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Research Article

Genetic Testing for Inherited Thrombophilia in Women with Recurrent Pregnancy Loss: Prevalence and Risk Assessment of Factor V Leiden, Prothrombin, and MTHFR Mutations

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Abstract: Genetic testing for inherited thrombophilia in women with recurrent pregnancy loss (RPL) may play an important role in detecting high-risk subjects. DNA was extracted from peripheral blood leukocytes. The high pure polymerase chain reaction template preparation kit and Reverse hybridization technique were used to detect the presence of factor V (G1691A, Leiden) (FVL), Factor II (prothrombin, G20210A), and 5,10-methylenetetrahydrofolate reductase (MTHFR, C677T) mutations. A total of 127 cases of pregnancy loss participated in the study, with the control group consisting of 26 multiparous women with no history of pregnancy loss. Women with early recurrent pregnancy loss (RPL) of unknown cause (n=76) experienced an average of 2.97 ± 1.15 miscarriages, with a mean fetal gestational age at the time of abortion of 7.79 ± 4.52 weeks. In this group, the prevalence of FVL, Factor II, and MTHFR was 50%, 1.3%, and 43.4%, respectively, compared to 15.4%, 0%, and 34.6%, respectively, in the control group. Among the tested mutations, only the prevalence of FVL was statistically different between the early RPL and control groups, with an odds ratio (OR) of 5.5 for early RPL of unknown cause. The early pregnancy loss group with a suspected cause (n=24) had hypothyroidism and respiratory infections as potential causes of abortion, contributing to 54.2% and 33.3% of cases, respectively. The prevalence of MTHFR, but not FVL or Factor II, was statistically different in this group compared to the control group, with an OR of 3.8. In conclusion, FVL mutation can be considered as a risk factor for early RPL of unknown cause.

Keywords: Abortion, Factor V Leiden, MTHFR, Recurrent Pregnancy Loss, Thrombophilia

Introduction

Physiological changes during a normal pregnancy lead to a hypercoagulable state (Alsheef *et al.*, 2020). These changes include elevated levels of several clotting factors (I, II, VII, VIII, IX, and XII), reduced protein S levels, and reduced activity of the overall fibrinolytic pathway (Reith *et al.*, 1993). Additionally, as pregnancy progresses, there is a significant reduction in the activity of activated protein C, a key anticoagulant. While these adaptations are vital for minimizing blood loss during childbirth, they also increase the risk of thromboembolism during pregnancy and the postpartum period (Alsheef *et al.*, 2020; Bremme, 2003).

The risk of pregnancy loss (spontaneous abortion or miscarriage) decreases as gestational age increases. Once the pregnancy reaches 8 weeks, the risk of miscarriage decreases substantially; conversely, the likelihood of a live birth approaches 97–98% (Musik *et al.*, 2021). Recurrent miscarriage, also known as recurrent pregnancy loss (RPL), is a medical condition characterized by the loss of three or more consecutive pregnancies before the 20th week of gestation (Al-Alami *et al.*, 2024). However, many experts consider two consecutive losses sufficient for diagnosis and suggest an assessment after each loss, with a thorough evaluation after three or more losses (Turesheva *et al.*, 2023). Although the primary cause of miscarriage is unknown in over 50% of cases (Oliveira *et al.*, 2020), miscarriage is influenced by a variety of risk factors, including embryonic chromosomal abnormalities (Elhady *et al.*, 2020), endometrial defects, genetic (Eslami *et al.*, 2020),

and health-related risks (Quenby *et al.*, 2021). Demographic factors such as maternal age, body mass index, and ethnicity also play a significant role. Lifestyle choices, including smoking, high caffeine intake, night shift work, and elevated stress levels, further increase the risk. Environmental exposures, such as air pollution, and clinical conditions like the presence of antiphospholipid antibodies, thyroid autoantibodies, uterine anomalies, and bacterial or viral infections, are additional contributors. Addressing these factors can help improve pregnancy outcomes (Quenby *et al.*, 2021).

Genetic factors play a significant role in elevating the likelihood of spontaneous miscarriage. Thrombophilic mutations carried by pregnant women have been reported to be associated with clot formation, potentially disrupting uterine blood flow and consequently impeding fetal development (Olimid, 2023). A genetic mutation in the factor V gene, known as factor V Leiden (FVL), particularly (G1691A) variant alters factor V, rendering it resistant to inactivation by activated protein C. FVL has been associated with abnormal blood clotting and an increased risk of recurrent miscarriages (Eslami *et al.*, 2020). Factor II (FII, Prothrombin) mutations, particularly the G20210A variant, have been linked to elevated prothrombin levels and a higher risk of venous thrombosis, which was related to recurrent miscarriages (Pietropolli *et al.*, 2014). Additionally, the 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C>T exchange mutation not only causes a prothrombotic predisposition but also affects essential metabolic processes in cells, such as DNA and protein methylation (Zhang *et al.*, 2020). Fortunately, studies suggest that pregnant women diagnosed with hereditary thrombophilia had a favorable outcome under properly administered anticoagulant treatment (Neamţu *et al.*, 2021).

Although many studies have shown an association between thrombophilia and fetal loss (Eslami *et al.*, 2020; Pietropolli *et al.*, 2014; Zhang *et al.*, 2020). Several other studies have not shown such an association (Abd Elhameed *et al.*, 2025; Vomstein *et al.*, 2021). This research seeks to explore the prevalence of FVL, FII, and MTHFR polymorphisms among Arabic women suffering from spontaneous abortion in the Jordanian population. Knowing the cause of abortion provides insights that may inform approaches for diagnosing and managing recurrent pregnancy loss.

Materials and methods

Ethical Considerations

Ethical approval was obtained from The Institutional Review Board (IRB) of the Royal Medical Services: Committee for Clinical and Pharmaceutical Research and Studies and Professional Ethics (IRB number 8/2024) in Aug 2024. Patient's data were carefully handled to safeguard the privacy of participants' information by excluding the national number and the patient's name that could identify them.

Patient Recruitment

The focus of the study was on women who had experienced abortion and were seeking care at the Gynecology Department of KHMC / Royal Medical Services and Princess Iman for Laboratory and Research Center. Hakeem's clinical data were extracted from the electronic medical record and included age, thrombotic events, number of recurrent abortions, and gestational age of the fetus at the time of abortion.

The study included cases of spontaneous pregnancy loss. Participants were excluded if their pregnancy loss was attributed to factors such as chromosomal abnormalities, immune system changes, infections, or structural issues in the uterus, whether they experienced early or late abortion. Women with early RPL of unknown cause had experienced two or more abortions before reaching the 20th week of pregnancy. The control group comprised age-matched multiparous women who had experienced uncomplicated childbirths and had no history of abortion.

Blood Sample Collection

Blood samples for DNA extraction were collected at the time of abortion using EDTA tubes from BD Vacutainer. Also, nitrocellulose membrane strips coated with oligonucleotides, DNAT Denaturation solution containing NaOH, hybridization, saline solution containing preservatives, wash solution, conjugate solution containing streptavidin labeled with alkaline phosphatase, stabilizers and preservatives, wash solution B containing preservatives, and color developer solution. polymerase chain reaction (PCR), DNA polymerase, a Mix solution containing oligonucleotides, an interpretation sheet, collector sheet for strip storage were obtained from ViennaLab Diagnostics (Austria). cell lysis solution, nuclei lysis solution, protein precipitation, and DNA rehydration solution were obtained from Promega (USA), while isopropanol was obtained from Sigma (USA).

The analysis consisted of three steps: DNA extraction, PCR of target DNA sequences using biotinylated primers, and hybridization of the amplified products with the oligonucleotide probes and colorimetric detection. Genomic DNA was extracted from EDTA samples according to standard procedures using Wizard Genomic DNA Purification kit (Promega, USA). For PCR reactions, the amplification of DNA samples was carried out using BIO-RAD iCycler Thermal Cycler. The thermal cycle amplification program was as follows: Preheat at 94°C for 5 minutes. Amplification 30 cycles: Denaturation at 95°C for 15 sec, Annealing at 58°C for 30 sec, and Extension at 72°C for 30 sec. Final elongation at 72°C for 3 minutes. Ending reaction at 8°C. For the simultaneous detection

of FVL (G1691A), FII (G20210A), and MTHFR(C677T), a reverse-hybridization test was performed, where specific oligonucleotide probes immobilized as parallel lines on membrane-based strips hybridize with biotinylated PCR products. The exact match between probes and amplified product generates a signal exploiting the bond between biotin and streptavidin-conjugated with alkaline phosphatase, and a subsequent color development. To identify the genotype of a sample, each strip was analyzed with the help of the interpretation sheet. To correctly align the strip and interpretation sheet, colored marker lines on the top and bottom were used. For each mutation, one of the following patterns should be observed: only the wild-type band (indicating a homozygous wild-type genotype), both wild-type and mutant bands (indicating a heterozygous genotype), or only the mutant band (indicating a homozygous mutant genotype).

Statistical Analysis

Descriptive statistics were applied to present the data, with categorical variables expressed as numbers (percentages) and numerical variables as mean ± standard deviation (SD). SPSS version 26 was employed to analyse the Spearman's correlation of the mother's age and the number of abortions or the age of the fetus at the time of abortion. The Fisher's Exact Test was utilized for the calculation of Monte Carlo 2-sided significance with 99% confidence. This test was used to determine if there is a significant difference in the prevalence of FVL, FII, and MTHFR C677T mutations between groups. A *p*-value of <0.05 was considered statistically significant.

Results

Prevalence of FVL, FII, and MTHFR Polymorphisms in Early RPL of Unknown Cause

The control group consisted of 26 women with multiple pregnancies and no previous history of abortion, with a mean age of 28.46 ± 3.24 years. The early RPL group of unknown cause included 76 participants with a mean age of 28.92 ± 6.86 years. No significant difference was found in age between the control group and the early RPL with unknown cause group at $p<0.05$.

The mean gestation age of the foetus at the time of abortion was 7.79 ± 4.52 weeks, and the average number of abortions in this group was 2.97 ± 1.15. There was no significant correlation between the mother's age and the number of abortions or the age of the fetus at the time of abortion.

In the early RPL group of unknown cause, the prevalence of FVL, Factor II, and MTHFR was 50%, 1.3%, and 43.4%, respectively, compared to 15.4%, 0%, and 34.6%, respectively, in the control group. A significant difference in the prevalence of FVL mutation between the control group and the RPL of unknown cause (p=0.006). The odds ratio (OR) was 5.5 with a 95% confidence interval (CI) of 1.73 to 17.481. Individuals in the early RPL of unknown cause group were 5.5 times more likely to have FVL (either homozygous or heterozygous) compared to the control group. The CI does not include 1, indicating a statistically significant result.

A high prevalence of the MTHFR C677T mutation in women affected by early RPL of unknown cause was observed, with 43.4% (30/76 heterozygous and 3/76 homozygous) of participants affected, compared to 34.6% (8/26 heterozygous and 1/26 homozygous) in the control group. On the other hand, none of the participants were positive for FII mutation except for one case in RPL of unknown cause. No significant difference in the prevalence of FII mutation or MTHFR C677T between the control group and the RPL group of unknown cause was found (Table 1).

Prevalence of FVL, FII, and MTHFR Polymorphisms in Early Abortion with Suspected Cause

The group with early abortion of suspected cause consisted of 24 participants, with a mean age of 33.8 ± 8.60 years. The study identified several medical conditions as known causes of abortion among the participants. These conditions included hypothyroidism (n=13, 54.2%), respiratory infections (n=8, 33.3%), hyperthyroidism (n=2, 8.3%), and HELLP (Hemolysis, Elevated Liver enzymes, and Low Platelets) syndromes (n=1, 4.2%).

No significant difference in the prevalence of FVL mutation between the early abortion group of suspected cause and the control group was found (Table 2). Homozygous mutation pattern of FVL was not detected in either group.

About 35% of women in the control group and 67 % of patients in the early abortion group of suspected cause had MTHFR C677T polymorphism (Table 2). Individuals in the early abortion group of suspected cause were 3.8 times more likely to have MTHFR polymorphism compared to the control group. Since the CI does not include 1, this finding is statistically significant, suggesting a meaningful difference in risk between the two groups (OR=3.8). Neither the cases with known causes of early abortion nor the control group had FII mutation (Table 2).

Among patients having early abortion with a suspected defined cause and having also FVL mutation, 4 patients had hypothyroidism and one patient had hyperthyroidism (Table 3). All 8 patients with abortion due to respiratory infection had MTHFR polymorphism, while 6 out of the 13 patients with abortion had hypothyroidism. Only 1 case with hyperthyroidism and one case with HELLP syndrome had the MTHFR polymorphism. None of the patients expressed 2 or 3 mutations except for one patient with hypothyroidism who was heterozygous for FVL and homozygous for MTHFR.

Table 1: Prevalence of FVL, FII, and MTHFR polymorphisms in the control and early RPL of unknown cause groups.

	Control Group (n = 26)	RPL of Unknown Cause Group (n = 76)	P-value (Fisher chi-square test)
Negative for FVL	22 (84.6%)	38 (50%)	0.006
FVL (Homozygous)	0 (0%)	5 (6.6%)	
FVL (Heterozygous)	4 (15.4%)	33 (43.4%)	
Negative for FII	26 (100%)	75 (98.7%)	1.000
FII (Homozygous)	0 (0%)	0 (0%)	
FII (Heterozygous)	0 (0%)	1 (1.3%)	
Negative for MTHFR	17 (65.4%)	43 (56.6%)	0.811
MTHFR (Homozygous)	1 (3.8%)	3 (3.9%)	
MTHFR (Heterozygous)	8 (30.8%)	30 (39.5%)	

Table 2: Prevalence of FVL, FII and MTHFR polymorphisms in control and early abortion with suspected cause.

	Control Group (n = 26)	Early Abortion Group with Suspected Cause (n = 24)	P-value (Fisher chi-square test)
Negative for FVL	22 (84.6%)	19 (79.2%)	0.721
FVL (Homozygous)	0 (0%)	0 (0%)	
FVL (Heterozygous)	4 (15.4%)	5 (20.8%)	
Negative for FII	26 (100%)	24 (100%)	---
FII (Homozygous)	0 (0%)	0 (0%)	
FII (Heterozygous)	0 (0%)	0 (0%)	
Negative for MTHFR	17 (65.4%)	8 (33.3%)	0.017
MTHFR (Homozygous)	1 (3.8%)	7 (29.2%)	
MTHFR (Heterozygous)	8 (30.8%)	9 (37.5%)	

Table 3: Prevalence of FVL, FII & MTHFR polymorphisms among early abortion cases of suspected cause.

Suspected Cause of Early Abortion	FVL	FII	MTHFR
HELLP (Hemolysis, Elevated Liver enzymes, Low Platelet count) Syndrome (n = 1)	-	-	1
Hyperthyroidism (n = 2)	1	-	1
Hypothyroidism (n = 13)	4	-	6
Respiratory Infection (n = 8)	-	-	8
Total	5	-	16

Table 4: Prevalence of FVL, FII, and MTHFR polymorphisms in control and late abortion.

	Control Group (n = 26)	Late Abortion Group (n = 27)	P-value (Fisher chi-square test)
Negative for FVL	22 (84.6%)	22 (81.5%)	1.000
FVL (Homozygous)	0 (0%)	0 (0%)	
FVL (Heterozygous)	4 (15.4%)	5 (18.5%)	
Negative for FII	26 (100%)	27 (100%)	----
FII (Homozygous)	0 (0%)	0 (0%)	
FII (Heterozygous)	0 (0%)	0 (0%)	
Negative for MTHFR	17 (65.4%)	16 (59.3%)	1.000
MTHFR (Homozygous)	1 (3.8%)	2 (7.4%)	
MTHFR (Heterozygous)	8 (30.8%)	9 (33.3%)	

Prevalence of FVL, FII, and MTHFR Polymorphisms in Late-Term Abortion

The late-term abortion group, which experienced abortions in the third trimester, included 27 participants; the mean age of this group was 31.56 ± 7.27 years. Pre-eclampsia toxemia (PET) was the most common cause, accounting for 44.4% (n=12) of cases. Placental abruption (n=6) and trauma (n=5) constituted 22.2% and 18.5% of cases, respectively. Baby malformation (n=1, 3.7%), hypercalcemia (n=1, 3.7%), and respiratory infections (n=2, 7.4%) were less common.

No significant difference in the prevalence of FVL mutation was found between the late abortion group and the control group. None of the groups had the homozygous mutation pattern of FVL mutation (Table 4). The analysis of the MTHFR C677T polymorphism revealed that 9 subjects in the control group and 11 patients in the late abortion group had this polymorphism (Table 4). None of the cases with suspected causes of early abortion, nor the control group, had FII mutation.

Among cases of late-term abortion with preeclampsia toxemia (PET), none had the FVL mutation, while four patients had the MTHFR C677T polymorphism. One

case out of 6 cases with placental abruption had FVL mutation, and one case had MTHFR C677T mutation. One case out of 5 cases having trauma had FVL mutation and 4 out of 5 of them had MTHFR C677T polymorphism. Two patients with respiratory infection had FVL mutation and 2 had MTHFR C677T polymorphism, while none of the other causes (baby malformation, hypercalcemia) had FVL or MTHFR C677T mutation. None of the patients carried two or more mutations, except for one patient with a history of trauma, who was heterozygous for both FVL and MTHFR. Also, none of the late abortion group had FII mutations among the late-term abortion group.

Thrombotic Events Among Women with Abortions

Five cases of thrombosis were reported in the early RPL of unknown cause group compared to one case in the early abortion group, with suspected causes, one case in the late-term abortion group, and none in the healthy control group.

Discussion

Pregnancy loss is a significant concern in reproductive health, and inherited thrombophilic mutations have been implicated in its pathogenesis (Gounain *et al.*, 2020; Taybeh *et al.*, 2023) . To the best of our knowledge, this study is among the few that report the prevalence of FVL, FII, and MTHFR C677T mutations among Arab women with pregnancy loss in Jordan. In neighboring regions, studies have suggested an association between some of these mutations and pregnancy complications in women of Arabic ethnicity (Gounain *et al.*, 2020; Najjar *et al.*, 2024).

The findings of this study suggest that FVL mutation is significantly more prevalent in the early RPL of unknown cause group (50%) compared to the control group (15.4%). This underlines the importance of this genetic mutation in early RPL of unknown cause. Previous studies reported a close prevalence of FVL mutation among healthy Jordanian subjects (15% and 21%) (Al-Zoubi *et al.*, 2021; Eid & Rihani, 2004).

An association between FVL and RPL of unknown cause (OR=5.5) was identified, which corroborates the role of this mutation in predisposing women to early RPL. The consequence of this mutation is resistance to activated protein C, and hence increases the risk of thrombosis (Dahlbäck, 2020). This thrombogenic tendency disrupts normal placental formation and results in compromised foetal development, thereby increasing the risk of miscarriage (Poort *et al.*, 1996). Different studies investigating the association of FVL with spontaneous abortion have produced varying outcomes. In some Arab countries, like Palestine and Algeria, a significant association was found between FVL and RPL (Najjar *et al.*, 2024; Nassour-Mokhtari *et al.*, 2020), while in other African countries, such as Egypt and Sudan, no such association was found (Babker *et al.*, 2024; Sayed *et al.*, 2022). A meta-analysis that included 62 studies, encompassing 10,410 cases and 9406 healthy controls, have shown a consistent association between FVL mutation and RPL across different populations. The increased susceptibility to RPL was observed in Iranian, Asian, European, and African populations, but not in South Americans (Eslami *et al.*, 2020).

The findings of the present study suggest that FVL detection may be a valuable part of the diagnostic workup in women with unexplained early RPL. The study showed a significantly higher FVL prevalence in affected women (50%) compared to controls (15.4%), with an OR of 5.5, indicating a strong association. While not a standalone diagnostic marker, FVL can help identify high-risk women who may benefit from closer monitoring and targeted interventions. FVL-positive women with RPL may be suitable for prophylactic anticoagulation, such as low molecular weight heparin (LMWH), to reduce future losses. Although universal FVL screening isn't currently recommended, selective testing in high-risk cases, such as idiopathic RPL or a history of thrombosis, is justified.

In the current investigation, one case out of 76 cases of women with RPL of unknown cause was a carrier of the FII mutation compared to 0% in the other groups, including the control, early abortion of suspected cause, and late-abortion groups. The very low prevalence or absence of this mutation among healthy Jordanians was reported earlier (Obeidat *et al.*, 2009). Similar to our findings, there was no significant correlation between the prevalence of FII (G20210A) and RPL in different Arab countries (Babker *et al.*, 2024; Najjar *et al.*, 2024; Sayed *et al.*, 2022). However, several studies in Mediterranean countries have demonstrated that the presence of the FII mutation increases the abortion risk in women due to thrombotic events within the placental circulation (Pietropolli *et al.*, 2014; Sehirali *et al.*, 2005).

No significant difference in MTHFR C677T polymorphism prevalence was found between the control group and the group affected by early RPL of unknown cause. The prevalence of the MTHFR C677T polymorphism in the control group in this study aligns with results published by other authors, who described a high prevalence of the MTHFR C677T variant in healthy Jordanians (Eid & Rihani, 2004). Similar to our findings, a case-controlled Korean study revealed that the genotype distribution of MTHFR C677T polymorphisms in the RPL group did not differ from those of the controls (Hwang *et al.*, 2017). In contrast, the association between the MTHFR C677T polymorphism and RPL has been documented in various populations, underscoring the need for genetic screening in women with RPL. For example, a study conducted in Nepal found that a significant association existed between MTHFR C677T polymorphism and the number of losses (Sah *et al.*, 2018). In Iran, it was reported that 40% of women with RPL carried the MTHFR C677T mutation, compared to

25% in the control group, suggesting a significant association between the mutation and RPL in the Iranian population (Bigdeli *et al.*, 2018). Similarly, in Chinese women an association between MTHFR and RPL was found (Zhang *et al.*, 2020). Supplementation of pregnant women with methylfolate, Vitamin B6 and B12 was effective for the lowering of hyperhomocysteinemia and prevented miscarriages in patients with MTHFR (Serapinas *et al.*, 2017).

In early pregnancy loss cases with known causes, hypothyroidism was identified as the most common cause, responsible for 54.2% of cases. Respiratory infections followed as the second most frequent cause, accounting for 33.3% of cases. This distribution underscores the importance of managing these medical conditions to potentially decrease the rate of early abortions. Surprisingly, all cases with respiratory tract infection (n=8) had MTHFR polymorphism (3 were homozygous and 5 heterozygous). More studies are needed to investigate the link between high homocysteine levels and immune response during the pregnancy period in the first trimester.

Several studies have reported causes of early pregnancy loss that align with our findings. Despite the existence of reports that thyroid autoimmunity and hypothyroidism significantly increase the risk of early RPL (Poppe & Glinoer, 2003) The relationship of hypothyroidism and anti-thyroid antibodies with first-trimester miscarriage remains a matter of debate (Alijotas-Reig *et al.*, 2015). Based on a double-blinded, placebo-controlled, randomized, multicenter international trial (the T4-LIFE study), levothyroxine treatment did not result in higher live birth rates compared to placebo in euthyroid women with recurrent pregnancy loss who tested positive for TPO antibody (van Dijk *et al.*, 2022). Therefore, the association between autoimmune thyroiditis and RPL warrants further investigation.

Preeclampsia toxemia (PET) was the leading cause of late abortion, accounting for 44% of cases. Placental abruption and trauma were also significant factors, with 22.2% and 18.5% of cases, respectively. Several studies have reported similar findings regarding the prevalence and causes of late-term abortion. A study found that PET was a major cause of late-term abortion (Norman & Davison, 2002). Similarly, research identified placental abruption as a critical factor in late-term abortion, accounting for a substantial proportion of cases (Gonen *et al.*, 2021). In the current investigation, none of the investigated mutations was a risk factor for late-pregnancy loss. Other studies have found that FVL homozygosity is more prevalent among pregnant women who experience first-trimester pregnancy loss, whereas protein C deficiency and Glycoprotein Ia polymorphism appear more frequently after the first trimester (Iordache *et al.*, 2022).

Conclusion

The findings of this study highlight the genetic risk associated with the FVL mutation in early RPL, while MTHFR appears to play a less significant role. Anticoagulation therapy, early monitoring, and preconception counseling are recommended to improve pregnancy outcomes. The extremely low prevalence of the FII mutation in the Jordanian population renders it undetectable in cases of RPL. These findings underscore the role of ethnic heterogeneity in shaping the prevalence of these mutations across different populations.

Conflict of Interest

The authors declare no conflicts of interest.

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Author's contributions

Lina M. Al Momani: Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation

Manal A. Abbas: Conceptualization, Supervision, Writing - Original Draft, Visualization

Ethics

This article is entirely original. The corresponding author certifies that all other authors have read and accepted the work and that there are no ethical issues.

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