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Original article

Association of genetic polymorphisms of *PON1* and *CETP* with the presence of metabolic syndrome; the effects of genotypes on their serum activity and concentrations

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ABSTRACT

Background: The Metabolic syndrome (MetS) is associated with an increased risk of cardiovascular disease and type 2 diabetes. *PON1* and *CETP* genes may be involved in the pathogenesis of lipid metabolism and thus MetS. Several single nucleotide polymorphisms of genes were demonstrated to affect their function. Curcumin (diferuloylmethane) is a yellow pigment of turmeric that has shown numerous pharmacological activity against obesity and related conditions through anti-oxidant/anti-inflammatory properties.
Objective: We aimed to assess the association of these polymorphisms with metabolic syndrome and to investigate if these genetic variants were associated with an altered activity of PON1 and the protein levels of CETP at base line and after Curcumin supplementation.
Methods: The genotypes of *PON1* and *CETP* polymorphisms were determined in 81 patients with MetS and 100 healthy individuals using ARMS-PCR and PCR-RFLP techniques.
Results: Individuals with different genotypes of the *PON1 rs662*, rs854560 and rs705379 polymorphisms did not differ with paraoxonase activity and *CETP* serum protein concentrations, either at baseline, or after intervention. Individuals with different *PON1 rs854560* genotypes differ significantly in serum arylesterase activities (p = .037). There were statistically significant differences in genotype frequencies between cases and controls for *CETP rs5882* genotypes (p-value = .034) but not in genotype frequencies and haplotypes for *PON1* studied polymorphisms (p-value < .05). The odds ratio for *CETP rs5882* was statistically significant using a dominant model. OR (95% CI) = 0.48 (025–0.92), p-value = .029.
Conclusions: There were no associations between the *PON1* polymorphisms, or haplotypes with MetS. There was an association between *CETPrs5882* and metabolic syndrome. AA genotype of *CETPrs5882* appeared to be protective against MetS in our studied population. There were no association between the *PON1* and *CETP* polymorphisms with PON1enzymatic activities and CETP protein levels at base line and after curcumin supplementation.
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1. Introduction

Metabolic syndrome (MetS) is defined by a clustering of cardiovascular risk factors including: central obesity, insulin resistance, hypertension and atherogenic dyslipidemia, and is associated with a greater risk of type 2 diabetes and cardiovascular disease (CVD) [1,2].

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The prevalence of (MetS) is increasing globally, and in Iran it has one of the highest occurrences worldwide. In the adult population, a prevalence of 30% in Tehran and 45% in Khorasan province has been reported [3].

MetS is a multifactorial disorder in which environmental factors as well as genetic factors play a role [4]. The heritability of MetS has been estimated to be 24–30% [5,6].

Two genes, *PON1* and *CETP* take part in pathways that may be involved in the pathogenesis of MetS [7,8].

Paraoxonase 1 (PON1) is involved in the antioxidant defence system and while it has major catalytic paraoxonase/arylesterase activities, its physiological substrates are lactones, oxidized-LDL and homocysteine thiolactone, and hence it may protect against cardiovascular diseases (CVD) [7].

The paraoxonase gene cluster comprises three members *PON1-PON2-PON3* that are located on chromosome 7q21.3 [9]. More than 160 polymorphisms (SNPs) have been reported for the *PON1* gene [10], of which the Q192R, L55M, -108C/T have been shown to be associated with serum enzyme protein concentrations and activity. The Q192R and L55M SNPs are in coding regions and cause an amino acid substitution. −108C/T is in the promoter of the gene and affects its expression [7,11,12].

Studies have demonstrated that the serum activity and concentrations of PON1 are reduced in conditions in which oxidative stress may be a contributory factor, such as CVD, Alzheimer, MetS, aging and higher levels of PON1 may be protective against these diseases [13].

Cholesterol ester transfer protein (CETP) is a plasma glycoprotein expressed in the liver and secreted into the plasma. The *CETP* gene is located on the chromosome 16q13, has 16 exons and is 26 kb long[14].

CETP is involved in the exchange of TG on LDL with cholesteryl esters on HDL, and results in HDL particles that can be degraded by hepatic lipase. CETP is also involved in reverse cholesterol transport (RCT) and therefore it is not completely clear whether increasing CETP activity would be pro- or anti-atherogenic [8,15–17].

Several SNPs of the CETP gene have been identified which the I405V causes an isoleusine for valine substitution. In genome wide association studies (GWAS) it has been found that variation at the *CETP* gene locus is associated with low levels of HDL-c and is related to CVD [18].

Curcumin is a polyphenolic compound derived from turmeric that has anti-inflammatory and antioxidant properties. It has poor bioavailability when given orally, but this can be improved by using a phytosomal complex [19,20]. Phytosomes are chemical complexes which are synthesized by one or two moles of synthetic or natural phospholipids mainly phosphatidylcoline, and one mole of botanical extracts [21].

The aim of this study was to investigate the association between *PON1* and *CETP* genetic polymorphisms and the presence of MetS. In addition, the associations of *PON1* and *CETP* genetic polymorphisms with PON1 enzymatic activities and the serum concentrations of CETP protein in response to curcumin supplementation was studied in the context of a clinical trial in MetS patients.

2. Subjects and methods

Blood samples of individuals with MetS were obtained from subjects who were randomised for a clinical trial study with registration number of IRCT2014052014521N3 that examined the efficacy of phytosomal curcumin in MetS patients. Volunteers referred to the Nutrition Clinic of Qaem hospital in Mashhad city between September to November 2015. A diagnosis of MetS was made using IDF criteria [1]. All participants gave their written informed consent to participate in this study, which received the approval of the Research Council of the Mashhad University of Medical Science. Patients with kidney disease, systemic lupus erythematosous (SLE), pregnant women and patients taking anti hyperlipidemic, or anti hyperglycaemic drugs during the previous 6 months were excluded from the study. The subjects were randomly allocated to three subgroups receiving either: 1-phytosomal Curcumin, 2-curcumin and 3-placebo. Then for this study, groups 1 and 3 were selected as cases (n = 81 of 120 subjects) of the randomised clinical trial study. The work has been carried out in accordance of the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans with IR.MUMS.REC.1394.209 reference number. In addition, 100 healthy individuals, sex and age matched with cases were selected as the control group to conduct this case control study. MetS patients were treated either with phytosomal curcumin (1mg per day; group1)/plain curcumin (1g per day-group2) or placebo (group3) for six weeks. Twenty ml of blood was taken before and after the intervention period. Anthropometric, demographic and biochemical parameters were recorded for each subject before and after the intervention period. The paraoxonase/arylestrase activity of paraoxonase 1 enzyme and levels of CETP protein before and after intervention were assessed in all MetS patients. The relationship between the *PON1 rs662, rs854560* and *rs705379* genotypes and serum enzymes paraoxonase/arylestrase activity, and the *CETP5882* genotypes with serum protein concentrations were determined before and after intervention.

2.1. DNA extraction

DNA was extracted using a DNA extraction kit (Pars tous, Iran) according to the manufacturer's instruction and the integrity of extracted DNA were examined by electrophoresis technique.

2.2. Genotyping

ARMS-PCR and PCR-RFLP were used to identify the various SNP genotypes, and sequencing was performed to confirm the validity of these techniques.

2.2.1. ARMS-PCR

ARMS-PCR with 2 different sets of primers was designed to determine genotypes of *PON1 rs662* and *CETP rs5882* in patients and controls. Three primers were used to determine the alleles of polymorphism and a pair of primers to amplify an internal control band. Primers to amplify *PON1 rs662* were 5′-ACTATTTTCTT GACCCCTACTTATG-3′ and 5′-CTATTTTCTTGACCCCTACTTTCA-3′ and 5′-AGTTCACATACTTGCCATCGG-3′ with an annealing temperature of 62^{0c} for 35 cycles yielding a 158 bp fragment for allele A and 159 bp for allele G. Primers to amplify *CETPrs5882* were 5′-GCAGAGCAGCTCCGAGTACG -3′, 5′-GCAGAGCAGCTCCGAGTACA -3′ and 5′-CCGCGGGGTGGCAAAGATAA-3′ with an annealing temperature of 63^{0c} for 35 cycles yielding a 311 bp fragment for both alleles A and G. We also designed a pair of primers to replicate a 408 bp of *HBB* gene as an internal control band to check the accuracy of PCR reactions. The primers were 5′-GAAGTCTGCCGTTACTGCCC-3′ and 5′-GATCCACGTGCAGCTTGTCA-3′. The PCR reaction was carried out for *PON1* rs662 in a total volume of 15 μl using Taq DNA Polymerase 2x Master Mix Red (Ampliqon) and 0.5 pmol/μl of allele specific primers and 0.3 pmol/μl of internal control primers and for *CETPrs5882* the total volume was 15 μl and the same PCR conditions as *PON1* rs662 but with a greater concentration of template DNA.

2.2.2. PCR-RFLP

The *PON1 rs854560* and rs705379 SNPs each was genotyped separately by PCR amplification and restriction digest. Primers

used for rs854560 were designed including 5′-ATGGGTATACA GAAAGCCTAAGTGA-3′ and 5′-GGAAAAAGCTCTAGTCCATCAA-3′ an annealing temperature of 61 °C for 1 min for 35 cycles. The 403 bp fragment was digested with *Hin1II* yielded 129 + 274 bp bands in presence of a T allele. Nevertheless, primers used for rs705379 were 5′-TGCAGCCGCAGCCCTGCTGGGGCAGCGCCGATTGGCCCG CCGC-3′ and 5′-GACCGCAAGCCACGCCCTCTGTGCACC-3′ with an annealing temperature of 71^{0c} for 1 min and 35 cycles [22]. The digested bands with *BstUI* were 42 and 67 bp in presence of C allele. The PCR product size was 109 bp. We also used a sequencing technique and read the sequence of 14 samples of all 4 SNPs to confirm the results of our genotyping. All results were the same as previously determined.

2.3. Statistical analysis

The data were analysed by using SPSS version 16. Statistical significance was considered at a P < .05. Discrete data were analysed by Chi-square test. Continuous data was assessed for normality using the Kolmogorov-Smirnov test. Independent sample T-Test and One – Way ANOVA tests were used to evaluate normally distributed variables and were expressed as means with their standard deviation. Mann-Whitney and Kruskal-Wallis tests were used for non-normally distributed variables and are expressed as medians and interquartile ranges. Hardy-Weinberg analysis were performed to assess the genotype distributions. Case-control genotypic and allelic distribution were calculated using χ^2 statistics. The Odds ratio (OR) with 95% confidence intervals (95% CI) was estimated in dominant, recessive and multiplicative models for all SNPs after determining risky alleles. The Bonferonni correction was made for multiple testing. Haplotype analysis was conducted by PHASE software (http://stephenslab.uchicago.edu/software.html#phase).

3. Results

Anthropometric, demographic and biochemical parameters of the studied population are shown in Table 1. There was no difference in age between cases and controls as they were matched for this. Other parameters as expected were higher in patients, except for serum HDL-c that was lower in patients (Tables 2–5)

Statistical significance level P-value < .05. Normally distributed variables expressed as mean ± SD. Non-normally distributed variables are expressed as median and interquartile range.

HDL-C: high-density lipoprotein cholesterol, WC: waist circumference, SBP systolic blood pressure, DBP diastolic blood pressure, TG: triglycerides, FBG: fasting blood glucose.

3.1. Relationship of PON1 polymorphisms with Paraoxonase/ Arylesterase activity and CETP polymorphism with protein level

The relationship of *PON1 rs662,* rs854560 and rs705379 genotypes with enzyme paraoxonase activity and *CETP5882* genotypes with protein level before and after intervention were not significant. The differences of arylesterase activities between genotypes of *PON1 rs662* and rs705379 genotypes was not statistically significant. Nevertheless, rs854560 genotypes showed statistically significant differences in arylesterase activities before intervention (p = .037).

Table 1
Anthropometric, demographic and biochemical parameters of the study subjects.

Variable	Case	Control	P-value
BMI (kg/m^2)	26.47 ± 4.42	30.82 ± 4.79	<.05
Cholesteryl(mg/dl)	175.37 ± 3.04	240.79 ± 5.31	<.05
HDL-C (mg/dl)	48.71 ± 10.7	52.09 ± 11.92	<.05
Age(year)	42(16.25)	38(4.75)	**0.781**
Weight(kg)	79.60(16.90)	67(14.75)	<.05
WC(cm)	101(11)	88(13.75)	<.05
SBP(mmHg)	122(18)	110.67(14.92)	<.05
DBP(mmHg)	79(9)	75(10)	<.05
TG(mg/dl)	149.5(101.5)	87.5(46)	<.05
FBS(mg/dl)	95(26)	80(16.75)	<.05

Table 2
The relationship of PON1 and CETP polymorphisms with Paraoxonase activity and CETP protein levels.

PON1	Genotypes	Mean ± SD	P-value
rs662	AA	0.508 ± 0.176	.775
	AG	0.538 ± 0.167	
	GG	0.535 ± 0.184	
Total	75	0.527 ± 0.171	
rs854560	AA	0.513 ± 0.16	.767
	AT	0.531 ± 0.16	
	TT	0.566 ± 0.22	
Total	76	0.528 ± 0.17	
rs705379	CC	0.503 ± 0.17	.385
	CT	0.515 ± 0.15	
	TT	0.577 ± 0.19	
Total	75	0.527 ± 0.17	
CETPrs5882	AA	0.4338 ± 0.12	.842
	AG	0.4622 ± 0.15	
	GG	0.4365 ± 0.15	
Total	43	0.4525 ± 0.14	

The results of paraoxones1 activity before intervention are shown that they were not statistically significant. The results of CETP protein concentrations before intervention are demonstrate that they are not statistically significant.Comparisons are performed using one-way ANOVA test. Statistical significance level P-value < .05.

3.2. Association of PON1 polymorphisms with metabolic syndrome

PON1rs662, rs854560 and rs705379 genotypes were in Hardy-Weinberg equilibrium in cases and controls and in total studied population (p-value > 0.05). *CETP rs5882* genotypes in controls and studied population were in Hardy-Weinberg equilibrium (p-value > .05) but in cases genotypes were not in Hardy-Weinberg equilibrium (p-value = .035).

The difference of allele and genotype frequencies of *PON1rs662,* rs854560 and rs705379 polymorphisms between cases and controls was not statistically significant (p-value > .05). The Odds ratio estimated for the risky alleles for *PON1rs662,* rs854560 *and* rs705379 were not statistically significant (p-value > .05). There was however a statistically significant difference in genotypes frequencies between cases and controls in *CETP rs5882* (p-value = .034). The estimated odds ratio was statistically significant in the dominant model OR (95%CI) = 0.48 (0.25–0.92).

3.3. Haplotype analysis in the studied population

Haplotype frequencies of *PON1* desired polymorphisms in cases, controls and the whole population were estimated by PHASE software.

The p-value was estimated as 0.08 and showed that there was no statistically significant difference in haplotype distribution between cases and controls.

4. Discussion

Early diagnosis and treatment of the MetS can be one strategy to prevent cardiovascular disease and type 2 diabetes mellitus.

Table 3
The relationship of PON1polymorphisms with arylesterase activity.

SNP	Variable	Median	Percentile		P-value
			25th	75th	
rs662	arylesterase	145.54	89.05	206.106	0.331
rs854560	arylesterase	149.109	89.05	206.106	**0.037***
rs854560	Average Activity	156.87	89.05	206.106	**0.035***
rs705379	arylesterase	141.47	89.05	206.106	0.425

The results of arylesterase activity of rs662 and rs705379 genotypes before intervention was shown that these were not statistically significant. *The results of arylesterase activity of rs854560 genotypes was shown that before intervention and the average activities were significant. Comparisons are performed using Kruskal-Wallis test. Statistical significance level P-value < .05.

Table 4
Genotype Frequency distribution of *PON1rs662* of the study subjects.

Study Group (No.)	Genotypes			p-value	Alleles		p-value	OR(95%CI)	p-value
	N(%)	N(%)	N(%)		N(%)	N(%)			
PON1rs662	AA	AG	GG	0.15	A	G	0.09	1.46(0.94–2.30)	0.09
Case (79)	27(34.2)	42(53.2)	10(12.7)		96(60.75)	62(39.24)			
Control (100)	44(48.9)	37(41.1)	9(10)		125(69.44)	55(30.55)			
PON1rs854560	AA	AT	TT	0.93	A	T	1	1(0.65–1.55)	1
Case (80)	31(38.8)	42(52.5)	7(8.8)		104(65)	56(35)			
Control (100)	40(40)	50(50)	10 (10)		130(65)	70(35)			
PON1rs705379	CC	CT	TT	0.28	C	T	0.13	1.38(0.90–2.11)	0.13
Case (77)	17(22.1)	43(55.8)	17(22.1)		77(50)	77(50)			
Control (100)	32(32)	52(52)	16(16)		116(58)	84(42)			
CETPrs5882	AA	AG	GG	0.34	A	G	0.24	1.29(0.84–1.97)	0.24
Case (79)	20(25.3)	50(63.3)	9(11.4)		90(56.96)	68(43.03)			
Control (100)	41(41)	44(44)	15(15)		126(63)	74(37)			
*Dominant model	AA	AG + GG						0.48(0.25–0.92)	0.029 [0.1]+

Comparisons are performed using the $\chi 2$ test. Statistical significance level P-value < .05. +p-value after Bonferroni correction (multiple testing). *The odds ratio was of Statistical significance for *CETPrs5882* in the dominant model. All other models were analysed for odds ratio however there was no significant difference (data has only been shown for the Multiplicative model for *PON1rs662*, rs854560, and rs705379).

Table 5
Haplotype frequencies in Case, Control and studied population.

Haplotype	Total	Case	Control
111 (CAA)	0.303	0.298	0.307
112(CAG)	0.141	0.139	0.143
121 (CTA(	0.087	0.051	0.116
112 (CTG)	0.013	0.013	0.012
211 (TAA)	0.068	0.054	0.079
212 (TAG)	0.136	0.157	0.119
221 (TTA)	0.194	0.203	0.188
222 (TTG)	0.055	0.082	0.033

P-value for testing H0: Cases ~ Controls = 0.08, Statistical significance level P-value < .05.

Due to the complexity and uncertainties about this concept a comprehensive understanding of the underlying pathophysiology leading to the disease is required and genetic studies may be useful.

Our findings revealed that there was no association between *PON1rs662*, rs854560 and rs705379 and MetS.

One similar study has been conducted in the south-east of Iran in which it was reported that there was an association between the *PON1* polymorphisms and the risk of MetS. For *PON1rs662*, carriers of AG + GG and GG genotypes had an increased risk of MetS. However, the calculated risk for rs854560 and rs705379 was not statistically significant [23].

The different results of the two studies may be due to different sample sizes, allele frequencies and life styles (different Iranian minorities) which may therefore influence the risk of MetS. Moreover because of the lack of Hardy-Weinberg analysis, the interpretation of these results may be difficult. Allele frequencies of *PON1* polymorphisms have shown widely different distributions among populations. In the South-east of Iran, the minor allelic frequency of *PON1rs662* is 32%, rs854560 is 50% and rs705379 is 32% in healthy individuals [23] while in our study, they were 30.55%, 35% and 42% respectively. only allele frequencies of the *PON1rs662* was similar in these two populations. In our study population, the frequency of *rs662* allele A was 69.44% and G 30.55% similar to the South-east of Iran that had allele A 68% and G 32% frequent. Whereas, *rs854560* both alleles, A and T were 50% frequent and *rs705379* allele C 68% and T 32% frequent in the South-east of Iran. In our population, they were: A 65% and T 35% for *rs854560* and C 58% and T 42% for *rs705379*.

The allele distribution of rs662 and rs854560 in our population is similar to that reported in a study based in Spain (rs662 = 0.30%, rs854560 = 0.37%, rs705379 = 0.54%) [24] and to an Italian study where rs662, rs854560 and rs705379 allele frequencies have been reported as 0.35%, 0.34%, 0.43% respectively) [11]. In our study the minor allele frequencies of the *PON1rs854560* T allele was estimated as 35% in both cases and controls.

Other oxidative stress related conditions like cardiovascular diseases, Alzheimer's and cancer have also shown various prevalence and results of association with *PON1* polymorphisms in different ethnicities based on various studies [7]. Also, it is demonstrated that genetic confounding or population stratification effects explain contradiction of association studies [25].

We have also found that there was no significant difference among genotypes of each *PON1*polymorphisms in serum paraoxonase activity. Possible reasons for this result may be due to a small sample size, as when considering the means of enzyme paraoxonase activities there is a difference but it was not

statistically significant. There was an association between arylesterase activity and *PON1rs854560* but not with rs662 and rs705379. The mean arylesterase activities of AG genotypes of rs85450 was higher than other genotypes which may be due to the number of heterozygotes that was higher in this studied population or that this genotype is in linkage disequilibrium with allele C of rs705379 that shows a higher concentration and enzyme arylesterase activity.

In the present study, we found that there was no association between haplotypes of the *PON1*polymorphisms and the presence of MetS in this population.

Most of the association studies of haplotypes of *PON1* polymorphisms are focused on obesity, and their relationship with metabolic syndrome have not been assessed independently [26–28].

We found a weak association between *CETPrs5882* polymorphism and MetS. In our population two copies of allele A had a protective effect. However, after Bonferroni correction this was not significant.

Studies similar to our research have not been carried out. Few studies have demonstrated positive association of CETP level and obesity [29–31].

In various studies, the frequency of I405V alleles has been reported differently due to differences in sample size, ethnic groups and geographical places. However in every source, allele G has been known as a minor allele with 25% frequency [32]. The frequency of allele G was 0.37% in our population. It was 0.35% in Azerbaijan, almost similar to the Caucasian population with% 32 [33] in Ahvaz (the south-west of Iran) it was 0.37 [34] and Tehran 0.36% [35]. It was 0.617 in Asia and 0.611 in the African-American population [36].

In this study, there was no significant difference in CETP levels between genotypes. The protein level was measured in patients but was not done in controls.

Some studies have considered VV genotype as a risk factor for CHD [36] while others mentioned it as a protective effect against CAD [37].

It was thought that because CETP increases LDL level and decreases HDL-c, it may have a destructive effect on the heart and arteries despite the fact that the use of CETP inhibitor drugs increased stroke in patients in phase 3 clinical trials [37]. Therefore this protein cannot have a simple and direct relationship with lipid profile and risk of the diseases, and so more studies are essential. This result is consistent with previous studies reporting no association between certain genetic variants (e.g. TNF-α G-308A polymorphism) and the presence of Met.S [38].

The full explanation of the results of the randomised clinical trial study is not in the scope of our study. However, the non-significant results of the effect of genotypes on PON1 enzymatic activities and CETP concentrations is possibly due to insufficient sample size, the influence of life style, ethnicity or biochemical factors (HDL-c, TG, cholesterol) on enzymes.

5. Conclusions

In conclusion, we could not find any association between *PON1 rs662*, rs854560 and rs705379 genotypes and haplotypes with the risk of metabolic syndrome. However we found an association between *CETP rs5882* genotypes and the presence of metabolic syndrome. Also having two A allele for *CETP rs5882* may confer some degrees of protection. Moreover, it seems that neither CETP concentration nor PON1 activity may be affected by the genotype in this study.

Curcumin supplementation had no considerable effects on PON1 Paraoxonase/Arylesterase activity and CETP protein levels among three groups of MetS patients (1-phytosomal Curcumin, 2-plain curcumin and 3-placebo) before and after intervention.

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