ORIGINAL ARTICLE

Plasma concentration, genetic variation, and gene expression levels of matrix metalloproteinase 9 in Iranian patients with coronary artery disease

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Background: Matrix metalloproteinase 9 (MMP9) -1562C>T (rs3918242) polymorphism has been proposed as a risk factor for coronary artery disease (CAD) with conflicting results. The aim of the present study was to investigate the association of -1562C>T genetic polymorphism, gene expression and circulating levels of MMP9 with CAD risk in an Iranian subpopulation in in Zanjan City. **Materials and Methods:** This was a retrospective case–control study we investigated retrospectively 100 patients with angiographically verified CAD and 100 matched controls. Genotyping of -1562C>T polymorphism was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Gene expression levels and circulating levels of MMP9 was determined by real-time reverse transcription-PCR and enzyme immunoassay method, respectively. Statistical analysis was done using Student's *t*-test or Chi-square test by SPSS 16 software. **Results:** The mean circulating levels of MMP9 were significantly higher in CAD Group than control group ($P = 0.002$). Mean plasma levels of MMP9 were also significantly higher in triple vessel stenosis patients than double vessel or single vessel stenosis patients ($P < 0.001$). Moreover, mean plasma levels and gene expression levels of MMP9 were significantly higher in T allele carrier than C allele carrier of MMP9 -1562C>T polymorphism ($P = 0.002$, $P = 0.01$, respectively). However, genotype and allele frequencies of MMP9 -1562C>T polymorphism were similar between CAD patients and controls ($P > 0.05$). Additionally, the -1562C>T polymorphism of MMP9 gene didn't increase the risk of CAD in dominant ($P = 0.537$) or recessive ($P = 0.249$) genetic models. **Conclusion:** Our study demonstrated that circulating levels of MMP9 but not -1562C>T polymorphism of MMP9 gene may be a risk factor for development and severity of CAD in an Iranian subpopulation in Zanjan.

Key words: Coronary artery disease, matrix metalloproteinase 9, polymerase chain reaction-restriction fragment length polymorphism, polymorphism

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INTRODUCTION

Coronary artery disease (CAD) is a common multifactorial disease characterized by a high rate of morbidity and mortality. According to a study by Talaei *et al*., the annual incidence of CAD in an Iranian population was reported to be 1436 and 1168 per 100,000 persons-years in men and women, respectively.[1] Also, according to a study by Sadeghi *et al*., the prevalence of CAD based on the Rose questionnaire and Minnesota coding was 37.5% in women and 22.2% in men on a sample of Iranian population.[2] Moreover, the CAD mortality rate was reported to be 121.5–156.6 per 100,000 Iranian populations.[2] Despite the high prevalence of CAD in the worldwide, its underlying mechanism has not been fully understood.[3]

The main cause of CAD is atherosclerosis that is characterized by plaque formation in the inner wall of arterial.[3] Several groups of proteolytic enzymes, including matrix metalloproteinases 9 (MMP9) are involved in extracellular matrix remodeling and

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vascular atherosclerotic plaques destabilization and rupture.[4,5] Elevated levels of MMP9 have been observed in vulnerable regions of human atherosclerotic plaques and play a significant role in the progression of atherosclerosis and plaque rupture.[4] Genetic alterations in the MMP9 gene may modify the gene expression and circulating levels of the MMP9 and has been proposed as a risk factor for CAD.[6,7]

The human MMP9 gene is located on chromosome 20q12.2–13.1 and the -1562C>T promoter polymorphism (rs3918242) of MMP9 gene was shown to be associated with higher MMP9 circulating levels and an increased susceptibility to CAD.[8,9] However, conflicting results have been reported regarding the association of MMP9 -1562C>T polymorphism with CAD risk.[10-14] The association of -1562C>T polymorphism of MMP9 gene with the CAD risk has been studied at different populations. Some of these studies have reported MMP9 -1562C>T polymorphism as a risk factor for CAD development while other studies have not reported MMP9 -1562C>T polymorphism as a risk factor for CAD development. These results are considered as "conflicting results."[10-14] These controversial results are probably due to differences in the ethnic background of studied population.[12,15] Currently, there is limited data regarding the role of MMP9 -1562C>T polymorphism as a risk factor for CAD development from Northwest of Iran. So, in the present study the -1562C>T genetic variation, gene expression levels and circulating levels of MMP9 were assessed in CAD patients and control subjects.

MATERIALS AND METHODS

Study population

Our retrospective case–control study included a total of 200 unrelated individuals admitted for diagnostic angiography in the Mousavi Teaching Hospital of Zanjan, Iran. They were recruited from April 2014 to August 2014 for investigation of possible CAD. The patient's group included 100 individuals (56 male and 44 female) with a positive angiogram showing a minimum of 50% stenosis in at least one major coronary artery. Patients with previous myocardial infarction history showing positive angiogram result were also included in the study. Patients with congenital heart failure, valvular heart disease, cardiomyopathy and other organ failures were excluded from the study. The severity of CAD was determined based on the number of stenotic vessel. Control subjects (51 male and 49 female) were selected based on normal angiography results and the absence of any personal or family history of CAD or other reasons to suspect CAD. Also, control subjects with the evidence of concomitant diseases such as malignant diseases and febrile conditions were excluded. For all subjects, a complete medical history including questions about smoking habits, history of hypertension and diabetes and family history of heart disease was obtained by questionnaire.

The mean age of CAD patients and controls were59.4 ± 23.5 and 56.7 ± 29.5, respectively that were not statistically significant (P = 0.475). In CAD Group, the mean age of males and females were 56.10 ± 22.18 and 63.51 ± 24.67, that were statistically insignificant (P = 0.117). In control group, the mean age of males and females were 54.35 ± 27.21 and 59.23 ± 29.67, that were not statistically significant (P = 0.393).

The study sample size was estimated based on previous studies.[16,17] The minimum required sample size according to Cho *et al*.[16] study (TT+TC frequency in case and control group were 31% and 6%, respectively, α =0.05 and power = 0.9) was calculated by 55 persons in each group. The sample size was calculated by OpenEpi version 2.2 software (free online statistical software available at: www.openepi.com) and the following formula

$$(n=\frac{\left(z_{1-\frac{\alpha}{2}}+z_{1-\beta}\right)^2\left[P_1(1-P_1)+P_2(1-P_2)\right]}{(P_1-P_2)^2})$$ assuming $\alpha = 0.05$, $\beta = 0.1$, $P_1 = 0.06$, $P_2 = 0.31$.

All of the study subjects participated voluntarily in the study and gave written informed consent. The study was approved by Ethic Committee of Zanjan University of Medical Sciences (Ethical code: ZUMS.REC.1394.344), Zanjan, Iran.

Blood collection and processing

Fasting blood samples (10 ml) were collected in EDTA-containing tubes and immediately centrifuged. Plasma was separated and stored at −20°C until biochemical analysis and the cellular fraction was used for DNA and RNA extractions.

Biochemical analysis

Plasma levels of MMP9 were assayed by an ELISA kit (Shanghai Crystal Day Biotech Co., China) using double-antibody sandwich enzyme-linked immunosorbent assay technique. All assays were performed in duplicate according to manufacturer's protocol. The sensitivity limit of the assay kit for MMP9 was 0.015 ng/ml. Lipid profile and fasting glucose levels were determined by commercially available kits (Pars Azmoon Co., Iran) using Mindray Auto-analyzer. The low-density lipoprotein-cholesterol (LDL-C) was calculated by Friedewald formula.

Matrix metalloproteinase 9 -1562C>T polymorphism analysis

DNA was isolated from blood leukocytes using a DNA extraction kit (Viogene, Poland) according to instructions

of kit. Purified DNA was stored at −20°C until analysis. Genotyping of MMP9 -1562C>T polymorphism was conducted by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using the following primers, forward: 5′-GCC TGG CAC ATA GTA GGC CC-3′ and reverse: 5′-CTT CCT AGC CAG CCG GCA TC-3, as prviously described.[14] After amplification, 8 µl aliquot of PCR product was digested with 5 U of the restriction enzyme SphI (Fermentas, USA) at 37ºC for 8 h. The digested products subjected to electrophoresison a 3% agarose gel and subsequently stained with SYBR Green dye. Digestion of the PCR product in the presence of C allele results in a single noncleaved 435 bp fragment while in the presence of T allele, the 435 bp amplicon was cleaved into 188 bp and 247 bp fragments. Samples were analyzed and result presented only by code on the tubes, so the person responsible for PCR analysis was blind to the case/control condition for the sample.

Matrix metalloproteinase 9 gene expression analysis

Total RNA was extracted from blood leukocytes using TRIZOL reagents (Invitrogen, USA). Then, 500–1000 ng of total RNA was reversely transcribed to cDNA in a total volume of 20 µl according to the instruction of dART RT kit (EURx Ltd., Poland). Quantitative real-time reverse transcription PCR (RT-PCR) was performed using high ROS SYBR Green PCR Master Mix Kit (Ampliqon, Denmark) in an ABI 7300 instrument (Applied Biosystems, USA). Real-time PCR conditions were as follow: initial denaturation at 95°C for 15 min, and 35–40 subsequent cycles of denaturation at 95°C for 30 s, 65°C for 30 s and 72°C for 30 s. All samples were run in duplicate and β2 microglobulin (β2M) was amplified as an internal control to normalize target gene expression. The selection of β2M as an internal control was based on some previously published studies.[6,18] The sequence of real-time RT-PCR primers for MMP9 were as follows, forward: 5′-ACGACGTCTTCCAGTACCGA-3′, reverse: 5′-TCATAGGTCACGTAGCCCAC-3′ and for β2M were forward: 5′-TCTTTCTGGCCTGGAGGCTATC-3′, reverse: 5′-CGGATGGATGAAACCCAGACAC-3′. Fold changes in gene expression levels among different samples were determined by $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All statistical analysis was performed using SPSS 16 software (SPSS Inc, Chicago, IL., USA). In descriptive statistics categorical data were presented as number and percent; numerical data were presented as mean ± standard deviation. Normality test (Shapiro–Wilk test) showed that numerical variables are normally distributed. Independent sample *t*-test was used to compare the numerical variables between groups. Association between different categorical variables was done using Chi-square and Fisher exact tests. $P < 0.05$ was considered as statistical significance. Matching of case and controls for age and sex were not performed in this study, as the distribution of age and sex were not statistically different between groups.

Also, we had no missing data in the present study. Demographics and risk factors data have been obtained from all participants by questionnaire and medical reports. Additionally, genetic and biochemical analysis were successfully performed for all samples and resulting data were collected. Moreover, to minimize the potential sources of bias, strict selection criteria were considered for cases and controls, as previously described. Also, to minimize data collection bias all experimental analysis was done carefully and was repeated in case of necessity.

RESULTS

The comparison between clinical and demographic characteristics of the CAD patients and control subjects are listed in Table 1. There were no significant differences in the mean ages, sex distribution, triglyceride levels between the two groups ($P > 0.05$). However, compared with control group, CAD patients had significantly higher plasma levels of total cholesterol, LDL-C and lower plasma levels of high-density lipoprotein-cholesterol ($P < 0.05$). Also, there were significantly higher percentages of patients with diabetes ($P = 0.024$), hypertension ($P = 0.017$) and smoking habit ($P = 0.002$) in the CAD Group in comparison to control group. The mean circulating levels of MMP9 was significantly higher in CAD Group than control group (24.9 ± 8.5 vs. 16.7 ± 6.4, $P = 0.002$). Moreover, the mean plasma levels of MMP9 were significantly higher in triple vessel stenosis patients (36.15 ± 8.20) than double vessel (24.13 ± 6.70) or single vessel stenosis patients (21.36 ± 9.80) ($P < 0.001$). Additionally, the mean plasma levels of MMP9 were evaluated in patients with and without-1562C>T polymorphism. As shown in Figure 1, plasma levels of MMP9 was significantly

Table 1: Clinical characteristics of the coronary artery disease patients and the control subjects included in our study

Variables	CAD patients, *n*=100 (%)	Controls, *n*=100 (%)	*P*
Age (years)	59.4±23.5	56.7±29.5	0.475
Sex (male/female)	56/44	51/49	0.478
TG (mg/dl)	180.4±95.6	169.4±75.7	0.395
TC (mg/dl)	192.8±65.4	167.3±49.5	0.002
HDL, *n* (%)	38.6±10.5	43.3±14.8	0.010
LDL, *n* (%)	99.3±51.6	85.5±43.2	0.041
Hypertension, *n* (%)	21 (21)	9 (9)	0.017
Diabetes, *n* (%)	23 (23)	11 (11)	0.024
Smoking, *n* (%)	36 (36)	12 (12)	0.002
MMP9 (ng/ml)	24.9±8.6	16.7±6.4	0.002

TC = Total cholesterol; TG = Triglyceride; HDL = High-density lipoprotein; LDL = Low-density lipoprotein; CAD = Coronary artery disease; MMP9 = Matrix metalloproteinase 9

higher in heterozygote genotype and mutant homozygote genotype of MMP9 -1562C>T polymorphism than that of wild-type genotype ($P < 0.001$). As indicated in Table 2, no significant differences were observed regarding the frequency of heterozygote genotype (27% vs. 26%, $P = 0.768$), mutant homozygote genotype (5% vs. 2%, $P = 0.237$) and minor T allele (18.5% vs. 15%, $P = 0.348$) between CAD patients and controls. In addition, as shown in Table 3 no significant association was seen between MMP9 -1562C>T gene polymorphism and CAD risk under recessive genetic model (odds ratio [OR] =2.6, 95% confidence interval [CI]: 0.5–13.6, $P = 0.249$) or dominant genetic model (OR = 1.2, 95% CI: 0.7–2.2, $P = 0.537$) that were analyzed. Moreover, the association between MMP9 -1562C>T genotypes and severity of CAD were analyzed. As shown in Table 4, the heterozygote genotype and mutant homozygote genotype was significantly more common in triple vessel disease patients than single vessel disease patients ($P < 0.001$, $P = 0.007$, respectively). However, no significant differences were observed in the frequency of heterozygote and mutant homozygote genotype between CAD patients with one and two diseased vessels ($P > 0.05$).

The association between different genotypes of MMP9 -1562C>T polymorphism and gene expression levels of MMP9 indicated that fold increase in the mRNA expression levels of MMP9 was 2.25 ± 0.48 for heterozygote genotype and 2.98 ± 0.63 for mutant homozygote genotype as compared to wild type genotype of MMP9 -1562C>T polymorphism ($P = 0.03$; $P = 0.01$, respectively).

Table 2: Prevalence of matrix metalloproteinase 9 allele and genotypes in coronary artery disease patients and control subjects

MMP9-1562C >T gene polymorphism	CAD patients, *n*=100 (%)	Controls, *n*=100 (%)	OR (95%CI)	*P*
1562C allele	163 (81.5)	170 (85)	1	-
1562T allele	37 (18.5)	30 (15)	1.3 (0.76–2.2)	0.348
1562CC genotype	68 (68)	72 (72)	1	-
1562CT genotype	27 (27)	26 (26)	1.1 (0.6–2.1)	0.768
1562TT genotype	5 (5)	2 (2)	2.6 (0.5–14.1)	0.237

1562CC = Wild type; 1562CT = Heterozygote; 1562TT = Homozygote; CAD = Coronary artery disease; MMP9 = Matrix metalloproteinase 9; OR = Odds ratio; CI = Confidence interval

Table 3: Analysis of matrix metalloproteinase 9-1562C >T polymorphism in coronary artery disease patients and control subjects using dominant and recessive genetic models

Genetic model	Genotype	CAD patients, *n*=100 (%)	Controls, *n*=100 (%)	OR (95% CI)	*P*
Dominant	1562CC	68 (68)	72 (72)	1	
	1562 (CT+TT)	32 (32)	28 (28)	1.2 (0.7-2.2)	0.537
Recessive	1562 (CC+T)	95 (95)	98 (98)	1	
	1562TT	5 (5)	2 (2)	2.6 (0.5-13.6)	0.249

CAD = Coronary artery disease; MMP9 = Matrix metalloproteinase 9; 1562CC = Wild type; 1562CT = Heterozygote; 1562TT = Homozygote; OR = Odds ratio; CI = Confidence interval

DISCUSSION

The main findings of our study were that, (i) elevated circulating levels of MMP9 is a significant risk factor for development and severity of CAD in an Iranian subpopulation in Zanjan, (ii) the MMP9 -1562C>T gene polymorphism is associated with increased gene expression levels and plasma circulating levels of MMP9 in both heterozygote and mutant homozygote states, (iii) MMP9 -1562C>T polymorphism is not a significant risk factor for CAD development however, it is associated with the severity of CAD.

The association of -1562C>T polymorphism of MMP9 gene with the CAD risk has been studied at different populations;

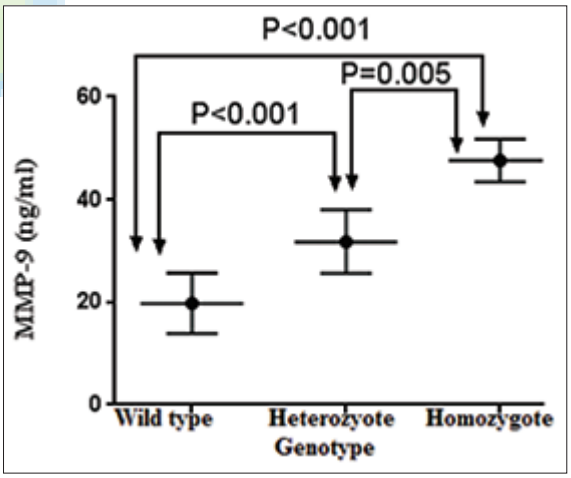

Figure 1: The association between different genotypes of matrix metalloproteinase 9 -1562C>T polymorphism and mean plasma levels of matrix metalloproteinase 9 in coronary artery disease patients

Table 4: Number of patients with 1, 2, and 3 stenotic coronary arteries according to matrix metalloproteinase 9-1562C >T genotypes

MMP9-1562C >T genotypes	With 1 stenotic vessel, *n* (%)	With 2 stenotic vessel, *n* (%)	With 3 stenotic vessel, *n* (%)	*P* (2 vs. 1 stenotic vessel)	*P* (3 vs. 1 stenotic vessel)
1562CC	31 (45.6)	33 (48.5)	4 (5.9)	Reference	Reference
1562CT	5 (18.5)	8 (29.6)	14 (51.9)	0.511	<0.001
1562TT	1 (20)	0 (0.0)	4 (80)	0.984*	0.007*
1562 (TT+CT)	6 (18.7)	8 (25)	18 (56.3)	0.704	<0.001

*Values were calculated using Fisher's exact test. MMP9 = Matrix metalloproteinase 9; 1562CC = Wild type; 1562CT = Heterozygote; 1562TT = Homozygote

however, the reported results are conflicting.[10-14] Our study demonstrated that -1562C>T polymorphism of MMP9 gene didn't increase the risk of CAD either in heterozygote, homozygote, dominant or recessive genetic models. Therefore, our study was in accordance with the Opstad *et al*. and Juan *et al*. studies that showed no correlation between this polymorphism and CAD development.[6,19] However, our results were inconsistent with those studies that reported a positive association between this polymorphism and CAD.[13,20-23] The reasons for these contradictory results may be related to variation in study design, different selection criteria for cases and controls, heterogeneity in sample size, gene-gene and gene-environmental interactions.[15] Also, ethnic differences may influence the impact of this polymorphism on CAD risk. Indeed, a recently published study by Wang and Shi[12] indicated an interethnic differences in the genotype distribution of MMP9 -1562C>T genetic polymorphism and demonstrated this genetic variant as a significant risk factor for CAD only in East Asian population but not in either West Asians or Western populations. Moreover, some specific interactions between genotype and environmental effects may explain the conflicting results of the association studies. According to some previously published studies, MMP9 -1562C>T polymorphism has been linked with increased CAD risk only in individuals with high levels of plasma apolipoprotein B, high levels of plasma apolipoprote in A and high fibrinogen levels.[23] Unfortunately, we were not able to assess these CAD risk factors in the current study.

In our study no significant association was found between MMP9 -1562C>T polymorphism and CAD risk, however, a positive association was seen between the -1562CT and -1562TT genotypes and severity of CAD. Similarly, in a study by Zhang *et al*.[8] it was reported that the severity of CAD, as determined by the number of diseased vessels showing more than 50% stenosis, was positively correlated with the -1562T allele.

In the present study, the gene expression levels of MMP9 gene were significantly higher in the heterozygote and mutant homozygote genotype compared with wild-type genotype of MMP9 -1562C>T polymorphism. This finding may be explained by the study of Morgan *et al*.[24] that showed an allele-specific effect of -1562C>T polymorphism on MMP9 gene expression levels. Also, according to some studies, the variant T allele of MMP9 C-1562T polymorphism had a higher promoter activity than the C allele resulting in increased expression of the gene and higher MMP9 plasma levels. This phenomenon may be attributable to the weaker binding of the transcriptional repressor protein to the T allele and preferential binding of the transcriptional repressor protein to the C allele.[8]

Moreover, our study demonstrated a positive correlation between heterozygote and mutant homozygote genotypes of -1562C>T polymorphism and circulating levels of MMP9 that was consistent with the Opstad *et al*., Izidoro-Toledo *et al*. and Wu *et al*. studies.[6,10,13] However, some other studies identified no close association between MMP9 -1562C>T polymorphism and plasma levels of MMP9 that were inconsistent with our study.[11,25,26] The explanation behind these contradictory findings may be related to use of some medications such as statins by CAD patients that can change plasma levels of MMP9 as indicated by some studies.[10,27]

In accordance with some previously published studies[6,13] our study indicated that MMP9 levels were significantly higher in CAD patients than control subjects. Similar to Kalela *et al*. study,[28] in our study circulating levels of MMP9 was significantly higher in triple vessel CAD patients than double vessel or single vessel CAD patients. These results confirmed the role of MMP9 in the development and severity of CAD.

Moreover, bias resulting from systematic errors can occur at any phase of research, including study design, data collection and in the process of data analysis.[29] However, we considered strict selection criteria to define case and control group to limit selection bias. Also, to minimize data collection bias, all experimental analysis was done carefully and was repeated in case of necessity. Also, the present study was conducted in Zanjan province (in the Northwest of Iran) where a significant proportion of populations are Azeri. So, we assume that generalization of the present results to other Iranian subpopulations with different ethnic and genetic background such as Fars, Kurdish, Lur, Baluch, Arabs, and Turkmens may require more investigations and should be done with more caution. We should mention that MMP9 -1562C>T polymorphism is a known mutation that has been widely studied in different studies by various genotyping methods such as PCR-RFLP method. I agree that sequencing further reinforce the quality of obtained data especially regarding the unknown mutations, however, in case of known mutations such as MMP9 -1562C>T polymorphism that has been widely studied in various articles with PCR-RFLP method, the lack of sequencing data may not impose considerable error in genotyping results.

Some of limitations of our study were as follows: (i) no gene-gene or gene-environment interaction was evaluated, so the potential susceptibility of MMP9 to CAD risk may be diluted or masked by other gene-gene or gene-environment interactions. (ii) The enzymatic activity of MMP9 was not assayed in this study. MMP9 activity assay may be more informative than MMP9 concentration assay, especially in the presence of MMP9 functional abnormalities. (iii) RFLP is an old method for detection of mutation. However, RFLP

is a widely used, and is perhaps the simplest method for detection of mutations conferring reliable results. The PCR-RFLP method allows simple and inexpensive detection of point mutations. This convenient and simple method is useful in a small basic research study. PCR-RFLP *per se* is not generally suitable for high-throughput SNP genotyping; however, this technique does have its advantages and still plays important role in many small labs.

CONCLUSION

Our study demonstrated that circulating levels of MMP9 but not -1562C>T polymorphism of MMP9 gene may be a risk factor for development and severity of CAD in an Iranian subpopulation in Zanjan. However, MMP9 -1562C>T polymorphism is a determinant factor for elevated levels of MMP9 and severity of CAD.

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Conflicts of interest

There are no conflicts of interest.

AUTHORS' CONTRIBUTION

- KM contributed to the conception and design of the work, drafting the work and revising it, approval of the final version of the manuscript and agreed for all aspects of the work.
- KK contributed to the conception and design of the work, the acquisition, analysis, or interpretation of data for the work and agreed for all aspects of the work.
- EK contributed in the conducting the study, conception of the work, approval of the final version of the manuscript, and agreed for all aspects of the work.
- MSS contributed in the conception and design of the work, conducting the study, final approval of the manuscript and agreed for all aspects of the work.

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