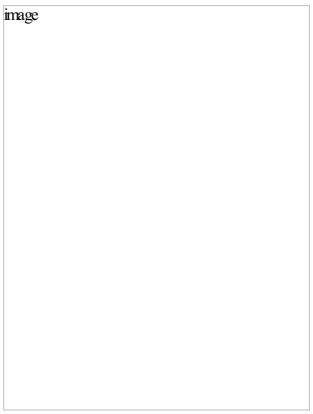

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The relationship between vascular endothelial growth factor cis- and trans-acting genetic variants and metabolic syndrome

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Running title: Association of 4 genetic variants and metabolic-syndrome

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Abbreviations:

MetS, metabolic syndrome; VEGF, vascular endothelial growth factor; CVDs, cardiovascular diseases; SNP, single nucleotide polymorphism; GWAS, genome-wide association study; WC, waist circumference; HC, hip circumference; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; IDF, International Diabetes Federation; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; hs-CRP, high sensitivity C-reactive protein; FBG, fasting blood glucose; TG, triglyceride.

ABSTRACT

Background: We have investigated the association between 4 cis- and trans-genetic variants (*rs6921438, rs4416670, rs6993770* and *rs10738760*) of the vascular endothelial growth factor (VEGF) gene and metabolic syndrome (MetS) and its individual components in an Iranian population.

Material & Method: Three hundred and thirty-six subjects were enrolled and MetS was defined according to the International-Diabetes-Federation (IDF) criteria. Genotyping was carried out in all the individuals for 4 VEGF genetic variants using an assay based on a combination of multiplex PCR and biochip array hybridization.

Results: As may be expected, patients with MetS had significantly higher levels of serum high-sensitivity C-reactive protein, waist circumference, hip circumference, body mass index; Fat percentage, systolic blood pressure, diastolic blood pressure and triglyceride, while the high-density lipoprotein cholesterol levels were significantly lower, compared to the control group ($P<0.05$). We also found that 1 of the VEGF- level associated genetic variants, rs6993770, was

associated with the presence of MetS; the less common T allele at this locus was associated with an increased risk for MetS. This association remained significant after adjustment for confounding factors (P= 0.007). Individuals with MetS carrying the *AT+TT* genotypes had markedly higher levels of fasting blood glucose, triglyceride and systolic blood pressure (P<0.05).

Conclusions: We have found an association between the *rs6993770* polymorphism and MetS. This gene variant was also associated with serum VEGF concentrations. There was also an association between this variant and the individual components of the MetS, including triglyceride, fasting blood glucose and systolic blood pressure.

Key words: metabolic syndrome; vascular endothelial growth factor; genetic polymorphism; single nucleotide polymorphism

Introduction

Metabolic syndrome (MetS) is characterized by abdominal obesity, glucose intolerance, dyslipidemia, and elevated blood pressure, and is associated with an increased risk of developing cardiovascular disease (CVD), diabetes, chronic kidney disease and related diseases (1-4). The prevalence of MetS is increasing globally, including in Iran (5, 6). The association between MetS and chronic kidney disease (CKD) has been reported in adults from USA, China, Thailand, and Japan (7-11). There is a need to identify patients who are susceptible to MetS. Moreover, the early detection and treatment of MetS may be a strategy for prevention of CAD and other MetS associated diseases.

There is increasing evidence for a role of vascular endothelial growth factor (VEGF) in angiogenesis, vascular permeability, and hematopoiesis (12). It has been suggested that adipocytes could produce VEGF (13) and an increased VEGF level has been reported in patients with ischemic heart disease, stroke, type 2 diabetes and heart failure. However, the involvement of VEGF in MetS has not been extensively studied. Several other studies have suggested the important role of VEGF circulating levels and MetS as well as the association of some genetic variants with MetS (14-18). We have recently investigated 4 single nucleotide polymorphisms (SNPs) (*rs6921438, rs4416670, rs6993770* and *rs10738760*) that explain the variability of serum VEGF concentrations, as detected by a genome-wide association study (GWAS) (19). As a relationship between VEGF and MetS has been previously reported, these SNPs may be considered to be candidate genetic markers for MetS and its components. Therefore, in the current study we further examined the possible associations of MetS with VEGF cis- and trans-acting genetic variants in an Iranian population with and without MetS.

Materials and Methods

Phenotypic Definition of MetS

MetS was defined according to the International Diabetes Federation (IDF) criteria (20): waist circumference (WC) ≥94 cm in men or ≥80 cm in women plus any 2 of the 4 following criteria (1) triglyceride ≥1.7 mmol/l, (2) high-density lipoprotein cholesterol (HDL-C) < 1.03 mmol/l in men or <1.3 in women, (3) systolic blood pressure (SBP) ≥ 130 mmHg or diastolic blood pressure (DBP) ≥ 85 mmHg, (4) fasting blood glucose (FBG) ≥ 5.6 mmol/l.

Population

Three hundred and thirty six subjects were enrolled from the Mashhad Stroke and Heart Atherosclerotic Disorder (MASHAD) study (21). Individuals with known acute or chronic diseases such as stroke, myocardial infarctions, hypertension, dyslipidemia or cancer and also drug therapy for these conditions were excluded from current study. All data were collected during the first visit of the MASHAD study. Data were collected using standard questionnaire including information about lifestyle such as smoking, personal medical history and drug history. The study protocol was approved by the Ethics Committee of the University and all participants (n=336) provided written informed consent for their participation in the study. This study complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Anthropometric and Biochemical parameters

Anthropometric parameters (including height, weight, waist and hip circumference, etc) were assessed in all the subjects as MASHAD study (21). SBP and DBP were calculated as the mean of 3 measurements taken under standardized conditions with a sphygmomanometer, with the subject in a supine position (22). Body mass index (BMI) was calculated according to the Quetelet's formula: weight (kg)/height (m^2). Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), high sensitivity C-reactive protein (hs-CRP), and fasting blood glucose (FBG) were measured as described previously (4, 23, 24).

Genotyping

DNA was isolated from peripheral blood leukocytes using QIAamp® DNA Mini-Kit (Qiagen, San Diego, CA) according to the manufacturer's protocol. Genotyping of *rs6921438, rs4416670,*

rs6993770 and *rs10738760* were performed by Randox Ltd (Crumlin, UK) (Evidence Investigator ®) using an assay based on a combination of multiplex PCR and biochip array hybridisation (25).

Statistical analysis

Data was analyzed using IBM SPSS Statistics 19 (SPSS Inc., IL, USA). The normality of data distribution and equality of variances were assessed using Kolmogorov-Smirnov and Levene's test, respectively. Descriptive statistics including mean±standard deviation (SD) were determined for normally distributed variables or as the median (interquartile range (IQR)) for not normally distributed variables. For normally distributed variables, the t-student test was used, while Bonferonni correction was applied for multiple comparisons. The Mann-Whitney U test was used for continuous variables if they were not normally distributed. Chi-square or Fisher exact tests were used for categorical variables. Logistic regression and multivariate analysis was used to calculate the association of MetS factors and genetic polymorphism. All the analyses were 2-sided and statistical significance was set at $P<0.05$.

Results

Characteristics of the population

The characteristics of the population with and without MetS are summarized in table 1. Subjects with MetS were significantly ($p<0.05$) heavier, with a higher fat percentage, WC, HC, body mass index (BMI), TG, SBP, DBP and hs-CRP, while the levels of high-density lipoprotein cholesterol (HDL-C) and height were significantly lower in the MetS group versus control group

(Table 1).

Polymorphisms and MetS

In order to investigate whether there was an association between serum VEGF- level and the genetic-variants, *rs6921438, rs4416670, rs6993770* and *rs10738760*, and MetS, we performed genotyping on extracted DNA. Genotyping was successfully performed in most DNA samples. The wild-type genotype (AA) of the *rs6993770 polymorphism* was found in 36% of the samples, whereas the AT and TT genotypes were found in 49% and 15% of the MetS group, respectively. The corresponding frequencies in the control group were 51% (AA), 38% (AT), and 11% (TT). Similar results were also observed for other SNPs. All the polymorphisms were consistent with the Hardy–Weinberg equilibrium (Table 2).

***Relationship between MetS and* VEGF trans- and cis-acting genetic variants**

Furthermore, the association of 4 VEGF-level associated genetic variants and MetS was evaluated using logistic regression before and after adjustment with confounding variables including age, sex and smoking. This analysis revealed that *rs6993770* was associated with MetS (Table 3). Subjects carrying a AT genotype had an increased risk for MetS (OR = 1.87, 95%CI: 1.13-3.10, P= 0.015). Individuals with the TT genotype also had an increased risk of MetS. Additionally, we observed the majority of these results remained statistically significant for this genetic polymorphism after adjustment for age, sex and smoking and subjects carrying T was associated with increased risk of MetS (e.g., OR of MetS subjects with TT genotype was 2.35 [95%CI: 1.06-5.20], P=0.034). Conversely no statistically relationship was detected for other SNPs and MetS in our population.

Relationship between VEGF trans- and cis-acting genetic variants and biometric and clinical characteristics of population

We further evaluated the association of biometric and clinical characteristics of our population assuming a dominant model of *rs6993770* variant (Table 4). These analyses showed that subjects with *AT+TT* genotypes had a significantly increased likelihood of the presence of MetS defining criteria, including FBG, TG and SBP (Table 4 and figure 1).

Discussion

To the best of our knowledge, this is the first study demonstrating an association between a VEGF trans-acting genetic variant, *rs6993770*, with an increased risk of MetS in an Iranian population. This polymorphism was also associated with higher levels of FBG, TG and SBP ; components of MetS.

Recent evidence indicates that an increase in adipose tissue VEGF-induced angiogenesis is a protective phenomenon against obesity-induced hypoxia and consequently insulin resistance (26). There is growing evidence for the involvement of VEGF in MetS and CAD (12, 27). It has been reported that a high level of VEGF in adipose tissue, which might suggest its protective role against obesity-induced hypoxia and insulin resistance (26). Tarantino *et al* (28) revealed the higher circulating level of VEGF in individuals with MetS and its role in hyperlipidemia and type 2 diabetes patients (29, 30). Several mechanisms have been documented to influence the level of VEGF, including genetic polymorphisms. Recently, we have reported that *rs10738760* is associated with an up-regulation of VEGF and explains around 5% of variation of circulating VEGF (19). Pathway analyses revealed the plausible biological among VEGF and VLDLR, which plays a key role in VLDL-triglyceride metabolism (31). In our previous study we found an

association between a genetic variant that was related to serum VEGF, *rs10738760*, and MetS in a population from Nancy, France.

This study revealed that *rs6993770* SNP is associated with the presence of MetS (P=0.007), high systolic blood pressure, hypertriglyceridemia (P=0.048) and fasting blood glucose (P=0.020). Facemire *et al* (32) reported a critical role of VEGF in blood pressure control, possibly by promoting nitric oxide synthase expression and NO activity. Therefore VEGF causes promotion of vasodilatation by inducing the production of NO by endothelial cells (33). As for the association rs6993770 and VEGF levels (19) it may be concluded that this SNP influences blood pressure control. Therefore, our study suggests a possible link between *rs6993770* and SBP through VEGF.

Rs6993770 SNP is an intronic genetic variant located in a zinc finger protein, multitype 2 (ZFPM2) gene in a region of 6p21.1 and 980.4 kb away from the low-density lipoprotein receptor-related protein 12 gene (LRP12) (19). One of the activities of ZFPM2 (FOG2) protein is involvement in adipogenesis (34). It is reported that at the beginning of adipogenesis, FOG1 and FOG2 genes are down-regulated. Differentiation of cells into lipid-containing cells is suppressed by overexpression of FOG1 or FOG2. Those demonstrate Fog2 expression is down-regulated during cell differentiation to adipocytes and knockdown of Fog2 expression impairs preadipocyte function (34). It may be *rs6993770* SNP effect in down-regulation of FOG2 during differentiation of cells into lipid-containing cells and preadipocytes differentiate to adipocytes to greater extent. Since adiposity has a central role in MetS, it is not an unexpected association of this genetic variant with MetS in our study.

However, we have recently reported a lack of association between the *rs6921438* and *rs10738760* and fasting glucose, fasting insulin, the risk of diabetes type 2, diabetic nephropathy

and retinopathy (35). These results were also observed in the current study, where no association of these SNPs was found for the MetS and its component traits related to glucose metabolism and central obesity. Concerning the traits associated with lipids metabolism, we have previously shown an association between VEGF-related SNP rs6921438 with LDL-C and HDL-C, but again the rs10738760 was not significantly associated with lipid phenotypes (36). This finding supports the results of the present study that rs10738760 has no direct effect on the risk of MetS.

We found that *rs6993770* was associated with MetS, and individuals carrying the AT genotype had an increased risk of MetS. These findings are in agreement with our previous study about the role of this genetic variant with MetS and its components (19). In addition, subjects carrying T had an increased risk of MetS after adjustment, which was related to the increased levels of FBG, TG and SBP. Moreover no association was observed between other VEGF genetic variants with MetS which is in line with previous studies, showing the lack of associations rs6921438 and rs10738760 with fasting glucose, fasting insulin, the risk of diabetes type 2, diabetic nephropathy and retinopathy (37-39).

A strength of the present study is that it was carried out in a large number of patient's samples, while the main limitation was the gender difference between groups. However gender, age and smoking were adjusted for in the logistic regression model. Another limitation of the study is the lack of the evaluation of VEGF in term of expression and serum level of VEGF in our population as well as the validation of our markers in an independent cohort. However we have investigated the serum VEGF levels in 3527 participants and identified the association of these 4 SNPs with circulating VEGF levels (19).

Hence, individuals carrying the T allele had an increased risk of MetS and higher FBG, TGs and blood pressure, supporting further investigation on the role of this genetic polymorphism in a larger population.

The results should be replicated in larger studies before making final conclusions. Furthermore, the mechanisms that explain the risk effect of *rs6993770* still require clarification.

Conclusions

In conclusion, we have identified an unpublished risk allele of a VEGF-related genetic variant, *rs6993770*, in MetS individuals. Given the complex actions of VEGF, the multifactorial nature of MetS, and the unexplained interconnections between them, the identification of a common variant with pleiotropic effect such as the *rs6993770* could be useful as a marker of increased risk of MetS. It could also provide a novel research starting point for the assessment of the implicated mechanisms between angiogenesis and metabolic syndrome.

Acknowledgements

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Author Contributions

M.A.N., M.G.M. and S.V.S. conceived and designed the study; S.R.M., M.S. and H.M. collected the data; H.M.M., A.V., N.C.N., A.B., M.R., B.H., J.H. and P.F. contributed data or analysis tools; S.R.M., A.V. and G.F. performed the analysis and wrote the paper. All authors discussed the results and contributed to the final manuscript.

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Figure 1. Association of rs6993770 as a vascular endothelial growth factor regulatory genetic variant and triglyceride, fasting blood glucose and systolic blood pressure.

Comparison of **(A**, **D)** triglyceride, **(B**, **E)** fasting blood glucose and **(C**, **F)** systolic blood pressure of metabolic syndrome patients in versus control subjects in AA and AT+TT genotypes.

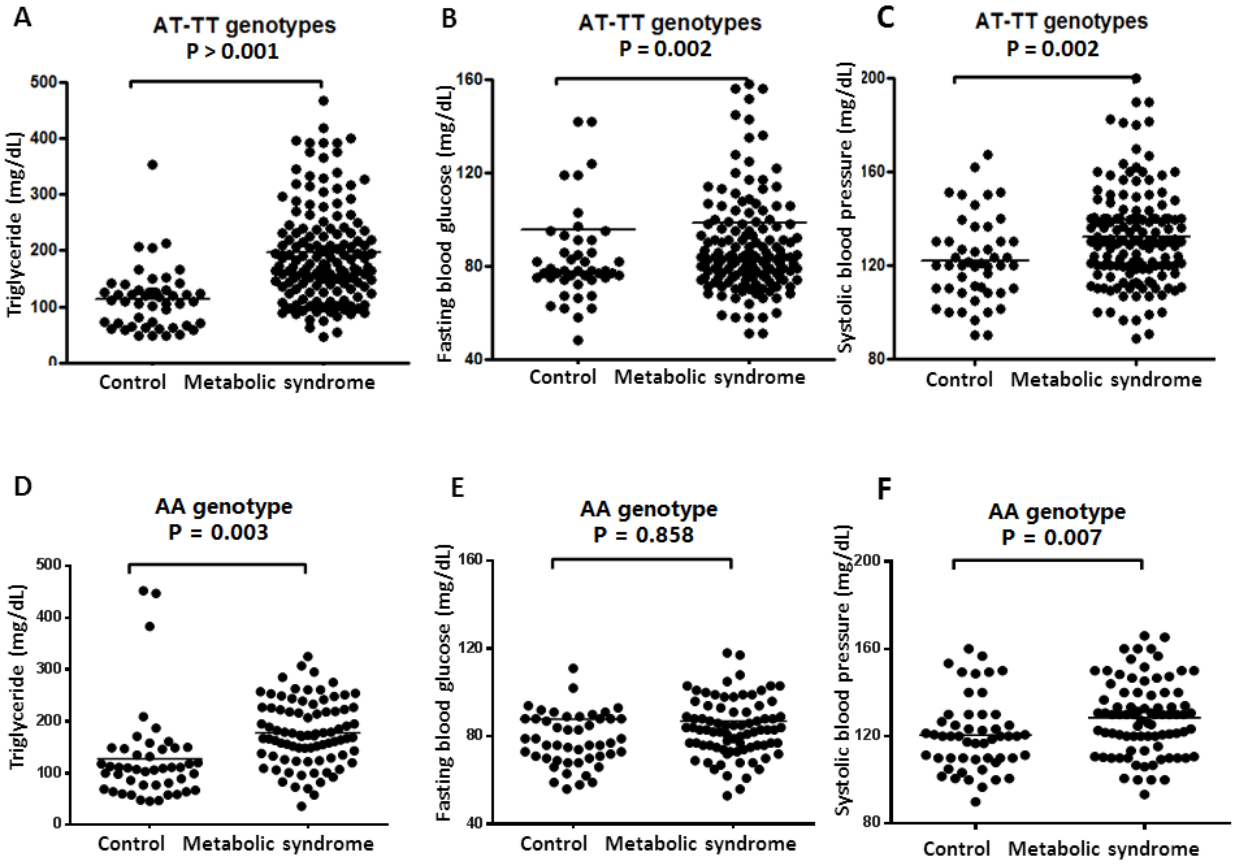
A
AT-TT genotypes
P > 0.001
Triglyceride (mg/dL)
500
400
300
200
100
0
Control
Metabolic syndrome
B
AT-TT genotypes
P = 0.002
Fasting blood glucose (mg/dL)
160
120
80
40
Control
Metabolic syndrome
C
AT-TT genotypes
P = 0.002
Systolic blood pressure (mg/dL)
200
160
120
80
Control
Metabolic syndrome
D
AA genotype
P = 0.003
Triglyceride (mg/dL)
500
400
300
200
100
0
Control
Metabolic syndrome
E
AA genotype
P = 0.858
Fasting blood glucose (mg/dL)
160
120
80
40
Control
Metabolic syndrome
F
AA genotype
P = 0.007
Systolic blood pressure (mg/dL)
200
160
120
80
Control
Metabolic syndrome

Table 1: Baseline and biochemical data from all subjects in each group

Characteristics		MetS- (n= 101)	MetS+ (n= 235)	P value
Age (year)		50.9±8.1	51.5±7.3	0.515
Sex (%)	Male	46.5	23.8	<0.001
	Female	53.5	76.2	
Current Smoker (%)	Yes	14.9	19.6	0.303
	No	85.1	80.4	
Former Smoker (%)	Yes	24.8	27.7	0.581
	No	75.2	72.3	
Height (m)		1.6±0.1	1.5±0.1	0.005
hsCRP (mg/dl)		1.6(1.0-4.1)	2.1(1.3-4.0)	0.044
LDL-C (mg/dl)		115.8±33.1	117.3±41.0	0.739
TC (mg/dl)		189.9±38.5	197.9±42.5	0.095
Weight (kg)		70.7±12.2	76.4±12.1	<0.001
BMI (kg/m2)		27.3±4.4	30.5±4.1	<0.001
Fat percentage		33.1±10.3	39.4±8.6	<0.001
WC (cm)		93.5±11.6	101.0±9.6	<0.001
HC (cm)		102.6±8.9	106.8±9.2	<0.001
FBG (mg/dl)		91.7±44.0	94.2±44.3	0.048
TG (mg/dl)		110.0(71.0-134.2)	173.0(135.0-231.0)	<0.001
HDL-C (mg/dl)		45.5±10.8	38.7±6.9	<0.001
SBP (mmHg)		121.1±17.1	130.6±19.5	<0.001
DBP (mmHg)		78.4±9.8	84.3±12.4	<0.001

Data is presented as mean± SD for normally distributed variables, while non-normally distributed variables such as TG and hS-CRP are expressed as Median (Interquartile range [IQR]).

The T-student test and Mann-Whitney U test were used to compare the demographic and clinical characteristics in normally and non-normally distributed variables, respectively.

FBG: Fasting blood glucose, TG: Triglyceride, IQR: Interquartile range, HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, SBP: Systolic blood pressure, DBP: Diastolic Blood Pressure. IQR: Interquartile range, BMI: Body mass index, WC: Waist circumference, HC: Hip Circumference.

Table 2: Single-SNP analysis

			All subjects		MetS+		MetS-	
			Count	Proportion	Count	Proportion	Count	Proportion
rs4416670	Allele frequencies	C	357	0.53	256	0.54	101	0.50
		T	315	0.47	214	0.46	101	0.50
	Genotype frequencies	C/C	96	0.29	69	0.29	27	0.27
		C/T	165	0.49	118	0.50	47	0.47
		T/T	75	0.22	48	0.20	27	0.27
	HWE	P value		0.83		0.9		0.55
rs6921438	Allele frequencies	A	407	0.61	284	0.6	123	0.61
		G	265	0.39	186	0.4	79	0.39
	Genotype frequencies	A/A	125	0.37	88	0.37	37	0.37
		A/G	157	0.47	108	0.46	49	0.49
		G/G	54	0.16	39	0.17	15	0.15
	HWE	P value		0.73		0.59		1
rs6993770	Allele frequencies	A	425	0.63	283	0.6	142	0.7
		T	247	0.37	187	0.4	60	0.3
	Genotype frequencies	A/A	136	0.4	84	0.36	52	0.51
		A/T	153	0.46	115	0.49	38	0.38
		T/T	47	0.14	36	0.15	11	0.11
	HWE	P value		0.73		0.79		0.34
rs10738760	Allele frequencies	A	382	0.58	264	0.56	118	0.6
		G	282	0.42	204	0.44	78	0.4
	Genotype frequencies	A/A	115	0.35	76	0.32	39	0.4
		A/G	152	0.46	112	0.48	40	0.41
		G/G	65	0.2	46	0.2	19	0.19
	HWE	P value		0.26		0.69		0.14

HWE: Hardy-Weinberg Equilibrium

Table 3: Association of SNPs related to VEGF level and metabolic syndrome

SNPs	Genotype	MetS+	MetS-	OR	P value	Adj-OR (95% CI)	P-value
rs4416670	C/C	69 (29.4%)	27 (26.7%)	1	0.446	1.00	0.451
	C/T	118 (50.2%)	47 (46.5%)	1.41(0.80-2.52)	0.244	1.46(0.80-2.67)	0.215
	T/T	48 (20.4%)	27 (26.7%)	1.43(0.75-2.74)	0.273	1.37(0.70-2.68)	0.355
rs6921438	A/A	88 (37.5%)	37 (36.6%)	1.00	0.885	1.00	0.884
	A/G	108 (46%)	49 (48.5%)	0.84 (0.42-1.45)	0.656	0.85 (0.42-1.72)	0.656
	G/G	39 (16.6%)	15 (14.8%)	0.91 (0.45-1.85)	0.860	0.93 (0.45-1.94)	0.860
rs6993770	A/A	84 (35.7%)	52 (51.5%)		0.027	1.00	0.007
	A/T	115 (48.9%)	38 (37.6%)	1.87(1.13-3.10)	0.015	2.17(1.28-3.69)	0.004
	T/T	36 (15.3%)	11 (10.9%)	2.02(0.94-4.32)	0.068	2.35(1.06-5.20)	0.034
rs10738760	A/A	76 (32.5%)	39 (39.8%)	1.00	0.404	1.00	0.320
	A/G	112 (47.9%)	40 (40.8%)	1.43(0.84-2.43)	0.179	1.52 (0.88-2.63)	0.131
	G/G	46 (19.7%)	19 (19.4%)	1.24(0.64-2.40)	0.519	1.27 (0.64-2.50)	0.491

The adjusted odds ratios (OR) for metabolic syndrome in subjects carrying the rare alleles of these polymorphisms were assessed by logistic regression model after correction for age, sex and smoking in codominant, dominant, recessive, overdominant and log-additive models using homozygous for the common allele as reference. Results showed no evidence for any significant increased or decreased risk for metabolic syndrome exception rs6993770 that was associated with decrease risk of MetS in codominant, dominant, over dominant and log-additive models.

Table 4: Biometric and clinical characteristics based on the dominant model of rs6993770 variants

Characteristics	AA	AT+TT	P value
hs-CRP (mg/dl) (Median(IQR))	1.9(1.2-3.9)	2.9(1.2-3.9)	0.962
LDL-C (mg/dl) (Mean ± SD)	118.9±40.4	115.5±37.6	0.436
TC (mg/dl) (Mean ± SD)	196.2±41.7	195.0±41.3	0.799
BMI (kg/m2) (Mean ± SD)	29.6±4.6	29.5±4.3	0.955
Weight (kg) (Mean ± SD)	73.9±12.3	75.3±12.4	0.312
Fat percentage	38.0±9.5	37.2±9.6	0.444
WC (cm) (Mean ± SD)	98.6±10.9	98.9±10.8	0.832
HC (cm) (Mean ± SD)	105.0±9.1	105.9±9.5	0.349
FBG (mg/dl) (Mean ± SD)	87.2±29.8	97.6±51.2	0.020
TG (mg/dl) (Median(IQR))	149.0 (102.0-195.0)	158.0 (111.2-215.5)	0.048
HDL-C (mg/dl) (Mean ± SD)	41.5±8.8	40.2±8.8	0.180
SBP (mmHg) (Mean ± SD)	125.4±17.0	129.7±20.9	0.048
DBP (mmHg) (Mean ± SD)	81.5±11.1	83.2±12.5	0.198

Data is presented as mean± SD, while TG and HS-CRP are expressed as Median (Interquartile range [IQR])
The T-student test and Mann-Whitney U test were used to compare the demographic and clinical characteristics in normally and non-normally distributed variables, respectively.
FBG: Fasting blood glucose, TC: Total Cholesterol, TG: Triglyceride, IQR: Interquartile range, HDL-C: High-density lipoprotein cholesterol, LDL-C: Low-density lipoprotein cholesterol, SBP: Systolic blood pressure, DBP: Diastolic Blood Pressure. IQR: Interquartile range, BMI: Body mass index, WC: Waist circumference, HC: Hip Circumference.