Interaction of genetic risk factors confers increased risk for metabolic syndrome: the role of peroxisome proliferator-activated receptor γ

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Interaction of Genetic Risk Factors Confers Increased Risk for Metabolic Syndrome: The Role of PPARγ

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Running title

Genetic Risk Factors and Metabolic Syndrome

Abstract

The aim of the study was to estimate the influence of interactions between peroxisome proliferator-activated receptor γ (*PPARγ*) and target genes lipoprotein lipase (*LPL*), interleukin 6 (*IL6*), angiotensin converting enzyme (*ACE*), and angiotensin II type 1 receptor (*AT1R*) on metabolic syndrome (MetSy) and its traits.

Methods: The study included 527 participants (263 with MetSy and 264 controls). Genotyping of *PPARγ* Pro12Ala, *LPL* PvuII (-/+), *IL6* -174G>C, *ACE* I/D and *AT1R* 1166A>C was performed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based methods.

Results: Interaction between *PPARγ* Pro12Ala and *LPL* (Pvu-/+) improved prediction of MetSy over and above prediction based on a model containing no interactions (χ^2=7.22; df=1; p=0.007). In the group of participants with *PPARγ* Pro12Ala or Ala12Ala genotypes, those with *LPL* Pvu (-/+) or (+/+) genotype had greater odds for MetSy (OR=5.98; 95% CI: 1.46-24.47, p=0.013). Interaction between *PPARγ* Pro12Ala and *IL6* -174G>C improved prediction of high fasting blood glucose (χ^2=13.99; df=1; p<0.001). *PPARγ* Ala12 variant was found protective in patients with *IL6* -174GG genotype (OR=0.10; 95% CI: 0.02-0.57, p=0.01), while in the case of *IL6* -174C allele carriers, for *PPARγ* Ala12 carriers larger odds for high glucose levels compared with Pro12 variant were observed (OR=2.39; 95% CI: 1.11-5.17, p=0.026). Interactions of *PPARγ* and *ACE* were significant for BMI. In the group with *ACE* DD genotype, those with *PPARγ* Pro12Ala or Ala12Ala genotype have greater odds for obesity (OR=9.98; 95% CI: 1.18-84.14, p=0.034).

Conclusions: *PPARγ* gene variants can, in interaction with some of its target genes, modulate physiological processes leading to the development of MetSy.

Key words: Metabolic syndrome, Genetic polymorphisms, Peroxisome proliferator-activated receptor γ gene, Lipoprotein lipase, Interleukin 6, Angiotensin converting enzyme, Angiotensin II type 1 receptor

INTRODUCTION

The pathogenesis of metabolic syndrome (MetSy) has been associated with the effect of genetic predisposition in combination with environmental factors (Lusis et al. 2008; Lin et al., 2005). Whereas the importance of genetic factors for the development of MetSy has been widely recognized, the contribution of genes has not yet been fully clarified. Many association studies have been conducted with inconsistent results (Monda et al., 2010; Park et al. 2009). Considering the central role of adipose tissue in MetSy, different adipocyte-related genes have been studied as possible candidates in MetSy, including peroxisome proliferator-activated receptor γ (*PPARγ*), lipoprotein lipase (*LPL*), renin-angiotensin system (RAS)-related genes - angiotensin converting enzyme (*ACE*) and angiotensin II type 1 receptor (*AT1R*), and interleukin 6 (*IL6*), which exhibit considerable variability.

PPARs are transcription factors implicated in different biological pathways ranging from lipid and glucose homeostasis and insulin sensitization, to control of cell proliferation/differentiation, inflammation, and immunity (Sharma and Staels, 2007; Olefsky, 2000). Endogenous ligands are thought to bind PPARγ and promote downstream gene target transcription (Ide et al., 2003; Bell-Parikh et al., 2003). Because of alternative mRNA splicing, two protein isoforms occur: PPARγ1 and PPARγ2. While PPARγ1 is found ubiquitously in the body, PPARγ2 is largely found in adipose tissue. Studies have demonstrated that PPARγ has a critical role in regulating adipocyte differentiation and lipid accumulation (Rangwala and Lazar, 2009). The *PPARγ* Pro12Ala variant (rs1801282) has been found to modulate transcriptional activity and has a reduced affinity for the response element in target genes, which leads to the less efficient stimulation of PPARγ target genes (He, 2009). The frequency of the 12Ala allele ranges from 2% to 18% in healthy people (Paracchini et al., 2005). PPARγ function is a key factor in mediating conditions such as dyslipidemia, obesity, and insulin resistance (Huang et al., 2011; Gouda et al., 2010), and has also been implicated in the development of cardiovascular diseases (Azhar, 2010).

LPL plays the major role in the metabolism and transport of lipids. Distinct physiological activities of LPL together regulate the supply of fatty acids to various tissues for either storage or oxidation. Insulin has a major effect on LPL activity in adipose tissue during adipocyte differentiation by increasing LPL gene transcription (Semenkovich et al., 1989). Glucose also increases adipose tissue LPL activity. Several mutation loci have been detected in *LPL* gene and investigated for their associations with plasma lipid level and the development of cardiovascular diseases (Angelakopoulou et al., 2012). Polymorphism *LPL Pvu*II (-/+) (rs285) is caused by the absence (-) or presence (+) of a C>T transition at position 497 in intron 6, and may interfere with correct splicing of mRNA, diminishing the enzyme activity.

Interleukin 6 (IL6) is a pleiotropic inflammatory cytokine derived from diverse tissues. In chronic inflammation it has rather proinflammatory properties. There is evidence that variations in IL6 gene are associated with cytokine and

metabolic modulation, leading to impaired glucose and lipid homeostasis and increasing cardiometabolic risk (Stephens et al., 2004). Polymorphism -174G>C (rs1800795) is most prevalent and of significant biological importance since it affects the IL6 transcription (Curti et al., 2011). The frequencies of -174G>C variants were estimated to be 0.57 in Caucasians and 0.93 in Afro-Americans (Huang et al., 2007). Higher circulating IL6 levels have been associated with obesity and visceral fat deposition (Qi et al., 2007; Stephens et al., 2007), increased risk of impaired glucose tolerance, type 2 diabetes mellitus (T2DM) (Sattar et al., 2003), and high blood pressure (Fernandez-Real et al., 2001).

PPARs modulate RAS by transcriptional control of all its components (Roszer and Ricote, 2010). Polymorphism in *ACE* and *AT1R* gene has been found significant for variability in many pathophysiological processes connected with RAS, including hypertension (Tiret et al., 1998), diabetes, and cardiovascular disease (Pujia et al., 1994). RAS might be involved in the pathophysiology of obesity (Engeli et al., 2000). The most common *ACE* gene polymorphism is the insertion/deletion (I/D) (rs4646994) of 287-bp *Alu* repeats located in intron 16.

A nucleotide substitution (1166A>C) in 3′-UTR of the *AT1R* gene (rs1272176) results in increased expression of the receptor gene (Bonnardeaux et al., 1994). The increased frequency of the *AT1R* 1166C allele has been associated with essential hypertension (Tiret et al., 1998), cardiac hypertrophy (Pujia et al., 1994), myocardial infarction (Engeli et al., 2000). But opposite findings have also been published where *AT1R* 1166CC genotype predisposes to favorable anthropometric and metabolic traits relative to cardiovascular risk (Möllsten et al., 2008).

The influence of gene variants on phenotype expression seems to be population specific because of possible differences in environmental factors and genetic background (Groop, 2000).

In this paper we consider the combined effects of *PPARγ, LPL, IL6, ACE* and *AT1R* that might confer a higher risk for MetSy or for some of its traits than individual gene variants in Croatian population.

MATERIAL AND METHODS

Subjects

A total of 527 subjects (343 female, 184 male) of Croatian origin participated in the study, including 265 patients with MetSy and 262 controls (without MetSy criteria). Participants with MetSy were recruited among patients admitted to the University Hospital Center Zagreb, and control subjects were selected among hospital staff and other Zagreb citizens appointed for routine check up. The study was performed in the period between March 2010 and October 2012. MetSy was defined using the criteria established in the third report of the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment in Adults (NCEP-ATPIII, 2001). An individual with a combination of any three or more of the following risk factors was classified as having MetSy: waist circumference (WC), male >102 cm, female >88 cm; TG ≥1.7 mmol/L; HDL-C <1.0 in men and <1.3 mmol/L in women; systolic

blood pressure (SBP) or diastolic blood pressure (DBP) ≥ 130/85 mm Hg, or use of anti-hypertensive medications; and fasting blood glucose ≥ 6.1 mmol/L or use of antidiabetic medication with the age of diagnosis of T2DM ≥ 40 years. In the case of participants' use of medications, corrections of medicated traits were made (Kraja et al., 2006). For control group we selected healthy subjects without MetSy and without any other serious illness. Data were collected on clinical variables including age, height, weight, and WC. Body mass index (BMI) was calculated as weight (kg)/height (m^2). Participants with BMI between 25-30 kg/m^2 were considered overweight, and those with BMI > 30 kg/m^2 are obese. Blood samples for biochemical analyses (total cholesterol, TG, LDL-C, HDL-C and glucose) were collected after overnight fasting and were analyzed by using routine laboratory methods. All participants signed informed consent forms, and the study protocol was approved by Ethics Committee of the University Hospital Center Zagreb.

Genotyping

Genomic DNA was extracted from leukocytes using the salting out procedure (Miller et al., 1988), and genotyping of *PPARγ* Pro12Ala, *LPL* PvuII (-/+), *IL6* -174G>C, *ACE* I/D and *AT1R* 1166A>C was performed according to previously published methods based on PCR or PCR-RFLP procedures (Oh et al., 2000; Xu et al., 2008; Jamie et al., 2005; Rigat et al., 1992; Hilgers et al., 1999, respectively). PCR amplifications were performed in 25 μL final volume in GeneAmp PCR System 9600 (Applied Biosystems, USA).

Statistical analysis

The level of statistical significance was set to 5% ($p<0.05$), and in all instances two-tailed tests of statistical significance were used. Means and standard deviations were used as measures of central tendency and variability for continuous variables, and independent samples t-test was used for their comparison. If variances were heterogeneous, t-test for unequal variances with corrected degrees of freedom was used. Univariate and multivariate prediction of MetSy, its traits and BMI was carried out by means of logistic regression, and odds ratios with 95% confidence intervals were given for each variable. To examine gene-gene interactions, total predictive model was built using hierarchical backward elimination approach (Kleinbaum and Klein, 2002), with initial model containing all variables and all possible pairs of gene-gene interactions. Interaction with the highest level of statistical significance from the full model was considered first so that logistic regression model without that interaction was compared to the model containing that interaction. If the difference between two models was not statistically significant, interaction was dropped out of the model and the procedure was repeated for the least significant interaction from the reduced model. If gene-gene interaction proved to be statistically significant, regression coefficients for each polymorphism et each of the categories

of the other one were examined by redefining their reference groups, since coefficients of variables in interaction are conditioned to the reference group of the other variable from that interaction (Jaccard, 2001). The analyses were carried out using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) statistical software package.

RESULTS

Baseline characteristics

Baseline characteristics of study participants are given in Table 1. Patients had significantly higher BMI, WC, higher levels of TG, total cholesterol, LDL-C, glucose and significantly higher blood pressure and lower levels of HDL-C compared to controls ($p<0.001$). MetSy and control group did not differ significantly with respect to age and gender.

Table 1. Baseline characteristics of study participants

	Patients *Mean +/- SD*	*Controls* *Mean +/- SD*	*P*
Age (yrs)	53.3 +/- 10.51	53.1 +/- 10.72	0.874
Gender (N of male participants/All)	99/265	85/262	0.238
Body mass index (kg/m^2)	32.1 +/- 4.77	24.6 +/- 3.44	**<0.001**
Waist circumference (cm)	107.3 +/- 11.15	85.1 +/- 11.42	**<0.001**
Triglycerides (mmol/L)	2.82 +/- 2.21	1.21 +/- 0.73	**<0.001**
Cholesterol (mmol/L)	6.0 +/- 1.11	5.5 +/- 1.02	**<0.001**
High-density lipoprotein cholesterol (mmol/L)	1.22 +/- 0.33	1.69 +/- 0.41	**<0.001**
Low-density lipoprotein cholesterol (mmol/L)	3.7 +/- 1.14	3.3 +/- 0.88	**<0.001**
Glucose (mmol/L)	6.24 +/- 2.21	5.0 +/- 0.77	**<0.001**
High Blood pressure (N of subjects with high BP/All)	235/260	71/255	**<0.001**
Systolic blood pressure	152.4 +/- 26.0	124.8 +/- 14.24	**<0.001**
Diastolic blood pressure	94.4 +/- 15.87	79.5 +/- 8.60	**<0.001**

Differences between groups were evaluated by t-test, Mann-Whitney test or chi-square test depending on distribution normality. Significant values are indicated in bold. $P<0.05$ was considered statistically significant.

Associations of *PPARγ, LPL, IL6, ACE* and *AT1R* gene variants with MetSy or its trait

We found no differences in *PPARγ* Pro12Ala, *LPL* PvuII (-/+), *IL6* 174G>C, *ACE* I/D and *AT1R* 1166A>C genotype distributions between MetSy cases and controls (Table 2). We found no departure from the Hardy-Weinberg equilibrium.

Table 2. Genotype frequencies in case and control group

Gene/Genotype	Patients n(%)	Controls n(%)	OR (95% CI)
ACE			
DD	72 (27.2)	81 (30.9)	1
ID	132 (49.8)	130 (49.6)	1.14 (0.77-1.70)
II	61 (23.0)	51 (19.5)	1.35 (0.83-2.19)
PPAR			
Pro12Pro	200 (75.5)	199 (76.0)	1
Pro12Ala	62 (23.4)	60 (22.9)	1.03 (0.69-1.53)
Ala12Ala	3 (1.1)	3 (1.1)	1.03 (0.69-1.53)
IL6			
GG	85 (32.1)	91 (34.7)	1
GC	130 (49.1)	137 (52.3)	1.02 (0.69-1.49)
CC	50 (18.9)	34 (13.0)	1.57 (0.93-2.67)
AT1R			
AA	136 (51.3)	134 (51.1)	1
AC	114 (43.0)	110 (42.0)	1.02 (0.72-1.46)
CC	15 (5.7)	18 (6.9)	0.82 (0.40-1.70)
LPL			
-/-	62 (23.4)	71 (27.1)	1
-/+	124 (46.8)	132 (50.4)	1.08 (0.71-1.64)
+/+	79 (29.8)	59 (22.5)	1.53 (0.95-2.48)

ABBREVIATIONS: OR = univariate odds ratio; 95% CI = 95% confidence interval for odds ratio

When tested for the influence of interactions between *PPARγ* and its target genes *LPL, IL6, ACE* and *AT1R* on MetSy and its traits, separately in the group with and without MetSy, several statistically significant interactions were observed (Table 3).

Table 3. Hierarchical backward elimination of gene-gene interactions

	Least significant interaction / feature	P (interaction)	P (difference between models)
Full model*			
Reduced model**			
1	*PPARγ* x *LPL* PvuII / MetSy	**0.008**	**0.007**
2	*PPARγ* x *ACE* I/D / BMI	**0.010**	**0.004**
3	*PPARγ* x *IL6* -174G>C / Glucose	**0.001**	**<0.001**

ABBREVIATIONS: MetSy- metabolic syndrome, BMI-body mass index, p (interaction) = level of statistical significance for interaction, results of Wald χ^2 test; p (difference between models) = level of statistical significance, comparison of model without interaction and model with interaction

*Full model with all possible pairs of interactions included

**Model from which interaction with highest level of statistical significance from previous model was excluded

Interaction of PPARγ and LPL and MetSy

Interaction between *PPARγ* Pro12Ala and *LPL* PvuII (-/+) significantly improved prediction of MetSy, over and above prediction based on model containing no interactions (χ^2=7.22; df=1; p=0.008) (Table 4).

In the group of participants with *LPL* PvuII (-/-) genotype, participants with *PPARγ* Pro12Ala or Ala12Ala genotype had smaller odds for MetSy compared to those with Pro12Pro genotype (OR=0.12; 95% CI: 0.03-0.52, p=0.005). In the group of those participants with *PPARγ* Pro12Ala or Ala12Ala genotypes, those with *LPL* PvuII (-/+) or PvuII (+/+) genotype had greater odds for MetSy (OR=5.98; 95% CI: 1.46-24.47, p=0.013).

In multivariate prediction, when adjusted for all other variables, age (p=0.012) and BMI (p<0.001) were also statistically significantly associated with MetSy. With each year increase in age, odds for MetSy decrease by 3% (OR = 0.97; 95% CI = 1.54 – 1.85) and with each unit increase in BMI the odds for MetSy increase more than 1.5 times (OR = 1.69; 95% CI = 1.54 – 1.85).

Table 4. Total predictive model for MetSy with all polymorphisms

	Patients n(%)	Controls n(%)	OR_{mv} (95% CI)	P
Age*	53.3 (10.51)	53.1 (10.72)	**0.97 (0.94-0.99)**	**0.012**
Gender				
female	166 (62.6)	177 (67.6)	1	
male	99 (37.4)	85 (32.4)	1.27 (0.70-2.29)	0.427
total	265 (100.0)	262 (100.0)		
ACE				
DD	72 (27.2)	81 (30.9)	1	
ID or II	193 (72.8)	181 (69.1)	1.04 (0.56-1.92)	0.909
total	265 (100.0)	262 (100.0)		
PPARγ if LPL Pvu (-/-)				
Pro12Pro	50 (80.6)	52 (73.2)	1	
Pro12Ala or Ala12Ala	12 (19.4)	19 (26.8)	**0.12 (0.03-0.52)**	**0.005**
total	62 (100.0)	71 (100.0)		
PPARγ if LPL (-/+) or (+/+)				
Pro12Pro	150 (73.9)	147 (77.0)	1	
Pro12Ala or Ala12Ala	53 (26.1)	44 (23.0)	1.11 (0.54-2.25)	0.781
total	203 (100.0)	191 (100.0)		
IL6				
GG	85 (32.1)	91 (34.7)	1	
GC or CC	180 (67.9)	171 (65.3)	0.80 (0.45-1.44)	0.803
total	265 (100.0)	262 (100.0)		
AT1R				
AA	136 (51.3)	134 (51.1)	1	
AC or CC	129 (48.7)	128 (48.9)	0.69 (0.39-1.20)	0.185
total	265 (100.0)	262 (100.0)		
LPL PvuII if *PPARγ* Pro12Pro				

(-/-)	50 (25.0)	52 (26.1)	1	
(-/+) or (+/+)	150 (75.0)	147 (73.9)	0.67 (0.32-1.41)	0.290
total	200 (100.0)	199 (100.0)		
LPL PvuII if *PPARγ* Pro12Ala or Ala12Ala				
(-/-)	12 (18.5)	19 (30.2)	1	
(-/+) or (+/+)	53 (81.5)	44 (69.8)	**5.98 (1.46-24.47)**	**0.013**
total	65 (100.0)	63 (100.0)		
BMI*	32.1 (4.77)	24.6 (3.44)	**1.69 (1.54-1.85)**	**<0.001**
Total cholesterol*	6.0 (1.11)	5.5 (1.02)	1.32 (0.82-1.12)	0.248
LDL cholesterol*	3.7 (1.14)	3.3 (0.88)	1.41 (0.86-2.31)	0.170
PPARγ x *LPL*			8.92 (1.77-44.88)	0.008

ABBREVIATIONS: OR_{mv} = multivariate odds ratio; 95% CI = 95% confidence interval for odds ratio; *Mean (standard deviation)
Significant values are indicated in bold. P<0.05 was considered statistically significant.

Interaction of PPARγ and IL6 and fasting blood glucose

Interaction of *PPARγ* Pro12Ala and *IL6* -174G>C statistically significantly improved prediction of high fasting blood glucose in the group of MetSy patients, over and above model containing no interactions (χ^2=13.99; df=1; p=0.001). Inclusion of this interaction to the model containing no interactions increased Nagelkerke R square from 0.21 to 0.27 (Table 5). In the group of patients with *IL6* -174GG genotype, *PPARγ* Pro12Ala or Ala12Ala genotype carriers (taken together) had smaller odds of having high glucose compared to Pro12Pro genotype carriers (OR=0.10; 95% CI: 0.02-0.57, p=0.01). In the group of patients with *IL6* -174GC or CC genotype, *PPARγ* Pro12Ala or Ala12Ala genotype carriers (taken together) had larger odds of having high glucose compared to Pro12Pro genotype carriers (OR=2.39; 95% CI: 1.11-5.17, p=0.026). In the group of patients with *PPARγ* Pro12Pro genotype, *IL6* -174GC or CC genotype carriers (taken together) had smaller odds of having high glucose compared to *IL-6* -174GG genotype carriers (OR=0.41; 95% CI: 0.20-0.83, p=0.013). In multivariate prediction, age (p=0.001), gender (p=0.045), and TGs (p=0.001) were also associated with high fasting blood glucose. With each year increase in age, odds for high glucose increase by 6% (OR = 1.06; 95% CI = 1.02 – 1.09), and male patients had more than two times larger odds of having high glucose (OR = 2.08; 95 % CI = 1.02 – 4.26). Each unit increase in TGs increases the odds for high glucose 1.3 times (OR = 1.3; 95% CI = 1.11 – 1.53).

Table 5. Multivariate prediction of high fasting blood glucose in MetSy group

	High n(%)	Normal n(%)	OR_{mv} (95% CI)	P
Age*	56.3 (9.25)	51.6 (10.87)	**1.06 (1.02-1.09)**	**0.001**
Gender				
female	52 (53.6)	113 (68.5)	1	
male	45 (46.4)	52 (31.5)	**2.08 (1.02-4.26)**	**0.045**
total	97 (100.0)	165 (100.0)		
ACE				
DD	24 (24.7)	47 (28.5)	1	
ID or II	73 (75.3)	118 (71.5)	1.92 (0.95-3.89)	0.071
total	97 (100.0)	165 (100.0)		
PPARγ if *IL6* -174GG				
Pro12Pro	33 (94.3)	35 (70.0)	1	
Pro12Ala or Ala12Ala	2 (5.7)	15 (30.0)	**0.10 (0.02-0.57)**	**0.010**
total	35 (100.0)	50 (100.0)		
PPARγ if *IL6* -174GC/CC				
Pro12Pro	39 (62.9)	90 (78.3)	1	
Pro12Ala or Ala12Ala	23 (37.1)	25 (21.7)	**2.39 (1.11-5.17)**	**0.026**
total	62 (100.0)	115 (100.0)		
IL6 if *PPARγ* Pro12Pro				
GG	33 (45.8)	35 (28.0)	1	
GC or CC	39 (54.2)	90 (72.0)	**0.41 (0.20-0.83)**	**0.013**
total	72 (100.0)	125 (100.0)		
IL6 if *PPARγ* ProAla/AlaAla				
GG	2 (8.0)	15 (37.5)	1	
GC or CC	23 (92.0)	25 (62.5)	**10.13 (1.66-61.89)**	**0.012**
total	25 (100.0)	40 (100.0)		
AT1R A1166C				
AA	49 (50.5)	86 (52.1)	1	
AC or CC	48 (49.5)	79 (47.9)	0.96 (0.53-1.74)	0.893
total	97 (100.0)	165 (100.0)		
LPL PvuII				
(-/-)	21 (21.6)	39 (23.6)	1	
(-/+) or (+/+)	76 (78.4)	126 (76.4)	1.01 (0.49-2.05)	0.989
total	97 (100.0)	165 (100.0)		
BMI	32.3 (5.06)	32.1 (4.65)	1.01 (0.92-1.12)	0.781
Total cholesterol	5.9 (1.05)	6.0 (1.14)	0.73 (0.56-1.12)	0.214
LDL cholesterol	3.7 (1.01)	3.8 (1.22)	1.14 (0.84-1.55)	0.400
Waist circumference	108.7 (11.11)	106.4 (11.07)	1.01 (0.97-1.06)	0.615
Triglycerides	3.1 (2.97)	2.7 (1.60)	**1.30 (1.11-1.53)**	**0.001**
HDL cholesterol	1.3 (0.37)	1.2 (0.31)	3.60 (0.91-2.23)	0.101
Systolic blood pressure	156.5 (28.40)	150.3 (24.32)	1.02 (1.00-1.03)	0.120
Diastolic blood pressure	95.1 (16.01)	94.3 (15.65)	0.98 (0.95-1.02)	0.330
PPARγ x *IL6*			**24.81 (3.49-176.38)**	**0.001**

ABBREVIATIONS: OR_{mv} = multivariate odds ratio; 95% CI = 95% confidence interval for odds ratio; *Mean (standard deviation)
Significant values are indicated in bold. P<0.05 was considered statistically significant.

Interaction of PPARγ and ACE and obesity

PPARγ Pro12Ala and *ACE* I/D gene interaction statistically significantly improved prediction of obesity in the group of MetSy patients, over and above prediction based on model containing no interactions (χ^2=8.08; df=1; p=0.004). Inclusion of this interaction to the model containing no interactions increased Nagelkerke R square from 0.51 to 0.53 (Table 6). In the group of patients with *PPARγ* Pro12Ala or Ala12Ala genotype, patients with *ACE* ID or II genotype had 89% smaller odds for obesity compared to those with DD genotype (OR=0.11; 95% CI: 0.01-0.88, p=0.038). In the group of those patients with *ACE* DD genotype, those with *PPARγ* Pro12Ala or Ala12Ala genotype had about ten times greater odds for obesity (OR=9.98; 95% CI: 1.18-84.14, p=0.034).

In multivariate prediction, gender (p=0.009), WC (p<0.001), and HDL-C (p=0.043) were also associated with obesity. Male patients had smaller odds for obesity (OR = 0.30; 95% CI = 0.12 – 0.74). With each unit increase in WC the odds for obesity increase 1.26 times (OR = 1.26; 95% CI = 1.18 – 1.34), and with each unit increase in HDL-C the odds for obesity decrease 76% (OR = 0.24; 95% = 0.06 – 0.96).

Table 6. Multivariate prediction of obesity in MetSy group

	Yes	No	OR_{mv} (95% CI)	P
Age*	53.1 (9.97)	53.7 (11.57)	0.99 (0.96-1.03)	0.582
Gender				
female	104 (59.8)	61 (67.8)	1	
male	70 (40.2)	29 (32.2)	**0.30 (0.12-0.74)**	**0.009**
total	174 (100.0)	90 (100.0)		
ACE if *PPARγ* Pro12Pro				
DD	32 (24.6)	20 (29.0)	1	
ID or II	98 (75.4)	49 (71.0)	2.36 (0.93-6.01)	0.072
total	130 (100.0)	69 (100.0)		
ACE if *PPARγ* Pro12Ala/Ala12Ala				
DD	18 (40.9)	2 (9.5)	1	
ID or II	26 (59.1)	19 (90.5)	**0.11 (0.01-0.88)**	**0.038**
total	44 (100.0)	21 (100.0)		
PPARγ if *ACE* DD				
Pro12Pro	32 (64.0)	20 (90.9)	1	
Pro12Ala or Ala12Ala	18 (36.0)	2 (9.1)	**9.98 (1.18-84.14)**	**0.034**
total	50 (100.0)	22 (100.0)		
PPARγ if *ACE* ID/DD				
Pro12Pro	98 (79.0)	49 (72.1)	1	
Pro12Ala or Ala12Ala	26 (21.0)	19 (27.9)	0.44 (0.17-1.15)	0.094
total	124 (100.0)	68 (100.0)		
IL6				
GG	51 (29.3)	33 (36.7)	1	
GC or CC	123 (70.7)	57 (63.3)	1.16 (0.55-2.46)	0.698

total	174 (100.0)	90 (100.0)		
AT1R				
AA	90 (51.7)	45 (50.0)	1	
AC or CC	84 (48.3)	45 (50.0)	1.14 (0.56-2.31)	0.722
total	174 (100.0)	90 (100.0)		
LPL PvuII				
(-/-)	43 (24.7)	18 (20.0)	1	
(-/+) or (+/+)	131 (75.3)	72 (80.0)	0.77 (0.33-1.81)	0.550
total	174 (100.0)	90 (100.0)		
Total cholesterol*	5.9 (1.11)	6.1 (1.12)	0.91 (0.49-1.70)	0.777
LDL cholesterol*	3.7 (1.15)	3.8 (1.13)	0.93 (0.50-1.74)	0.813
Waist circumference*	111.7 (10.44)	98.9 (6.83)	**1.26 (1.18-1.34)**	**<0.001**
Triglycerides*	2.91 (2.27)	2.6 (2.12)	1.04 (0.83-1.30)	0.730
HDL cholesterol*	1.2 (0.32)	1.3 (0.36)	**0.24 (0.06-0.96)**	**0.043**
Systolic blood pressure*	153.7 (26.50)	149.7 (25.13)	1.02 (0.99-1.04)	0.197
Diastolic blood pressure*	94.8 (15.09)	93.2 (17.17)	0.99 (0.96-1.03)	0.719
Fasting blood glucose*	6.3 (2.28)	6.1 (2.09)	0.90 (0.73-1.11)	0.309
PPARγ x *ACE* I/D			**0.04 (0.004-0.47)**	**0.010**

ABBREVIATIONS: OR_{mv} = multivariate odds ratio; 95% CI = 95% confidence interval for odds ratio; *Mean (standard deviation)
Significant values are indicated in bold. P<0.05 was considered statistically significant.

Single gene correlations in the control group

1. In multivariate prediction *PPARγ* Pro12Ala or Ala12Ala genotype carriers had almost four times larger odds of having high WC compared to those with *PPARγ* Pro12Pro genotype carriers (OR=3.86; 95% CI: 1.24-12.06; p = 0.020) (Table 7).

2. In multivariate prediction *AT1R* 1166AC or CC genotype carriers had about eight times larger odds of having high TGs compared to *AT1R* AA genotype carriers (OR=8.05; 95% CI: 1.85-35.0; p = 0.005).

Table 7. Associations of single gene variants in the control group

Feature	Genotype	High n (%)	Normal n (%)	OR_{mv} (95% CI)	P
Waist circumference	***PPARγ* Pro12Ala**				
	Pro12Pro	27 (65.9)	142 (77.2)	1	
	Pro12Ala/Ala12Ala	14 (34.1)	42 (22.8)	**3.86 (1.24-12.06)**	**0.020**
Triglycerides	***AT1R* 1166A>C**				
	AA	8 (26.7)	112 (53.1)	1	
	AC or CC	22 (73.3)	99 (46.9)	**8.05 (1.85-35.0)**	**0.005**
	total	30 (100.0)	211 (100.0)		

ABBREVIATIONS: OR_{mv} = multivariate odds ratio; 95% CI = 95% confidence interval for odds ratio
Significant values are indicated in bold. P<0.05 was considered statistically significant.

DISCUSSION

Assuming a modest role of single polymorphisms as markers of complex traits and diseases, the study of variant gene combinations could provide complementary information that could be clinically important. The obtained results confirmed the significance of the interacting effects of the *PPARγ* polymorphisms with some of its target genes.

Although *PPARγ* Pro12Ala variant in this study showed no influence on MetSy when tested separately, its interaction with *LPL* PvuII (-/+) variants seems to be relevant. Carriers of low activity alleles, *PPARγ* Ala and *LPL* PvuII (+), had six times greater odds for MetSy. The PPARs have been implicated in the regulation of adipocyte differentiation and lipid and fatty acid metabolism. As both central obesity and serum lipids were found to be related with LPL activity, it is reasonable to assume that those two gene interactions contribute to the development of MetSy. Considering transcriptional regulation, the activity of the LPL promoter has been studied extensively. The 5' regulatory region contains a large number of specific *cis*-acting elements, among others the PPAR response element (Schoonjans et al., 1996). Because the *LPL* promoter was shown to be ~ 30% less efficiently transactivated in the presence of the *PPARγ* Ala allele in a study in vitro, Schneider group proved that the Pro12Ala substitution may lead to decreased LPL activity in vivo (Schneider et al, 2002).

Our results further pointed to the importance of the interacting role of the *PPARγ* with target gene *IL6* influencing the glucose level. The role of PPARs has been confirmed in glucose homeostasis but with different results. When analyzed as a single marker for T2DM risk, *PPARγ* Ala variant was considered protective in different studies (Gouda et al., 2010). However, for association between PPARγ and T2DM, environmental factors as dietary lipids (Scacchi et al., 2007) and even intrauterine condition (de Rooij et al., 2006) were pointed to be relevant in addition to gene variants. In our study *PPARγ* Ala variant was found protective in patients with *IL6* -174GG genotype, while in the case of *IL6* -174C allele carriers, *PPARγ* Ala12 variant lost its protective role, and Ala12 carriers had even larger odds for high glucose levels compared to Pro12 variant. In Asian Indian population *PPARγ* Ala12Ala genotype was associated with obesity and insulin resistance (Bhatt et al., 2012), indicating that ethnicity with different genetic and environmental background can have dominant role. For Croatian population, multivariate prediction revealed that cholesterol and TGs were also associated with high fasting blood glucose, where each unit increase in TGs increased the odds for high glucose 1.3 times. The relationship between insulin resistance and metabolic risk factors is complex and abdominal obesity with dysfunctional adipose tissue might be one of risk predispositions. Variants of *IL6* gene -174G>C are associated with cytokine and metabolic modulation that can lead to impaired glucose and lipid homeostasis and increased metabolic risk. Association of the C allele with obesity has been documented (Berthier et al., 2003). Due to lower energy expenditure and insulin sensitivity, carriers of the CC genotype could be more prone to insulin resistance

and obesity (Fernandez-Real et al., 2000; Kubaszek et al., 2003). Our results are in accordance to such published data. Again, other opposite results can be found in the literature. It has been found that *IL6* -174C allele carriers had significantly lower fasting glucose (Hutch et al., 2009). In our study, association between *IL6* -174CC genotype and higher WC was observed in the control group, which is in accordance with published data and points to the association between C allele and obesity (Berthier et al., 2003). Further, we found interactions of *PPARγ* and *ACE* genotypes significant for BMI, specifically in the group of patients with *ACE* DD genotype where *PPARγ* Ala allele carriers had greater odds for obesity, which is in accordance with the data from previous studies (Passaro et al., 2011) in which have also been reported *ACE* DD / *PPARγ* Ala female carriers having a higher BMI and fat distribution, but not MetSy. Association of the *ACE* DD genotype with BMI can be explained by the observation that *ACE* DD subjects have increased levels of plasma ACE (Rigat et al., 1990; Tiret et al., 1992), and therefore potentially increased level of angiotensin II which is considered a trophic factor in the differentiation of preadipocytes to mature adipocytes (Saint-Marc et al., 2001). Previously published data also suggest that the RAS might be involved in the pathophysiology of obesity (Engeli et al., 2000). The data on the influence of *PPARγ* 12Ala variant on BMI are inconsistent, being associated with decreased BMI (Deeb et al., 1998; Damcott et al., 2004) or increased BMI (Masud and Ye, 2003), and increased risk of obesity (Ochoa et al., 2004). In our study, it was observed for the control group in multivariate prediction, that *PPARγ* 12Ala allele carriers had larger odds of having high WC compared to those with *PPARγ* Pro12 allele carriers.

Presented data confirmed that PPARγ gene variants, interacting with some of its polymorphic target genes, can modulate pathophysiological processes in the development of MetSy in Croatian population. We have also confirmed that between-study heterogeneity can be at least partly attributed to ethnicity and gene-gene interactions.

Study Limitations

The size of our population might be a limitation. Another limitation might be that the effects of environmental factors and controlling the risk of obesity and MetSy were not evaluated in this study.

Although further studies are needed to confirm our results, the interplay between genes seems to be significant.

Conflict of interest

The authors declare that they have no conflict of interest.

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