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Association of tissue inhibitor of metalloproteinase-3 (TIMP-3) serum level and its genetic polymorphism with pregnancy outcome of patients undergoing *in vitro* fertilization and embryo transfer

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ABSTRACT

Objectives: Tissue inhibitors of metalloproteinase-3 (TIMP-3) and matrix metalloproteinases (MMPs) play a major role in embryo implantation and placentation. This study aimed to investigate the relationship between TIMP-3 serum level and TIMP-3 genetic polymorphism with pregnancy outcome in patients undergoing *in vitro* fertilization and embryo transfer (IVF-ET).

Material and methods: This project included 100 infertile women who became pregnant after IVF (IVF^+) and 100 infertile women who failed to conceive after IVF (IVF^-). Genotyping was performed using restriction fragments length polymorphism polymerase chain reaction (PCR-RFLP), and the serum level was measured by Enzyme-Linked Immunosorbent Assay (ELISA).

Results: The frequencies of TT, TC, and CC in the IVF^+ group were 41%, 37% and 22%, respectively, while in the IVF^- group were 18%, 43% and 39%, respectively. The C and T allele frequencies were 40.5% and 59.5% in the IVF^+ group and 60.5% and 39.5% in IVF^- group, respectively. The C allele conferred a 2.25-fold increased risk of IVF failure (OR 2.25; 95% CI 1.5–3.35; $p = 0.0001$). Also, there was a significant increase in TIMP-3 serum levels in the IVF^- group (193.29 ± 29.50 ng/mL), which was higher than the IVF^+ group (166.74 ± 17.60 ng/mL; $p = 0.00002$), was demonstrated. It was shown that the TT genotype is associated with decreased TIMP-3 serum levels in IVF^- group (CC, CT, and TT, values were 143.19 ± 88.49 ng/mL, 117.55 ± 15.73 ng/mL, 61.17 ± 44.36 ng/mL, respectively).

Conclusions: It is concluded that there is a relationship between TIMP-3 gene polymorphism and its serum concentration with IVF-ET outcome. We also suggest that the TT genotype might be involved in IVF-ET outcome.

Key words: TIMP-3; genetic polymorphism; *in vitro* fertilization embryo transfer; serum

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INTRODUCTION

Infertility is a disease defined as the failure to achieve a clinical pregnancy after 12 months of regular and unprotected sexual intercourse. It is estimated that between 8 and 12% of reproductive-aged couples worldwide are affected by infertility [1]. Infertility can be due to many factors such as genetics, age, obesity, low body weight, smoking, infectious or immunological diseases, physiological disorders, and abnormalities in gametes. In most cases, the cause of infertility in most patients (approximately 30% of infertile couples) is unexplained because it is difficult to assess the genetic cause of reduced fertility. Certain genotypes and karyotypes are associated with infertility phenotypes, and the study of genes influencing the reproductive process in humans and model systems can reveal more knowledge about the genetic and multifactorial basis of infertility [2]. Infertility is

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recognized in Iran as one of the main concerns of young couples. In 2019, the overall prevalence of primary infertility was 20.2% which estimates a prevalence of infertility higher than the global average [3]. Fertility involves the production of a viable oocyte, transportation through the fallopian tube, and fertilization with a viable spermatozoan. Having failure in completing any of these stages will lead to infertility. *In vitro* fertilization and embryo transfer (IVF-ET) is one of the most effective infertility treatments. IVF-ET is a two-part assisted reproductive technology procedure performed to manage patients who have problems during pregnancy [3]. Despite progress in assisted reproductive technology, including IVF-ET, people undertaking IVF often face a considerable risk of failure. Implantation is a complex process that requires synchronization of events in the developing embryo and receptive endometrium and involves immune cells, cytokines, growth factors, and cell adhesion molecules.

Metalloproteinases (MMPs) can degrade the extracellular matrix components and are important to many physiological and pathological processes, including embryo implantation and cyclic endometrial breakdown [4] we investigated the epidemiologic features of Kawasaki disease (ICD-9-CM code 446.1. At least 25 members of the MMP family have been identified in human beings [5]. Tissue inhibitors of MMPs (TIMPs) are produced by trophoblastic and decidual tissues throughout gestation. TIMP genes are among the first to be expressed in the developing embryo, preparing for implantation. Embryo implantation is accompanied by extensive cellular and extracellular matrix (ECM) remodeling within the endometrium, which is crucial for the success of this process and placental development. MMPs play a pivotal role in tissue remodeling and the breakdown of the ECM during implantation and placentation in humans. But TIMPs act as regulatory factors to control the site and extent of ECM degradation. There is now much evidence to suggest that the regulation of MMP activity in maternal-fetal dialogue is critical for successful embryo implantation and placentation. Therefore, any perturbation in MMPs and their inhibitors, TIMPs, expression could affect embryo implantation and cause implantation failure [6].

It has been suggested that MMPs and TIMPs are essential during implantation and mediate *in vitro* trophoblast penetration [7]. MMP-3, also known as stromelysin-1, belongs to zinc-dependent proteolytic enzymes. It is a 54 kDa protein produced by various cells, including endothelial cells [8]. MMP3 is situated alongside MMP1 on chromosome 11q22. The dormant form of the enzyme is preserved by the 80-amino acids propeptide containing a zinc-interacting thiol group, which keeps the catalytic domain intact [9]. Tissue inhibitors of TIMPs are locally produced and inhibit specifically active forms of MMPs in the extracellular space. The expression of TIMP-3 was detected in the early phase of pregnancy and induced by progesterone [10] which contains the tripeptide sequence Arg-Gly-Asp (believed to be a specific interaction site for cell-attachment, suggesting that TIMP-3 could be the main regulator inhibiting trophoblast invasion.

TIMP-3 is expressed in the lumen and glandular epithelial cells and uterine stromal cells [11]. Genetic polymorphism play an important role in the susceptibility to diseases [12]. As TIMP-3 was shown to be important in embryo implantation, and the most important polymorphisms identified within the promoter of TIMP-3 is rs9619311 which regulates the TIMP-3 expression, we studied the association of maternal genotype of TIMP3 rs9619311 and its serum level with IVF-ET outcome in infertile women.

MATERIAL AND METHODS

Study design

The current project includes 100 infertile women who became pregnant after IVF (IVF^+) and 100 infertile women who failed to conceive after IVF (IVF^-). None of the women in both groups included in this study were drinkers and smokers. Infertile patients were diagnosed by a gynecologist using available historical information from interviews and clinical records. The age range of IVF^+ was 23–41 and mean age of 33 ± 7 years, and IVF^- with the age range of 24–40 and with an average age of 34 ± 6 years ($p > 0.05$). All participants gave their informed consent before their inclusion in the study, and the contributors were intentionally asked to answer the questionnaire for blood sampling. Furthermore, maternal pathology or genetic anomaly, maternal inflammatory disease, uterine malformation, diabetes, lupus erythematosus, and embryonic aneuploidy were excluded from the study. Blood samples from both groups were collected from Alzahra Hospital, IVF section, Rasht, Iran, from September 2017 to November 2019. This study was approved by the University Ethics Committee and has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Genomic DNA extraction and genotyping

All blood samples (2 mL) were collected in EDTA-coated Tubes (Venoject, Belgium), which were used to extract DNA from peripheral blood samples (leukocytes) using the Triton X100 extraction method. Extracted DNA was confirmed by electrophoresis on one percent agarose gel containing a safe stain solution, and DNA was kept at −20°C until used.

For genotyping the TIMP-3 polymorphism (rs9619311), the restriction fragments length polymorphism polymerase chain reaction (RFLP-PCR) method was used. The Macrogen Company, South Korea, synthesized the PCR primers. The primers were designed by the Oligo7 software (version 7.54, USA).

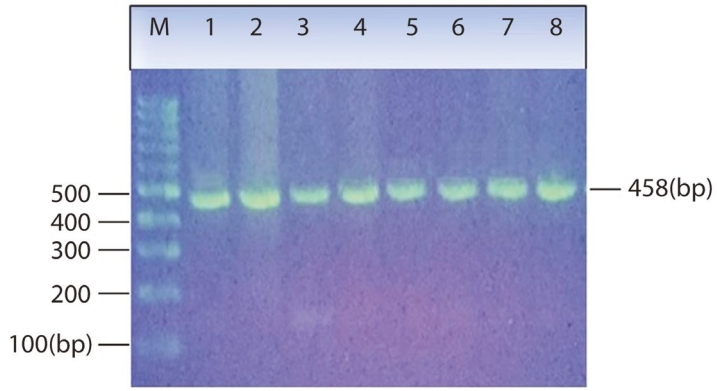

Figure 1. Agarose gel electrophoresis after polymerase chain reaction (PCR) amplification of TIMP33 rs9619311. "M" represents molecular marker. The PCR product size was 458 bp; PCR — polymerase chain reaction; TIMP-3 — tissue inhibitor of metalloproteinase-3

(Forward: 5′-TGGCCACCAATCATCCCATC-3′ and Reverse: 5′-TCCTCGCTGAGAAGTGGACAA-3′).

In this method, SacI restriction endonuclease was used for enzymatic digestion of the PCR product, which identifies and cleaves the GAGCTC specific sequence of DNA strands. In this polymorphism, T replaces C in the promoter region. After incubation, a gel docking machine was used to observe the enzymatic digestion products on 2% agarose gel. Then, the genotypes were interpreted based on the enzyme's effect in the presence of the T or C allele at the site. Three-band patterns were seen based on the site of enzyme cleavage. The CC homozygote showed a single band of 458 bp, the heterozygote (CT) showed three bands of 458 bp, 298 bp, 168 bp, and the TT homozygote showed two bands of 298 bp and 160 bp.

For the quality control, genotyping of 5% of samples was randomly repeated, yielding 100% similarity.

Serum TIMP-3 concentration

Circulating TIMP-3 levels were measured using ELISA and antiserum against human TIMP-3. The Human TIMP-3 ELISA Kit (Abcam Corporation, ab119608) was used for the measurement of TIMP-3 serum concentration, according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using χ^2 by Med Calc ver. 12.1.4 Software (Mariakerke, Belgium) to predict the association or absence of relationship between TIMP-3 promoter (rs9619311) polymorphism of TIMP-3 gene and the outcome of *in vitro* fertilization. Odds ratios were calculated together with their 95% confidence intervals (CI). Data were analyzed using chi-square test, analysis of variance (ANOVA), and by calculating odds ratios (OR) and 95% CI. A p value of less than 0.05 was considered statistically significant.

RESULTS

TIMP-3 genotyping by PCR-RFLP

This study included 100 infertile women who became pregnant after IVF (IVF^+) and 100 infertile women who failed to conceive after IVF (IVF^-). The undigested PCR product size was 458 bp for TIMP-3 rs9619311 (Fig. 1). The CC homozygote showed a single band of 458 bp, the heterozygote (CT) showed three bands of 458 bp, 298 bp, 168 bp, and the TT homozygote showed two bands of 298 bp and 160 bp (Fig. 2).

The genetic and allele frequencies of the rs9619311 polymorphism of TIMP-3 were also analyzed. All information about allele and genotype frequencies and associated ORs (95% CI) for (IVF^+) and IVF^- cases are presented in Table 1.

The frequencies of TT, TC, and CC genotypes in IVF^+ were 41%, 37%, and 22%, respectively, while in IVF^- were 18%, 43%, and 39%, respectively ($p < 0.05$). We have also analyzed the allele frequency in both groups. The T and C allele frequencies in the IVF^+ group were 59.50% and 40.50%, respectively, while the amount in IVF^- was 39.50% and 60.50%, respectively. There was a significant difference between T and C allele frequency between IVF^+ and IVF^- groups ($p = 0.0001$). The frequency of the C allele was significantly higher in the IVF^- than in the IVF^+ (59% vs 41%; $\chi^2 = 8.36$; $p = 0.003$), suggesting that the C allele is a risk factor for IVF-ET outcome and C allele carriers are more than two times at the risk of IVF-ET failure [OR 2.25 (1.5–3.35)].

Serum levels of TIMP-3 between IVF^+ and IVF^- groups

Serum levels of TIMP-3 in IVF^- and 100 sex and age-matched IVF^+ are shown in Table 2 and Figure 3. The serum level of TIMP-3 was significantly higher in IVF^- group when compared to the IVF^+ group (193.29 ± 29.50 ng/mL vs 166.74 ± 17.60 ng/mL, $p = 0.00002$). We have also shown that the TT genotype is significantly associated with

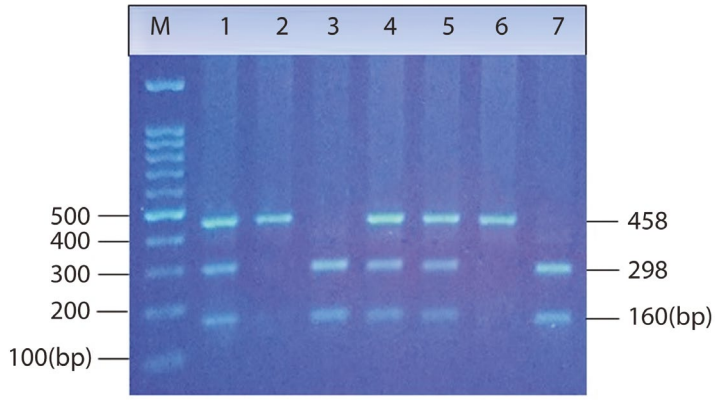

Figure 2. Agarose gel electrophoresis of tissue inhibitor of metalloproteinase-3 (TIMP-3) polymorphism; M — molecular marker, 2 and 6: CC homozygote, 1, 4 and 5: TC heterozygote, 3 and 7: TT homozygous

Table 1. Allele and genotype frequencies of rs9619311 polymorphism among IVF$^+$ and IVF$^-$ groups

	IVF$^+$ (n = 100)	IVF$^-$ (n = 100)		p
	n (%)	n (%)	OR (95% CI)	
Allele				
T	119 (59.5)	79 (39.5)	1.00 (reference)	0.0001*
C	81 (40.5)	121 (60.5)	2.25 (1.5–3.35)	
Genotypes				
TT	41 (41%)	18 (18%)	1.00 (reference)	
TC	37 (37%)	43 (43%)	2.64 (1.03–5.36)	0.007*
CC	22 (22%)	39 (39%)	4.03 (1.88–8.64)	0.0003*

*Significant at 5% level of significance ($p < 0.05$); CI — confidence interval; IVF — *in vitro* fertilization; OR — odds ratio

Table 2. Serum levels of TIMP-3 in IVF$^+$ and IVF$^-$ groups

IVF$^-$ (n = 100) Mean ± SD	IVF$^+$ (n = 100) Mean ± SD	p
193.29 ± 29.50	166.74 ± 17.60	0.00002*

*Significant at 5% level of significance ($p < 0.05$); IVF — *in vitro* fertilization; SD — standard deviation

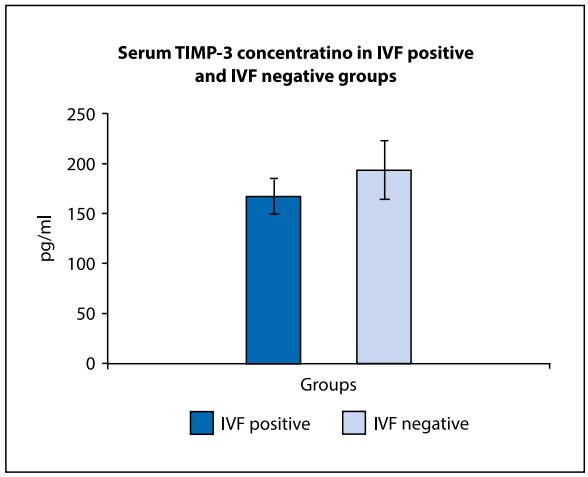

Figure 3. Comparison of tissue inhibitor of metalloproteinase-3 (TIMP-3) concentrations in the serum of IVF$^+$ and IVF$^-$ subjects; IVF — *in vitro* fertilization

decreased TIMP-3 serum concentration in the IVF$^-$ group (CC, CT, and TT, the values were 143.19 ± 88.49 ng/mL, 117.55 ± 15.73 ng/mL, 61.17 ± 44.36 ng/mL respectively) (Fig. 4 and Tab. 3). CC genotype seems to be associated with higher serum TIMP-3 concentration in IVF$^-$ group and may be related to the IVF-ET outcome.

DISCUSSION

In this project, we investigated the relationship between TIMP-3 serum concentration and its genetic promoter (rs9619311) polymorphism with pregnancy outcomes of patients undergoing IVF-ET. We showed a significant relationship between TIMP-3 gene polymorphism and its serum concentration with IVF-ET outcome. We also showed that TT genotype might be involved in pregnancy outcomes after IVF-ET. In addition to assessing female factors in the process of artificial insemination, male factors such as age,

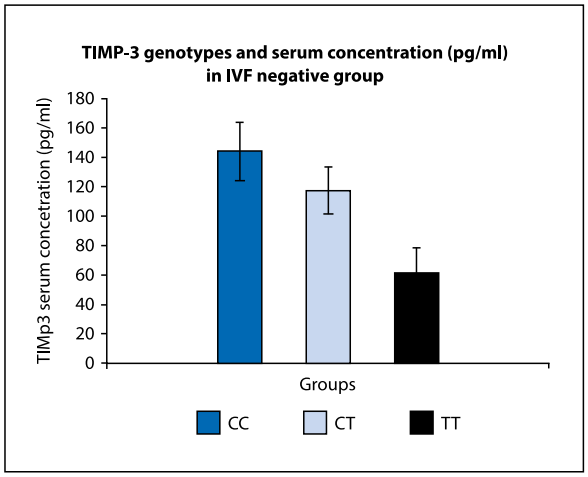

Figure 4. Association of tissue inhibitor of metalloproteinase-3 (TIMP-3) serum concentration and genotypes IVF⁻ group. CC genotype is associated with increased TIMP-3 expression in IVF⁻ group (TT, CT and CC serum levels were 61.17 ± 44.36, 117.55 ± 15.73 and 143.19 ± 88.49 mg/mL, respectively); IVF — *in vitro* fertilization

lifestyle, sperm count, motility, and natural morphology are traditional criteria for assessing semen quality [13]. Angiogenesis, invasion and decidualization play important roles in implantation and embryo development. Many genes, including MMPs and TIMPs, have been shown to be implicated in the embryo implantation and IVF-ET outcome. MMPs and TIMPs play a critical role in embryo-endometrium crosstalk and embryo implantation [14]. The expression of MMPs and TIMPs mRNA has been shown in the decidual cells [15]. MMPs and TIMPs play central roles in the reproductive organs' structural and functional properties. Dysregulation of MMPs and TIMPs contributes to recurrent implantation failure [14]. It has been documented that TIMP-3 in the extracellular proteolysis relates to the implantation of the early embryo [16]. It has been suggested that TIMP could be a candidate gene for embryo implantation and embryo survival [17].

Furthermore, TIMP mRNAs and proteins are widely and highly expressed in the endometrium [18]. TIMP is considered a good marker in the formation of the decidua. At the time of implantation, the TIMP protein is secreted by the granular epithelium and is thought to be involved in diagnosing and implementing the blastocyst [19]. MMP-9 and TIMP-1 mRNA have been documented to play central roles in implantation. The decreased expression of their gene might be one of the main causes of unexplained infertility [20].

It has been shown that serum MMP-3 value is positively correlated with age, and a contrary tendency was found for MMP-10 and TIMP-2 serum levels. Gender is a significant factor modifying MMP/TIMP potential, except for the MMP-10 level [21]. MMP-3 expression levels are positively correlated with blood pressure in obese individuals and metabolic syndrome. Moreover, MMP-1, MMP-2, TIMP-1, and TIMP-2 levels increase in obese people; hence MMP-1, MMP-3, and MMP-9 levels with several parameters related to obesity [22]. MMPs regulated by active mast cells and eosinophils play an important role in menstruation. For example, MMP-1, -3, and -9 are associated with degraded tissue in the menstrual endometrium. The activation of MMPs is a prerequisite for tissue destruction during menstruation. It is regulated by progesterone uptake and paracrine factors from epithelial and stromal cells and mast cells [23]. MMPs expression change during the physiological menstrual cycle. MMPs are essential for maintaining the physiological stability of the endometrium and are involved in the menstrual cycle [24].

Wysocka et al. [25] for the first time showed that there is a correlation between recurrent pregnancy loss and TNF rs1800629 and MMP3 rs35068180 gene polymorphisms in the polish women. They concluded that the maternal GG TNF and 5A/5A MMP3 genotypes occur significantly more frequently in cases with repeated miscarriages. The association of gene polymorphisms with pregnancy outcomes of patients undergoing IVF-ET has been demonstrated [26].

Roshankhah et al. [27] have shown that the polymorphism of the TIMP-2 gene and its interaction with the MMP9 gene might be related to male infertility. They suggested that further studies are required in different ethnicities and with a larger sample size in order to confirm these findings. It has been shown that the MMP3 polymorphism is involved in advanced endometriosis cases and female infertility [28]. In 2017, Kurzawski [29] and colleagues suggested a link between MMP9 and TIMP-2 SNPs with sperm

Table 3. Serum TIMP-3 concentration in three genotypes in IVF⁺ and IVF⁻ groups

	CC	CT	TT
Average	143.19	117.55	61.17
SD	88.49	15.73	44.36
p value	CC v CT CC v TT CT v TT		0.006 5.79×10^{-7} 2.17×10^{-5}

IVF — *in vitro* fertilization; SD — standard deviation

parameters. Abnormal MMP and TIMP expression has been suggested to be involved in the pathogenesis of endometriosis [30]. Matrix metalloproteinases activity in the endometrium has been shown to depend on the phase of the menstrual cycle and the levels of steroid hormones, whereas TIMP-1 and -2 are expressed at relatively stable levels in proliferative and secretory endometrium. However, marked increases have been observed in the expression of TIMP-3 during the secretory phase, suggesting a role in the preparation of the endometrium for decidualization and implantation [31]. Shabanipour et al. [26] in 2015, conducted a study on the association of MMP9 gene polymorphism with the results of pregnancy after IVF-ET. They suggested that MMP9 gene polymorphism may not be associated with IVF-ET outcomes in the Iranian population [27]. Another study showed that SNP 1562C/T of MMP9 is not related to the risk of recurrent early pregnancy loss (REPL) in the Indian population and suggested further study in other people will verify whether it is associated with REPL risk or not. REPL is a multifactorial pathology, and other genetic or environmental factors may contribute to the complex etiology of recurrent early pregnancy loss [32]. It was suggested that changes in TIMP and MMP expression are implicated in embryo implantation dysfunction (EID) [33]. Changes in MMP-9 serum and follicular fluid concentrations have been shown in female patients undergoing IVF treatment. They could be a good predictor of the successful IVF outcome (pregnancy), which was proven for serum and follicular MMP-9 levels. MMP-9 and TIMP-1 mRNA have been documented to play a central role in implantation. The decreased expression of their gene might be one of the main causes of unexplained infertility [20]. It was demonstrated that serum TIMP-1 could be used to predict pregnancy outcomes following IVF-ET [34]. Malvezzi et al. [35] explained that advanced pelvic endometriosis severity is connected to higher serum MMP-2 concentrations.

The results of our study showed that there is a connection between TIMP-3 gene polymorphism with the outcome of IVF-ET in the Iranian population. However, in some studies, no association has been observed between MMPs and TIMPs gene polymorphism with the results of IVF-ET. Since genetic polymorphisms often vary among different ethnic groups, the different results in different populations may be due to the differences in sample sizes, genetic backgrounds and impact of environmental factors.

This was a case-control study with 200 samples, but it has several limitations that must be considered. First, only one polymorphic site was investigated, which may not represent the entire gene. Second, potential selection bias might have occurred because of the hospital-based case-control study. Third, our population was not large enough. Fourth, numerous factors such as age, lifestyle, and genetic factors act individually and together to influence pregnancy outcomes after IVF-ET. Thus, we should involve more elements in our future work.

CONCLUSIONS

In this study, we showed a significant association between TIMP-3 (rs9619311) gene polymorphism and its serum concentration with IVF-ET outcome. We also suggest that the TT genotype is associated with decreased TIMP-3 concentration and may play a role in IVF-ET outcome. Larger studies with more patients and controls are needed to confirm these results.

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Authors' contribution conceptualization

Conception or design of the work: FM, Data collection: MM and ZZ, Data analysis and interpretation: FM, MM, ZZ and AE, Drafting of the article: MM and FM, Critical revision of the article: FM, supervision, FM, ZZ and AE.

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Conflict of interest

The authors declare there is no conflict of interest.

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