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Original Article

Alteration in expression of matrix metalloproteinase-9 in peripheral blood mononuclear cells could be considered for estimating the severity of coronary artery stenosis

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Abstract

Background and aims: Atherosclerosis is one of the main reasons why people die from cardiovascular disease. The pathogenesis of atherosclerosis may have been aided by the deregulation of cellular and molecular events in peripheral blood mononuclear cells (PBMCs). This study aimed to investigate the alteration of the expression of matrix metallopeptidase-9 (MMP-9) in PBMCs of subjects who underwent angiography.

Methods: Following a thorough clinical examination and anthropometric assessments, 90 individuals were divided into two groups: 56 coronary artery disease (CAD) participants (subjects with coronary artery stenosis ≥ 50%) and 34 non-CAD participants (subjects with coronary artery stenosis ≤ 30%). Then, this study evaluated fasting serum glucose (FSG), total cholesterol (Chol), high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and triglyceride (TG). Next, the severity of coronary artery stenosis was recorded. Additionally, real-time polymerase chain reaction (PCR) was used to assess the gene expression of MMP-9. MMP-9 protein level was also assessed using western blot techniques. The overexpression of MMP-9 and elevated level of FSG were positively associated with coronary artery stenosis.

Results: Our results revealed that MMP-9 was upregulated both at the level of transcription and translation. Moreover, the upregulation of MMP-9 had a significant positive correlation with the severity of coronary artery stenosis.

Conclusion: Significant correlation between the overexpression of MMP-9 and coronary artery stenosis confirms our hypothesis that the upregulation of MMP-9 in PBMCs has an important role in the pathogenesis of atherosclerosis before monocyte recruitment and its subsequent processes.

Keywords: Matrix metalloproteinase-9, Atherosclerosis, Coronary stenosis, Peripheral blood mononuclear cell

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Introduction

The global trend of cardiovascular disease mortality is attributed to atherosclerosis disease (1). A large body of data was reported to manage the atherosclerosis process during the past years (2). Although this issue is still the leading global health threat, the global spread creates an urgent need to understand the underlying molecular mechanism of atherosclerosis. Therefore, identifying the molecular mechanism of atherosclerosis helps us manage it and create prospects for its reduction. Thus, experimental studies elucidated molecular and cellular aspects of atherosclerotic cardiovascular disease (3). Meanwhile, macrophages as a differentiated form of circulating monocytes play central roles in the pathophysiology of atherosclerosis (4). For example, macrophages recruit leukocytes in the atherosclerotic plaque and secrete pro-inflammatory cytokines through regulating atherosclerotic-related inflammation that participates pivotally in the maintenance of the local inflammatory response and development of atherosclerotic plaque (5).

The development of an effective diagnostic and therapeutic approach is crucial to improve the prognosis of coronary artery disease (CAD) (6). Therefore, it is important to identify the contributing variables to the development of atherosclerosis. Peripheral blood mononuclear cells (PBMCs) expression analysis has recently been promoted as a diagnostic technique for determining the state of local and systemic inflammation. PBMCs are readily available reporter cells allowing identification of atherosclerosis events in early stages and can be considered an important candidate for novel therapeutic approaches (7-10). As such, evaluating the cellular and molecular events in PBMCs via the progression of atherosclerosis has become a hot research topic in recent years.

Matrix metallopeptidase 9 (MMP-9), also known as gelatinase B, is a member of the zinc-metalloproteinase family that is released by macrophages and endothelial

cells and contributes to the development of atherosclerosis (11). It also degrades a wide range of extracellular matrix proteins (12).

Prior research indicates that MMP-9 levels rise as CAD progresses (13). For instance, MMP-9 levels rise immediately following a heart attack and remain elevated for the first week. However, the analysis of MMP-9 in PBMCs and its correlation with other inflammatory factors may be valuable for clinical use (14). It has also been demonstrated that increased circulating levels of MMP-9 are associated with coronary atherosclerosis in subjects with type 2 diabetes (15).

On the other hand, according to earlier research, the overexpression of MMP-9 in combination with other MMPs encourages the recruitment of monocytes and macrophages into vascular lesions (16). Furthermore, clinical animal model studies by Lalu et al have demonstrated that MMP inhibitors can significantly preserve cardiac mechanical function (17), but it is unclear whether PBMC-derived MMP-9 has a direct role in the onset and development of atherosclerosis.

Investigating the expression profile of PBMCs can become a non-invasive diagnostic tool for the identification of CAD. Therefore, we examined MMP-9 expression in the PBMCs of patients with CAD in the present research. We also evaluated the relationship among changes in MMP-9 expression, the degree of coronary artery stenosis, serum biochemical markers, and demographic data.

Materials and Methods

Study population and anthropometric data collection

The study population comprised 90 Iranian subjects who had coronary angiography at the Hajar hospital, which is connected to the Shahrekord University of Medical Sciences in the Iranian province of Chaharmahal and Bakhtiari. Between May 2021 and November 2021, a total of 56 individuals with CAD and 34 age- and gender-matched non-CAD participants were recruited. Typical coronary angiography was used to make the diagnosis of CAD. All participants completed a questionnaire that asked about their medical history as well as demographic information and drugs they had used in the past. Weight (kg)/Height^2 (m) was the formula used to compute the body mass index (kg/m^2). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured. Following angiography, blood was taken from the artery sheaths of participants according to standard operating procedure. All participants provided written informed permission, and the research adhered to the Declaration of Helsinki.

Participants in the study should not have any signs or a history of cancer, renal impairment, autoimmune diseases such as rheumatoid arthritis, liver disorders, the prehistory of insulin injection, or any other chronic illnesses. A cardiologist examined each angiography. A main coronary artery with a stenosis ≥ 50% in at least one coronary artery was considered to belong to the CAD category. Additionally, an angiography test revealed that the non-CAD group had stenosis ≤ 30% in coronary arteries. With a few modifications, the Gensini scoring system was conducted to identify the severity of stenosis in the main coronary arteries, including the left anterior descending coronary artery (LAD), left circumflex (LCX), and right coronary artery (RCA). This method assigned 1 point to lumen stenosis ≤ 30%, 4 points to lumen stenosis of 51% to 75%, 8 points to lumen stenosis of 76% to 90%, and 16 points to lumen stenosis of more than 90% (18).

Measurement of serum biochemical parameters

Fasting serum glucose (FSG), total cholesterol (Chol), high-density lipoprotein-cholesterol (HDL-C), and triglyceride (TG) were measured by commercial kits (Pars Azmoon, Iran) according to the manufacturer's protocols, and low-density lipoprotein-cholesterol (LDL-C) concentrations were calculated by Friedewald's formula.

PBMC isolation

PBMCs have been isolated from 10 mL ethylenediaminetetraacetic acid (EDTA)-containing whole blood tubes by Ficoll-Hypaque density-gradient centrifugation as previously described with a little modification (19). Briefly, the whole blood was diluted by an equal volume of sterile phosphate-buffered saline (PBS). Then, Ficoll at half the volume of whole blood, was added carefully to a plastic sterile disposable Pasteur pipette and subsequently was centrifuged at 800 × g for 40 minutes at room temperature. The cloud layer containing mononuclear cells between the plasma and Ficoll solution was removed using a sterile disposable Pasteur pipette.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from PBMCs using RNX- Plus solution (Cinnagen, Iran) according to the manufacturer's instructions. The purity and concentration of total RNA were determined using a nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc, USA). The isolated RNA was reverse transcribed to cDNA by using the cDNA preparation kit (Ana Cell, Iran). Real-time PCR was carried out using SYBR Green master mix (Parstous, Iran) in the LightCycler instruments (Roto Gene 6000, Germany). All reactions were performed in triplicates, and the relative expression level of mRNAs was determined by Pfaffl methods and was normalized to the housekeeping gene (GAPDH) (20), and the specific primers were designed by an online software tool called Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The designed primers were synthesized (GenScript, China) and listed as follows:

MMP-9 Forward: 5' GTATGGGATCCGGTCTAGCA 3´;
Reverse: 5′ ATTCCCAGCACCCACAGTAA 3´.
GAPDH Forward: 5′ CCAGGCAGTCAGATCATCTTC-

3′; Reverse: 5′ AGCTGCCCCTCAGCTTGA 3′.

Western blot analysis

Total protein was extracted from PBMCs by urea–thiourea solution. The total protein content was measured by the Bradford method (21). The equal amounts of protein extracts (100 µg) were separated on 10% SDS-PAGE and then transferred to a nitrocellulose membrane. Then, blocking was performed for two hours at room temperature with 5% Non-fat skimmed milk powder (Santa Cruz Biotechnology) that was dissolved in tris-buffered saline with tween-20 (TBST). After blocking, the membrane was incubated with primary antibodies: MMP-9 (EP1255Y, Abcam) and GAPDH (sc25778, Santa Cruz) overnight at 4°C. After washing the membrane with tris-buffered saline with tween-20 buffer, incubation was performed with secondary horseradish peroxidase-conjugated antibody (ab6721, Abcam) at room temperature for 90 minutes. Finally, antibodies bound to proteins were detected with a chemiluminescent kit (Bio-Rad, Germany), and western blotting signals were visualized using a C-DiGit chemiluminescence western blot scanner (LI-COR Biosciences, US). Densitometry analysis of western blot bands was performed via the ImageJ software (National Institutes of Health), and GAPDH protein was used as a loading control.

Statistical analysis

The statistical analysis of anthropometric measurements and serum biochemical parameters was performed using SPSS 20.0 software (IBM, Armonk, NY, USA). The gene expression and western blot data were analyzed by using GraphPad Prism 8.00 (GraphPad Software, LaJolla, CA, USA). Further, normality was evaluated using the Kolmogorov–Smirnov test. Student's t-test or Mann–Whitney U-test was carried out to compare the data between the two groups depending on the normality of the data. Moreover, Pearson's and Spearman's correlation coefficient analyses were carried out to identify the correlation between study variables' depending on the normality of the data. Moreover, the correlation between quantitative variables and ordinal variables was calculated by Kendall's tau method. All data were expressed as Mean ± standard deviation, and $P \leq 0.05$ was considered significant.

Results

Demographic and biochemical characteristics

Demographic and biochemical characteristics are presented in Table 1. When compared to another group, the subjects with remarkable coronary stenosis had considerably greater FSG levels in their serum ($P \leq 0.05$). In contrast, there was no significant difference in the serum levels of Chol, VLDL, LDL-C, HDL-C, and TG between the two groups. As presented in Table 1, significant differences were observed in the pre-existing type 2 diabetes ($P \leq 0.05$), but smoking history between the two groups was not significantly different.

Table 1. Anthropometric indices, clinical characteristics, and serum biomarkers of the study population

Characteristic	Non-CAD	CAD	*P* value
Number	34	56	-
Age, years	51.62 ± 11	59.70 ± 8	0.06
Gender (Male (%))	41	45	0.68
Height	168.68 ± 12	169.02 ± 9	0.86
Weight	78.04 ± 12	76.91 ± 13	0.68
BMI, kg/m^2	27.38 ± 3	26.89 ± 3	0.55
SBP, mm Hg	128.44 ± 20	134.42 ± 16	0.13
DBP, mm Hg	78.38 ± 16	83.77 ± 14	0.12
FSG, mg/dL	107.21 ± 21	173.72 ± 25	0.00*
TG, mg/dL	114.50 ± 65	132.09 ± 95	0.34
Chol, mg/dL	123.24 ± 25	135.11 ± 40	0.13
HDL-C, mg/dL	38.43 ± 12	40.94 ± 13	0.37
LDL-C, mg/dL	61.91 ± 25	67.75 ± 33	0.38
Smoking (%)	29	28	0.68

Note. BMI: Body mass index; Chol: Total cholesterol; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FSG: Fasting serum glucose; TG: triglyceride; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein-cholesterol; CAD: Subjects with coronary artery stenosis ≥ 50%; Non-CAD: Subjects with coronary artery stenosis ≤ 30%; SD: Standard deviation.
Values are Mean ± SD. *$P < 0.05$: $P \leq 0.05$ was considered statistically significant.

Alteration of the gene expression and protein level of MMP-9

As compared with non-CAD participants, the gene expression of MMP-9 was considerably greater in PBMCs of CAD patients ($P \leq 0.05$). Further, as illustrated in Figure 1, the densitometry analysis of western blot results about protein levels of MMP-9 showed that MMP-9 is overexpressed in the CAD group than in the non-CAD group ($P \leq 0.05$). Additionally, there was not a significant difference between male and female subjects in terms of the MMP-9 expression (Figure 2a). A similar result was detected in CAD subjects, in which the overexpression of MMP-9 did not show any significant difference between male and female subjects (Figure 2b). Among male participants, gene expression of MMP-9 in CAD subjects was significantly higher than that in non-CAD subjects (Figure 2c). Interestingly, a more significant difference was observed among female participants (Figure 2d).

The association among the severity of coronary artery stenosis, biochemical parameters, blood pressure, diabetes, and alteration of expression of MMP-9

As illustrated in Figure 3, there was not any significant correlation between SBP and DBP with the severity of coronary artery stenosis. Prehistory of diabetes declared a strong correlation and severity of LAD and RCA coronary artery stenosis. Furthermore, among lipid profile parameters, Chol was more positively correlated with the severity of coronary artery stenosis, and the

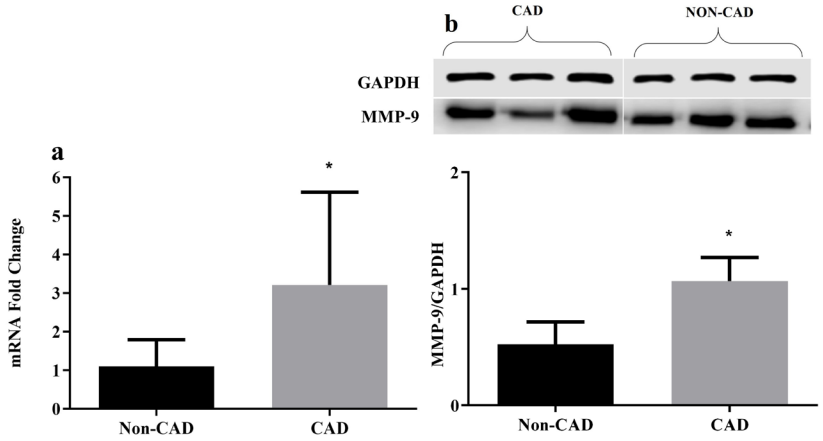

Figure 1. Relative Expression (a) and Protein Level (b) of the MMP-9 in the Non-CAD and CAD subjects. The expression of MMP-9 in the PBMCs of CAD subjects was higher than that in Non-CAD subjects at transcription and protein level. *Note.* MMP-9: Matrix metallopeptidase-9; PBMCs: Peripheral blood mononuclear cells; CAD: Subjects with coronary artery stenosis ≥ 50%; Non-CAD: Subjects with coronary artery stenosis ≤ 30%; SD: Standard deviation. * Compared to the Non-CAD group ($P \leq 0.05$). Data were expressed as Mean ± SD

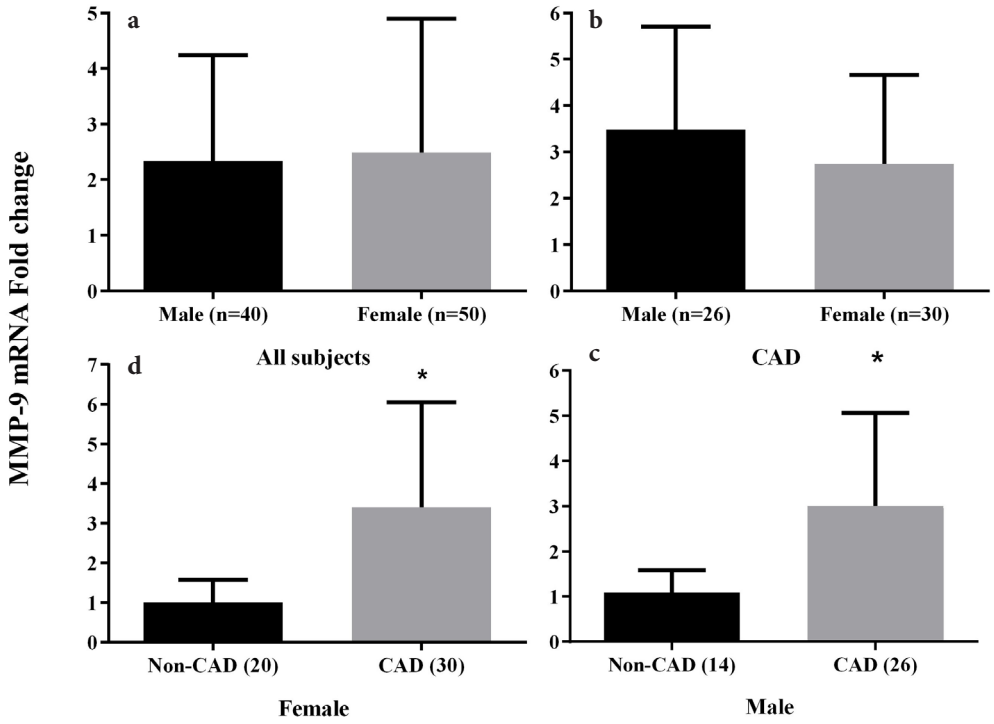

Figure 2. Comparison of mRNA levels of MMP-9 in PBMCs of study participants among all subjects (a), CAD participants (b), male participants and (c), female participants (d). There was no significant difference in the expression of MMP-9 between male and female participants. In CAD patients any significant difference was observed between males and females. Gene expression analysis of MMP-9 in male and female subjects declared significant overexpression in CAD patients than in non-CAD subjects. *Note.* MMP-9: Matrix metallopeptidase-9; PBMCs: Peripheral blood mononuclear cells; CAD: Subjects with coronary artery stenosis ≥ 50%; Non-CAD: Subjects with coronary artery stenosis ≤ 30%; SD: Standard deviation. * Significant difference. Data were expressed as Mean ± SD

expression changes of MMP-9 in the PBMCs were positively correlated with their protein levels. In addition, the overexpression of MMP-9 and augmented protein level of MMP-9 were related to the severity of stenosis of LAD, RCA, and LCX.

Discussion

In the present study, we examined the expression and protein levels of MMP-9 in the PBMCs of study participants to assess their possible involvement in the pathophysiology of atherosclerosis in CAD patients. We also evaluated their relationship to serum biochemical markers and the degree of coronary artery stenosis. The main finding of this study is increased baseline expression of MMP-9 in PBMCs of CAD participants. Another finding is that the overexpression of MMP-9 in PBMCs was significantly associated with the severity of arteries stenosis. Moreover, among serum biochemical

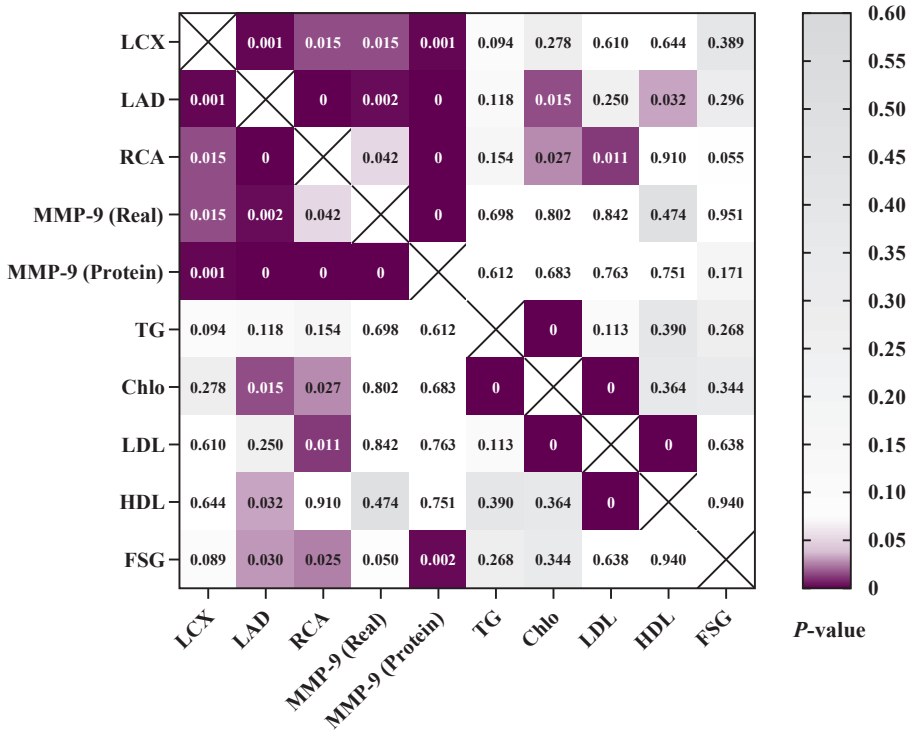

Figure 3. The correlation coefficient matrix visualizes the correlation among serum biochemical parameters, SBP, DBP, the prehistory of diabetes, protein and gene expression of MMP-9, and the severity of coronary artery stenosis. *Note*. LCX: Left circumflex artery; LAD: Left anterior descending; RCA: Right coronary artery; MMP-9: Matrix metallopeptidase-9; TG: Triglyceride; Chol: Total cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; FSG: Fasting serum glucose; DBP: Diastolic blood pressure; SBP: Systolic blood pressure. $P \leq 0.05$ was considered a statistically significant correlation by Pearson, spearman, and Kendall's tau correlation. The change in color from white to purple is accompanied by a significant increase

parameters, hyperglycemia was associated with the severity of coronary artery stenosis and upregulation of MMP-9.

Additionally, the analysis of the relationship between coronary artery stenosis and biochemical serum parameters revealed that hyperglycemia has the highest correlation to coronary artery stenosis. These data highlight the crucial connection between the frequency of cardiovascular problems and the accuracy of hyperglycemia. It goes without saying that managing diabetes reduces mortality from cardiovascular disease.

Contrary to our expectations, lipid profile parameters did not increase significantly in people with clogged arteries above 50% (Table 1). The present finding can be discussed from different perspectives. A substantial amount of evidence over the last several decades revealed the connection between abnormalities in the serum lipid profile and the onset of CAD (22,23). However, it is now commonly accepted that changed lipid profile characteristics such as oxidized low-density-lipoprotein (Ox-LDL) are more strongly linked to atherosclerotic events such as macrophage activation and foam cell formation (24). Goldstein, et al declared that in the presence of a high concentration of the native form of LDL-C, foam cell formation from macrophages did not occur significantly (25). They implied that the high concentration of LDL-C may lead to the downregulation of LDL-C receptors expressed in macrophages. Consequently, uptake of LDL-C by macrophage is reduced. Although the recognition and internalization of Ox-LDL into the macrophages occurred through scavenger receptors, in the presence of a high concentration of the native form of LDL-C, foam cell formation is more affected by Ox-LDL than by LDL-C. Moreover, Soumyarani and Jayakumari found that the treatment of cells with oxidized HDL can enhance the release of MMP-9 from arterial monocyte macrophages, favoring the destabilization of atherosclerotic plaque (26). Therefore, measuring the serum level of Ox-LDL has a higher priority than that of LDL-C in subsequent studies. On the other hand, the association between lipid profile disturbance and cardiovascular diseases depends on whether the early or advanced stage of cardiovascular diseases and the lipid profile parameters are measured. Herein, Zhang et al demonstrated that the plasma level of Ox-LDL might be employed as a potent risk factor for cardiovascular disease in their early stages but not in their later stages (27).

Investigating the participants' medical records showed that the pre-history of hyperglycemia is higher in participants undergoing angiography, especially in people with artery stenosis above 50% (28).

Furthermore, there was no significant difference in smoking history between the two groups. However, drug addiction, especially opium, was much higher in CAD than non-CAD subjects. In general, it can be concluded that the diabetes history at the beginning of the atherosclerosis process as well as drug addiction play key roles in the advanced stages of atherosclerosis. Undoubtedly, the simultaneous presence of diabetes type

2 and opium addiction increases the risk of incidence of cardiovascular disease.

Moreover, SBP and DBP did not vary substantially between the two groups, which may not have been the result that was anticipated. It should be mentioned that individuals had angiography and taken hypertension medication. On the other hand, it is possible that they did not carefully check their blood pressure changes (Table 1).

The migration of monocytes/macrophages into the subendothelial is known as the most important effective step in the promotion of early stages of atherosclerosis and the formation of atherosclerotic plaques (29). In the current study, it was discovered that MMP-9 is highly overexpressed in PBMCs of people with coronary artery stenosis, both transcriptionally and translationally (Figures 1 and 2). Hence, the alteration of the gene expression of MMP-9 along with other involving genes in the PBMCs may be an important factor in the onset of atherosclerotic events such as their uptake into the subendothelial space of coronary arteries (30). It is suggested that measuring the expression of other members of the MMP family as well as evaluating upstream regulatory factors of MMP-9 such as monocyte chemoattractant protein-1 (MCP-1) can be helpful in declining or confirming our findings (31).

Coronary arteries stenosis, especially LAD and RCA were correlated with increased serum FSG in CAD subjects. Moreover, the upregulation of MMP-9 in the PBMCs of CAD participants was positively correlated with increased serum FSG, but there was not a significant correlation among other biochemical parameters even serum levels of Chol and LDL. It was also found that constant hyperglycemia plays a crucial role in the pathogenesis of atherosclerosis, as depicted in Figure 3 (32).

In the present study, stenosis of the LAD coronary artery was more common than of LCX and RCA arteries among study participants. In addition, the correlation analysis of the obtained results indicated that LAD coronary artery stenosis has a more significant correlation with other coronary stenoses. Since the LAD artery is the most important coronary artery in terms of blood supply to the myocardium, it is more prone than other coronary arteries to recruit and accumulate of PBMCs and inflammatory agents.

Our findings demonstrated a strong association between the upregulation of MMP-9 and the severity of coronary artery stenosis. Indeed, the study of expression changes of molecular markers in PBMCs could possibly be used as a diagnostic biomarker in patients with CAD.

Finally, the current study has some important limitations that should be considered. First, it included a small sample size, which might result in limited statistical power. Secondly, regarding the lack of access to atherosclerotic plaque specimens and ethical considerations, it was not possible to measure the concentration of MMP-9 in atherosclerotic plaques. Furthermore, no certain clinical data were available on participants how long the medication lasted before angiography. In addition, the two groups of the study were adjusted for some cardiovascular risk factors such as the history of smoking and age, and we excluded participants with autoimmune dismisses. Nevertheless, the possibility of residual confounding by unmeasured factors cannot be ignored. Finally, it was not possible to measure other metabolites related to MMP-9 as well as inflammatory factors affecting the atherosclerosis process.

Conclusion

In conclusion, our study provided molecular evidence supporting the alteration of PBMCs metabolism through atherosclerosis progression. The research hypothesis that the upregulation of involved extracellular proteases in PBMCs plays a crucial role in the pathogenesis of atherosclerosis before monocyte recruitment and its further process was confirmed by a significant correlation between the overexpression of MMP-9 and the severity of coronary artery stenosis. Taken together, the obtained data revealed that MMP-9 may be considered a useful biomarker for the diagnosis and prognosis of atherosclerosis. Such investigations can provide precise information about the specific function of the PBMCs in the pathophysiology of atherosclerosis as well as new information regarding the association between PBMC metabolic regulation and the severity of coronary stenosis.

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Competing Interests

All authors declare that they have no conflict of interests.

Ethical Approval

All participants provided written informed permission, and the research adhered to the Declaration of Helsinki. Ethical clearance was received from the Shahrekord University of Medical Sciences Research Ethics Committee (IR.SKUMS.REC.1400.036).

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