Udruženost polimorfizama Pro12Ala i His477His gena *PPARG* s inzulinskom rezistencijom u bolesnica sa sindromom policističnih jajnika

Association between Pro12Ala and His477His polymorphisms in *PPARG* gene and insulin resistance in patients with polycystic ovary syndrome

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Sažetak

Uvod: Inzulinska rezistencija je obilježena oslabljenim tkivnim odgovorom na djelovanje inzulina, a udružena je s pretilošću, šećernom bolesti tipa 2, metaboličkim sindromom, lipodistrofijama, sindromom policističnih jajnika (engl. *polycystic ovary syndrome*, PCOS) i kroničnom infekcijom. Inzulinska rezistencija je prisutna kod 50–70% bolesnica s PCOS. Peroksizomni proliferatorom aktivirani receptor gama (engl. *peroxisome proliferator-activated receptor γ*, PPARγ) je jezgreni receptor koji kontrolira transkripciju gena uključenih u metabolizam slobodnih masnih kiselina i lipogenezu, a važan je za diferencijaciju i preživljenje adipocita. Cilj ovoga probnog istraživanja bio je ispitati povezanost polimorfizama Pro12Ala i His477His *PPARG* gena s inzulinskom rezistencijom kod bolesnica s PCOS.

Bolesnice i metode: U istraživanju je sudjelovalo 69 bolesnica s PCOS. Metodom PCR-RFLP analizirane su frekvencije genotipova. Glukoza natašte i glukoza nakon oralnog testa opterećenja glukozom (engl. *oral glucose tolerance test,* OGTT), inzulin, hsCRP (engl. *high sensitivity C-reactive protein*), tjelesna masa, visina, opseg struka, sistolični i dijastolični krvni tlak, indeks tjelesne mase (engl. *body mass index*, BMI) i indeks procjene modela homeostaze (engl. *homeostasis model assessment*, HOMA) izmjereni su rutinskim metodama ili su izračunati iz podataka.

Rezultati: Pronađena je značajna povezanost između alela Ala polimorfizma Pro12Ala i nižeg BMI ($P = 0{,}040$) te između alela T polimorfizma His477His i niže koncentracije hsCRP ($P = 0{,}047$). Polimorfizmi Pro12Ala i His477His pokazali su značajnu neravnotežu povezanosti (engl. *linkage disequilibrium*) ($D' = 0{,}727$). Analiza diplotipova nije pokazala da postoji povezanost.

Zaključak: U ovom preliminarnom istraživanju pronašli smo značajnu povezanost između alela Ala polimorfizma Pro12Ala i nižeg BMI te alela T polimorfizma His477His i nižeg hsCRP. Međutim, niti jedan od ova dva polimorfizma, pojedinačno ili u kombinaciji (diplotip), nije bio povezan s koncentracijama inzulina i glukoze natašte u plazmi, kao ni s indeksom HOMA, koji su znakoviti za inzulinsku rezistenciju. Stoga zaključujemo kako istraživani polimorfizmi nisu povezani s inzulinskom rezistencijom kod bolesnica s PCOS.

Ključne riječi: PPARγ; polimorfizam; inzulinska rezistencija; PCOS; hsCRP

Abstract

Background: Insulin resistance is characterized by attenuated response of tissues to insulin action and is associated with obesity, type 2 diabetes mellitus, metabolic syndrome, lipodystrophies, polycystic ovary syndrome (PCOS) and chronic infection. Insulin resistance is present in 50%–70% of PCOS patients. Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor which controls transcription of genes involved in free fatty acid uptake and lipogenesis, and is essential for differentiation and survival of adipocytes. The aim of our pilot study was to investigate the association between Pro12Ala and His477His polymorphisms in *PPARG* gene with insulin resistance in PCOS patients.

Patients and methods: The study included 69 PCOS patients. Genotype frequencies were analyzed by PCR-RFLP methods. Fasting glucose and glucose after oral glucose tolerance test, insulin, and high sensitivity C-reactive protein (hsCRP), body mass, height, waist circumference, systolic and diastolic blood pressure, body mass index (BMI) and homeostasis model assessment (HOMA) index were measured by routine methods or calculated from the findings obtained.

Results: A significant association was found between the Ala allele of the Pro12Ala polymorphism and lower BMI ($P = 0.040$) and between the T allele of the His477His polymorphism and lower hsCRP ($P = 0.047$). The Pro12Ala and the His477His polymorphisms were in considerable linkage disequilibrium ($D' = 0.727$). Diplotype analysis showed no association.

Conclusions: In our preliminary study, we found a significant association between the Ala allele of the Pro12Ala polymorphism and lower BMI, and between the T allele of the His477His polymorphism and lower hsCRP. However, none of the two polymorphisms, individually or in combination (diplotype), was associated with fasting plasma insulin and glucose concentrations and HOMA index that are characteristic of insulin resistance. We therefore conclude that the polymorphisms investigated are not associated with insulin resistance in PCOS patients.

Key words: PPARγ; polymorphism; insulin resistance; PCOS; hsCRP

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Uvod

Sindrom policističnih jajnika (engl. *polycystic ovary syndrome*, PCOS) je metabolički i reproduktivni poremećaj koji zahvaća 5–10% žena u reproduktivnim godinama (1). Obilježava ga povećano izlučivanje androgena iz jajnika i nadbubrežne žlijezde, hiperandrogenični simptomi (hirzutizam, akne, alopecija), neredoviti menstrualni ciklusi i inzulinska rezistencija (2) koja je prisutna kod 50–70% bolesnica s PCOS. Uz inzulinsku rezistenciju prisutne su često i druge značajke metaboličkog sindroma [pretilost, dislipidemija, hipertenzija i poremećena tolerancija glukoze (engl. *impaired glucose tolerance*, IGT). Uz PCOS i metabolički sindrom, inzulinska rezistencija povezana je i s pretilošću, šećernom bolesti tipa 2, lipodistrofijama i kroničnom infekcijom. Podaci govore kako je sveukupna učestalost inzulinske rezistencije 10–25% (1). Inzulinsku rezistenciju obilježava slab tkivni odgovor na djelovanje inzulina, a može biti uzrokovana poremećajima na bilo kojoj razini inzulinskog signalnog puta. Klinički biljezi inzulinske rezistencije su visceralna pretilost, crnkasta akantoza, akne, prekomjerna dlakavost i jetrena steatoza. Hiperinzulinemijska euglikemijska spona (engl. *hyperinsulinemic euglycemic clamp*) smatra se zlatnim standardom za procjenu inzulinske rezistencije. Zbog invazivnosti ove metode razvijaju se i alternativna mjerenja, kao npr. mjerenje koncentracije inzulina u plazmi natašte, indeks procjene modela homeostaze (engl. *homeastasis model assessment*, HOMA), kvantitativni indeks provjere inzulinske osjetljivosti (engl. *quantitative insulin sensitivity check index*, QUICKI) i McAuleyev indeks (1).

Receptori PPAR (engl. *peroxisome proliferator-activated receptors*) su jezgreni receptori koji kontroliraju transkripciju gena. Postoje tri tipa PPAR: PPARα, PPARβ (također poznat kao PPARδ) i PPARγ. PPARγ ima važnu ulogu kao regulator ekspresije adipocitnog gena. Specifična aktivacija PPARγ izaziva diferencijaciju predadipocita u zrele adipocite u staničnim linijama ljudi i glodavaca (3). PPARγ regulira transkripciju proteina uključenih u metabolizam slobodnih masnih kiselina (engl. *free fatty acids*, FFA) (aP2 protein koji veže FFA specifične za adipocit, lipoproteinska lipaza, proteini transporteri masnih kiselina FATP i CD36, adipofilin, jetreni X receptor α) i lipogenezu (dugolančana acil-CoA sintaza, fosfoenolpiruvat-karboksikinaza) (4). Uz diferencijaciju adipocita, PPARγ je isto tako bitan za preživljenje zrelih adipocita *in vitro* (5).

Gen *PPARG* nalazi se na kromosomu 3p25. Ima 146 kbp i sastoji se od 9 eksona. Kodira 4 različite mRNA koje se prepisuju u 2 različite proteinske izoforme (PPARγ1 and PPARγ2) pomoću različitih promotora i alternativnog izrezivanja. Izoforma PPARγ2 ima 30 dodatnih aminokiselina na svom N-terminalnom kraju i pokazuje pet do šest puta povećanu funkciju aktiviranja ovisnu o ligandu (5). Polimorfizam Pro12Ala nalazi se u eksonu B i stoga je prisutan

Introduction

Polycystic ovary syndrome (PCOS) is a metabolic and reproductive disorder affecting 5%-10% of women of reproductive age (1). It is characterized by increased ovarian and adrenal androgen secretion, hyperandrogenic symptoms (hirsutism, acne, alopecia), menstrual irregularities and insulin resistance (2), which is present in 50%-70% of PCOS patients. In addition to insulin resistance, other features of metabolic syndrome are often also present (obesity, dyslipidemia, hypertension and impaired glucose tolerance). Besides PCOS and metabolic syndrome, insulin resistance is also associated with obesity, type 2 diabetes mellitus, lipodystrophies and chronic infection. The overall prevalence of insulin resistance is reported to be 10%-25% (1). Insulin resistance is characterized by attenuated response of tissues to insulin action and can be due to impairments at any level of insulin signaling. Clinical markers of insulin resistance are visceral obesity, acanthosis nigricans, acne, hirsutism and hepatic steatosis. Hyperinsulinemic euglycemic clamp is considered to be the gold standard for evaluating insulin resistance. Because of the invasiveness of this method, other alternative measures like fasting plasma insulin concentration, homeostasis model assessment (HOMA) index, quantitative insulin sensitivity check index (QUICKI) and McAuley index have also been developed (1).

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors which control gene transcription. There are three types of PPARs: PPARα, PPARβ (also named PPARδ) and PPARγ. PPARγ has an important role as an adipocyte gene expression regulator. Specific PPARγ activation induces preadipocyte differentation into mature adipocyte in rodent and human cell lines (3). PPARγ regulates transcription of proteins involved in free fatty acid (FFA) uptake (adipocyte-specific FFA binding protein aP2, lipoprotein lipase, fatty acids transport proteins FATP and CD36, adipophilin, liver X receptor α) and lipogenesis (long chain acyl-CoA synthase, phosphoenolpyruvate carboxykinase) (4). Besides adipocyte differentiation, PPARγ is also essential for mature adipocyte survival *in vitro* (5).

PPARG gene is located on chrosomome 3p25. It spans over 146 kilobase pairs and consists of 9 exons. It codes 4 different mRNAs which translate to 2 different protein isoforms (PPARγ1 and PPARγ2) using different promotors and alternative splicing. The PPARγ2 isoform has 30 additional amino acids on its N-terminal end and has 5- to 6-fold increase in its ligand-dependent activating function (5). The Pro12Ala polymorphism lies in exon B and is therefore present only in PPARγ2 isoform. The His477His polymorphism lies in exon 6 and can be found in both protein isoforms (Figure 1). PPARγ containing the Ala allele has a lower transcriptional activity than the one contai-

samo u izoformi PPARγ2. Polimorfizam His477His leži u eksonu 6 i može se naći u obje izoforme proteina (Slika 1.). PPARγ koji sadrži alel Ala ima slabiju transkripcijsku aktivnost nego onaj koji sadrži alel Pro *in vitro*. To su potvrdila i 3 pojedinačna istraživanja *in vivo* (7).

Cilj našega preliminarnog istraživanja bio je utvrditi povezanost između polimorfizama Pro12Ala i His477His u genu *PPARG* i njihovih diplotipova s inzulinskom rezistencijom kod bolesnica s PCOS. Iako postoji mnogo istraživanja polimorfizma Pro12Ala i/ili His477His, često s oprečnim rezultatima, mi smo prvi proveli ovakvu vrstu istraživanja na slovenskoj skupini bolesnica s PCOS da bismo utvrdili udruženost diplotipova s hsCRP kod bolesnica s PCOS.

Bolesnice i metode

Bolesnice

Naše je istraživanje uključivalo 69 žena (bjelkinja) iz Slovenije s dijagnosticiranim PCOS prema kriterijima NICHD

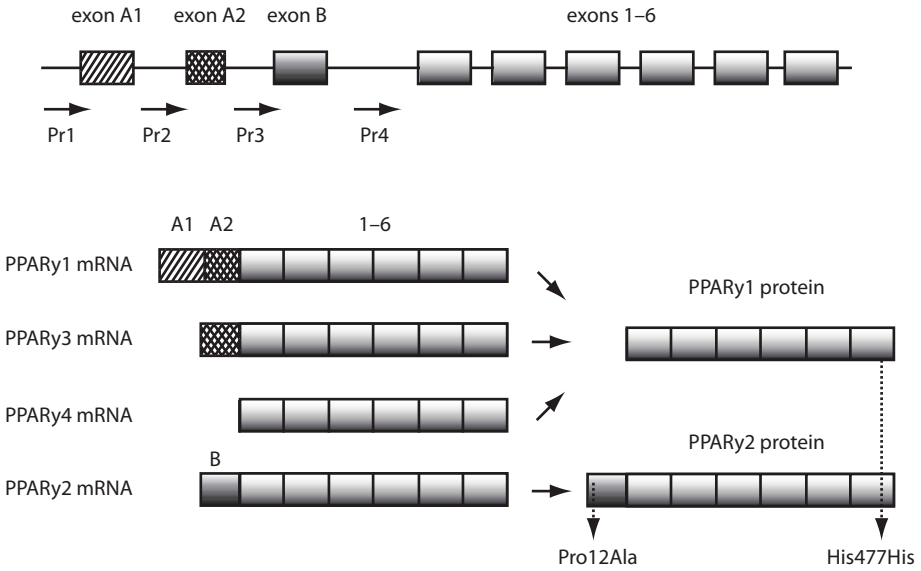
ning the Pro allele *in vitro*. This was confirmed by 3 individual *in vivo* studies (7).

The aim of our preliminary study was to determine the association of Pro12Ala and His477His polymorphisms in *PPARG* gene and their diplotypes with insulin resistance in PCOS patients. Although many studies have studied the Pro12Ala and/or His477His polymorphisms, often with opposing results, we were the first to conduct this kind of a study in a Slovene group of PCOS patients and to determine the association of diplotype with high sensitivity C-reactive protein (hsCRP) in PCOS patients.

Patients and methods

Patients

Our study included 69 Slovene (Caucasian) women, all of them diagnosed with PCOS according to NICHD (National Institute of Child Health and Human Development) crite-



Slika 1. Gen PPARγ i mRNAs. Izoforma PPARγ1 dugačka je 475 aminokiselina i rezultat je transkripcije PPARγ1, PPARγ3 i PPARγ4 mRNA. Izoforma PPARγ2 ima dodatnih 30 aminokiselina na svojem N-terminalnom kraju. PPARγ1 izražen je ubikvitarno dok je PPARγ2 adipozno specifičan. PPARγ3 je izražen u makrofazima, adipoznom tkivu i debelom crijevu. Lokalizacija ekspresije PPARγ4 još nije definirana. (Prema ref. 6).

Figure 1. PPARγ gene and mRNAs. The PPARγ1 isoform is 475 amino acids long and is a result of translation of PPARγ1, PPARγ3 and PPARγ4 mRNA. The PPARγ2 isoform has 30 additional amino acids on its N-terminal end. PPARγ1 is expressed ubiquitously, whereas PPARγ2 is adipose-specific. PPARγ3 is expressed in macrophages, adipose tissue and colon. Localization of PPARγ4 expression is not yet defined. (Adapted from ref. 6)

(engl. *National Institute of Child Health and Human Development*). Ispitivanje je odobrio Etički odbor i sve su bolesnice potpisale obaviješteni pristanak.

Metode

Antropometrijska mjerenja uključivala su mjerenje tjelesne mase, visine i opsega struka. Rutinskim se metodama bolesnicama mjerio sistolički i dijastolički tlak. Za genetska istraživanja i određivanje biokemijskih parametara vadila se periferna krv natašte. Glukoza se izmjerila nakon oralnog testa opterećenja glukozom (OGTT) metodom GOD-PAP (Roche Hitachi 917, Roche, Mannheim, Njemačka). Kemilumiscentna imunokemijska metoda primijenjena je za mjerenja koncentracija inzulina (Liaison Insulin, Diasorin, Saluggia VC, Italija) i hsCRP (Immulite, DPC Inc., Los Angeles, SAD). Svi su parametri izmjereni u certificiranom laboratoriju rutinski provjerenim metodama. Indeks HOMA izračunao se iz podataka prema slijedećoj jednadžbi:

HOMA-IR = inzulin natašte (mU/L) × glukoza natašte (mmol/L)/22,5 (8).

DNA je izolirana iz periferne krvi pomoću FlexiGene DNA Kit (Qiagen, Hilden, Njemačka). Genotipovi su određeni metodama PCR-RFLP. Polimorfizam Pro12Ala je genotipiziran uporabom početnice s krivo sparenim bazama i restrikcijskog enzima *Hpa*II (New England Biolabs, Ipswich, MA, SAD) koji su prethodno opisali Globočnik i sur. (9). Polimorfizam His477His (druga imena: C161T, C1431T, His447His i CAC477CAT) genotipiziran je pomoću početnica PPARG-Ex6_F: CAGGTTTGCTGAATGTGAAGC i PPARG-Ex6_R: TGGCTCAGGACTCTCTGCTAGT (dizajniran programom Primer3) i restrikcijskim enzimom *Nla*III (New England Biolabs, Ipswich, MA, SAD). U genotipiziranju pomoću RFLP uvijek se rabila negativna kontrola i najmanje je 10% uzoraka rađeno u duplikatu.

Statistička analiza

Hardy-Weinbergova ravnoteža određena je Courtlabovim kalkulatorom (10). Podaci su analizirani programom SPSS 13.0 na operativnom sustavu Windows. Vrijednost $P < 0,05$ smatrala se statistički značajnom. Normalnost raspodjele podataka testirala se Kolmogorov-Smirnovljevim testom. Povezanost genotipa s kliničkim biljezima ispitivala se Studentovim t-testom za varijable s normalnom raspodjelom ili Mann-Whitneyevim testom za varijable koje nisu imale normalnu raspodjelu. Frekvencije haplotipova izračunale su se programom LDA 1.0 (11). Haplotipovi su se pridruživali programom HAP (12). Udruženost diplotipova s kliničkim biljezima uz homogenu varijancu analizirala se pomoću testa ANOVA i *post hoc* testa (Bonferroni, Scheffe, Tukey HSD). Udruženost diplotipova s kliničkim biljezima s nehomogenom varijancom analizirala se Kruskal-Wallisovim testom.

ria. The study was approved by the Ethics Committee and all patients signed an informed consent.

Methods

Anthropometric measurements included body weight, height and waist circumference. Subject systolic and diastolic blood pressures were measured using routine methods. Peripheral blood was drawn in fastening state for genetic studies and measurement of biochemical parameters. Glucose was also measured after oral glucose tolerance test (OGTT) using GOD-PAP method (Roche Hitachi 917, Roche, Mannheim, Germany). Chemiluminescent immunochemical method was used for insulin (Liaison Insulin, Diasorin, Saluggia VC, Italy) and hsCRP (Immulite, DPC Inc., Los Angeles, USA) measurements. All parameters were measured in a certified laboratory using routine validated methods. HOMA index was calculated from the findings using the following equation:

HOMA-IR = fasting insulin (mU/L) × fasting glucose (mmol/L)/22.5 (8).

DNA was isolated from peripheral blood using FlexiGene DNA Kit (Qiagen, Hilden, Germany). Genotypes were determined using PCR-RFLP methods. The Pro12Ala polymorphism was genotyped using mismatch primers and restriction enzyme *Hpa*II (New England Biolabs) previously described by Globočnik *et al*. (9). The His477His polymorphism (also named C161T, C1431T, His447His and CAC477CAT) was genotyped using primers PPARG-Ex6_F: CAGGTTTGCTGAATGTGAAGC and PPARG-Ex6_R: TGGCTCAGGACTCTCTGCTAGT (designed using Primer3 software) and restriction enzyme *Nla*III (New England Biolabs). Negative control was always used in RFLP genotyping and duplicate analysis were performed on at least 10% of samples.

Statistical analysis

Hardy-Weinberg equilibrium was determined using Courtlab calculator (10). Data were analyzed with SPSS 13.0 for Windows. A $P < 0.05$ was considered statistically significant. Data distribution normality was tested using Kolmogorov-Smirnov test. Genotype association with clinical markers was tested using Student's t-test for variables with normal distribution or Mann-Whitney test for variables that didn't show normal distribution. Haplotype frequencies were calculated with LDA 1.0 program (11). Haplotypes were assigned using HAP program (12). Diplotype association with clinical markers with homogenic variance was analyzed using ANOVA and post-hoc tests (Bonferroni, Scheffe, Tukey HSD). Diplotype association with clinical markers with non-homogenic variance was analyzed with Kruskal-Wallis test.

Rezultati

U našoj su skupini ispitanica frekvencije genotipova za oba polimorfizma bile unutar Hardy-Weinbergove ravnoteže. Frekvencije genotipova i alela prikazane su u tablici 1. Prema indeksu HOMA koji je bio iznad 2,0, 34 bolesnice s PCOS imale su inzulinsku rezistenciju. Značajna je razlika u BMI pronađena među podskupinama genotipa Pro12Ala (tablica 2.), gdje su nosioci alela Ala imali niži BMI (Pro/Pro BMI = 28,9 ± 7,2 kg/m^2 prema Pro/Ala BMI = 25,2 ± 5,2 kg/m^2; P = 0,040). Polimorfizam Pro12Ala nije bio povezan sa sistoličkim i dijastoličkim tlakom, opsegom struka, koncentracijom glukoze natašte i nakon OGTT, koncentracijom inzulina natašte, indeksom HOMA ili hsCRP.
Značajna razlika nađena je i kod polimorfizma His477His u odnosu na hsCRP (tablica 2.). Nosioci alela T imali su ni-

Results

Genotype frequencies for both SNPs were in Hardy-Weinberg equilibrium in our study group. Genotype and allele frequencies are shown in Table 1. Thirty-four PCOS patients had insulin resistance according to HOMA index above 2.0. A significant difference was found between Pro12Ala genotype subgroups according to body mass index (BMI) (Table 2), where carriers of the Ala allele had lower BMI (Pro/Pro BMI = 28.9 ± 7.2 kg/m^2 *vs*. Pro/Ala BMI = 25.2 ± 5.2 kg/m^2; P = 0.040). The Pro12Ala polymorphism was not associated with systolic or diastolic blood pressure, waist circumference, fasting glucose, glucose after OGTT, fasting insulin, HOMA index or hsCRP.
A significant difference was also found for the His477His polymorphism according to hsCRP (Table 2). Carriers of

Tablica 1. Frekvencije alela i genotipova polimorfizama Pro12Ala i His477His kod bolesnica s policističnom bolesti jajnika (PCOS)

Table 1. Allele and genotype frequencies of Pro12Ala and His477His polymorphisms in polycystic ovary syndrome (PCOS) patients

	Allele frequencies (%)		Genotype frequencies (%)		
Pro12Ala (C>G)	C	G	CC	GC	GG
	86,2	13,8	72,5	27,5	/
His477His (C>T)	C	T	CC	CT	TT
	87,7	12,3	76,8	21,7	1,5

Tablica 2. Klinički i biokemijski parametri u podskupinama prema genotipovima Pro12Ala i His477His polimorfizama

Table 2. Clinical and biochemical parameters in Pro12Ala and His477His polymorphism subgroups

Clinical/biochemical marker (mean ± SD)	Pro12Ala		P	His477His		P
	Pro/Pro	Pro/Ala		CC	CT + TT	
BMI (kg/m^2)	28.9 ± 7.2	25.2 ± 5.2	**0.040**	28.7 ± 7.2	25.2 ± 4.9	0.076
Systolic pressure (mmHg)	120.1 ± 16.4	116.1 ± 12.5	0.148	121.1 ± 15.9	114.4 ± 10.5	0.116
Waist circumference (cm)	87.3 ± 15.4	81.2 ± 12.5	0.122	87.2 ± 15.5	80.4 ± 11.2	0.105
Fasting glucose (mmol/L)	4.51 ± 0.54	4.37 ± 0.39	0.312	4.48 ± 0.54	4.43 ± 0.35	0.698
Glucose after OGTT (mmol/L)	5.65 ± 1.73	5.19 ± 1.05	0.283	5.63 ± 1.67	5.19 ± 1.21	0.336
Fasting insulin (mU/L)	12.1 ± 7.0	11.1 ± 8.3	0.615	11.9 ± 7.0	11.9 ± 8.6	0.994
HOMA index	2.49 ± 1.53	2.18± 1.67	0.479	2.42 ± 1.53	2.35 ± 1.71	0.875
hsCRP* (mg/L)	1.33 † (0.32 – 4.62)	0.72 † (0.30 – 1.60)	0.064	1.22 † (0.39 – 4.15)	0.60 † (0.25 – 1.45)	**0.047**
Diastolic pressure* (mmHg)	70.0 † (70.0 – 80.0)	70.0 † (65.0 – 80.0)	0.319	70.0 (70.0 – 80.0)	70.0 (66.3 – 75.0)	0.166

*log transformed prior to statistical analysis; †median (1st-3rd quartile); BMI – body mass index; OGTT – oral glucose tolerance test; hsCRP – high sensitivity C-reactive protein; HOMA index – homeostasis model assessment index

že koncentracije hsCRP i time manji rizik od razvoja srčanožilnih bolesti (podaci su iskazani kao medijan (gornji kvartil – donji kvartil); C/C hsCRP = 1,22 (0,39–4,15) mg/L prema C/T + T/T hsCRP = 0,60 (0,25–1,45) mg/L; P = 0,047). Polimorfizam His477His nije bio povezan s BMI, sistoličkim i dijastoličkim tlakom, opsegom struka, koncentracijom glukoze natašte i nakon OGTT, koncentracijom inzulina natašte ili indeksom HOMA.

Također je napravljena i analiza haplotipova. Polimorfizmi Pro12Ala i His477His bili su u značajnoj neravnoteži povezanosti (engl. *linkage disequilibrium*, LD) (D' = 0.727) (Tablica 3.). Od deset teoretski mogućih diplotipova našli smo ih samo pet. Samo je kod jedne bolesnice nađen Ala-T/Pro-T diplotip te je ona isključena iz daljnje statističke analize. Za ostale diplotipove ispitivale su se povezanosti kako bi se pronašli klinički i biokemijski biljezi inzulinske rezistencije. Testiranje je pokazalo kako nema nikakve značajne povezanosti (podaci nisu prikazani).

the T allele had lower hsCRP and therefore a lower risk of developing cardiovascular disease (data displayed as median (1st quartile-3rd quartile); C/C hsCRP = 1.22 (0.39-4.15) mg/L *vs*. C/T + T/T hsCRP = 0.60 (0.25-1.45) mg/L; P = 0.047). The His477His polymorphism was not associated with BMI, systolic or diastolic blood pressure, waist circumference, fasting glucose, glucose after OGTT, fasting insulin or HOMA index.

In addition, haplotype analysis was performed. The Pro12Ala and His477His polymorphisms were in considerable linkage disequilibrium (D' = 0.727) (Table 3). Out of ten theoretically possible diplotypes, only five diplotypes were found. Only one subject had the Ala-T/Pro-T diplotype and was excluded from further statistical analysis. For the rest, diplotype association was tested for clinical and biochemical markers of insulin resistance, yielding no significant association (data not presented).

Tablica 3. Frekvencije haplotipova i diplotipovi

Table 3. Haplotype frequencies and diplotypes

Haplotype	Frequency	Diplotype	N
Pro-C	0.833	Pro-C/Pro-C	47
Pro-T	0.029	Pro-C/Ala-T	12
Ala-C	0.043	Pro-C/Ala-C	6
Ala-T	0.094	Pro-C/Pro-T	3
D' = 0.727		Ala-T/Pro-T	1

Rasprava

Pronašli smo značajnu povezanost između alela Ala polimorfizma Pro12Ala i niskog BMI te između alela T polimorfizma His477His i niske vrijednosti hsCRP, ali niti jedan od ta dva polimorfizma nije bio povezan s koncentracijama glukoze i inzulina natašte niti s indeksom HOMA. Analizom diplotipova također nije pronađena povezanost s antropometrijskim i biokemijskim biljezima inzulinske rezistencije kod PCOS.

Pro12Ala je čest polimorfizam; frekvencija alela Ala ovisna je o rasi i kreće se od 1% do 16%. Naši rezultati kod bolesnica s PCOS pokazali su frekvenciju alela Ala od 13,8%. Te su frekvencije slične onima utvrđenima kod populacije Danske (13), Škotske (14,15), Finske (16), Francuske (17), Indije (18), SAD (19) i Slovenije (9). Niže frekvencije alela (8%) pronađene su kod bjelkinja u SAD, te 1–4% kod Amerikanki afričkog podrijetla (20), te kod žena u Kini, Maleziji (18), Koreji (21) i Italiji (22). U više se studija ispitivao utjecaj alela Ala na tkivnu osjetljivost na inzulin. Neke studije su

Discussion

We found significant association between the Ala allele of the Pro12Ala polymorphism and lower BMI, and between the T allele of the His477His polymorphism and lower hsCRP but none of the two polymorphisms was associated with fasting plasma insulin and glucose concentrations and HOMA index. Diplotype analysis showed no association with anthropometric and biochemical markers of insulin resistance in PCOS.

Pro12Ala is a common polymorphism; Ala allele frequency is race dependent and spans from 1% to 16%. Our results in PCOS patients showed an allele frequency of 13.8% for the Ala allele. These frequencies are similar to those established in Danes (13), Scots (14,15), Finns (16), French (17), Indians (18), Americans (19) and Slovenes (9). A lower allele frequency (8%) was found in USA Caucasian females, and between 1% and 4% in African Americans (20), Chinese, Malayans (18), Koreans (21) and Italian females (22). A number of studies explored the influence of the

pokazale da alel Ala poboljšava tkivnu osjetljivost na inzulin, neke nisu našle nikakvu povezanost, a zanimljivo je da niti jedno istraživanje nije pronašlo negativan utjecaj alela Ala. Rezultati su joj više neujednačeni glede šećerne bolesti. Neka su istraživanja povezala alel Ala sa smanjenim rizikom, druga pak s povećanim rizikom za šećernu bolest tipa 2, dok neka nisu našla nikakvu povezanost (7).
Polimorfizam His477His može se naći pod raznim imenima: C161T, C1431T, His447His i CAC477CAT. Frekvencija alela T kreće se od 6,5% do 25,2% ovisno o rasi. Ustanovljena frekvencija alela T za polimorfizam His477His kod bolesnica s PCOS u našem istraživanju bila je 12,3%. Slične su frekvencije alela pronađene kod populacija Francuske (17,23), SAD (19) i Škotske (14,15). Nešto više frekvencije alela zabilježene su kod žena u Australiji (24) i Indiji, a još veće (19–25%) u Kini, Maleziji (18) i Finskoj (16).
Statistička analiza je pokazala značajnu povezanost između alela Ala polimorfizma Pro12Ala i BMI, gdje su nosioci alela Ala imali niži BMI. Neka od objavljenih korelacijskih istraživanja nisu pokazala značajnu povezanost između alela Ala i BMI (13,21); tri takva istraživanja su provedena u žena oboljelih od PCOS (20,22,25). Druga su ustanovila da nosioci alela Ala imaju viši BMI (16,18,19). Sva su ta istraživanja provedena na 2 do 60 puta većem broju ispitanica nego u našoj studiji. Meta-analiza koja je uključivala podatke iz 30 istraživanja (~19.000 ispitanika) pronašla je značajnu povezanost alela Ala kod ispitanika s BMI višim od 27 kg/m^2 (26). Druga je meta-analiza uključivala podatke iz 57 istraživanja (~32.000 ispitanika) i pokazala da polimorfizam Pro12Ala nije povezan sa značajkama šećerne bolesti, no u određenim podskupinama (bijelci, pretili ispitanici), alel Ala bio je povezan s višim BMI i višom inzuliskom osjetljivošću (27).
U našem istraživanju polimorfizam Pro12Ala nije pokazao povezanost s opsegom struka, dijastoličkim i sistoličkim tlakom, što se slaže s rezultatima Frederiksena i sur. (13), ali se ne slaže s onima Valve i sur. i Wei i sur. (16,19) gdje je alel Ala bio udružen s većim opsegom struka, dakle, višim BMI. U niti jednom istraživanju (13,16,21,22,25), kao niti u našemu, nije pronađena povezanost između alela Ala i koncentracije glukoze i inzulina natašte i/ili indeksa HOMA. Hara i sur. (20) nisu pronašli značajnu povezanost alela Ala i koncentracije glukoze natašte i nakon OGTT, no kod nosioca alela Ala pronađene su značajno niže koncentracije inzulina i niži indeks HOMA. Slično kao i u našoj studiji, alel Ala nije bio povezan s koncentracijom inzulina natašte niti u istraživanjima autora Tai i sur. (18) i Wei i sur. (19), ali je bio povezan s višim [Tai i sur., (18)] odnosno nižim [Wei i sur., (19)] koncentracijama glukoze. U literaturi nema izvješća o istraživanjima povezanosti polimorfizma Pro12Ala i hsCRP kod bolesnica s PCOS.
Naša je statistička analiza polimorfizma His477His pokazala značajnu povezanost s hsCRP; kod nosioca alela T koncentracija hsCRP bila je niža. Slično kao Meirhaeghe i

Ala allele on tissue insulin sensitivity. Some showed the Ala allele to improve tissue insulin sensitivity, some failed to find any connection and, interestingly, none found a negative impact of the Ala allele. Results are even more inconsistent regarding diabetes. Some studies linked the Ala allele with a decreased risk, some with an increased risk of type 2 diabetes and some found no connection (7).
The His477His polymorphism can be found under different names: C161T, C1431T, His447His and CAC477CAT. The T allele frequency ranges from 6.5% to 25.2%, depending on race. The determined T allele frequency for His477His polymorphism in our study was 12.3% in PCOS patients. Similar allele frequencies were found in French (17,23), Americans (19) and Scots (14,15). Slightly higher allele frequencies (16%-17%) were found in Australians (24) and Indians, and even higher (19%-25%) in Chinese, Malayans (18) and Finns (16).
Statistical analysis showed a significant association between the Ala allele of the Pro12Ala polymorphism and BMI where the carriers of the Ala allele had lower BMI. Some of the published correlation studies showed no significant association of the Ala allele with BMI (13,21), three of them including women with PCOS (20,22,25). Others found the Ala allele carriers to have higher BMI (16,18,19). All of these studies were conducted on a 2- to 60-fold larger number of subjects compared to our study. A meta-analysis including data from 30 studies (~19,000 subjects) showed a significant connection of the Ala allele in subjects with BMI over 27 kg/m^2 (26). Another meta-analysis including data from 57 studies (~32,000 subjects) showed the Pro12Ala to have no association with diabetes related traits but in certain subgroups (Caucasians, obese subjects) the Ala allele was associated with higher BMI and greater insulin sensitivity (27).
In our study, the Pro12Ala polymorphism was not associated with waist circumference and diastolic and systolic blood pressure, which is consistent with the findings of Frederiksen *et al*. (13) but inconsistent with Valve *et al*. and Wei *et al*. (16,19), where the Ala allele was connected with higher waist circumference and higher BMI as well. Like our study, none of those studies (13,16,21,22,25) found any association between the Ala allele and fasting glucose, fasting insulin and/or HOMA index. Hara *et al*. (20) found no significant association of the Ala allele and fasting glucose or glucose after OGTT, but carriers of the Ala allele had a significantly lower insulin concentration and HOMA index. Similar to our study, the Ala allele was not associated with fasting insulin in studies conducted by Tai *et al*. (18) and Wei *et al*. (19), but it was associated with higher and lower glucose, respectively. Studies of the Pro12Ala polymorphism in association with hsCRP in PCOS patients cannot be found in the literature.
Our statistical analysis on the His477His polymorphism showed a significant association with hsCRP; carriers of

sur. (23), nismo našli povezanost alela T s BMI. Suprotno našim rezultatima, u nekoliko je istraživanja (16,18,19,22) pronađena povezanost alela T s višim BMI. U našem istraživanju, kao i kod Wei i sur. (19), alel T nije bio povezan s opsegom struka ili je bio povezan s većim opsegom struka (16), što je suprotno našim rezultatima. Meirhaeghe i sur. (23) nisu, kao ni mi, našli povezanost između alela T i sistoličkog i dijastoličkog tlaka. U našem istraživanju nismo pronašli povezanost alela T s koncentracijom glukoze i inzulina natašte te, što je slično rezultatima nekih istraživanja (16,18,19,22,23). Pokazali smo da je alel T povezan s smanjenim hsCRP i stoga smanjenim rizikom od razvoja srčanožilnih bolesti. Slične su rezultate objavili i Wang i sur. kod kojih su nosioci alela T imali niži rizik od razvoja srčanožilnih bolesti, povezanost koja se vidi osobito kod C/T heterozigota (24).

Proveli smo analizu diplotipova i ustanovili stupanj neravnoteže povezanosti (engl. *degree of linkage disequilibrium*) D′ = 0,727, što je slično onom što ga kod bolesnika s šećernom bolesti opisuju Doney i sur. iz Škotske (D′ = 0,697) (14). Nešto niži D′ pronađeni su u drugim istraživanjima među bolesnicima s šećernom bolesti iz Škotske (D′ = 0,663), zdravom djecom (D′ = 0,638) i zdravim odraslim osobama (D′ = 0,608) (15), bijelim Amerikancima (D′ = 0,65) (19), Kinezima (D′ = 0,555) i Malezijcima (D′ = 0,572). Viši stupanj neravnoteže povezanosti pronađen je kod Indijaca (D′ = 0,799) (18). Frekvencije haplotipova kod naših ispitanica (Tablica 3.) bile su slične onima što su našli Doney i sur. u dvama istraživanjima na populaciji Škotske (14,15).

Naši su rezultati pokazali da diplotip nije povezan niti s jednim od antropometrijskih ili biokemijskih biljega. Štoviše, Doney i sur. su pokazali da je haplotip Pro-T povezan s višim BMI u usporedbi s haplotipovima Pro-C ili Ala-C kod škotskih ispitanika sa šećernom bolesti i bez nje (14).

Uzimajući u obzir činjenicu da su rezultati nekoliko ispitivanja nedosljedni, moguće je da polimorfizmi Pro12Ala i His477His imaju tek manji utjecaj na inzulinsku rezistenciju. U našem probnom istraživanju pojedinačnih polimorfizama pokazali smo kako nosioci alela Ala polimorfizma Pro12Ala imaju niži BMI, a nosioci alela T polimorfizma His477His niži hsCRP, što su dva rizična čimbenika za razvoj inzulinske rezistencije. Međutim, povezanost s indeksom HOMA i koncentracijom inzulina natašte, koji su oba biljezi inzulinske rezistencije, nije zabilježena u našem istraživanju. Stoga zaključujemo kako istraživani polimorfizmi nisu povezani s inzulinskom rezistencijom kod bolesnica s PCOS.

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the T allele had lower hsCRP. Similar to Meirhaeghe *et al.* (23), we found no association of the T allele with BMI. In contrast to our findings, the T allele was associated with higher BMI in several studies (16,18,19,22). In our study, the T allele was not associated with waist circumference, which is similar to the results reported by Wei et *al*. (19). In the study by Valve *et al.* (16), it was associated with higher waist circumference (16), which is in contrast to our results. Meirhaeghe *et al*. (23) found no association between the T allele and systolic and diastolic blood pressure, which is consistent with our findings. Fasting glucose and insulin concentrations were not associated with the T allele in our study, which is similar to the findings reported elsewhere (16,18,19,22,23). We showed the T allele to be associated with decreased hsCRP, therefore representing a lower risk for cardiovascular disease development. Similar findings have been reported by Wang *et al*., where carriers of the T allele had a lower risk of developing cardiovascular disease, an association seen especially in C/T heterozygotes (24).

We performed a diplotype analysis and established the degree of linkage disequilibrium D′ = 0.727, which was similar to the one reported by Doney *et al*. in diabetic Scots (D′ = 0.697) (14). Somewhat lower D's were found in another study in diabetic Scots (D′ = 0.663), healthy children (D′ = 0.638) and healthy adults (D′ = 0.608) (15), Caucasian Americans (D′ = 0.65) (19), Chinese (D′ = 0.555) and Malayans (D′ = 0.572). A higher degree of linkage disequilibrium was found in Indians (D′ = 0.799) (18). Haplotype frequencies in our subjects (Table 3) were similar to those found by Doney *et al*. in Scots in two studies (14,15).

Our results showed that diplotype was not associated with any of the anthropometric or biochemical markers. On the contrary, Doney *et al*. demonstrated that Pro-T haplotype was connected with higher BMI compared to Pro-C or Ala-C haplotype in diabetic and non-diabetic Scots (14).

Considering the fact that the results of several studies are inconsistent, it is possible that the Pro12Ala and His477His polymorphisms only have a small influence on insulin resistance. In our pilot study on individual polymorphisms we showed that carriers of the Ala allele of the Pro12Ala polymorphism had a lower BMI and carriers of the T allele of the His477His polymorphism had a lower hsCRP, which are two risk factors for the development of insulin resistance. However, the association with the HOMA index or fasting insulin, two of insulin resistance markers, was not observed in our study. We therefore conclude that the polymorphisms investigated are not associated with insulin resistance in PCOS patients.

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