observed in the second trimester. However, Mahjoub et al. [16] found that FVL was associated with early-late abortions. A systematic meta-analysis where late loss was defined as a pregnancy loss after the 24th week demonstrated a significant risk for early loss in homozygous FVL but a lower, non-significant risk in heterozygous FVL. With respect to late loss (3rd trimester), there was a significant risk in heterozygous FVL [31]. In fact, these differences can be explained by the heterogeneity in study designs, different definitions of late pregnancy loss among studies, and population heterogeneity.

Several studies have also investigated the link between other thrombophilic mutations—such as the methylene-tetrahydrofolate reductase (MTHFR) and the plasminogen activator inhibitor-1 (PAI-1) polymorphisms—and the risk of RPL; however, the results remain controversial. For example, Dell’Edera et al. study indicated the absence of association between the two polymorphisms (C677T and A1298T) of the gene encoding the MTHFR and the RPL risk [32]. Another study realized by Li et al. suggested that PAI-1 4G/5G polymorphism might be associated with RPL development in Caucasians [33].

Regarding the association of PC and S with RPL, Gris et al. [34] and Parand et al. [12] found a significant association of RPL with PS deficiency and a non-significant correlation with PC deficiency. In our study, the frequency of these deficiencies was only determined in the study group so the comparison was not possible, but we have found the same frequencies for both proteins (7.5%).

In terms of limitations, the study had subjects only from one ethnic group at only one center; also the number of participants was limited due to financial considerations. A larger sample size might be required to achieve an adequate statistical power. However, we permitted ourselves to evaluate the causal link using this small sample size since the results obtained are in alignment with previous works in this research area. In fact, our data provide further insight about the importance of testing for FVL mutation in women who have experienced pregnancy losses and may be useful for further investigation about the role of anticoagulants in RPL.

In this subject, Leaf et al. [35] reported that the role of anticoagulants in the prevention of pregnancy complications, including recurrent miscarriage and late fetal loss, continues to be an area of active research. Although

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### Table 3

| Parameter | Frequency | Mean | Range | Normal range |
|-----------|-----------|------|-------|--------------|
| APCR | 5 (6.25%) | 228.71 | 70.4–300 | 120–300 |
| PC | 6 (7.5%) | 90.13 | 55–143 | 70–130 |
| PS | 6 (7.5%) | 91.33 | 30–143 | 55–140 |
| AT | 3 (3.75%) | 96.62 | 46–119 | 80–120 |

### Table 4

| Parameter | Patients (n = 80) | Controls (n = 100) | p value |
|-----------|------------------|--------------------|---------|
| PT | 76 | 100 | 0.024 |
| APTT | 77 | 100 | 0.051 |
| Fibrinogen | 60 | 83 | 0.275 |
| APCR | 75 | 100 | 0.011 |
| Prothrombin | 78 | 99 | 0.434 |

### Table 5

| Genotype | Patients (n = 60) | Controls (n = 45) | p value |
|----------|------------------|------------------|---------|
| FVL | Wild type (GG) | 55 | 45 | 0.047 |
| Heterozygous (AG) | 5 | 0 |
| Homozygous (AA) | 0 | 0 |
| Prothrombin G20210A | Wild type (GG) | 55 | 40 | 0.631 |
| Heterozygous (AG) | 5 | 5 |
| Homozygous (AA) | 0 | 0 |
prophylactic anticoagulation with heparin and aspirin is considered the standard of care in obstetric antithrombophilic disorder and its perceived safety in pregnancy [37].

Conclusion

Our study demonstrates the implication of FVL mutation in the occurrence of RPL; for that, further study which will investigate the role of anticoagulants for pregnant women with a known FVL mutation is recommended and anticoagulation could be considered for future pregnancies of women with a RPL history with known FVL mutation.

Competing interests

All authors declare that they have no competing interests.

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