# Factor V Leiden G1691A and Prothrombin G20210A mutations are associated with repeated spontaneous miscarriage in Northern area of Saudi Arabia

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**ABSTRACT.** Maternally inherited thrombophilia could be one of the causes of recurrent spontaneous miscarriage (RSM). We aimed to investigate the frequency of three thrombotic gene variants; factor V Leiden (FVL; G1691A), prothrombin (PTH; G20210A), and methylenetetrahydrofolate reductase (MTHFR; C677T) in Saudi patients diagnosed with RSM. A case control study was conducted on 96 RSM patients and 96 age-matched controls. Genotyping was based on polymerase chain reaction followed by hybridization with variant-specific oligonucleotide probes using FV-PTH-MTHFR Strip Assay. There was a significantly higher frequency of the AA genotype of FVL in the RSM group when compared to controls (P <

0.001, OR (95% CI) = 15.7 (3.6-68.5). For the PTH G20210A mutation, the heterozygous genotype (GA) showed significantly higher frequency in patient group than control group (P < 0.0001, OR (95% CI) = 3.8 (2.05-6.9). For the MTHFR C677T mutation, there was no significant difference in the distribution of genotypes and alleles among the patients and controls. There was a significant association between the combined genotypes; FVL AA, PTH GA, and MTHFR CC as well as FVL GA, PTH GA, and MTHFR CT and patients group when compared to the control group. The FVL and PTH variants could be helpful in identifying RSM risk among Saudi women in the Northern area of Saudi Arabia. The combination of a panel of variants may be more conclusive in prediction of the disease risk compared to the small effect of individual mutation

**Key words:** FVL; MTHFR; PTH; RSM; Saudi Arabia

## INTRODUCTION

Recurrent spontaneous miscarriage (RSM) is defined as ≥ 3 spontaneous successive early (before the 12th gestational week) miscarriages (Hossain et al., 2013), although previous studies considered it as two or more spontaneous abortions (Branch et al., 2010).

RSM is the most common complication of pregnancy in Saudi Female. Spontaneously aborted women represent about 18% of all clinically recognized pregnancies (Gawish and Al-Khamees, 2013). Although some known disorders in reproductive, endocrine and immunological systems as well as some infectious diseases and chromosomal aberrations were implicated in some cases (Teremmahi et al., 2013), more than 50 % of cases remain unexplained. When the aforementioned disorders are excluded, thrombophilia was speculated as possible cause of RSM (Martínez-Zamora et al., 2012). This latter condition is a status in which hypercoagulability is prominent, which may be acquired or inherited (Yokuş et al., 2010).

Hereditary thrombophilia may cause infarcts secondary to placental vascular micro-thrombosis that result in low placental perfusion and eventually recurrent miscarriages and intrauterine fetal death (Carrington et al., 2005). Most women with RSM, placental thrombosis may be the final common pathophysiological event (Kasparova and Fait, 2009).

Cao et al. (2013) have suggested that mutations of Factor V Leiden (FVL), prothrombin (PTH) and methylene tetrahydrofolate reductase (MTHFR) genes are the most common thrombogenic mutations that can cause inherited thrombophilia.

FVL mutation involves replacement of guanine by adenine (factor V Arg534Gln or factor V Leiden) (G>A) at the nucleotide 1691 gene position in exon 10 (Table 1), leads to resistance of factor V to the cleavage effect of activated protein C (APC) (McNamee et al., 2012). Meanwhile, PTH gene mutation resulted in a genetic variant in the 3`UTR (untranslated region) at position 20210 caused by substitution of adenine for guanine (Table 1), leading to hyper-prothrombinemia and increased thrombin generation (Castoldi et al., 2007).

Another common mutation is MTHFR in which there is a substitution at position 677 of T instead of C (Table 1); leading to a reduced enzyme activity and subsequent hyperhomocysteinemia in homozygous cases that could predispose to thrombosis and RSM (Leclerc et al., 2013)

As there are no previous studies of the prevalence of the aforementioned mutations among Saudi women residents in the Northern Border area (NBA) of Saudi Arabia, this preliminary study aimed to detect the frequencies of these mutations in a group of Saudi females presented with unexplained RSM and to correlate these frequencies, with the available clinical and laboratory data of the patients.

This could help in refining the risk prevention and highlighting the importance of thromboprophylaxis in an attempt to improve the outcome of pregnancy in inherited thrombophilia affected women in the current area.

**Table 1.** General characteristics of the studied single nucleotide polymorphisms.

| Gene (ID) | SNP ID | Chromosome | | CDS | | Protein | | Function | Poly Phen annotation (score) |
|---|---|---|---|---|---|---|---|---|---|
| | | No | Position | Position | Allele change | Position | Residue change | | |
| *FVL* (2153) | Rs6025 | 1 (exon 10) | 169549811 minus | 1601 | CGA ⇒ CAA | 534 | R[Arg] ⇒ Q[Gln] | Missense | Probably damaging (0.984) |
| *PTH* (2147) | Rs1799963 | 11 (3'UTR) | 46739505 plus | *97G>A | 20210G>A | NA | NA | 3 prime UTR variant | NA |
| *MTHFR* (4524) | Rs1801133 | 1 (exon 5) | 11796321 minus | 667 | GCC ⇒ GTC | 222 | A[Ala] ⇒ V[Val] | Missense | Probably damaging (0.996) |

Coagulation factor 5 Leiden (FVL); Prothrombin (PTH); Methylenetetrahydrofolate reductase (MTHFR); single nucleotide polymorphism (SNP); number (No); untranslated region (UTR) [Data source: ensembl.org using Human Genome Assembly GRCh38.p2, Annotation release: 107].

# MATERIALS and METHODS

## Study subjects

A case-control study was conducted on 96 patients with RSM (20-35 years) and 96 age-matched controls. A past history of ≥ 2 losses of pregnancy before the 20th gestational week has been confirmed for all patients who were enrolled from the Arar Obstetrics and Gynecology Hospital, Northern Border Area, Saudi Arabia. Other causes of RSM (e.g. endocrine, autoimmune, infectious, uterine factors, etc.) have been excluded.

Developmental or acquired reproductive system abnormalities, in addition, have been excluded by abdominal/pelvic ultrasound. Routine screening for hyperprolactinemia, thyroid dysfunction, diabetes, corpus luteum insufficiency and polycystic ovary syndrome have been done clinically and by laboratory testing for patient hormone profiles, including basal follicle stimulating hormone, luteinizing hormone, estradiol, luteal phase progesterone, thyroid stimulating hormone, free tri-iododthyronine and free tetra-iodothyronine. Patients with positive lupus anticoagulant and anti-phospholipid antibodies were also excluded.

Age-matched, healthy Northern Border University (NBU) employees' females without a history of any pregnancy loss or chronic disease were allocated in the control group. Previous history of thrombosis has been excluded in both study groups. The study was conducted in accordance with the guidelines in the Declaration of Helsinki and was approved by the Medical and Bioethics local committee of NBU. All participants provided written informed consent to participate in the study after being informed with its purpose.

## Blood sample collection

Whole blood sample (3 ml) was drawn from each participant; 2 ml collected on Trisodium citrate for hemostatic tests (prothrombin time and the INR) and 1 ml collected on ethylenediaminetetraacetic acid (EDTA) for subsequent extraction of DNA.

## Genotyping

### DNA extraction and amplification

DNA extraction from EDTA tubes of blood specimens has been done following the manufacturer's instructions, using FV-PTH-MTHFR StripAssay kit (ViennaLab Labordiagnostika GmbH, Cat. No. 4-260, Vienna, Austria). All extracted DNA was kept frozen at -20°C until the time of amplification. FVL (G1691A), PTH (G20210A) and MTHFR (C677T) genes were amplified by polymerase chain reaction (PCR) and labeled with biotin simultaneously in a single (multiplex) amplification reaction. The PCR reaction mix for each amplified sample was containing Amplification Mix (15 µl), diluted Thermus Aquaticus DNA polymerase (1U), and DNA template (200 ng).

The PCR has been run on the thermocycler (C1000, BIO-RAD) following the manufacturer's recommended program (i.e. 2 minutes initial DNA denaturation at 94°C followed by incubation at 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; repeated for 30 cycles, then a final extension step for 3 minutes at 72°C). The PCR products were stored at 2-8°C until the time of hybridization in the following day.

| | | | | |
|---|---|---|---|---|
| A allele | 98 (51.0) | 120 (62.5) | | **1.6 (1.06-2.4)** |
| ***PTH* G20210A** | | | | |
| G allele | 159 (82.8) | 126 (65.6) | **< 0.001** | Ref. |
| A allele | 33 (17.2) | 66 (34.4) | | **2.5 (1.6-4.1)** |
| ***MTHFR* C677T** | | | | |
| C allele | 181 (94.4) | 171 (89.1) | 0.069 | Ref. |
| T allele | 11 (5.6) | 21 (10.9) | | 2.0 (0.95-4.3) |

Coagulation factor 5 Leiden (FVL); Prothrombin (PTH); Methylenetetrahydrofolate reductase (MTHFR); odds ratio 95% confidence interval (OR 95 % CI), OR was adjusted for confounding variables (age, duration of marriage, family history). Bold values indicate statistically significant at $P < 0.05$.

Furthermore, analysis of the combined genotypes constructed by FVL, PTH and MTHFR mutations, revealed higher frequency of FVL AA, PTH GA, and MTHFR CC as well as FVL GA, PTH GA, and MTHFR CT in RSM patients than controls in the study population (Figure 2).

Figure 2. Frequency of genotype combinations in patients and controls. Combination of the three variants (1) FV G169A, (2) PTH G20210A, and (3) MTHFR C677T.

On employing association analysis between the frequencies of PTH G20210A and MTHFR C677T mutations and the available patients´ clinical and laboratory data, there were no considerable significant relations could be drawn (Table 4).

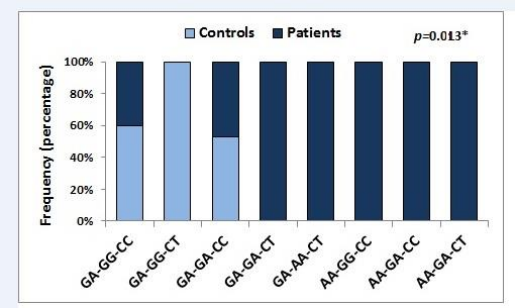


**Figure 2.** Frequency of genotype combinations in patients and controls. Combination of the three variants (1) *FV* G169A, (2) *PTH* G20210A, and (3) *MTHFR* C677T.

## DISCUSSION

Inherited thrombophilic mutations have been reported as one of the main causes of RSM. These mutations lead to disturbance of the trophoblast differentiation and placental vascularisation causing restrictions of the fetal growth, failure of pregnancy, placental insufficiency and therefore miscarriages (Incebiyik et al., 2014).

In the current study, the homo-mutant AA genotype of FVL and the heterozygous mutant GA genotype of PTH were significantly associated with RSM compared to the controls ($P < 0.0001$). These results support the relative high incidence of thrombophilic mutations in the Northern area of Saudi Arabia as reported in other areas of KSA in previous studies (Gawish and Al-Khamees, 2013; Saour et al., 2009; Gawish, 2015; Turki et al., 2016). In agreement with this finding, both mutations have been implicated as common genetic variants that predispose to early and/or late RSM in other populations as reported in Egyptian (Mohamed et al., 2010; Settin et al., 2011), Palestinian (Abu-Asab et al., 2011), Syrian (Mohammadi et al., 2007) and Turkish (Isaoglu et al., 2014) women.

**Table 4.** Association of PTH G20210A and MTHFR C677T mutations with the clinical characteristics of RSM women.

| Characteristics | *PTH* G20210A | | | | *MTHFR* C677T | | | |
|---|---|---|---|---|---|---|---|---|
| | GG | GA+AA | P value | OR (95% CI) | CC | CT+TT | P value | OR (95% CI) |
| **Total number** | 33 | 63 | | | 75 | 21 | | |
| Age | | | | | | | | |
| ≤35 y | 30 (90.9) | 57 (90.5) | 0.944 | 1.0 | 69 (92.0) | 18 (85.7) | 0.389 | 1.0 |
| >35 y | 3 (9.1) | 6 (9.5) | | 1.05 (0.25-4.50) | 6 (8.0) | 3 (14.3) | | 1.91 (0.44-8.4) |
| **FH of abortion** | | | | | | | | |
| Negative | 30 (90.9) | 57 (90.5) | 0.945 | 1.0 | 66 (88.0) | 21 (100) | 0.218 | 1.0 |
| Positive | 3 (9.1) | 6 (9.5) | | 1.05 (0.25-4.50) | 9 (12.0) | 0 (0.0) | | 0.163 (0.0-2.9) |
| **Obstetric history** | | | | | | | | |
| Duration of marriage | 9.0 (3.0-11.0) | 9.0 (5.0-11.0) | 0.969 | | 7.0 (3.0-10.5) | 9.0 (5.0-13.0) | 0.151 | |
| No. of living children | 1.0 (1.0-3.0) | 1.0 (0.5-4.0) | 0.639 | | 1.0 (1.0-4.0) | 4.0 (0.0-4.0) | 0.359 | |
| No. of abortions | 3.0 (2.0-3.5) | 5.0 (3.0-6.0) | **0.031** | | 3.0 (2.0-5.0) | 3.0 (2.0-5.0) | 0.762 | |
| **Medical disorder** | | | | | | | | |
| Negative | 30 (90.9) | 54 (85.7) | 0.468 | 1.0 | 66 (88.0) | 18 (85.7) | 0.780 | 1.0 |
| Positive | 3 (9.1) | 9 (14.3) | | 1.67 (0.42-6.63) | 9 (12.0) | 3 (14.3) | | 1.22 (0.30-5.0) |
| **Drug history** | | | | | | | | |
| No drug intake | 21 (63.6) | 33 (52.4) | | 1.0 | 45 (60.0) | 9 (42.9) | | 1.0 |
| Aspirin | 6 (18.2) | 27 (42.9) | 0.053 | 2.78 (0.98-7.85) | 21 (28.0) | 12 (57.1) | **0.041** | **2.85 (1.04-7.83)** |
| COCP | 12 (36.4) | 9 (14.3) | 0.156 | 0.47 (0.17-1.33) | 18 (24.0) | 3 (14.3) | 0.801 | 0.83 (0.20-3.44) |
| Clexane | 12 (36.4) | 6 (9.5) | 0.050 | 0.32 (0.10-0.97) | 12 (16.0) | 6 (28.6) | 0.139 | 2.5 (0.74-8.41) |
| **Biochemical investigations** | | | | | | | | |
| PT | 11 (10.6-11.9) | 11 (10.5-11.7) | 0.434 | | 11 (10.5-11.8) | 11.1 (10-11.8) | 0.684 | |
| INR | 0.9 (0.92-1.07) | 0.9 (0.90-1.0) | 0.411 | | 0.9 (0.92-1.05) | 0.95 (0.9-1.06) | 0.655 | |
| PTT | 31 (25.7-33.2) | 30 (28.7-32.1) | 0.667 | | 30 (27.5-33.1) | 31 (28.5-32.5) | 0.765 | |

Data is presented as a number (percentage), mean ± standard deviation, and median (quartiles). OR (95% CI), odds ratio (95% confidence interval); No., number; PT, prothrombin time, PTT, partial thromboplastin; INR, international normalized ratio; COCP, combined oral contraceptive pill. Two-sided Chi-square, student's *t*, and Mann-Whitney U tests were used for comparison. The genetic association dominant model was used for calculating odds ratio. Bold values indicate statistically significant at $P < 0.05$.

The substitution of guanine to adenine at nucleotide 1691 in the exon 10 of factor V gene, leads to the single amino-acid replacement Arg506Gln which causes resistance to cleavage by the natural anticoagulant activated protein C and hence increased susceptibility to clotting (McNamee et al., 2012). The mutation in factor V Leiden is responsible for about 75% of inherited activated protein C resistance, which is the most common inherited thrombotic risk factor associated to repeated abortion (Mierla et al., 2012). On the other side, the replacement of adenine for guanine at position 20210 leading to hyper-prothrombinemia and subsequent an increase in thrombin generation. It has been also reported that carriers of PTH mutation have increased risk of venous thrombosis, arterial diseases and RSM 5-fold than normal (Jacobsen et al., 2010; Incebiyik et al., 2014; Fawzy et al., 2017). In addition, both of these mutations have been reported to be associated with other obstetric disorders such as abruptio placentae, intrauterine growth retardation or death (Turki et al., 2016). The findings of the previous studies and the current one could support the presumption that both FVL and PTH mutations are major risk factors for RSM. The actual mechanism by which inherited thrombophilia affecting recurrent pregnancy loss is still unknown. It has been suggested that thrombosis of maternal blood vessels may be related to the complications in association with thrombophilias (Mierla et al., 2012).

However, several studies did not report a significant association between these mutations and RSM (Kobashi et al., 2005; Altintas et al., 2007; Mierla et al., 2012; Poursadegh Zonouzi et al., 2013). This could be explained by the differences in ethnicity, the study sample size and design as well as the other interacting genetic and environmental factors that affect the final thrombophilic phenotype of the RSM patients (Almawi et al., 2005; Awad et al., 2013).

Regarding the MTHFR (C677T) mutation, our findings elucidated no significant difference in the frequency of this mutation between controls and patient group, and absence of homozygous mutant genotype in all study groups. This finding was in line with Gawish (2015), Turki et al. (2016), Isaoglu et al. (2014) as well as Osman and Abulata (2015) in Saudi, Turkish and Egyptian women. On contrast to our findings, others reported the association between this mutation and RSM (Jeddi-Tehrani et al., 2011). The MTHFR (C677T) mutation is involved in thrombophilia as the replacement of alanine by valine at codon 222 in the N-terminal of the protein resulting in decreased activity of MTHFR enzyme which leads to hyperhomocysteinemia which could predispose to thrombosis and RSM (Saour et al., 2009; Cao et al., 2013). Folic acid supplementation

during conception as a general practice could be a reason of masking the effect of the MTHFR mutation in RSM patients as speculated by Turki et al. (2016) and Li et al. (2015).

Many studies showed that concomitant of two or more mutations have more unfavourable effects on pregnancy compared to the presence of a single hereditary thrombophilic factor (Coulam et al., 2006). This hypothesis is in agreement with our findings that showed a significant increase in specific combined mutation prevalence in RSM patients in comparison to normal controls. Multiple gene mutation concept as an RSM risk factor, has been reported previously (Coulam et al., 2006; Vora et al., 2008), but disagreed with others (Carp et al., 2002; Jaslow et al., 2010).

The role of thrombophilic gene variants in RSM has been speculated by many studies to be due to the presence of enhanced uncontrolled coagulation in the placental inter-villous spaces which in turn could induce deposition of fibrin within the fetal circulation, leading to multithrombotic events such as stem vessels thrombosis, infarction of the placenta, and spontaneous abortion (Osman and Abulata, 2015). Most of the results reported by different investigators for the same population showed inconsistency and this again may be due to the bias in patients' selection and/or ethnic heterogeneity among the patients (Mohammadi et al., 2007). In addition, many authors suggest that methodological diversity, sample size and clinical heterogeneity may have a role in this discrepancy and contradiction.

## CONCLUSION

The current study concludes that FVL and PTH gene mutations, but not MTHFR were significantly prevalent and associated with RSM in the study population. In addition, combined inheritance may have more accordance with the occurrence of thrombophilic events related to repeated abortions in the current area. An appropriate therapy for inherited and acquired thrombophilia will improve the pregnancy outcomes as recommended by De Santis et al. (2006). Furthermore, "a routine screening national medical program for these two genes in RSM patients in Saudi Arabia is highly recommended" (Turki et al., 2016).

We confirm that these preliminary findings will require further complementary studies that include other thrombogenic variants to help in planning a set of candidate genes which could be implicated in RSM of unknown aetiology in the study population.

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## CONFLICT OF INTEREST

All authors declare they have no conflicts of interest

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