DOI: 10.4274/MMJ.galenos.2022.45057
Medeni Med J 2022;37:306-312

Original Article

Smoking Induces the Circulating Levels of Matrix Metalloproteinase-9 and Its Association with Cardiovascular Risk in Young Smokers

Sigara, Gençlerde Matriks Metalloproteinaz-9'un Dolaşımdaki Düzeylerini ve Kardiyovasküler Riskle İlişkisini İndüklemektedir

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ABSTRACT

Objective: Smoking causes cardiovascular risk, which may alter the stability between the production and degradation of the extracellular matrix. Matrix metalloproteinase-9 (MMP-9) is a zinc-containing endopeptidase that degrades the extracellular matrix and is involved in tissue remodelling and several physiological processes. As a result, smoking-induced elevated serum MMP-9 levels, particularly at a younger age, raise the risk of coronary heart disease (CHD). Thus, this study aimed to determine the possible relationship between smoking-induced circulating MMP-9 and the risk of cardiovascular disease in young smokers.

Methods: In this cross-sectional study, the patients were divided into three groups. Each group contains 120 study participants. Group one consisted of 120 healthy individuals with no physical and mental illness, group two consisted of 120 active smokers with a heart disease, and group three consisted of 120 active smokers with a heart disease and diabetes, who attended Sri Ramaswamy Memorial Hospital for cardiology checkup at the age of 20-55 years. The serum MMP-9, high-sensitivity C-reactive protein (hs-CRP), and apolipoprotein-E (APO-E) levels were analyzed using the ELISA method, and the lipid levels were measured enzymatically using AU480 automatic analyzer (Beckman Coulter).

Results: Compared with non-smokers, the study shows that the mean serum MMP-9, hs-CRP, and APO-E levels were significantly higher in smokers ($p<0.001$). A strong relationship was also found between MMP-9 and hs-CRP, APO-E, smoking load, and smoking intensity.

Conclusions: A significant association was found between cigarette smoking with MMP-9, and relative exposure to circulating inflammation markers plays a potential role in the pathogenesis of CHD.

Keywords: APO-E, cardiovascular disease, hs-CRP, matrix metalloproteinase-9, smoking

ÖZ

Amaç: Sigara içmek, hücre dışı matriksin üretimi ve degradasyonu arasındaki stabiliteyi değiştirebilen kardiyovasküler riske neden olur. Matriks metalloproteinaz-9, hücre dışı matriksi bozan ve doku yenilenmesinde ve çeşitli fizyolojik süreçlerde yer alan çinko içeren bir endopeptidazdır. Sonuç olarak, sigaranın neden olduğu yüksek serum MMP-9 seviyeleri, özellikle genç yaşta, koroner kalp hastalığı (KKH) riskini artırır. Bu nedenle, bu çalışma genç sigara içenlerde, sigaranın neden olduğu dolaşımdaki MMP-9 ile kardiyovasküler hastalık riski arasındaki olası ilişkiyi belirlemeyi amaçlamıştır.

Yöntemler: Bu kesitsel çalışmada hastalar üç gruba ayrıldı. Her grup 120 katılımcı içerdi. Birinci grup, fiziksel ve ruhsal hastalığı olmayan 120 sağlıklı bireyden, ikinci grup kalp hastalığı olan 120 aktif sigara içicisinden ve üçüncü grup, Sri Ramaswamy Memorial Hastanesi'ne kardiyoloji kontrolü için başvuran, kalp hastalığı ve diyabeti olan 20-55 yaşlarındaki 120 aktif sigara içicisinden oluştu. Serum MMP-9, yüksek hassasiyetli C-reaktif protein (hs-CRP) ve apolipoprotein-E (APO-E) seviyeleri ELISA yöntemi kullanılarak analiz edildi ve lipit seviyeleri AU480 otomatik analizörü (Beckman Coulter) kullanılarak enzimatik olarak ölçüldü.

Bulgular: Sigara içmeyenlerle karşılaştırıldığında, çalışma ortalama serum MMP-9, hs-CRP ve APO-E düzeylerinin sigara içenlerde anlamlı olarak daha yüksek olduğunu gösterdi ($p<0,001$). MMP-9 ile hs-CRP, APO-E, sigara içme yükü ve sigara içme yoğunluğu arasında da güçlü bir ilişki bulundu.

Sonuçlar: Sigara içmek ile MMP-9 arasında anlamlı bir ilişki bulundu. Dolaşımdaki enflamasyon belirteçlerine nispi maruziyet KKH patogenezinde potansiyel rol oynamaktadır.

Anahtar kelimeler: APO-E, kardiyovasküler hastalık, hs-CRP, matriks metalloproteinaz-9, sigara içmek

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Received: 04 August 2022
Accepted: 29 September 2022
Online First: 02 November 2022

Cite as: Nath D, Shivasekar M, Vinodhini VM. Smoking Induces the Circulating Levels of Matrix Metalloproteinase-9 and Its Association with Cardiovascular Risk in Young Smokers. Medeni Med J 2022;37:306-312

INTRODUCTION

Smoking is an environmental risk factor that is associated with different genetic factors and multifactorial disorders[1]. The mechanism by which smoking induces cardiovascular risk is still unclear to some extent[2]. Smoking can increase the risk of cardiovascular disease, which is adversely related to the smoking burden and smoking intensity[3]. Other than active smoking, passive smoking also increases the cardiovascular risk by altering the serum lipid levels in young smokers[4]. The chemical components of cigarette smoke, especially nicotine, can increase the fatty acid level by stimulating the release of adrenaline, leading to an increase in cardiovascular risk[5]. Free fatty acids can increase the secretion of triglycerides (TGL) and low-density lipoprotein (LDL) and the release of cholesterol from the hepatic circulation[6]. Smoking also produces free radicals that can alter the coagulation system[7] and may accelerate the formation of plaque in the arteries[8]. Moreover, smoking promotes inflammation, which leads to the formation of atherosclerotic lesions and the progression of cardiovascular events. A recent study found that chronic inflammation results in atherosclerotic plaque development and eventual rupture[8]. However, regarding the predictors of cardiovascular disease, the markers for general inflammation are related to acute-phase reactants and proinflammatory cytokines including high-sensitivity C-reactive protein (hs-CRP)[9]. Specifically, the tissue necrosis factor-α and proinflammatory cytokines such as interleukin-1 (IL-1) regulate the synthesis of matrix metalloproteinase-9 (MMP-9) through the mesenchymal stem cells[10]. A recent study reported that smoking may modify the integrity of the extracellular matrix by shifting the balance between MMP synthesis and breakdown, potentially leading to the development of cardiovascular disease[11]. MMP is the family of zinc-containing zymogene endopeptidases that degrade extracellular matrix proteins, play an important role in the extracellular matrix for tissue remodeling, and contribute to various physiological processes[12,13]. MMP-9 is a collagenase enzyme with a molecular weight of 92 kDa. In the basement membrane, MMP-9 breaks proteoglycan proteins, type 4 collagen, and interstitial proteins[14]. It is now the most researched protein in connection to cardiovascular disease. Another study showed that in a high inflammatory setting, the activity of MMP-9 led to the increase in the formation of atherosclerotic plaque[15]. Thus, increased MMP-9 levels are linked to a higher risk of cardiovascular disease development, especially in smoking-induced inflammatory conditions. MMP-9 can be used as a therapeutic target and biomarker for future CHD[16].

In Tamil Nadu, which is located the southern part of India, the smoking rate is likely to increase, which may enhance the prevalence of patients with CHD because of lifestyle changes and increased smoking rate at an early age. Thus, this study aimed to investigate the link between circulating MMP-9 levels and CHD risk in young smokers.

MATERIALS and METHODS

Study Subjects and Pattern

In this cross-sectional study, the patients were divided into three groups. Each group contained 120 study participants. Group one consisted of 120 healthy individuals with no physical and mental illness, group two consisted of 120 active smokers with a heart disease, and group three consisted of 120 active smokers with a disease and diabetes, who attended Sri Ramaswamy Memorial Hospital for cardiology checkup at the age of 20-55 years. During regular cardiovascular health assessments, a standard questionnaire is used to acquire information about the patient's history and lifestyle. To determine whether patients fit the inclusion/exclusion criteria and are qualified to take part in the trial, a simple questionnaire was initially administered to each participant.

Inclusion criteria: Men aged 20-55 years who were either non-smokers or smoked at least five cigarettes per day for more than 1 year were included. CHD was defined as coronary angiography revealing >50% stenosis.

Exclusion criteria: The study excluded participants with hepatic disorders, renal disease, cardiovascular accidents, and systemic illness. Previous smokers, alcoholics, individuals with a family history of cardiovascular disease, and individuals taking medications such as steroids and lipid-lowering drugs were also excluded.

Quantifying smoking exposure: Smoking behaviors were determined by self-report. Smoking status, smoking burden (smoking duration) and smoking intensity (number of cigarettes smoked per day) were calculated. Smoking was defined as smoking at least five cigarettes per day for more than 1 year[17].

Ascertainment of Covariates

Age, sex, educational background, and other covariates, as well as prior medical and health histories, were based on self-reports. Standard techniques were used to acquire anthropometric measures such as height and body weight. Blood pressure at rest was measured after 2 min of relaxation. Systolic blood pressure of >140 mmHg and diastolic blood pressure of >90 mmHg are

considered signs of hypertension. In this study, fasting levels of plasma glucose >126 mg/dL or a glycated hemoglobin level >6.5% were used to diagnose diabetes mellitus.

Data Collection

Individuals were advised to report to the hospital between 8:00 and 9:00 a.m. before laboratory measurements. After a 12-h fast, 5 mL of venous blood was aseptically extracted in a plane tube using a vacutainer. The serum samples were obtained by centrifuging blood samples at 2000 × g for 10 min at 4 °C, which were then stored at -20 °C before further analysis.

Laboratory Assays

The AU480 automated analyzer (Beckman coulter) was used to quantify fasting blood glucose (FBG), total cholesterol (TC), TGL, high-density lipoprotein-cholesterol (HDL-C), LDL-C, and very-LDL-cholesterol levels enzymatically. A sandwich enzyme-linked immunosorbent assay was used to assess the serum MMP-9, hs-CRP, and apolipoprotein-E (APO-E) concentration in accordance with the manufacturing protocol (Abbkine, Inc. China).

Ethical Consideration

The study was approved by the Ethical Committee of SRM Hospital, SRMIST, and India (IEC no. 1763, date: 22.08.2019). All study participants provided written consent.

Statistical Analysis

Statistical analysis was conducted using IBM SPSS Statistics, version 22 (IBM Corp., Armonk, NY, USA). Numeric values are expressed as the mean and standard deviation (SD). A One-Way analysis of variance (ANOVA) was used to evaluate the differences between the three groups. For $p<0.001$, $p<0.05$, and $p>0.05$, differences were judged as extremely significant, significant, or non-significant, respectively. The correlations between variables were determined using Pearson's correlation coefficient (r) or Spearman correlations, and linear regression analysis was performed to show the relationship between MMP-9 and hs-CRP, APO-E, smoking load, and smoking intensity.

RESULTS

Demographic and Quantitative Parameters of the Study Population

Tables 1 and 2 show the demographic and baseline characteristic data of all the three groups. A significant difference was found in participants' weight, body mass index, waist-hip ratio, systolic blood pressure, number of cigarettes smoked per day (smoking intensity), and smoking duration (smoking load). Compared with group 1, groups 2 and 3 had significantly higher FBG and lipid profile. The study group did not receive any kind of lipid-lowering treatment.

The serum MMP-9, hs-CRP, and APO-E levels were presented as mean ± SD. As shown in Table 2, group 2

Table 1. Demographic details of the study groups.

Parameters	Non-smokers Group 1 (n=120)	Smokers (CHD) Group 2 (n=120)	Smokers (CHD+DM) Group 3 (n=120)	p-value
Age	31.45±11.57	37.65±9.16	42.45±8.08	<0.0001
Height (m)	171.11±3.37	171.15±2.97	171.43±4.31	0.8740
Weight (kg)	67.75±7.97	71.4±4.9	74.31±7.64	<0.0001
BMI (kg/m^2)	23.18±2.00	24.25±1.50	25.29±1.97	<0.0001
WC (cm)	86.95±5.54	91.03±4.81	92.15±4.73	<0.0867
HC (cm)	99.8±2.6	100.36±4.54	99.8±5.00	0.683
W/H ratio	0.86±0.04	0.90±0.04	0.91±0.04	<0.0001
Systolic blood pressure (mmHg)	117.9±4.2	124.8±4.4	128.3±3.7	<0.0001
Diastolic blood pressure (mmHg)	80±2.1	79.8±4.1	77.4±5.8	0.0662
Number of cigarettes smoked per day	0	8.9±3.03	10.05±4.01	<0.0001
Smoking duration (years)	0	10.9±6.06	14.61±7.23	<0.0001

A p-value of <0.05 is significant. ANOVA calculation. BMI: Body mass index, HC: Hip circumference, WC: Waist circumference, W/H: Waist/hip ratio, CHD: Coronary heart disease, DM: Diabetes mellitus

was found to have higher blood MMP-9, hs-CRP, and APO-E levels, followed by group 3, which were gradually rising when compared with those of group 1 (p<0.001), indicating that smoking induces inflammation.

Correlation Analysis

Table 3 and Figure 1 show Pearson's correlation between serum MMP-9, hs-CRP, and APO-E levels in the CHD group. A positive correlation was found between MMP-9 and hs-CRP (r=0.3776, p<0.0001), APO-E (r=0.4039, p<0.0001), TC (r=0.3204, p=0.0003), TGL (r=0.3881, p<0.0001), LDL-C (r=0.5003, p<0.0001), number of cigarettes smoked per day (r=0.3411, p=0.00013) and smoking duration (r=0.3175, p=0.0004).

Table 4 and Figure 2 also show a correlation between the serum MMP-9, hs-CRP, and APO-E levels in group 3. A positive correlation was found between MMP-9 and hs-CRP (r=0.3776, p<0.0001), APO-E (r=0.4614, p<0.0001), TC (r=0.4858, p<0.0001), TGL (r=0.3917, p<0.0001), LDL (r=0.4689, p<0.0001), number of cigarettes smoked per day (r=0.4287, p<0.0001), and smoking duration (r=0.3638, p<0.0001), and a negative correlation was found with HDL-C levels (r=-0.3705, p<0.0001).

DISCUSSION

To our knowledge, this is the first-ever report on MMP-9 in young smokers. Smoking habits continuously

Table 2. Biochemical parameters of the study groups.

Parameters	Non-smokers Group 1 (n=120)	Smokers (CHD) Group 2 (n=120)	Smokers (CHD+DM) Group 3 (n=120)	p-value
FBG (mg/dL)	96.37±8.11	99.3±11.5	217.76±66.84	<0.0001
TC (mg/dL)	160.01±22.48	222.86±27.02	230.51±47.67	<0.0001
TGL (mg/dL)	91.21±34.74	164.45±94.97	200.61±90.44	<0.0001
HDL (mg/dL)	45.76±8.44	40.45±6.41	39.81±6.88	<0.0001
LDL (mg/dL)	106.21±17.59	155.03±22.56	157.76±28.08	<0.0001
VLDL (mg/dL)	18.03±6.97	32.08±15.14	38.56±15.79	<0.0001
TC/HDL-C	3.56±0.64	5.60±1.03	5.83±1.12	<0.0001
LDL/HDL-C	2.36±0.51	3.92±0.85	4.00±0.75	<0.0001
MMP-9 (ng/mL)	26.10±12.17	58.09±27.78	91.87±31.64	<0.0001
hs-CRP (mg/L)	0.7789±0.4179	1.9507±0.9531	4.0250±2.2819	<0.0001
APO-E (ng/mL)	56.78±9.76	46.84±13.19	36.94±6.71	<0.0001

A p-value of <0.05 is significant. ANOVA calculation. FBG: Fasting blood glucose, TC: Total cholesterol, TGL: Triglycerides, HDL-C: High-density lipoprotein-cholesterol, LDL: Low-density lipoprotein, MMP-9: Matrix metalloproteinase-9, hs-CRP: High-sensitivity C-reactive protein, APO-E: Apolipoprotein-E, CHD: Coronary heart disease, DM: Diabetes mellitus, VLDL: Very low-density lipoprotein

Table 3. Correlation of all different biochemical parameters in the CHD group.

Parameter	Smokers (CHD)					
	MMP-9		hs-CRP		APO-E	
	r	p	r	p	r	p
TC (mg/dL)	0.3204	0.0003	0.3180	0.0004	0.4651	<0.0001
TGL (mg/dL)	0.3881	<0.0001	0.3278	0.0003	0.3805	<0.0001
HDL (mg/dL)	-0.1774	0.0525	-0.1701	0.0428	-0.1843	0.051
LDL (mg/dL)	0.5003	<0.0001	0.5065	<0.0001	0.5058	<0.0001
VLDL (mg/dL)	0.1177	0.370	0.1455	0.0767	0.2033	0.0259
MMP-9 (ng/mL)	-	-	0.3776	<0.0001	0.4039	<0.0001
hs-CRP (mg/L)	0.3776	<0.0001	-	-	0.4039	<0.0001
APO-E (ng/mL)	0.4039	<0.0001	0.4039	<0.0001	-	-
Number of cigarettes smoked per day	0.3411	0.00013	0.3362	0.00017	0.4854	<0.0001
Smoking duration (years)	0.3175	0.0004	0.2825	0.0017	0.374	<0.0001

A p-value of <0.05 is significant. TC: Total cholesterol, TGL: Triglycerides, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, MMP-9: Matrix metalloproteinase-9, hs-CRP: High-sensitivity C-reactive protein, APO-E: Apolipoprotein-E, CHD: Coronary heart disease, VLDL: Very low-density lipoprotein

increase the risk of cardiovascular disease and peripheral vascular disease[18]. Modifiable risk factors such as high blood pressure or high cholesterol levels do not explain clearly the relationship between cigarette smoking and CHD risk[19]. However, certain studies revealed that smoking increases the levels of circulating MMP-9, which raises the likelihood of cardiovascular disease[20].

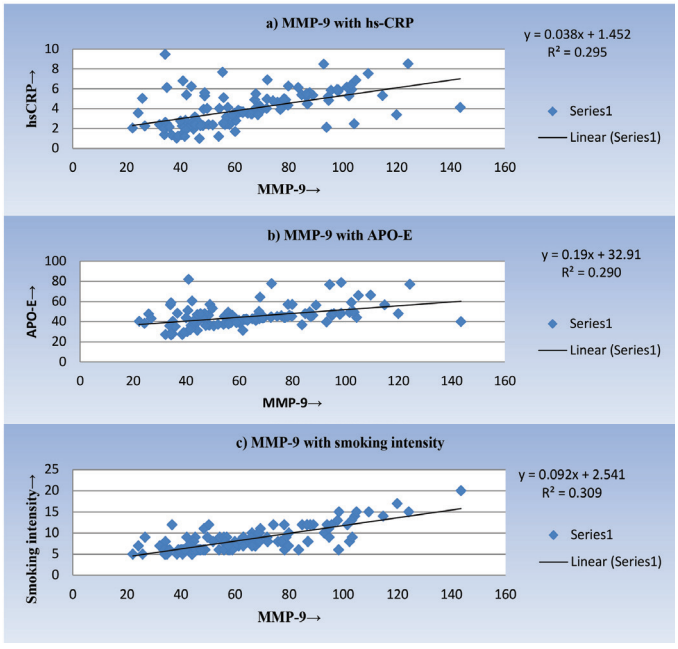

Figure 1. Regression analysis of MMP-9 with hs-CRP, APO-E, smoking load, and smoking intensity in smokers and CHD.

hs-CRP: High-sensitivity C-reactive protein, APO-E: Apolipoprotein-E, CHD: Coronary heart disease, MMP-9: Matrix metalloproteinase-9

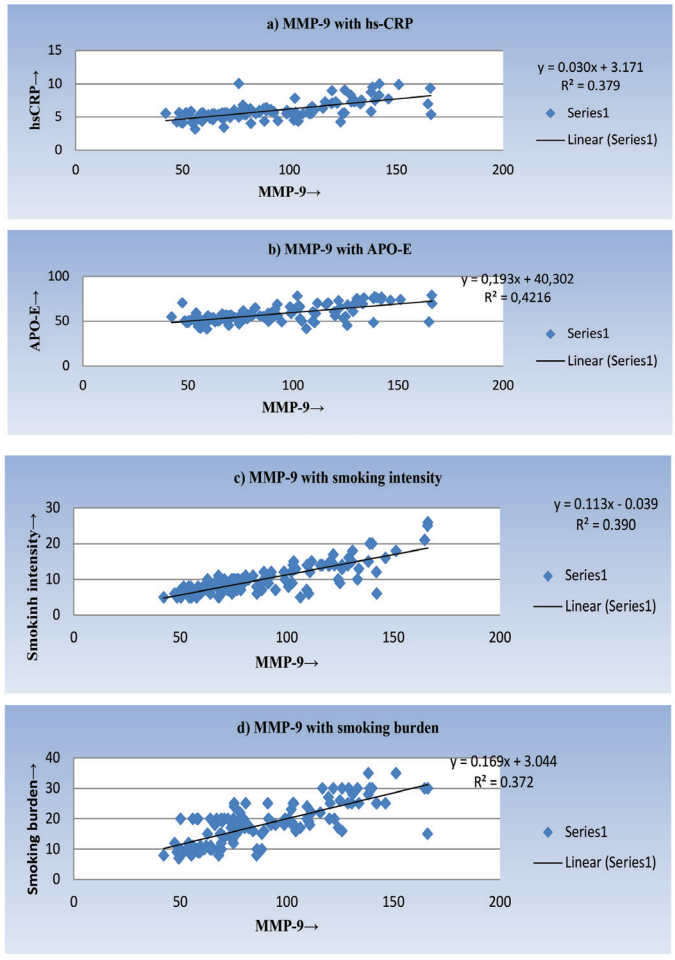

Figure 2. Regression analysis of MMP-9 with hs-CRP, APO-E, smoking load, and smoking intensity in smokers and participants with CHD and diabetes.

hs-CRP: High-sensitivity C-reactive protein, APO-E: Apolipoprotein-E, CHD: Coronary heart disease, MMP-9: Matrix metalloproteinase-9

Table 4. Correlation of all different biochemical parameters in the CHD and diabetes group.

Parameter	Smokers (CHD+DM)					
	MMP-9		hs-CRP		APO-E	
	r	p	r	p	r	p
TC (mg/dL)	0.4858	<0.0001	0.4773	<0.0001	0.513	<0.0001
TGL (mg/dL)	0.3917	<0.0001	0.2257	0.013	0.3021	0.0189
HDL (mg/dL)	-0.3705	<0.0001	-1762	0.0542	-0.1903	0.0007
LDL (mg/dL)	0.4689	<0.0001	0.7314	<0.0001	0.6983	<0.0001
VLDL (mg/dL)	0.1287	0.161	0.1964	0.0315	0.1579	0.084
MMP-9 (ng/mL)	-	-	0.3686	<0.0001	0.4614	<0.0001
hs-CRP (mg/L)	0.3686	<0.0001	-	-	0.5253	<0.0001
APO-E (ng/mL)	0.4614	<0.0001	0.5253	<0.0001	-	-
Number of cigarettes smoked per day	0.4287	<0.0001	0.3845	<0.0001	0.7335	<0.0001
Smoking duration (years)	0.3638	<0.0001	0.3899	<0.0001	0.7629	<0.0001

A p-value of <0.05 is significant. TC: Total cholesterol, TGL: Triglycerides, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, MMP-9: Matrix metalloproteinase-9, hs-CRP: High-sensitivity C-reactive protein, APO-E: Apolipoprotein-E, CHD: Coronary heart disease, VLDL: Very low-density lipoprotein

The results of this study reveal a significant positive link between cardiac risk factors, notably cigarette smoking, hs-CRP, and MMP-9. Furthermore, some studies have shown a correlation between higher circulating MMP-9 levels, smoking status, and inflammatory markers including hs-CRP and IL-6[21]. The study also showed that MMP-9 significantly correlated with APO-E levels. This may be due to the shedding of the lipoprotein receptor by MMP-9. MMP-9 is an endopeptidase that can bind and proteolyze (i.e., shedding) lipoprotein receptors[22].

In this study, smokers with CHD (both with and without diabetes) had significantly higher blood MMP-9 levels than normal controls (non-smokers) ($p<0.001$). Compared with groups 2 and 1, group 3 had significantly higher MMP-9 levels, with a significance value of 0.001. This may reflect abnormal extracellular matrix metabolism group 3[23]. MMP-9 is also a novel regulator of cholesterol metabolism. Furthermore, the findings show that the dysregulation of MMP-9 activity alters hepatic transcriptional responses to dietary cholesterol, potentially leading to metabolic disorders such as atherosclerosis and CHD[24].

Smoking accelerates inflammation and the oxidative modification of lipids and prospectively slows down the activity of MMPs at various levels. By activating inflammatory transcription factors, smoking increases the MMP expression[25]. Moreover, smoking increases the monocyte expression of IL-beta cells[26]. Cigarette smoke contains nicotine, and the predominant metabolite cotinine increases the production of vascular smooth muscle cell collagenase and gelatinase, which may lead to plaque rupture[27].

Different mechanisms have been anticipated to explain how smoking stimulates MMP both *in vitro* and *in vivo*[28]. *In vitro*, tobacco smoke induces MMP-9 expression through the endothelial cells[29]. Similarly, exposure to smoking induces MMP-1 expression through the human fibroblasts. Moreover, tobacco smoke induces proteolysis when the cadmium present in the smoke is inhaled, and increased exposure may lead to cardiovascular disease as it increases in the aorta of smokers[12].

The main limitation of this study is the small sample size, despite the solid conclusion on the link between smoking and CHD risk. Some factors such as age, sex, individual condition, environment, and experimental procedure may affect the results, which may influence the interpretation of the results. Analyzing the activities of endogenous tissue inhibitors of metalloproteinase-1 (TIMP-1) is necessary because it creates a balance between MMP-9 and TIMP-1[30]. However, this study did not analyze TIMP-1 concentration. Our finding regarding MMP-9 in young smokers gives evidence that cigarette smoking increases the circulating MMP-9 levels at a young age.

CONCLUSION

This study reveals a substantial link between serum MMP-9 levels and CHD risk in young smokers. The results of this study indicate that an increase in MMP-9 levels, particularly in smoking-induced inflammatory conditions, is related to CHD risk in young smokers.

Acknowledgment: We thank the Department of Medicine, Cardiology, Translational Medicine, and Master Health checkup unit for the approval and support.

Ethics

Ethics Committee Approval: The study was approved by the Ethical Committee of SRM Hospital, SRMIST, and India (IEC no. 1763, date: 22.08.2019).

Informed Consent: All study participants provided written consent.

Peer-review: Externally and internally peer-reviewed.

Author Contributions

Concept: M.S., Design: D.N., M.S., V.M.V., Data Collection and/or Processing: D.N., Analysis and/or Interpretation: D.N., Literature Search: D.N., M.S., V.M.V., Writing: D.N., M.S., V.M.V.

Conflict of Interest: The authors have no conflict of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

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