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Mixed Lineage Kinase Domain-Like Pseudokinase (MLKL) Gene Expression in Human Atherosclerosis with and without Type 2 Diabetes Mellitus

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ABSTRACT

Background: MLKL, one of the main downstream components of the necroptosis or programmed necrosis has recently been demonstrated in advanced atherosclerotic lesions. However, its precise role in the atherosclerosis pathogenesis still requires more elucidation. Our study was set to delineate both the changes in peripheral MLKL gene expression and its influence on disease severity in atherosclerotic patients with and without type 2 DM. **Methods:** The study involved 50 patients (20 non-diabetics and 30 diabetics) undergoing coronary artery bypass graft and 20 apparently healthy controls. Taqman RT-PCR was used to quantify MLKL mRNA expression levels, while ELISA was employed to estimate serum insulin and hsCRP levels. **Results:** Compared with the control group, MLKL gene was up regulated significantly in CVD ($p \leq 0.001$). Higher MLKL expression was demonstrated in diabetic CVD group than non-diabetic group ($p < 0.05$). Correlation studies reported positive associations between MLKL and markers of dyslipidemia, inflammation, and insulin resistance. Multiple regression analysis revealed that FBG levels, hsCRP levels, and total WBCs count were significant predictors for MLKL levels. ROC showed a significant diagnostic value of MLKL for CVD. Moreover, regression analysis demonstrated that MLKL and hsCRP were independent predicting factors for the severity of CVD. **Conclusion:** MLKL is linked to hallmarks of atherosclerosis and could explain increased cardiovascular risk in diabetic patients. Thus, it can be a potential drug target for treatment of atherosclerotic patients. ***DOI: 10.52547/ibj.25.4.265***

Keywords: Atherosclerosis, Diabetes mellitus, Inflammation, Insulin resistance, Necroptosis

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INTRODUCTION

Atherosclerosis, an inflammatory disease, is the prime reason for the majority of CVDs, the central cause of global deaths[1]. Type 2 DM, mainly characterized by hyperglycemia due to insulin resistance, is the most common metabolic disease worldwide and is increasing drastically in developing countries[2]. Diabetes is deeply implicated in the pathogenesis of atherosclerosis and increasing the risk of CVD by up to three folds[3].

Beyond the clear role of smooth muscle and endothelial cells in atherogenesis, many immunologic components were identified as crucial mediators in

List of Abbreviations:
AUC, area under curve; **BMI,** body mass index; **CAD**, coronary artery diseases; **CVD,** cardiovascular diseases; **DM,** diabetes mellitus; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **HbA1c%**, glycated hemoglobin; **HDL-C,** high-density lipoprotein cholesterol; **HOMA-IR,** homeostasis model assessment of insulin resistance; **hsCRP,** high sensitivity C-reactive protein; **LDL-C,** low-density lipoprotein cholesterol; **MLKL,** mixed lineage kinase domain-like pseudokinase; **QUICKI,** quantitative insulin sensitivity check index; **QUICKI,** quantitative insulin sensitivity check index; **RIPK,** receptor-interacting protein kinase; **ROC,** Receiver operating characteristic curve; **WBCs,** white blood cells

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atherosclerosis in which the inflammatory process is a pivotal regulator[4]. The inflammatory response could be speculated by many circulating inflammatory biomarkers such as hsCRP, a poor prognostic marker in atherosclerosis[5]. Inflammation is closely related to cell death as many of the receptors that drive inflammatory cytokine expression can also induce the cell death[6].

Activated immune cells not only mediate fibrous cap degradation and subsequent plaque rupture but also mediate apoptotic cell death in atherosclerotic plaques[7]. The concept of cells equipped with an internal self-destruction mechanism was first proposed in 1965, highlighting the essential role of programmed cell death in many inflammatory-driven diseases[8]. Since then, the magnitude and the mechanism of cell death have extensively been studied. Nowadays, apoptosis, necrosis, and necroptosis are the three well characterized and extensively studied cell death pathways[9].

Necroptosis or programmed necrosis is triggered by many signals such as death receptor ligands. RIPK 1 and 3 as well as MLKL, which constitute the regulatory necrosome complex, are the central regulators of necroptosis[10]. The first member of necroptosis-induced death receptors, RIPK1, was first described by Holler *et al*.[11] in 2000. However, the term necroptosis was introduced by Degterev and coworkers[12] in 2005. The main players of the necroptotic pathway are RIPK3 and MLKL[13]. The latter was first described by Sun *et al*.[14] in 2012 as a RIP3 substrate in the necroptotic pathway. Although necroptosis can protect cells against pathogens, its regulation impairment has been recognized as an emerging important pathway in many autoimmune and/or inflammatory diseases[6] as a consequence of immunogenic cellular content release, such as cytokines and damage-associated molecular patterns to initiate inflammatory responses[13].

The activation of RIPK1/3 by death receptors or toll-like receptors induces necrosome formation, which in turn phosphorylates MLKL, the necroptotic executioner[15]. This phosphorylation induces MLKL oligomerization and bind to phosphatidylinositol phosphates of the cell membrane with subsequent formation of a pore-forming complex. These events lead to rapid necrotic membrane disruption[16] and the release of potential damage-associated molecular patterns[17]. An alternative suggested mechanism is the translocation of phosphorylated MLKL to the plasma membrane with calcium or sodium influx that trigger the rupture of necrotic membrane as a result of increased osmotic pressure[18]. Although RIPK1 is fundamental for necroptosis, recent studies have proven that MLKL can directly induce necroptosis[13].

Necroptotic MLKL signaling has been reported in different inflammatory diseases, such as tumor necrosis factor-induced shock[19], obesity[20], and ischemia-reperfusion injuries[21]. Since MLKL is an essential element in necroptosis, it may be considered as a promising target for restraining necroptosis and associated inflammation, the major hallmark of atherosclerosis. Although phosphorylated MLKL has been demonstrated in advanced atherosclerotic lesions[22], and its functional knockdown showed a significant decrease in plaque necrotic core[23], the exact relations between MLKL and key processes of atherogenesis along with the influence of DM are still unclear. Accordingly, we aimed to investigate the expression pattern changes of the human MLKL gene in circulating leukocytes and their link to atherosclerosis severity. Moreover, the association of MLKL with inflammatory response and insulin resistance in addition to other risk factors in atherosclerosis accompanied by DM were assessed.

MATERIALS AND METHODS

Sample size calculation

Using PASS 13 for sample size calculation and data from previous studies[22,24], a sample size of 20 patients per group achieved 81% power to detect differences among the MLKL means versus the alternative of equal means using an F test with a 0.05 significance level.

Subjects

From May 2018 to August 2019, a total of 50 patients (29 males and 21 females) undergoing coronary artery bypass grafting were selected from Maadi Military Hospitals, Egypt. Using angiography, the CAD severity was evaluated in patients according to the number of atherosclerotic blood vessels. Based on patients' history and postprandial and fasting plasma glucose levels, the patients' group was divided into non-diabetic (20 CVD/non-diabetic patients; 12 males and 8 females) and diabetic (30 CVD/diabetic patients; 17 males and 13 females) subgroups. The control group was composed of 20 (13 males and 7 females) age- and sex-matched healthy volunteers. Subjects with acute coronary syndrome, type 1 DM, kidney diseases, liver diseases, malignancy, acute and chronic inflammatory diseases, or autoimmune disorders were excluded.

Blood samples

Fasting peripheral blood samples were obtained from

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controls and CVD subjects before the surgical operation. Blood samples were divided into three aliquots for whole blood, plasma, and serum preparation. Serum was used for the assay of lipids parameters, insulin, and hsCRP. Whole blood was used for the determination of HbA1c%, full blood count, and total RNA extraction, while plasma was used for the FBG assay.

Biochemical analysis

Sysmex® XT 2000i autoanalyzer (Japan) was used to determine full blood count, whereas Cobas® 8000 Modular automated analyzer (Roche Diagnostics, Germany) was employed to determine the levels of FBG, total cholesterol, triglycerides, HDL-C, and LDL-C. The Bio-Rad VARIANT II Turbo Hemoglobin Testing System (Bio-Rad Laboratories, Hercules, CA, USA) was utilized to determine HbA1c% levels. Serum hsCRP and fasting insulin were measured by ELISA technique using MyBioSource ELISA kits (USA). The HOMA-IR and QUICKI were calculated as follows: HOMA-IR= fasting insulin (µU/mL) × FBG (mg/dL)/405[25] and QUICKI= 1/[log(fasting insulin) + log(FBG)][26], respectively.

RNA isolation and quantitative Taqman RT-PCR

Total RNA was extracted and purified using QIAamp RNA Blood Mini extraction kit (Qiagen, Germany) as the manufacturer's protocol. After the determination of RNA concentration and purity using DeNovix Donating Platinum Edition DS-11 (Wilmington, Delaware, USA), a total RNA of 500 ng was reversely transcribed to cDNA by High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (catalog number: 4374967) supplied by Applied Biosystems (USA) using Biometra TProfessional Standard Thermocycler (Biometra, Germany). Taqman RT-PCR was carried out on Rotor-gene Q (Qiagen, Germany) using Taqman® Universal Master Mix II (Applied Biosystems, USA). Human MLKL and GAPDH predesigned ready-to-use primers and probe Taqman® assays were supplied by Applied Biosystems (Assay IDs: Hs04188505_m1 and Hs02786624_g1, respectively). Each Taqman® gene expression assay contained 18 uM of corresponding forward and reverse primers and 5 uM of specific probe in 20× format. cDNA template (50 ng), Taqman® gene expression assay (1 µL), and Taqman® universal master mix (10 µL) were used to perform Taqman RT-PCR. The used reaction protocol involved uracil-N-glycosylase activation at 50 °C for 2 min, then polymerase activation at 95 °C for 10 min, followed by 40 amplification cycles (at 95 °C for 15 s and at 60°C for 1 min). Each experiment included a negative control. The relative MLKL gene expression levels were normalized and analyzed by the $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct = (Ct_{MLKL}-Ct_{GAPDH})$ sample - $(Ct_{MLKL}-Ct_{GAPDH})$ control[27].

Statistical analysis

The IBM SPSS statistics (V.23, USA) was used to perform all the statistical analyses. Nominal data were represented as numbers (percentages) and performed by Chi-square test. Numerical data were represented as mean ± SEM. Student's t-test or Mann-Whitney U test was used to compare two independent mean groups for parametric and nonparametric variables, respectively. More than two groups were compared by one-way ANOVA with a post-hoc Tukey test or Kruskal–Wallis test for parametric and nonparametric variables, respectively. Correlations were estimated by Pearson or Ranked Spearman tests. ROC curve analysis was performed to evaluate the diagnostic value of MLKL gene expression. Finally, multiple regression analysis was conducted to investigate parameters associated with MLKL expression and to assess predictors that contribute to CAD severity. *p* values of 0.05 or less were considered of a statistical significance.

Ethical statement

The above-mentioned sampling protocol was approved by the Ethical Committee of Faculty of Pharmacy, Ain Shams University, Egypt (ethical code: 2/2018-52). The study followed the regulations and recommendations of the Declaration of Helsinki[28]. A written consent indicating the use of blood samples for research purposes and the aim of the study was obtained from each participant.

RESULTS

Demographic and clinicopathological data of the studied groups

Table 1 summarizes the subjects' demographic and clinicopathological data. No statistical difference was observed between the control and CVD/non-diabetic and CVD/diabetic group regarding age, gender, BMI, and monocyte and eosinophil percentages, while a statistical difference was reported between the studied groups with respect to the rest of the measured parameters. Comparing with non-diabetic group, CVD/diabetic group showed significantly the higher levels of lipid profile parameters, including triglyceride, total cholesterol, LDL-C, cardiovascular risk ratios, FBG, HbA1c%, HOMA-IR index, and hsCRP, as well as significantly lower levels of red blood cells count and QUICKI index.

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Table 1. Demographic data, clinicopathological parameters, and MLKL gene expression levels of the studied groups

Data	Control group (n= 20)	CVD/non-diabetic group (n=20)	CVD/diabetic group(n=30)
Age (years)	51.95 ± 0.60	54.65 ± 1.18	54.90 ± 0.84
Gender (Males/Females)	13/7	12/8	17/13
BMI (Kg/m^2)	25.84 ± 0.17	26.41 ± 0.53	27.02 ± 0.56
Hypertension (n, %)	0 (0%)	14 (70.0%)[a]	22 (73.3%)[a]
Current smoking (n, %)	2 (10%)	10 (50.0%)[a*]	17 (56.7%)[a*]
TG (mg/dL)	84.56 ± 4.83	125.30 ± 7.74[a*]	165.33 ± 9.78[a,b#]
TC (mg/dL)	148.94 ± 3.08	171.50 ± 4.18[a*]	195.27 ± 5.22[a,b#]
HDL-C (mg/dL)	53.06 ± 1.42	36.75 ± 1.69[a]	32.30 ± 1.03[a]
LDL-C(mg/dL)	78.94 ± 3.13	109.60 ± 3.37[a]	129.85 ± 4.49[a,b#]
TC/HDL	2.83 ± 0.09	4.82 ± 0.20[a]	6.23 ± 0.26[a,b]
LDL/HDL	1.51 ± 0.08	3.10 ± 0.17[a]	4.17 ± 0.21[a,b]
FBG (mg/dL)	86.40 ± 1.37	98.41 ± 2.08	190.33 ± 8.19[a,b]
HbA_{1c} (%)	5.11 ± 0.07	5.72 ± 0.05	8.83 ± 0.26[a,b]
Insulin (μIU/mL)[¶]	3.15 ± 0.26	9.86 ± 1.86[a#]	8.61 ± 1.12[a#]
QUICKI	0.42 ± 0.01	0.36 ± 0.01[a]	0.31 ± 0.02[a,b#]
HOMA-IR[¶]	0.68 ± 0.06	2.38 ± 0.46[a]	4.23 ± 0.67[a,b#]
hsCRP (mg/L)[¶]	0.76 ± 0.11	5.55 ± 0.83[a]	11.34 ± 1.53[a,b*]
RBCs (10^6 cells/cm^3)	5.07 ± 0.12	4.38 ± 0.11[a]	4.71 ± 0.10[a,b#]
WBCs (10^3 cell/cm^3)	5.57 ± 0.23	7.48 ± 0.40[a*]	8.12 ± 0.50[a]
Neutrophils (% of WBCs)	51.75 ± 1.17	62.40 ± 2.54[a#]	61.33 ± 2.03[a#]
Lymphocytes (% of WBCs)	37.44 ± 1.00	27.00 ± 2.08[a#]	29.03 ± 1.92[a#]
Monocytes (% of WBCs)	7.22 ± 0.33	7.75 ± 0.57	7.10 ± 0.30
Eosinophils (% of WBCs)	2.56 ± 0.27	2.70 ± 0.33	2.34 ± 0.25
Basophils (% of WBCs)	0.78 ± 0.18	0.15 ± 0.08[a]	0.19 ± 0.07[a]
mRNA MKLK levels (fold expression)[¶]	1.00 ± 0.01	4.28 ± 0.30[a]	7.01 ± 0.70[a,b*]

Data are presented as mean ± SEM or numbers of patient (percentages); [¶]Variables analyzed by Kruskal Wallis test. [a]considered statistically significant from the control group at $p \leq 0.001$; [b]considered statistically significant from CVD/non-diabetic group at $p \leq 0.001$; [#]statistically significant at $p \leq 0.01$; [*]statistically significant at $p \leq 0.05$; RBCs, red blood cells; TC, total cholesterol; TG, triglycerides

MLKL gene expression and its diagnostic value in the studied groups

As shown in Table 1 and Figure 1, MLKL gene expression was statistically elevated in CVD subjects than control subjects at $p < 0.0001$. Moreover, MLKL expression was significantly higher in CVD/diabetic group (7.01 ± 0.70 folds) than CVD/non-diabetic group (4.28 ± 0.30 folds) at $p < 0.05$. Besides, statistical analysis of MLKL gene expression levels regarding risk factors, including age, gender, BMI, hypertension, smoking, and hsCRP levels revealed a significant overexpression in patients with BMI ≥25 kg/m^2 compared to those with BMI <25 kg/m^2 (7.07 ± 0.61 versus 4.07 ± 0.55, $p = 0.001$; Fig. 2). There was also a significantly higher expression in patients having hsCRP levels ≥3 mg/L compared to patients having hsCRP levels <3 mg/L (6.44 ± 0.54 versus 3.53 ± 0.40, $p = 0.008$). Regression analysis of parameters affecting MLKL gene expression indicated that FBG, hsCRP, total WBCs count, and BMI were positive independent predictors for MLKL gene expression levels (β = 0.468, 0.259, 0.299, and 0.283 at $p < 0.05$, respectively), as depicted in Table 2, highlighting the possible role of these parameters in regulating MLKL expression. We also evaluated the diagnostic value of MLKL using ROC analysis, where MLKL showed an excellent diagnostic value for discrimination between CVD patients and controls (AUC = 0.93, 95% CI: 0.87-0.99, sensitivity = 92%, and specificity = 95% for cut-off value >1.16; Fig. 3A). However, ROC analysis for discrimination between CVD/diabetic patients and CVD/non-diabetic patients showed a moderate diagnostic value (AUC = 0.71, 95% CI: 0.56–0.85, sensitivity 60%, specificity= 75% for a cut-off criterion >4.465; Fig. 3B).

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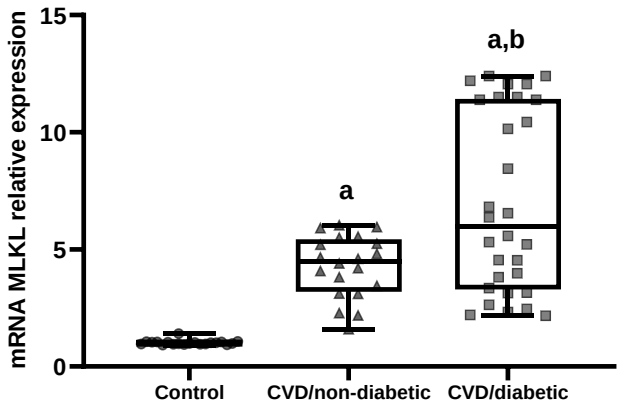

Fig. 1. Scatterplot illustrating MLKL relative expression levels in the studied groups. Groups are compared using Kruskal Wallis test. [a]significantly different from the control group at $p < 0.001$; [b]significantly different from CVD/non-diabetic group at $p < 0.05$.

Correlations between MLKL gene expression and other demographic and clinicopathological parameters

As illustrated in Figure 4, MLKL gene expression showed positive correlations with total cholesterol, LDL-C, total cholesterol/HDL-C ratio, and LDL-C/HDL-C ratio at $p < 0.01$ (r = 0.393, 0.579, 0.424, and 0.493, respectively) and negative association with HDL-C (r= -0.296, at $p < 0.05$). Moreover, MLKL gene expression levels showed positive relationship with insulin, glucose, HbA1c%, HOMA-IR index, hsCRP, and WBCs count at $p < 0.01$ and also displayed negative association with QUICKI index.

Multivariate regression analysis for the CAD severity

Concerning the severity of CAD, we found that MLKL gene expression levels were elevated in 22 patients having four atherosclerotic arteries as compared to its levels in 26 patients having three atherosclerotic arteries (8.23 ± 0.80 versus 4.20 ± 0.26, at $p < 0.001$). To explore the possible variables associated with CAD severity, a multiple regression analysis utilizing the affected arteries number as a dependent parameter was performed. Three models were established by age, gender, BMI, DM, current smoking, hypertension, hsCRP, and MLKL gene expression as independent parameters. Patients' demographic data (age and gender) and MLKL expression levels were included in Model 1, while Model 2 includes atherosclerosis classical risk factors in addition to Model 1. In Model 3, we included hsCRP as an independent variable in addition to Model 2. As summarized in Table 3, both hsCRP and MLKL gene expression levels were independent predicting factors for CAD severity in Model 3 (β = 0.360 and 0.438, at $p < 0.05$, respectively).

DISCUSSION

When the tight balance between cell proliferation and death is disrupted, pathological events, including major CVD as atherosclerosis, occur [6]. DM increases the risk of CVD by two to three folds[3] as a result of insulin resistance and other associated processes of dyslipidemia, inflammation, and cell death[29,30].

Apart from apoptosis and necrosis, necroptosis or programmed necrosis is a novel proinflammatory type of cell death that has recently been demonstrated as a critical player in cancer and several inflammatory diseases, including atherosclerosis[10,31]. Necroptosis activation leads to the phosphorylation of MLKL and subsequent cell lysis[16]. However, phosphorylated

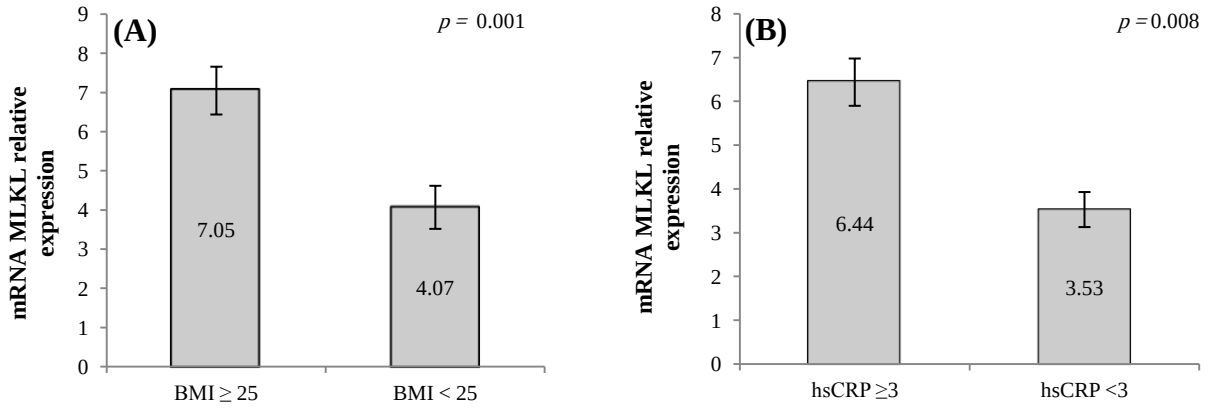

Fig. 2. MLKL gene expression levels with respect to (A) BMI ≥ 25 and <25 kg/m^2 and (B) serum hsCRP levels ≥3 and <3 mg/L. Data are expressed as mean ± SEM. Groups are compared using Mann-Whitney U test.

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Table 2. Multivariate regression analysis for predicting factors affecting mRNA levels of MLKL

Parameters	Multivariate full regression		Multivariate stepwise regression	
	B	***p***	***β***	***p***
Age	0.107	0.271	……	……
Gender	-0.070	0.383	……	……
BMI	0.244	0.009*	0.283	0.001**
Hypertension	0.032	0.699	……	……
Current smoking	0.134	0.156	……	……
HDL-C	-0.123	0.160	……	……
LDL-C	0.045	0.598	……	……
hsCRP	0.218	0.031*	0.259	0.006**
Total WBC count	0.222	0.022*	0.299	0.001**
FBG	0.424	< 0.001**	0.468	< 0.001**
Insulin	0.204	0.025*	…….	……

R^2 for multivariate full regression and stepwise regression models for dependent variables were 79.2% and 72.1% respectively. *statistically significant at $p \leq 0.05$; **statistically significant at $p \leq 0.01$

MLKL has lately been detected in advanced atherosclerotic lesions[23] and diabetic mouse models[20]; its certain function in atherogenesis with the influence of diabetes needs more elucidation. Therefore, in this study we evaluated the alterations MLKL gene expression levels, together with its association with atherosclerosis-related hallmarks as well as disease severity in CVD patients.

The current study showed that MLKL gene expression levels significantly increased in both CVD groups when compared to the controls. Accumulating animal studies have proved that necroptosis is involved in the pathogenesis of CVD, e.g. acute coronary syndrome, abdominal aortic aneurysms, and atherosclerosis[32,33]. However, the number of human studies in this field is still limited. Lin *et al.*[33] reported elevated RIP3 within necroptotic cells of advanced atherosclerotic lesions in murine models of atherosclerosis. Subsequently, Karunakaran *et al.*[22] reported elevated RIP3, MLKL, and phosphorylated MLKL in advanced human carotid atheroma. Necroptosis inhibition in the vasculature has been proven to have a protective outcome[31], and hence, necroptosis is probably a novel promising therapeutic target to halt the atherogenesis. Additionally, MLKL gene expression was significantly elevated in CVD/diabetics than non-diabetics. To our best knowledge, this is the first work reporting circulating MLKL gene expression in human atherosclerotic diabetic patients. The elevated MLKL expression in diabetic patients could be attributed to sustained hyperglycemia, which is prevalent in diabetics and causes the production of excessive amounts of intracellular reactive oxygen species that potentiates systemic oxidative stress accelerating both apoptotic and proinflammatory processes[34]. Moreover, downstream necroptotic pathways include the production of advanced glycation end products and oxygen free radicals, depending on glycolysis. This evidence might explain the link between increased intracellular glucose and necroptosis induction[35]. Such relation is supported by a positive correlation

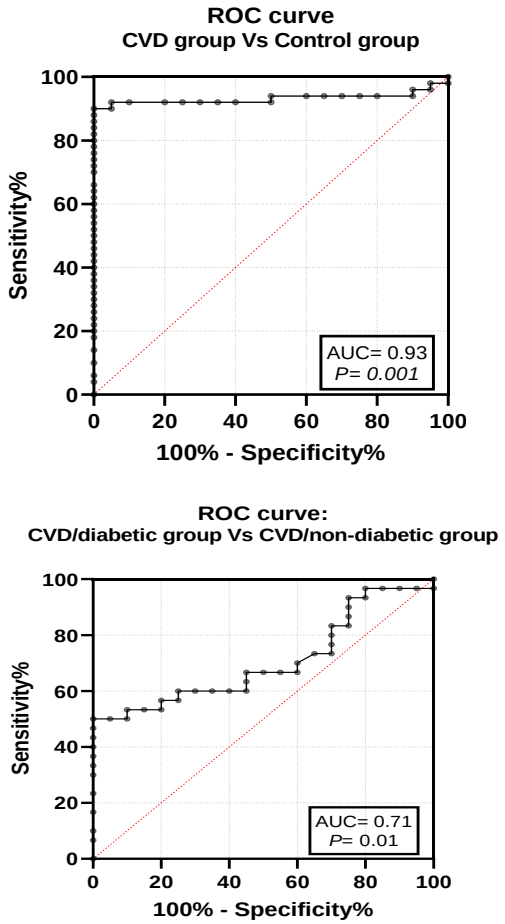

Fig. 3. ROC curve analysis for the diagnostic value of MLKL expression.

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between MLKL mRNA levels and HbA1c%, a precursor for hemoglobin- advanced glycation end products[36].

In our study, diabetic and non-diabetic CVD patients showed an obvious insulin resistance manifested by elevated serum insulin and increased HOMA-IR index. This result complies with that of Karrowni *et al.*[37] who reported elevated HOMA-IR index in CVD non-diabetic patients and could be ascribed to chronic subclinical inflammation, a prevalent risk factor for atherosclerosis. It has also been suggested that hsCRP could be an independent predictor of insulin resistance[38]. With regard to diabetes and its associated metabolic disturbance, correlation analysis demonstrated that MLKL gene expression had significant positive correlations with FBG, insulin, and HOMA-IR index and negative correlation with QUICKI index. Moreover, multivariate regression analysis identified FBG as a positive independent predictor for MLKL gene expression. This finding may approve the study of Xu and his colleges[20] who revealed that hepatic MLKL could impair insulin

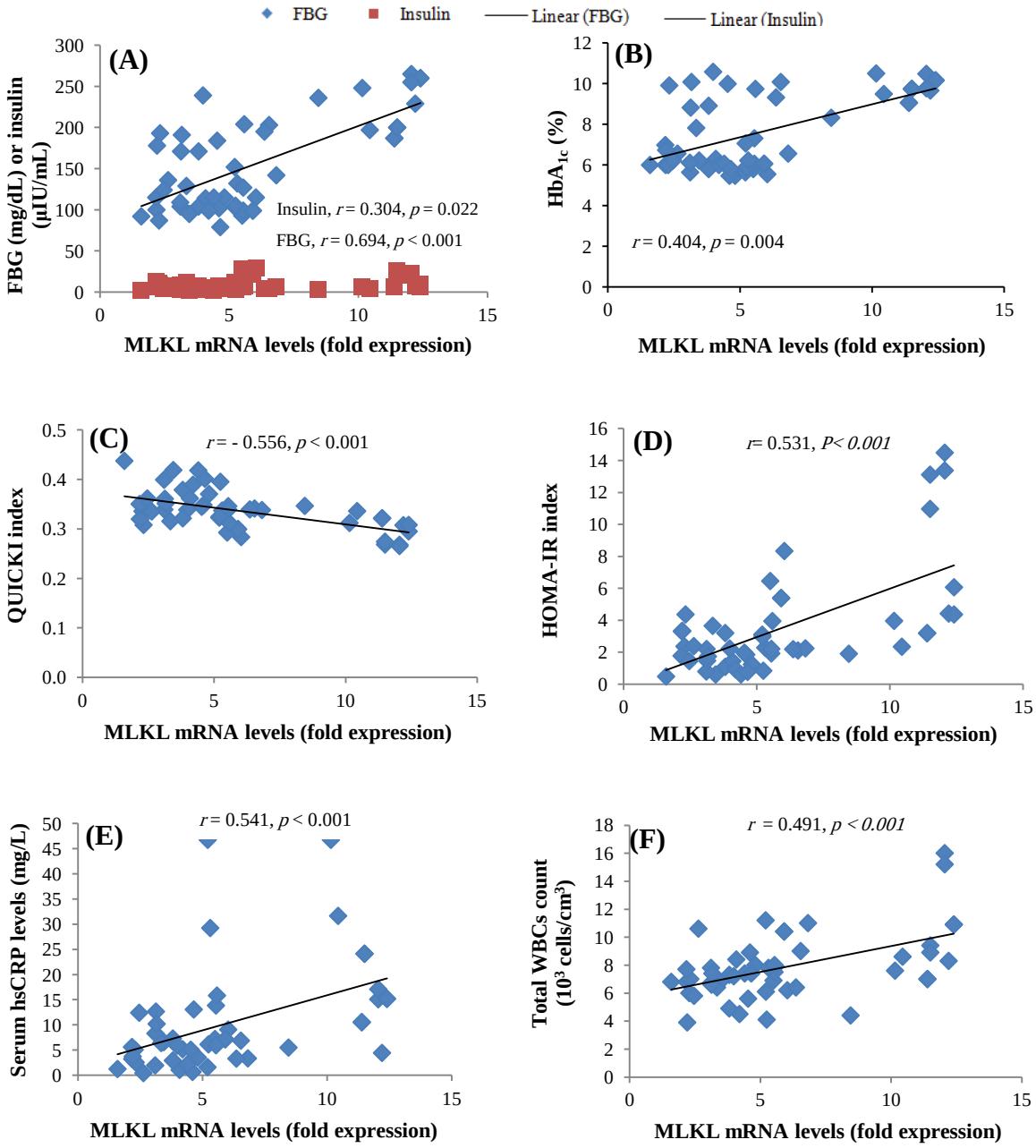

Fig. 4. Correlation between MLKL gene expression levels and other biochemical parameters (A) FBG and insulin levels, (B) HbA1c%, (C) QUICKI index, (D) HOMA-IR index, (E) hsCRP levels, and (F) total WBCs count.

Table 3. Predicting factors for severity of CAD

Parameters	*β coefficient*	*p* value
Model 1: R^2= 36.6%		
Age	-0.147	0.218
Gender	0.071	0.549
MLKL gene expression levels	0.594	< 0.001
Model 2: R^2= 43.5%		
Age	-0.262	0.162
Gender	0.099	0.424
BMI	-0.082	0.572
DM	0.108	0.388
Hypertension	0.174	0.163
Current smoking	-0.194	0.166
MLKL gene expression levels	0.630	<0.001
Model 3: R^2= 49.5%		
Age	-0.089	0.562
Gender	0.091	0.444
BMI	-0.071	0.609
DM	0.033	0.788
Hypertension	0.164	0.170
Current smoking	-0.156	0.245
hsCRP	0.360	0.032
MLKL gene expression levels	0.438	0.010

signaling via controlling insulin-stimulated AKT phosphorylation pathway, in an inflammation-independent mechanism. Besides, regression analysis recognized BMI as a positive modulator for MLKL expression. This outcome declares the possible role of MLKL as a key mediator of saturated free fatty acids signaling in obesity-related insulin resistance and subsequent activation of cJun NH2-terminal kinase[39]. Increased hepatic MLKL levels have also been reported in obese diabetic mice[20]. The current study demonstrated that MLKL expression elevated in patients having BMI ≥25 kg/m^2 than those having BMI less than 25 kg/m^2. This observation highlights the link between MLKL and obesity, the primary risk factor for the Type 2 DM.

Necroptosis and inflammation are strongly connected. Necroptosis is recognized as a potent proinflammatory type of cell death as it could participate in the augmentation and chronicity of inflammation. Many cytokines produced during the immune response are robust cell death inducers[13,40]. It has been found that both necroptosis and inflammation could be avoided by RIPK3 and/or MLKL reduction[21,40]. Cumulative studies have shown the crucial function of necroptosis in inflammation and its implication in the pathogenesis of many inflammatory diseases[6,13]. For this reason, MLKL gene expression level could be predicted by the level of some inflammation markers. We demonstrated that MLKL gene expression had strongly positive correlation with chronic inflammatory markers (hsCRP and WBCs count), which supports its proinflammatory role in atherosclerosis. This relation is also supported by multivariate regression analysis that identified both hsCRP and total WBCs count as predicting factors affecting mRNA levels of MLKL. It is important to mention that chronic inflammation could indirectly explain the link between MLKL and BMI, a marker of obesity, because of known association between obesity and chronic low-grade inflammation[41]. The current study also evaluated the diagnostic value of MLKL gene expression using ROC analysis. It has been found that MLKL is an excellent diagnostic value for discrimination between CVD patients and controls and a moderate diagnostic value for differentiation between diabetic and non-diabetic CVD patients. Therefore, we could assume that MLKL expression might be a promising diagnostic biomarker for CVD patients. Interestingly, we demonstrated that MLKL gene expression was significantly higher in patients having four atherosclerotic arteries as compared to patients having three atherosclerotic arteries. Moreover, regression analysis revealed that both hsCRP and MLKL gene expression levels were positive predictors for the CAD severity. Hence, further studies may be required to address the possible diagnostic value of MLKL as a marker for CAD severity. Based on these findings, we recommend that more effort should be devoted towards developing MLKL inhibitors targeting the necroptotic machinery, which might be a radical treatment for many CVD including atherosclerosis.

Based on our knowledge, this is an initial work to assess circulating MLKL gene expression in human atherosclerotic patients with the impact of type 2 DM. Overexpression of MLKL in atherosclerosis is correlated with the hallmarks of atherosclerosis such as dyslipidemia, inflammation, and insulin resistance. Furthermore, MLKL is an independent predictor of CAD severity. Therefore, the inclusion of MLKL inhibitors might provide a novel therapeutic hope for atherosclerotic patients.

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CONFLICT OF INTEREST. None declared.

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