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The association of vascular endothelial growth factor, metalloproteinases and their tissue inhibitors with cardiovascular risk factors in the metabolic syndrome

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Abstract. – OBJECTIVE: The present study was proposed to examine the matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and vascular endothelial growth factor (VEGF) in patients with metabolic syndrome (MetS), and to compare these parameters with healthy controls. We also compared the possible association of the circulating levels of MMP-2, MMP-9, TIMP-1, TIMP-2, and VEGF with cardiovascular risk factors in patients with MetS. We also compared the possible association of the circulating levels of MMP-2, MMP-9, TIMP-1, TIMP-2, and VEGF with cardiovascular risk factors in patients with MetS.

PATIENTS AND METHODS: A total of 45 patients with MetS and 17 healthy controls with a body mass index (BMI) less than 25 kg/m^2 were enrolled in the study. Plasma MMP-2, MMP-9, TIMP-1, TIMP-2, and VEGF levels were determined using ELISA.

RESULTS: TIMP-1,-2, MMP-2,-9 levels were significantly higher in patients with MetS compared with healthy controls ($p < 0.001$). Carotid intima-media thickness and serum VEGF levels were found to be significantly increased ($p < 0.01$, $p < 0.05$ respectively) in MetS compared with healthy controls. According to the ROC curves, TIMP-1 levels were both sensitive (93.3%) and specific (81.2%).

CONCLUSIONS: We observed that the patients with MetS have increased circulating concentrations of MMP-9, MMP-2, and TIMP-1, TIMP-2 that are associated with increased concentrations of VEGF. These findings suggest that MMP-2 may have a role in the increased cardiovascular risk of MetS patients.

Key Words:
Metabolic syndrome, Matrix metalloproteinases, Tissue inhibitors of metalloproteinases, Vascular endothelial growth factor, Carotid Intima-Media Thickness.

Introduction

Metabolic Syndrome (MetS) includes a group of clinical disorders (obesity, insulin resistance, glucose intolerance, hypertension, and dyslipidemia, etc.) that are related to each other in certain aspects[1]. Hypercoagulation, chronic inflammation, endothelial dysfunction, oxidative stress, and reduced bioavailability of insulin-like growth factor-1 are the remaining abnormalities that are related to MetS and have been associated with the pathogenesis and progression of chronic diseases[2].

Information exchange between the cells is enabled by chemical messengers and signals, and the protein macromolecules which constitute the extracellular matrix (ECM)[3]. Degradation of ECM in time has importance during development, morphogenesis, tissue repair and remodeling. Different types of proteinases are included during ECM degradation process. However, ma-

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trix metalloproteinases (MMPs) (matrixins) are considered the main enzymes. Activation of the precursor zymogens and inhibition by endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) regulate matrixin activities, besides other factors. As a result, MMP and TIMP balance is important for ultimate ECM remodeling in the tissue[4]. Berg et al[5,6] found higher plasma activity of MMP-2 in women with MetS, which correlates with other soluble molecules involved in the plaque development like sVCAM. Patients with MetS have increased circulating concentrations of pro-MMP-9, MMP-8, and TIMP-1 that are associated with increased concentrations of pro-inflammatory mediators and adhesion molecules[7]. Hopps et al[8] showed an increase in plasma concentrations of MMP-2 and MMP-9 and their inhibitors (TIMP-1 and TIMP-2), in diabetic and non-diabetic subjects with MS.

The vascular endothelial growth factor (VEGF) is originally a basic 45-kDa heparin-binding glycoprotein. VEGF is a highly specific mitogen for vascular endothelial cells. VEGF is a well-known potent stimulator of angiogenesis in both physiological and pathological conditions. The expression of VEGF is potentiated in response to hypoxia, by activated oncogenes, and by a variety of cytokines[9]. VEGF has also been shown to stimulate the expression of MMPs[10,11].

No data are available concerning possible association of the circulating levels of MMP-2, MMP-9, TIMP-1, TIMP-2, and VEGF with intima-media thickness (IMT) as cardiovascular risk factor in patients with MetS. Different components of MetS have been identified as possible stimuli for the synthesis and activity of MMPs. Several authors[5-8] found strong associations of MMP-2, MMP-9, TIMP-1, and TIMP-2 with components of MetS. However, circulating MMP-2,-9 and TIMP-1,-2 levels of patients with MetS have been reported to be controversial results in various studies. Therefore, in the present study, our aim was to investigate the influence of MetS on MMP-2,-9, TIMP-1,-2 and VEGF levels and their relationship with the components of MetS.

Patients and Methods

Paatients

We evaluated 45 patients with MetS, admitted to Istanbul Education and Research Hospital Internal Medicine Clinic. Metabolic syndrome was diagnosed as the presence of at least 3 of the following parameters, according to ATP III[12] criteria: abdominal obesity (waist circumference > 102 cm for males and >88 cm for females), hypertension (SBP > 130 mmHg and/or DBP > 85 mmHg) or history of antihypertensive usage, hypertriglyceridemia (≥150 mg/dL) or presence of treatment for this disorder, low HDL-C (<40 mg/dL in males and <50 mg/dL in females). Smokers and patients who have cardiovascular disease were excluded.

The control group consisted of 17 healthy subjects who were selected from the hospital staff and did not have a personal or family history of diabetes or dyslipidemia and had normal thyroid, hepatic and renal function. The BMI of each subject was calculated using the following formula: weight (kg)/height $(m)^2$. Waist circumference was measured twice to the nearest 0.1 cm with a flexible tape measure at the level of the minimum circumference, which was usually at the level of the navel.

All participants were informed about the survey and freely signed and dated the consent form. The protocol was approved by the Ethics Committee of Istanbul Education and Research Hospital (no: 852, date: 18.01.2012) and was conducted in accordance with the Declaration of Helsinki.

Ultrasonographic Examinations: Measurement of Carotid Intima-Media Thickness (IMT)

The extracranial carotid arteries were examined using a standardized protocol. IMT was measured by Doppler ultrasound (LOGIQ e9, GE Healthcare, Cheshire, UK) of the common carotid arteries (CCA), as described previously[13]. All measurements were made at the time of scanning on frozen images of longitudinal scans by using the machines electronic caliper.

Laboratory Analysis

Sample collection and preparation

Drugs were administered at least 24 h prior to blood collection. Clinical parameters, including routine biochemical parameters, were measured using standard protocols. Blood samples were collected in EDTA-containing tubes and anticoagulant-free tubes after an overnight fast. After immediate centrifugation (3000 g) for 10 min at 4 °C, plasma was separated in Eppendorf tubes and frozen immediately at -80 °C until analysis.

Routine parameters were determined on the Olympus AU 800 analyzer by enzymatic methods using commercial kits (Roche Diagnostics, GmbH, Mannheim, Germany). Plasma insulin was measured by radioimmunoassay using a commercial kit (DSL-1600, Webster, TX, USA). The homeostasis model assessment (HOMA) was used to detect the degree of insulin resistance (IR) by measuring the levels of basal (fasting) glucose and insulin. HOMA-IR was calculated using the following formula: HOMA-IR = (fasting glucose [mg/dL] x fasting insulin [μU/mL]) / 405.

Plasma MMP-2 levels were measured by a commercially available enzyme-linked immunosorbent assay kit (RayBio, GA, USA, Cat no; ELH-MMP-2–001). The coefficients of intra- and inter-assay variation were 5.3% (n=10) and 7.5% (n=10), respectively. Plasma MMP-9 concentrations were measured by enzyme-linked immunoassay using a commercially available kit (eBiosience, Vienna, Austria, BMS2018). The coefficients of intra- and inter-assay variations were 4.8% (n=10), and 5.6% (n=10), respectively. Plasma TIMP-1 concentrations were measured by enzyme-linked immunoassay using a commercially available kit (RayBio, GA, USA, Cat no; ELH-TIMP–1–001). The coefficients of intra- and inter-assay variations were 5.9% (n=10), and 6.9% (n=10), respectively. Plasma TIMP-2 concentrations were determined by enzyme-linked immunoassay using a commercially available kit (RayBio, GA, USA, Cat no; ELH-TIMP–2–001). The coefficients of intra- and inter-assay variations were 4.1% (n=15), and 4.9% (n=15), respectively. Plasma VEGF activity was measured by enzyme-linked immunoassay using a commercially available kit (Invitrogen, CA, USA, Cat no; KHG0111). The coefficients of intra- and inter-assay variations were 5.7% (n=10), and 6.9% (n=10), respectively.

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA). The results are expressed as the mean ± standard deviation. Unpaired Student's *t*-test was used to compare the mean values between the groups. Pearson's correlations were used to test the relationship among variables. To assess the diagnostic accuracy, we performed receiver operating characteristic (ROC) curve analysis. The area under the ROC curve (AUC) was then estimated. TIMP-1, TIMP-2, MMP-2, MMP-9. Cut-off values were also determined according to ROC analysis. $p < 0.05$ was considered statistically significant.

Results

The general characteristics of the studied groups are shown in Table I. No significant differences were observed between all patients and

Table I. Anthropometric and biochemical parameters of MetS patients and control.

	Control (n: 17)	Metabolic Syndrome (n: 45)	*p*
Age (year)	49.07 ± 5.11	53.67 ± 8.28	NS
BMI (kg/m^2)	26.2 ± 2.6	31.03 ± 4.35	<0.001
Waist circumference (cm)	92.6 ± 7.13	101.98 ± 13.04	<0.01
Total cholesterol (mmol/L)	5.06 ± 0.92	5.3 ± 1.3	NS
Triglicerides (mmol/L)	1.28 ± 0.54	2.48 ± 1.13	<0.001
HDL (mmol/L)	1.16 ± 0.22	1.1 ± 0.27	NS
LDL (mmol/L)	3.14 ± 0.56	3.19 ± 0.95	NS
Total Protein (g/L)	75.6 ± 3.8	74.7 ± 4	NS
Albumin (g/L)	41.5 ± 1.7	41.8 ± 5.8	NS
Creatinine (μmol/L)	82.21 ± 18.56	76.91 ± 13.26	NS
Urea (mmol/L)	4.73 ± 1.08	5.34 ± 1.76	NS
Insulin (pmol/L)	54.45 ± 20.28	89.87 ± 65.7	<0.05
Glucose (mmol/L)	5.17 ± 0.49	9.81 ± 4.07	<0.001
HOMA-IR	1.79 ± 0.65	5.89 ± 5.08	<0.001
IMT (mm)	0.65 ± 0.17	1.09 ± 1.56	<0.01
hsCRP (nmol/L)	2.86 ± 2.57	5.81 ± 4.29	<0.01

BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; HOMA-IR, insulin resistance; IMT, intima-media thickness; hsCRP, high sensitive C-reactive protein; NS, non-significant.

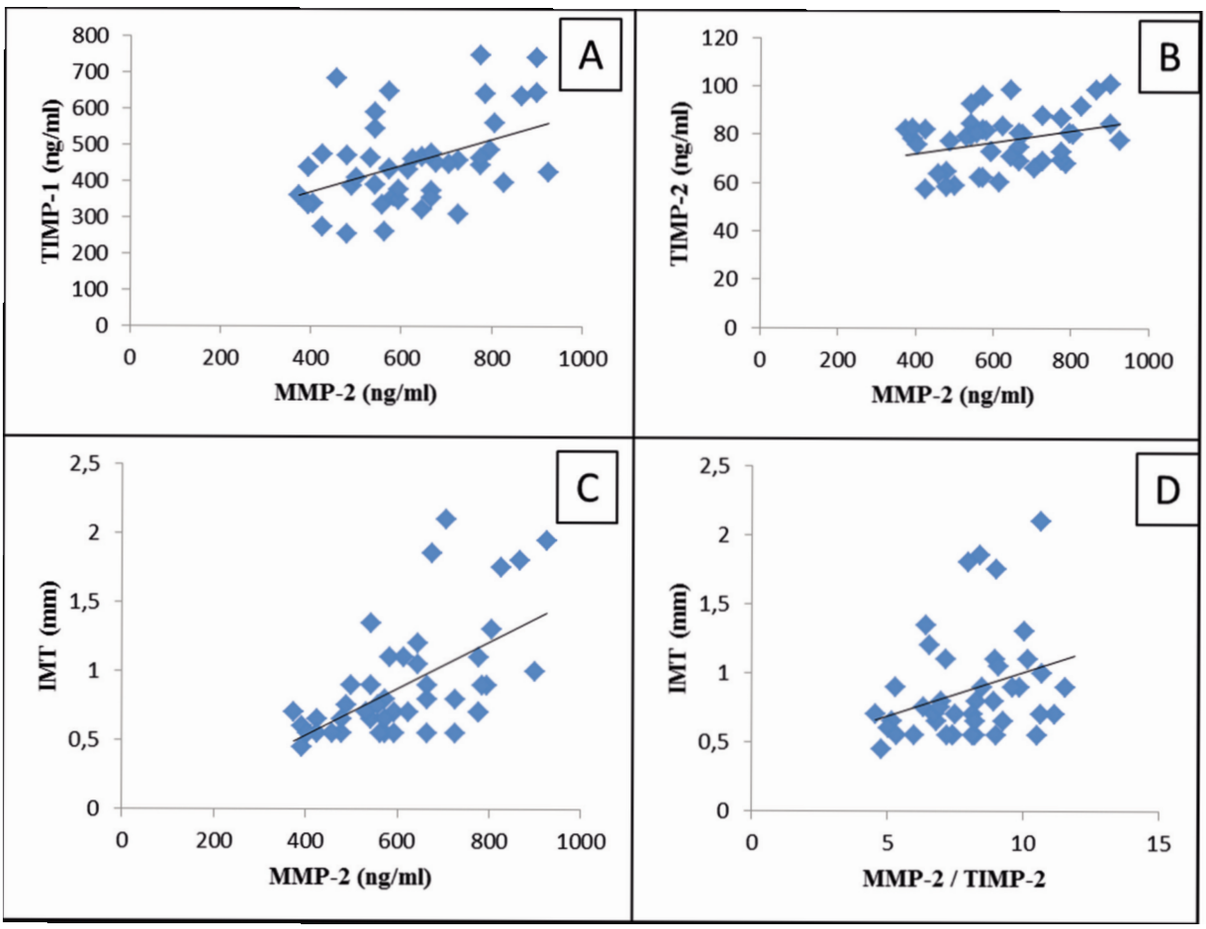

Figure 1. The relationship between MMP-2with TIMP-1 ***(A)***, TIMP-2 ***(B)***, IMT ***(C)*** in MetS patients. The relationship between MMP-2/TIMP 2 with IMT ***(D)*** in MetS patients.

control group, control group with regards to age, total cholesterol, total protein, albumin, urea, creatinine, LDL-C and HDL-C levels. As expected, a comparison of the diagnostic criteria between the two groups revealed significant differences in hyperlipidemia, hyperglycemia, obesity, waist circumference. Patients in the MetS group had significantly higher levels of triglyceride (p<0.001), glucose (p<0.001), insulin (p<0.05), HOMA-IR (p<0.001), hsCRP (p<0.01) than healthy subjects in the control group.

TIMP-1, TIMP-2, MMP-9, MMP-2 levels were significantly higher in patients with MetS than healthy control (p<0.001). Plasma VEGF levels were also found to be significantly increased in patients with MetS compared to controls (p<0.05) (Table II).

Total cholesterol was positively correlated with TG (r=0.449; p<0.01), LDL-C (r=0.906; p<0.001) in MetS patients. MMP-2 was positively correlated with IMT (r=0.386; p<0.01), TIMP-1 (r=0.357; p<0.05) and TIMP-2 (r=0.320; p<0.05) in MetS patients. TIMP-2 was positively correlated with TIMP-1 (r=0.297; p<0.05), HOMA-IR (r=0.362; p<0.05). TIMP-2 was negatively correlated with VEGF (r=-0.370; p<0.05) in MetS patients . IMT was positively correlated with MMP-2/TIMP-2 (r=0.355; p<0.05) in MetS patients (Figure 1).

A comparison of the ROC curves for sensitivity, specificity, AUC, cut-off and asymptotic significance of MMP-2,-9, TIMP-1, and TIMP-2 for predicting of MetS in all subjects is shown in Table III.

Discussion

In this study, the MetS group showed higher levels of triglyceride, glucose, insulin, HOMA-

Table II. Plasma concentrations of matrix metalloproteinases and tissue inhibitors in groups.

	Control (n: 17)	Metabolic Syndrome (n: 45)	p
TIMP-1 (ng/ml)	250.03 ± 61.66	462.89 ± 144.09	<0.001
TIMP-2 (ng/ml)	58.79 ± 8.26	77.79 ± 12.03	<0.001
MMP-2 (ng/ml)	411.27 ± 100.74	623.77 ± 149.65	<0.001
MMP-9 (ng/ml)	107.7 ± 53.96	207.91 ± 108.14	<0.001
VEGF (pg/ml)	521.68 ± 397.01	791.39 ± 392.24	<0.05

TIMP-1, tissue inhibitor of metalloprotease-1; TIMP-2, tissue inhibitor of metalloprotease-2; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9;VEGF, vascular endothelial growth factor.

Table III. Sensitivity, specificity, AUC, cut-off and asymptotic significance of TIMP 1, TIMP 2, MMP 9 and MMP 2 levels for predicting of MetS in all subjects.

	AUC (%)	Sensitivity (%)	Specifity	Cut-off	Asymptotic Sig.
TIMP-1	0.961	0.933	0.812	315.65	<0.001
TIMP-2	0.907	0.822	0.812	66.25	<0.001
MMP-2	0.883	0.800	0.937	483.9	<0.001
MMP-9	0.806	0.800	0.687	116.71	<0.001

TIMP-1, tissue inhibitor of metalloprotease-1; TIMP-2, tissue inhibitor of metalloprotease-2; MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9.

IR, MMP-2,-9, TIMP-1,-2 and VEGF when compared to healthy control group. No difference was observed between two groups in total cholesterol, LDL-C and HDL-C levels. The most important finding of this study was the demonstration that plasma MMP-2 levels were associated with IMT. IMT is a nonspecific cardiovascular risk factor; IMT was increased in patients with metabolic syndrome in this study. Interestingly, we did not find any association between components of MetS and circulating MMPs and TIMPs. We observed that MMPs and TIMPs, which are important regulators of extracellular matrix remodeling, have different mechanisms in MetS.

Specific MMPs and their inhibitors are essential in the regulation of collagen remodeling in cells and tissues. Consistent with the previous reports[7,14,15], the present study demonstrated that the levels of both MMP-2 and -9 were higher among patients with the MetS as compared with the control group. Signorelli et al[16] have observed considerably higher concentrations of the serum MMP-2 and MMP-9 in patients with type-2 diabetes and atherosclerosis of lower extremities. Likewise, Lee et al[17] have demonstrated increased activity of MMP-2 and -9 in type-2 diabetes. MMP-9 activity was associated with fasting plasma glucose levels (the highest correlation coefficient was noted between MMP levels and fasting plasma glucose levels, whereas lower correlation coefficients were noted between MMP-9 level and obesity indices)[15]. However, this situation was not observed in our study. In our study, MMP-2 and -9 was independently associated with the metabolic syndrome components. Our findings and other studies[14-19] suggest that MetS can be connected to the advanced dysmetabolic state. Plasma activity of MMP-2 is observed more in women with MetS. This situation is related to the cardiovascular risk factors and most common markers of MetS[6]. However, the results regarding MMP-2 expression under high glucose conditions are conflicting[20-22]. The prognostic value of this observation has to be evaluated.

In our study, MMP-2 was also positively correlated with IMT in MetS patients. Aydin et al[23] showed that mean IMT values of individuals with MetS, except morbid obese, were higher compared to individuals without MetS. Increased

IMT is found in young, middle and elderly subjects with MetS[24,25]. MMPs have been shown to induce the release of growth factors anchored in the extracellular matrix, suggesting a potential mechanism by which MMPs induce IMT. These results support the importance of screening and early intervention in MetS. Although the atherosclerotic process is not fully understood, MMP-2 and -9 can be regarded as nontraditional cardiovascular risk factors of the atherosclerotic process in MetS.

Alteration of the previously mentioned MMP-TIMP balance, which is a critical factor for matrix remodeling regulation, might contribute to many diseases[26]. In this study, an increase in TIMP-1,-2 levels was observed in MetS patients as a response to increase in levels of MMP-2,-9 , which are inhibitors of TIMP-1,-2 . MMP-2 levels were positively correlated with TIMP-1 and TIMP-2 levels in MetS patients. Plasma TIMP-1,-2 levels were also increased in diabetic patients which may reflect abnormal ECM metabolism[27]. Lewandowski et al[28] demonstrated that MMP-2, MMP-9, TIMP-1, TIMP-2 were positively correlated with fasting glucose levels in type-2 diabetic subjects. Goncalves et al[7] demonstrated that TIMP-1 levels were found to be increased in MetS patients, where TIMP-2 levels did not change. Miksztowicz et al[6] observed an increased TIMP-1 level in postmenopausal women with MetS. Hopps et al[8] found increased plasma MMP-2,-9 and TIMP-1,-2 in the diabetic and non-diabetic subjects with MetS. In the whole group, MetS subjects showed a positive correlation between MMP-2, MMP-9, TIMP-2 and waist circumference. There was a positive correlation between MMP-2 and BMI. Negative correlation between MMP-2, MMP-9, TIMP-1, TIMP-2, and lipid profile was observed. They also noted higher concentrations of all the measured parameters in the diabetic patients with MetS in comparison with the non-diabetic subjects with MetS. Their results are partially similar to our findings. In another study[29], TIMP-1,-2 were found significantly higher in MetS patients than in healthy controls. Increased plasma MMP-9 and TIMP-1 levels were correlated with BMI, waist circumference, triglyceridemia, and HDL-C. Plasma TIMP-1,-2 levels could be significant determinants and/or diagnostic markers of MetS. Our results are partially in accordance with these data, as we observed TIMP-2 was positively correlated with TIMP-1 and HOMA-IR, in the MetS subjects. IMT was also positively correlated with MMP-2/TIMP-2 in MetS patients. TIMP-2 can promote insulin resistance in the presence of MetS. Increased TIMP-2 was associated with increases in IMT in MetS patients. We suggest that increased IMT can also result from greater increases in arterial blood pressure.

We found that the diagnostic value of plasma TIMP-1 as a disease marker were: sensitivity 93% and specificity 81%. TIMP-1 is considered a new candidate adipokine. Pro-inflammatory cytokines upgregulate TIMP-1 expression and secretion in obese patients and *in vitro*, which implies that during obesity TIMP-1 might affect sustaining adipose tissue mass[30]. TIMP-1 levels are influenced by all MMPs and not just by one MMP. According to the work done by Abdelaziz et al[31] the plasma levels of TIMP-1 and TIMP-2 showed 96.7%, 93.3% sensitivity respectively and both showed 100% specificity in differentiating the NASH group from controls. NAFLD is considered the hepatic manifestation of the metabolic syndrome. It is strongly associated with insulin resistance and hyperglycemia and thus linked with simultaneous presence of type-2 diabetes mellitus and other features of MetS. However, we have not evaluated whether our patients have NASH or not.

VEGF (referred to also as VEGF-A) induces the proliferation of endothelium at both the microvascular and macrovascular levels. VEGF has also been shown to stimulate the expression of MMPs[10,11]. Plasma VEGF levels increased in patients with MetS and were negatively correlated with TIMP-2 in the present study. Mitogenic response of human microvascular endothelial cells to VEGF-A *in vitro* and angiogenesis *in vivo*[32-34] is inhibited by TIMP-2, according to recent research. The mechanism of this effect is independent of metalloproteinase inhibition. Consistent with Kowalski et al[35], we demonstrated that the plasma VEGF levels were increased in subjects with MetS. In MetS patients, main cause for vascular complications is found to be endothelial dysfunction, which is seen as an increase in plasma VEGF levels[35]. In another study[36], serum VEGF levels were correlated with multiple factors including waist circumference, hip circumference, waist/hip ratio, fasting blood glucose, fasting insulin, and insulin resistance index in children and adolescents with MetS. VEGF increased in direct proportion to levels of MetS components in rural Bangladeshi women[37]. A positive association was observed between plasma VEGF levels and MetS components using a large sample size from South Asia. But no correlation was found between

VEGF and the components of MetS in our study. Dandona et al[38] demonstrated that both plasma VEGF and MMP-9 concentrations decreased significantly following insulin infusion and continued to be inhibited at 6 h, even after the cessation of insulin infusion to obese, nondiabetic subjects. Insulin acutely decreases plasma VEGF and MMP-9 concentrations.

There are some limitations of the study. First, the study group is small. Secondly, we did not investigate some of the important metalloproteinases (MMP-1, -7, -8) in MetS patients.

Conclusions

The patients with MetS have increased circulating concentrations of MMP-9, MMP-2, and TIMP-1, TIMP-2 that are associated with increased concentrations of VEGF. Several authors[6-8,14,15,29,35] are consistent with our findings. We conclude that that MMPs may have a role in the increased cardiovascular risk of MetS patients. VEGF is also linked to MetS. We suggest that, in the future, MMP-9, MMP-2, and TIMP-1, TIMP-2 and VEGF may serve an attractive field for cardiovascular risk factors as the components of MetS. Further clinical studies are needed to support our current findings and conclusions.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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