

Effects of Leptin, Resistin, and *PPAR-Gama* Gene Variants on Obese Patients with Acute Coronary Syndrome in the Turkish Population

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ABSTRACT

Objective: Obesity and acute coronary syndrome (ACS) are common health problems of recent years. There are many candidate genes related to the genetic infrastructure of ACS and obesity. This study aimed to investigate the association of Leptin glutamine to arginine substitution (Gln223Arg), Resistin-420 Cytosine/Guanine (C/G), and proliferator-activated receptor-gamma (PPAR- γ) proline to alanine substitution (Pro12Ala) polymorphisms in obese patients with ACS in the Turkish population.

Methods: Fifty obese patients concurrently diagnosed with ACS and 42 healthy controls were included in this study. These polymorphisms were analyzed using the polymerase chain reaction-restriction fragment length polymorphism and agarose gel electrophoresis methods.

Results: The PPAR- γ Pro12Ala polymorphism Pro/Ala genotype ($p=0.001$) was found to be higher in the obese patients with ACS, while the (Proline/Proline) genotype ($p=0.001$) was significantly higher in the control group. The GC genotype ($p=0.045$) distribution of Resistin-420 C/G was found to be significantly higher in the controls compared to the patient group.

Conclusion: Our study presents new findings that the PPAR- γ Pro12Ala polymorphism Pro/Ala genotype is a risk factor for ACS in obese individuals, whereas Resistin-420 C/G polymorphism GC genotype and PPAR- γ Pro12Ala polymorphism Pro/Pro genotype may be protective factors for ACS in obese individuals.

Keywords: Acute coronary syndrome, leptin, resistin, PPAR- γ , gene polymorphism, PCR-RFLP

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INTRODUCTION

Acute coronary syndrome (ACS) is the most common reason for emergency department visits and hospital admissions. ACS is one of the most important health problems causing morbidity, mortality, and reduced quality of life in today's society (1). Obesity is a multifactorial disease characterized by an excessive increase in the amount of body fat, adversely affecting health and the quality of life, reducing the lifespan, and causing many metabolic diseases (2). According to the World Health Organization's reports, obesity affects more than 700 million people worldwide and nearly 2.3 billion people are overweight (3). Obese individuals have a higher frequency of cardiovascular risk factors and have higher morbidity and mortality rates related to cardiovascular diseases (4). Previously, many studies showed that some gene polymorphisms are associated with obesity and cardiovascular diseases (5,6). Leptin, which is one of these genes, is expressed in adipocytes and regulates adipose-tissue mass, food intake, energy expenditure, and body weight (7). Many studies have demonstrated that the leptin gene or leptin receptor gene polymorphisms regulate obesity and cardiovascular pathogenesis (8,9). Resistin is a novel hormone that is secreted by adipocytes and its gene is located on chromosome 19p13.2 (10). Resistin-420 Cytosine/Guanine (C/G) polymorphism, located in the promoter region of the resistin gene, was reported to be associated with the regulation of resistin gene expression and serum resistin level (11). In addition, an association has been shown between the Resistin-420 C/G variant and diabetes, obesity, and cardiovascular disease in several studies (12-14). The peroxisome proliferator-activated receptor-gamma (PPAR- γ), localized on chromosome 3p25, is another related gene that is mainly expressed in adipose tissue, the colon, and macrophages. PPAR- γ plays a role in adipocyte differentiation and in the regulation of insulin responses, and it is linked to numerous diseases such as obesity, diabetes, atherosclerosis, and cancer (15).

It is suggested that Leptin Glutamine to Arginine substitution (Gln223Arg), Resistin-420 C/G, and PPAR- γ proline to alanine substitution (Pro12Ala) gene polymorphisms play an important role in the development of diabetes, obesity, and cardiovascular disease. However, there is no previous study that has investigated the combined effect of the variations of *Leptin*, *Resistin*, and *PPAR- γ* genes on the pathogenesis of ACS among obese patients in the Turkish population. Therefore, this study is the first to investigate the possible associations of *Leptin* Gln223Arg, *Resistin*-420 C/G, and *PPAR- γ* Pro12Ala gene polymorphisms together in obese patients with ACS.

METHODS

Ethical Approval

This research complies with all the relevant national regulations, institutional policies and is in accordance the tenets of the Helsinki Declaration, and has been approved by the İstanbul Faculty

of Medicine Ethical Committee, İstanbul University (approval number: 2012/1590-1251). All the participants' rights were protected and written informed consents were obtained before the procedures according to the Helsinki Declaration.

Study Group

The *Leptin* Gln223Arg, *Resistin*-420 C/G, and *PPAR- γ* Pro12Ala gene polymorphisms were investigated in 42 healthy subjects without any heart disease and 50 patients diagnosed with obesity and ACS. The patients were selected from the Medicana Bahçelievler Hospital, Clinic of Cardiovascular Surgery in İstanbul, Turkey.

The patient group consisted of obese patients [body mass index (BMI) ≥ 30] who were diagnosed with ACS in the hospital and whose samples were collected. ST segment elevated q positive or non-ST segment elevated, but enzyme positive patients were included into this group. Unstable angina pectoris patients were excluded. The control group consisted of people who were working in the same hospital, recruited via a survey. Particularly, the lack of a family history was the main criteria for maintaining the survey. While creating the control group, the age range was matched with that of the patient group.

DNA Isolation and Genotyping

In EDTA containing tubes, 10 mL of venous blood samples were obtained from the participants. Samples were stored at -20 °C until the genomic DNA isolation was performed using the salting out method (16). The PPAR- γ Pro12Ala, Resistin-420 C/G, and Leptin Gln223Arg polymorphisms were analyzed using the polymerase chain reaction (PCR)-restriction fragment length polymorphism methods. For detection of PPAR- γ Pro12Ala, Resistin-420 C/G, and Leptin Gln223Arg, 500 ng genomic DNA was amplified with 10x reaction buffer (10 mM Tris-HCl, 50 mM of KCl, 1.75 mM MgCl₂), 2.5 mM of each dNTP, 100 pmol/ μ L of each primer, and 0.1 unit Taq polymerase (Invitrogen) in a 25 μ L reaction volume. The annealing temperatures for Leptin Gln223Arg, Resistin-420 C/G, and PPAR- γ Pro12Ala polymorphisms were 64 °C, 63 °C, and 58 °C, respectively.

Used primers, restriction enzymes, and interpretations for determining Leptin Gln223Arg, Resistin-420 C/G, and PPAR- γ Pro12Ala polymorphisms are shown in Table 1.

Evaluation of the MspI, BpI, and BstUI Restriction Enzyme Digestion Results

The PCR yield of the Leptin Gln223Arg polymorphism was 421 bp and the bands obtained following digestion with MspI were 294 and 127 bp, only if the polymorphism was present. Therefore, a single band of 421 bp was appraised as Thymine/Thymine (TT) (wild type, TT), 294 and 127 bp as Cytosine/Cytosine (CC) (mutant type, CC), and 421, 294, and 127 bp as Cytosine/Thymine (CT) (heterozygous type, CT) (Figure 1).

In order to evaluate the Resistin-420 C/G polymorphism, BpI restriction enzyme was utilized. A single band of 533 was

appraised as Guanine/Guanine (GG) (329 and 204 bp as CC (mutant type, CC), and 533, 329 and 204 bp as Guanine/Cytosine (GC) (heterozygous type, GC) (Figure 2).

The PCR yield of the PPAR- γ Pro12Ala polymorphism was 270 bp and the bands obtained following digestion with BstUI were 227 and 43 bp, only if the polymorphism was present. Therefore, a single band of 270 bp was appraised as Proline/Proline (Pro/Pro) (wild type, Pro/Pro), 227 and 43 bp as Ala/Ala (mutant type, Ala/Ala: Alanine/Alanine), and 270, 227, and 43 bp as Proline/Alanine (Pro/Ala) (heterozygous type, Pro/Ala) (Figure 3).

Statistical Analysis

Statistical analysis was performed using SPSS version 11.5 (SPSS Inc, Chicago, USA). The chi-square (χ^2) test, Fischer's

exact test, and Student's t-test were used for comparison of the numerical variables between the groups. Allele frequencies were calculated by the gene counting method. Chi-square (χ^2) test was used for the comparison of clinical and non-clinical parameters and alleles. Student's t-test and ANOVA test were performed to compare the genotypes with more than two variables. Values of $p < 0.05$ were considered as statistically significant.

RESULTS

Fifty obese patients with ACS and 42 healthy controls were included in the analyses. The mean ages of the patient and control groups were 61.56 ± 8.92 and 60.34 ± 12.55 years, respectively. No significant difference was found between the patients and the

Table 1. Polymerase Chain Reaction–Restriction Fragment Length Polymorphism-Based Assay of Leptin GLN223ARG, Resistin-420 C/G, and PPAR- γ PRO12ALA SNPs

SNPs	Primers	Restriction enzymes	Interpretation (bp)
PPAR- γ PRO12ALA	Forward Primer: 5'-GCCAATTCAAGCCCAGTC-3' Reverse Primer: 5'-GATATGTTTGCAGCAAGTG AATCATAAGGAATCGCTTCCG-3'	BstUI	PP: 270 AA: 227+43 PA: 270+227+43
Resistin - 420 C/G	Forward Primer: 5'- TGTCATTCTCACCCACAG ACA-3' Reverse primer: 5'- TGGGCTCAGCTAACCAA ATC-3'	Bpil	GG: 533 CC: 329+204 GC: 533+329+204
Leptin GLN223ARG	Forward Primer: 5'-ACCCTTTAAGCTGGGTGT CCCAAATAG-3' Reverse Primer: 5'- CTAGCAAATATTTTGTAA GCAATT-3'	MspI	TT: 421 CC: 294+127 CT: 421+294+127

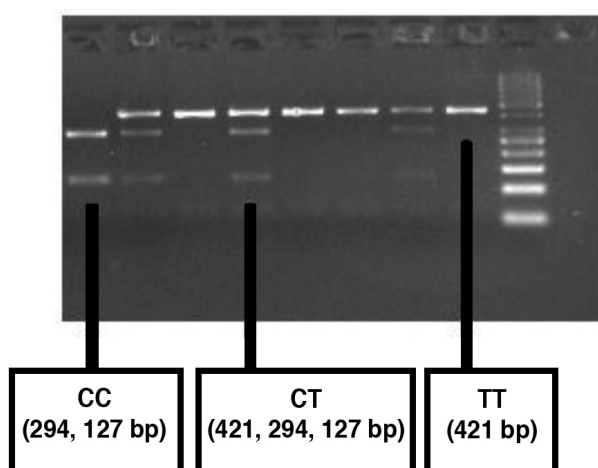


Figure 1. Gel photograph showing PCR-RFLP assay for genotyping of Leptin Gln223Arg polymorphism
PCR-RFLP: Polymerase chain reaction restriction-fragment length polymorphism, CC: cytosine-cytosine, CT: cytosine/thymine, TT: thymine/thymine

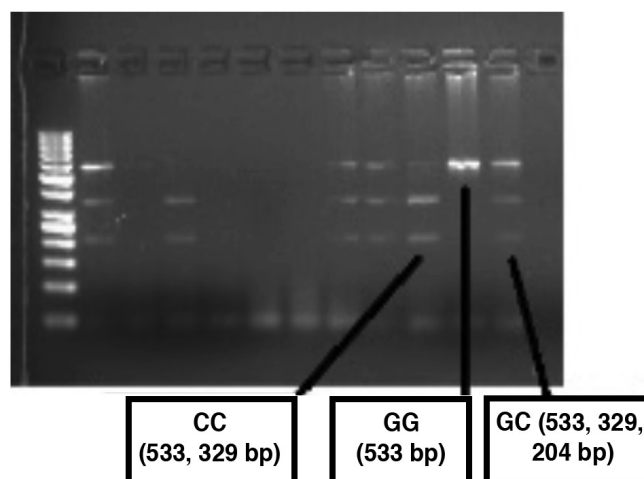


Figure 2. Gel photograph showing PCR-RFLP assay for genotyping of Resistin-420 C/G polymorphism
PCR-RFLP: Polymerase chain reaction restriction-fragment length polymorphism, CC: cytosine-cytosine, GG: guanine/guanine, GC: Guanine/Cytosine

controls in terms of age. Demographic parameters of the study groups are shown in Table 2. BMI [$p=0.001$, 95% confidence interval (CI): 5.63-8.25], very-low-density lipoprotein-cholesterol

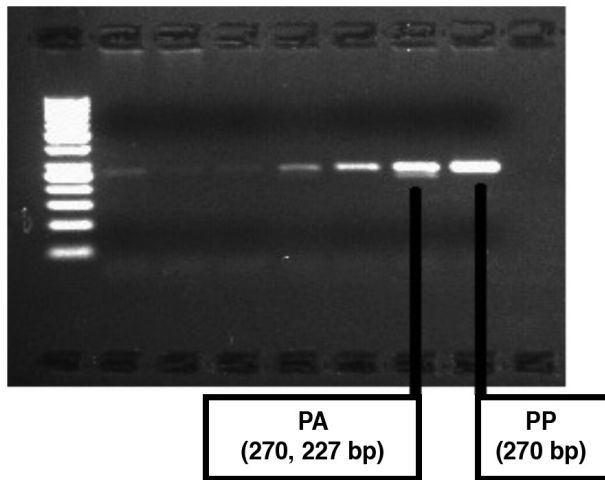


Figure 3. Gel photograph showing PCR-RFLP assay for genotyping of PPAR- γ Pro12Ala polymorphism
PCR-RFLP: Polymerase chain reaction restriction-fragment length polymorphism

($p=0.001$, 95% CI: 9.94-30.00), and triglyceride ($p=0.001$, 95% CI: 49.87-150.29) levels were found to be significantly higher in the patient group compared to the controls. Also, LDL-cholesterol ($p=0.002$, 95% CI: 10.77-44.15) and high-density lipoproteins-cholesterol ($p=0.001$, 95% CI: 8.65-18.28) levels were found to be higher in the controls than in the patient group. In addition, 66% of individuals in the patient group were also diagnosed with hypertension and 36% with diabetes mellitus (DM).

When we evaluated the study groups in terms of the PPAR- γ Pro12Ala genotype and allele frequencies, none of the individuals in either group showed the Ala/Ala genotype. In the patient group, Pro/Ala genotype was found to be significantly higher compared to the controls ($p=0.001$, 95% CI: 3.82-17.22). Due to the absence of the Ala/Ala genotype, carrying the mutant Ala/Ala allele presents as the Pro/Ala genotype. The Pro/Pro genotype frequency was determined to be significantly higher in the control group compared to the patient group ($p=0.001$, 95% CI: 0.004-0.202) (Table 3). The allele and genotype frequencies of Leptin Gln223Arg polymorphism were not significantly different between the patient group and controls (Table 3). According to the results of the statistical analysis of Resistin-420 C/G polymorphism, the GC genotype distribution was determined to be significantly

Table 2. Demographic characteristics of obesity with ACS patients and healthy controls

Demographic parameters	Patient (n=50)	Control (n=42)	p
Age (year)	(61.56 \pm 8.92)	(60.34 \pm 12.55)	-
Gender (F/M)	17/33	14/28	-
Body mass index (kg/m ²)	(32.75 \pm 2.76) ^a	(25.81 \pm 3.42)	0.001
Presence of hypertension (%)	66%	-	-
Presence of diabetes mellitus (%)	36%	-	-
Presence of KOAH (%)	8%	-	-
LDL-cholesterol (mg/dL)	(103.84 \pm 43.05)	(131.30 \pm 37.50) ^a	0.002
Triglyceride (mg/dL)	(216.18 \pm 168.22) ^a	(116.09 \pm 51.79)	0.001
Total cholesterol (mg/dL)	(185.41 \pm 61.83)	(206.38 \pm 43.21)	-
HDL-cholesterol (mg/dL)	(38.34 \pm 9.75)	(51.80 \pm 13.43) ^a	0.001
VLDL-cholesterol (mg/dL)	(43.23 \pm 33.64) ^a	(23.26 \pm 10.26)	0.001
AST (mg/dL)	(30.54 \pm 14.03)	-	-
ALT (mg/dL)	(36.89 \pm 20.41)	-	-
BUN (mg/dL)	(19.50 \pm 9.02)	-	-
Hematocrit	(40.49 \pm 5.18)	-	-
Platelet	(266.18 \pm 52.29)	-	-
Urea	(38.55 \pm 13.06)	-	-
WBC	(7.63 \pm 2.17)	-	-
INR	(1.06 \pm 0.14)	-	-
Sedimentation	(35.36 \pm 25.82)	-	-

Values are reported as number of patients, n: number of individuals, SD: standard deviation, ^a $p<0.05$ denoted statistically significant, F: female, M: male, ACS: acute coronary syndrome, KOAH: Chronic Obstructive Pulmonary disease, HDL: high-density lipoproteins, VLDL: very-low-density lipoprotein, AST: aspartate aminotransferase, ALT: alanine aminotransferase, BUN: blood urea nitrogen, WBC: white blood cell, INR: international normalized ratio

higher in the controls compared to the patient group [$p=0.045$, χ^2 : 4.033, odds ratio (OR): 1.364, 95% CI: 1.003-1.854] (Table 3).

When we investigated the association between demographic parameters and Leptin Gln223Arg, Resistin-420 C/G, and PPAR- γ Pro12Ala genotype distribution, the Leptin GG genotype carriers had significantly higher BMIs compared to heterozygote carriers in the patient group.

There were no significant differences between other polymorphisms and demographic parameters in both study groups (Table 3).

DISCUSSION

Leptin receptor Gln223Arg polymorphism is one of the most frequently encountered leptin receptor polymorphisms (17). Leptin Gln223Arg polymorphism falls within the region encoding the extracellular domain of the leptin receptor. Therefore, the amino-acid changes consecutively, leading to a change from a neutral to a positive charge of the molecule, in the extracellular domain of the receptor that represents a typical leptin-binding site and it was suggested that a change of charge could significantly affect the functionality of the receptor (18,19). Leptin Gln223Arg polymorphism affected the development process of coronary artery disease via facilitating the deposition of HDL cholesterol on blood vessel walls (20). While some studies have reported that Leptin Gln223Arg polymorphism has been associated with BMI, high blood pressure, obesity, lipids, and insulin resistance, other studies did not find any association with these parameters (21).

Although Leptin receptor Gln223Arg polymorphism has not been associated with lipid parameters in obese or normal-

weight persons before (22,23), some studies showed that it may increase the risk of obesity and/or obesity-related diseases in different populations (24-26). In obese children, no association was found between Leptin Gln223Arg polymorphism and obesity, leptin, insulin resistance, and metabolic abnormalities (27). Similarly, Okada et al. (28) have found no association between Leptin Gln223Arg polymorphism and serum lipid profiles of Japanese obese children. Leptin Gln223Arg polymorphism was found not to be associated with obesity in Turkish children with metabolic syndrome (29). There was no difference in the genotype frequencies of Leptin Gln223Arg polymorphism between obese and non-obese adolescents (18). Yang et al. (30) selected fifteen studies for their meta-analysis. They reported a significant association between decreased risk of obesity and the Leptin Gln223Arg polymorphism. Overweight or obese subjects had significantly higher frequencies of the Arg223 homozygous allele of the Leptin Gln223Arg polymorphism (31). In a study conducted in the Iranian population, it has been suggested that carrying the G allele increases the risk of non-ST-segment elevation myocardial infarction (32). In another study, Leptin Gln223Arg polymorphism has not been associated with the risk of coronary artery disease and hypertension in the Iranian population (33). Aijälä et al. (34) have reported that no impact on incidence for cardiovascular events or death was detected between Leptin Gln223Arg polymorphism. Also, Shi et al. (20) have demonstrated that Leptin Gln223Arg polymorphism showed a significant difference between coronary artery disease patients and healthy controls neither genotypes nor alleles in China population. In the represented study, we found no association between the Leptin Gln223Arg polymorphism and obese patients with ACS in the Turkish population. A meta-

Table 3. Genotype and allele frequencies of patients and controls for Leptin GLN223ARG, Resistin - 420 C/G, and PPAR- γ PRO12ALA polymorphisms

SNPs	Genotype and alleles	Patient (n=50) (n, %)	Control (n 42) (n, %)	p
Leptin GLN223ARG	AA	14 (28)	18 (42.90)	-
	GG	2 (4)	3 (7.10)	-
	AG	34 (68)	21 (50)	-
	A allele	62 (62)	57 (67.85)	-
	G allele	38 (38)	27 (32.15)	-
PPAR- γ PRO12ALA	PP	6 (12)	38 (97.40) ^a	0.001
	AA	0	0	-
	PA	44 (88) ^a	1 (2.60)	0.001
	P allele	56 (56)	77 (98.71)	-
	A allele	44 (44)	1 (1.29)	-
Resistin-420 C/G	GG	9 (20.5)	5 (12.5)	-
	CC	10 (22.7)	4 (10)	-
	GC	25 (56.8)	31 (77.5) ^a	0.045
	G	43 (48.86)	41 (51.25)	-
	C	45 (51.14)	39 (48.75)	-

Values are reported as number of patients (percentage of total group). ^a $p<0.05$ denoted statistically significant. PPAR- γ : peroxisome proliferator-activated receptor-gamma, GG: guanine/guanine, CC: cytosine-cytosine, GC: Guanine/Cytosine

analysis showed that Leptin Gln223Arg polymorphism was not significantly associated with cardiovascular disease risk, but have claimed that these findings are still unclear, because the frequency of the 223Arg allele was highly varied in different ethnicities (8).

The resistin gene coding resistin is found on chromosome 19p13; Resistin-420C/G polymorphism reported at promoter as well as on coding sequences. One of the most frequently studied polymorphisms, C to G substitution at -420 position in the 5' flanking region of the gene showed altered resistin gene expression (mRNA levels) in abdominal fat with increased serum resistin level (35). This polymorphism has independently been associated with cardiovascular risk factors, such as insulin resistance, type 2 diabetes mellitus (T2DM), obesity, hypertension, dyslipidemia, and metabolic syndrome, as well as with coronary heart disease (arteriosclerosis, coronary artery disease, idiopathic dilated cardiomyopathy) (36-39). In a meta-analysis, G allele of Resistin-420 C/G polymorphism was reported as a risk factor for obesity (40). The Resistin-420 GG genotype was significantly associated with obesity, impaired glucose tolerance, and T2DM in a Egyptian population (41). G/G genotypes and G alleles for Resistin-420 C/G polymorphism were significantly associated with T2DM and cardiovascular disease in Egyptian diabetic patients (12). Also, Nakashima et al. (42) have reported that carrying the GG genotype and G allele increased cardiovascular disease risk. In a Chinese population, subjects with CG and GG genotypes had an increased risk of coronary artery disease compared to CC carriers (11). Hoffman et al. (43) did not find any association between Resistin-420 C/G polymorphism and coronary artery disease in Caucasians. Hussain et al. (44) reported that elevated serum resistin levels and carrying the G allele for Resistin-420 C/G polymorphism may be associated with hypertrophic cardiomyopathy. In the Chinese population, no significant difference in the distribution of genotypes and allele frequencies of -420 C/G polymorphism has been found in T2DM patients and coronary heart disease patients (45). Resistin-420 C/G polymorphism has been found associated with increased obesity and metabolic syndrome, but it is not different in subjects with high cardiovascular diseases such as myocardial infarction (46). Resistin-420 C/G polymorphism was not associated with metabolic syndrome or coronary atherosclerosis in nondiabetic Caucasians (47). According to our results, the GC genotype distribution for Resistin-420 C/G polymorphism was determined to be significantly higher in the control group compared to obese patients with ACS; therefore, it can be said that GC genotype for Resistin-420 C/G polymorphism is protective for ACS in obese patients in the Turkish population.

PPAR is a member of the nuclear hormone receptor family. There are three subtypes of PPAR: α , δ , and γ (48). The PPAR- γ plays a pivotal role on local vasculature in several critical aspects of atherothrombosis, including lipid metabolism and foam cell responses (49). A point mutation found on the B exon of the NH2-terminal of PPAR- γ , substitution of proline with alanine at position 12, the Pro12Ala polymorphism, which causes an amino-acid substitution in its ligand-independent activation

domain, and a moderate decrease in its transcriptional activity (50,51). PPAR- γ expression has also been found in atherosclerotic lesions and macrophages, suggesting that PPAR- γ may influence atherogenic processes, and polymorphisms of PPAR- γ may modulate individual susceptibility to T2DM, insulin resistance, obesity, and related traits associated with coronary heart disease (48,49,52). Dedoussis et al. (53) reported that carrying the Ala allele for PPAR- γ Pro12Ala polymorphism is a risk factor for adiposity in children. Pro12Ala and/or Ala12Ala polymorphisms of the PPAR- γ gene have been found to be associated with obesity (54-57). The Ala/Ala genotype of the PPAR- γ gene was found to be associated with obesity and insulin resistance in Asian Indians (58). In a meta-analysis, it was notified that the PPAR- γ Pro12Ala polymorphism might be a risk factor for obesity susceptibility (59). On the contrary, some studies showed that the PPAR- γ Pro12Ala polymorphism is not associated with obesity in different populations (60,61). The relationship between the PPAR- γ Pro12Ala polymorphism and coronary diseases were investigated in previous studies. No association has been found between PPAR- γ Pro12Ala with ACS and coronary artery disease (62-64). In meta-analysis studies, the PPAR- γ Pro12Ala polymorphism was not associated with coronary heart disease (49,65). Wang et al. (66) reported that although the 12Ala is not an independent risk factor for obesity, the PPAR- γ Pro12Ala polymorphism is associated with increased risk of myocardial infarction in Han Chinese in Hohhot. In one study, the 12Ala allele in PPAR- γ correlated with a significantly increased coronary artery disease extent in men (67), whereas in another study, it was reported that this allele had a protective effect for cardiovascular disease (68). No association was found with coronary artery disease and PPAR- γ Pro12Ala polymorphism in Italian and Korean population (69,70), whereas in the Turkish population, it was reported that carrying the 12Ala allele increased the risk of coronary artery disease (71). In the represented study, we found that the PPAR- γ Pro12Ala polymorphism Pro/Ala genotype was found to be higher in the obese patients with ACS, while the Pro/Pro genotype was significantly higher in the control group. According to our results, it can be concluded that PPAR- γ Pro12Ala polymorphism Pro/Ala genotype may be a risk factor for ACS in obesity patients, while the Pro/Pro genotype may be a protective factor for ACS in obesity.

Finally, regarding the results of this study, and showing compliance with the previous ones, PPAR- γ Pro12Ala gene polymorphism may play a role in the development of ACS in obesity. Our results need to be confirmed in larger cohorts in order to improve the understanding of their role in the development of ACS in obesity patients.

Study Limitation

We believe that the small sample size in our study affected our results, and further studies with larger sample groups are needed to specifically clarify the role of the Leptin Gln223Arg, Resistin-420 C/G, and PPAR- γ Pro12Ala gene polymorphisms in the pathogenesis of ACS in obese patients. However, even with

the limited number of participants included in our study, we think that our findings will contribute to the understanding of the molecular mechanisms of ACS in obesity.

CONCLUSION

To date, no study had simultaneously evaluated the Leptin Gln223Arg, Resistin-420 C/G, and PPAR- γ Pro12Ala polymorphisms in obese patients with ACS. Thus, our study is the first to focus on the above-mentioned polymorphisms in obese patients with ACS and we believe that it might be a relevant source of data for the further studies.

Ethics Committee Approval: This research complies with all the relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration, and has been approved by the İstanbul Medical Faculty Ethical Committee, İstanbul University (approval number: 2012/1590-1251).

Informed Consent: All the participants' rights were protected and written informed consents were obtained before the procedures according to the Helsinki Declaration.

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