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Glutathione S-transferase (GST) polymorphism could be an early marker in the development of polycystic ovary syndrome (PCOS) — an insight from non-obese and non-insulin resistant adolescents

Polimorfizm S-transferazy glutationowej (GST) może być wczesnym markerem w rozwoju zespołu policystycznych jajników (PCOS) — doświadczenia nieotyłych dorosłych pacjentów z cukrzycą insulinoniezależną

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Abstract

Introduction: It has been supposed that endocrine disturbances might be responsible for polycystic ovary syndrome (PCOS)-associated oxidative stress, with special emphasis on hyperandrogenism. Considering the potential relationship between hyperandrogenism and increased free radical production, parameters of oxidative stress were determined in non-obese normoinsulinemic adolescent girls newly diagnosed with PCOS.

Materials and methods: Nitrotyrosine, thiol group concentrations, glutathione peroxidase, and superoxide dismutase activities were determined under fasting conditions and during oral glucose tolerance test (OGTT) in 35 PCOS patients and 17 controls. Insulin resistance was assessed by the homeostasis model (HOMA-IR), HOMA β, insulinogenic index (IGI), Matsuda insulin sensitivity index (ISI), and AUC for glucose. Glutathione S-transferases (GSTs) polymorphisms were determined by PCR.

Results: Under fasting conditions, no significant difference of oxidative stress parameters was found between PCOS and controls. Acute hyperglycaemia during OGTT induced significant alteration in parameters of oxidative protein damage in PCOS patients. Alteration in nitrotyrosine concentrations correlated with testosterone, DHEAS, androstenediones, FAI, and LH, while changes in thiol groups correlated with DHEAS. Significant inverse association was found between LH and ISI, as well as AUC glucose and thiol groups. PCOS girls, carriers of *GSTM1-null* genotype, had significantly lower testosterone in comparison to ones with *GSTM1-active* genotype.

Conclusions: PCOS girls exhibited high free radical production together with unchanged antioxidant enzymatic capacity, independently from obesity and insulin resistance. Based on associations between oxidative stress parameters and testosterone, DHEAS, and androstenedione, it can be suggested that increased free radical production, probably as a consequence of hyperandrogenaemia, is an early event in the development of PCOS. **(Endokrynol Pol 2018; 69 (4): 366–374)**

Key words: *oxidative stress, nitrotyrosine, testosterone, hyperandrogenism, polycystic ovary syndrome*

Streszczenie

Wstęp: Istnieją przypuszczenia, że zaburzenia hormonalne mogą odpowiadać za stres oksydacyjny, związany z zespołem policystycznych jajników (*polycystic ovary syndrome*; PCOS), ze szczególnym naciskiem na hiperandrogenizm. Biorąc pod uwagę potencjalny związek między hiperandrogenizmem a zwiększoną produkcją wolnych rodników, parametry stresu oksydacyjnego określono u nieotyłych, dorastających dziewcząt z normoinsulinemią, u których niedawno zdiagnozowano zespół policystycznych jajników (PCOS).

Materiał i metody: W warunkach na czczo oraz podczas doustnego testu obciążenia glukozą (*oral glucose tolerance test;* OGTT) określono stężenie nitrotyrozyny, stężenia grup tiolowych, aktywność peroksydazy glutationowej i dysmutazy ponadtlenkowej u 35 pacjentów z PCOS oraz 17 osób z grupy kontrolnej. Insulinooporność oceniano za pomocą modelu homeostazy (HOMA-IR), HOMA β, współczynnika insulinogennego IGI (*insulinogenic index*; IGI), wskaźnika insulinowrażliwości (*Matsuda insulin sensitivity index*; ISI) i pola pod krzywą (*area under the curve*; AUC) stężenia glukozy we krwi. Polimorfizmy S-transferaz glutationowych (GSTs) określono za pomocą metody PCR.

Wyniki: W warunkach na czczo nie stwierdzono istotnej różnicy parametrów stresu oksydacyjnego między pacjentami z PCOS a osobami z grupy kontrolnej. Ostra hiperglikemia podczas trwania OGTT wywołała istotne zmiany parametrów oksydacyjnych uszkodzeń białek u pacjentów z PCOS. Zmiana stężeń nitrotyrozyny korelowała ze stężeniem testosteronu, siarczanu dehydroepiandrosteronu (*dehydroepiandrosterone sulphate*; DHEAS), androstenedionów, współczynnikiem wolnych androgenów (*free androgen index*; FAI) oraz hormonem luteinizującym (*luteinising hormone*; LH), podczas, gdy zmiany stężeń grup tiolowych korelowały z DHEAS. Stwierdzono istotną odwrotną zależność między LH a ISI, jak również między AUC stężenia glukozy a grupami tiolowymi. Dziewczęta z PCOS, nosicielki genotypu *GSTM1-null*, wykazywały niższe stężenie testosteronu w porównaniu z dziewczętami z genotypem *GSTM1-active*.

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Wnioski: U dziewcząt z PCOS wykazano wyższą produkcję wolnych rodników wraz z niezmienioną enzymatyczną zdolnością antyoksydacyjną, niezależnie od otyłości i oporności na insulinę. Biorąc pod uwagę związek między parametrami stresu oksydacyjnego i testosteronu, DHEAS i androstenedionów, można uznać, że zwiększona produkcja wolnych rodników, prawdopodobnie jako konsekwencja hiperandrogenemii, występuje na wczesnym etapie rozwoju PCOS. **(Endokrynol Pol 2018; 69 (4): 367–374)**

Słowa kluczowe: *stres oksydacyjny, nitrotyrozyna, testosteron, hiperandrogenizm, zespół policystycznych jajników*

Introduction

Polycystic ovary syndrome (PCOS), when manifested with puberty, is characterised by all three of the following criteria: clinical or biochemical hyperandrogenism, oligo/anovulation, and polycystic ovaries [1, 2]. In addition to its relatively high prevalence, this syndrome has an important negative impact on health because it is frequently associated with infertility, obesity, insulin resistance, and metabolic syndrome, as well as higher risk for cardiovascular disease [3]. It has been suggested that increased gonadotropin-releasing hormone (GnRH) pulse frequency causes excessive secretion of luteinising hormone (LH) during the early phase of the development of the syndrome [4]. This further induces premature acquisition of LH receptors on growing ovarian follicles, leading to increased ovarian androgen production [5]. In addition to ovarian hyperthecosis and the resulting increase in androgen production, it has been underlined that different genetic, lifestyle, and environmental factors might significantly contribute to its pathophysiology [6].

It has been suggested that the parameters of oxidative stress might have complex associations with the etiopathogenesis, clinical manifestations, and possibly the development of unfavourable cardiometabolic outcomes in PCOS women [7]. In particular, hyperandrogenism seems to represent the main progenitor of increased production of reactive oxygen species (ROS). Oxidative stress, in turn, could stimulate the expression of ovarian steroidogenic enzymes involved in androgen production and proliferation of theca cells [7]. Furthermore, oxidative stress impairs insulin signalling, resulting in a compensatory hyperinsulinaemia, which can further stimulate thecal steroidogenesis. Overall, it can be concluded that oxidative stress, together with low-grade inflammation, insulin resistance, and/or hyperinsulinaemia, significantly contributes to hyperandrogenism and anovulation, as well as cardiovascular disorders accompanied by PCOS [8, 9].

Our previous results indicate that non-obese PCOS women are prone to oxidative stress induced primarily by glucose challenge, but this seems not to be related to the direct effect of hyperinsulinaemia during clamp [10, 11]. We also found that oxidative stress markers were related to indices of insulin resistance as well as circulating testosterone [10, 11]. However, the potential link between hormonal status and oxidative stress of adolescent, normoinsulinemic PCOS girls has not been investigated yet. Moreover, genetic polymorphisms of glutathione transferases (GSTs), enzymes involved in detoxification of different electrophilic compounds, including steroid hormones and free radicals, has not been addressed in this syndrome.

In this line, we investigated whether the hormonal status of non-obese adolescent girls with PCOS could affect level of oxidative stress before and after glucose challenge. During oral glucose tolerance test (OGTT) oxidative stress by-products, nitrotyrosine, and thiol groups, together with activity of key antioxidant enzymes, glutathione peroxidase (GPX), and superoxide dismutase (SOD), were measured. Genetic polymorphisms of main cytosolic GST classes in PCOS patients and age-matched healthy controls were also determined.

Materials and methods

Subjects

Thirty-five newly diagnosed adolescent girls with PCOS (age = 16.37 ± 0.94 years; mean ± SD) and 17 age- and BMI-matched healthy controls (16.71 ± 1.11 years old) (Table I) with regular ovulatory menstrual

Table I. ***Basal clinical parameters in non-obese PCOS patients and age-matched controls***

Tabela I. ***Podstawowe parametry kliniczne nieotyłych pacjentów z PCOS i dobranych pod względem wieku osób z grupy kontrolnej***

	PCOS (n = 35)	Controls (n = 17)	p
Age (years)	16.37 ± 0.94	16.71 ± 1.11	0.251
BMI [kg/m^2]	22.52 ± 2.45	22.53 ± 2.69	0.982
Waist circumference, WC [cm]	75.88 ± 6.42	75.23 ± 5.59	0.723
Hip circumference, HC [cm]	95.36 ± 5.61	94.80 ± 6.90	0.754
Waist-to-hip ratio, WHR [cm]	0.80 ± 0.05	0.79 ± 0.04	0.932

Values are presented as mean ± standard deviation. Statistical differences in the variables between groups were assessed by the t-test for independent samples. A p-value < 0.05 was considered statistically significant. PCOS, polycystic ovary syndrome; n, number of samples; BMI, body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio

cycles referred to the Department of Pediatric and Adolescent Gynecology, Mother and Child Health Care Institute of Serbia, Belgrade and were enrolled in this study. Diagnosis of PCOS was made on the basis of the revised 2003 Rotterdam ESHRE/ARSM consensus criteria (2004) [1] adapted for adolescence according to Carmina et al. [2]. Thus, all patients fulfilled all three of the Rotterdam criteria: oligo- or amenorrhoea and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovarian morphology on ultrasonographic examination. Oligomenorrhoea and amenorrhoea were defined according to Laven et al. [12]. Anovulation was diagnosed when progesterone concentrations measured on the 21th day of spontaneous or progestin-induced menstrual cycle were measured at less than 10 nmol/L. Hirsutism, a clinical sign of hyperandrogenism, was declared when the Ferriman-Gallwey (F-G) score was calculated as ≥ 8 [13]. Hyperandrogenemia, a biochemical sign of hyperandrogenism, was diagnosed when serum total testosterone concentrations were greater than 2 nmol/L. Ovaries were diagnosed as polycystic by transabdominal ultrasonography when the ovarian volume was greater than 10 mL and/or when 12 or more follicles measuring 2 to 9 mm in diameter were present [14]. Thus, the healthy controls had progesterone concentrations greater than 10 nmol/L on the 21th day of a regular menstrual cycle, no clinical or hormonal signs of hyperandrogenism, and no polycystic ovarian morphology on ultrasound examination.

Exclusion criteria for all the participants were as follows: time of menarche less than three years before inclusion in the study, pregnancy, Cushing's syndrome, impaired fasting glucose, hyperprolactinemia, thyroid dysfunction or other endocrine disorder as well as acute or chronic ailment or any rheumatologic diseases, and BMI > 30 kg/m^2. None of the participants had received any kind of antibiotics or any kind of hormonal treatment for at least six months prior to the beginning of the study. Patients and controls had no history of cigarette smoking or alcohol consumption.

Protocol

All subjects were seen in the early follicular phase of a spontaneous or progesterone-induced menstrual cycle (day 3–5) if not otherwise stated. At the initial visit a clinical examination was performed by the same researcher (I.M.). Body mass index (BMI, kg/m^2) was used as a marker of overall obesity. Abdominal obesity was calculated as waist-to-hip ratio (WHR). Waist circumference (WC, cm) was measured at the narrowest part of the torso as seen from the front, whereas hip circumference (HC, cm) was the maximum extension of the buttocks as seen from the side. Blood sampling was performed from the antecubital vein after 12-hour fasting between 8 a.m. and 9 a.m. Standard oral glucose tolerance test (OGTT) was performed after overnight fast, with 75 g of glucose. Glucose were determined at 0, 30, 60, 90, and 120 min of the test.

The study was approved by the Ethics Committee of the Mother and Child Health Care Institute of Serbia, and written, informed consent was obtained from all study participants or from their parents.

Biochemical and hormonal testing

If not stated differently, all biochemical and hormonal testing were determined using an automatic analyser Roche Hitachi 6000 Cobas c501 (Roche, Mannheim, Germany). Plasma glucose (mmol/L) was determined by enzymatic method. Plasma FSH (U/L), LH (U/L), total testosterone (nmol/L), progesterone (nmol/L), and insulin (mIU/L) were measured by electrochemiluminescence immunoassay (Cobas e411, Roche Diagnostics GmbH Marburg, Germany). Serum androstenedione (ng/mL), DHEAS (μmol/L) and SHBG (nmol/L) were measured by radioimmunoassay (R-GM-100, DHEAS-CT and SHBG-RIACT, respectively; CIS Bio International, Gifsur-Yvette, France). FAI was calculated from total testosterone and SHBG, using the formula FAI = (100 x T)/SHBG, and a value > 8 was considered elevated. Insulinogenic index as an indicator of beta cell response in first 60 minutes was calculated from OGTT [IGI = Insulin(mIU/L) 60 min — Insulin 0 min/Glucose(mmol/L) 60 min — Glucose 0 min] while insulin resistance (IR) was determined by the homeostasis model assessment [HOMA-IR = insulin(mIU/L) x glucose(mmol/L)/22.5] [15] and insulin sensitivity index-Matsuda [ISI (Matsuda) = 10000/(Fasting glucose, mg/dl x Fasting insulin, mIU/L) x (Mean glucose in OGTT, mg/dL x Mean insulin during OGTT, mIU/L)] [16]. The plasma concentration of nitrotyrosine (nmol/L) was monitored by enzyme immunoassay (Oxis International Inc.). The plasma thiol group content (μmol/l), glutathione peroxidase activity (GPX, U/L), and superoxide dismutase activity (SOD, U/L) were determined as described elsewhere [17, 18].

Genetic testing

Genomic DNA was isolated from whole peripheral blood using a DNA kit (*Qiagen, Chatswort, USA*). Genotyping was performed blinded to the case-control status, and blinded quality control samples were inserted to validate the genotyping identification procedures. Concordance for blinded samples was 100%. The DNA sequences of *GSTM1* and *GSTT1* were analysed by multiplex polymerase chain reaction according to the PCR protocol by Abdel-Rahman et al. [19]. The analysis of the SNP *GSTA1 C69T* (rs3957357) was performed using

PCR-restriction fragment length polymorphism (RFLP) according to the PCR protocol by Ping et al. [20]. For analyses of the SNP polymorphism *GSTP1 Ile105Val*, a 5′ nuclease TaqMan® SNP Genotyping Assays (*Life Technologies, Applied Biosystems, Carlsbad, USA, assay ID: C__3237198_20*) was used for amplification and detection of respective SNP alleles.

Statistical analyses

Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS, version 17.0; SPSS Inc., Chicago, Illinois, USA). The Kolmogorov-Smirnov test was used to evaluate the normality of the distributions of variables. Parametric continuous variables were presented as mean ± standard deviation (SD). Parametric t-test for independent samples was used to assess the differences between groups. Association between different variables were determined using the Pearson's coefficient for parametric variables. The area under the curve AUC was calculated according to the trapezoid method using Microsoft Excel software. The significance of the difference is indicated by the p-values. A p-value of < 0.05 was considered statistically significant.

Results

Biochemical and hormonal parameters in the PCOS and control groups

Clinical and biochemical parameters obtained from PCOS patients and age- and BMI-matched healthy controls are shown in Table I and Table II. There was no statistical difference in WC, HC, WHR, fasting blood glucose, indices of insulin secretion and resistance (basal insulin, HOMA-IR, HOMA β, IGI, ISI), as well as FSH between PCOS and the control group. Luteinising hormone, DHEAS and testosterone concentrations, as well as FAI values, were significantly greater ($p < 0.001$, $p = 0.004$, $p < 0.001$, $p < 0.001$, respectively) while progesterone and SHBG concentrations were significantly lower ($p < 0.001$, $p = 0.001$, respectively) in the PCOS adolescent girls in comparison to controls.

The baseline differences of oxidative stress parameters (nitrotyrosine, an indirect marker of peroxynitrite generation and thiol groups, established protein sacrificial antioxidants) and activity of antioxidant enzymes glutathione peroxidase (GPX) and superoxide dismutase (SOD) between PCOS and control subjects are reported in Table II. No significant difference was observed in GPX and SOD antioxidant activities or in nitrotyrosine and thiol groups, as markers of protein oxidative damage between groups ($p > 0.05$). Acute hyperglycaemia during OGTT induced alterations in both the parameters of oxidative damage of proteins

Table II. ***Basal biochemical parameters in non-obese PCOS patients and age-matched controls***

Tabela II. ***Podstawowe parametry biochemiczne nieotyłych pacjentów z PCOS i dobranych pod względem wieku osób z grupy kontrolnej***

	PCOS (n = 35)	**Controls (n = 17)**	*p*
Glucose; OGTT 0 min [mmol/L]	4.47 ± 0.89	4.79 ± 0.36	0.164
Glucose; OGTT 30 min [mmol/L]	7.25 ± 1.14	7.20 ± 0.87	0.870
Glucose; OGTT 60 min [mmol/L]	7.12 ± 1.52	6.58 ± 1.06	0.198
Glucose; OGTT 90 min [mmol/L]	6.44 ± 1.40	5.66 ± 1.07	0.050
Glucose; OGTT 120 min [mmol/L]	5.61 ± 1.16	5.50 ± 0.72	0.672
Insulin; OGTT 0 min [mIU/L]	9.35 ± 4.11	9.34 ± 5.11	0.993
Insulin; OGTT 30 min [mIU/L]	85.73 ± 38.50	79.96 ± 42.06	0.625
Insulin; OGTT 60 min [mIU/L]	98.22 ± 48.56	78.88 ± 45.38	0.175
Insulin; OGTT 90 min [mIU/L]	91.61 ± 51.21	66.14 ± 33.40	0.068
Insulin; OGTT 120 min [mIU/L]	73.46 ± 43.69	61.53 ± 35.51	0.333
HOMA-IR	1.93 ± 0.96	1.98 ± 1.02	0.825
HOMA β	188.73 ± 129.50	250.12 ± 417.34	0.427
IGI	41.32 ± 62.24	15.02 ± 103.11	0.258
$ISI_{(Matsuda)}$	4.93 ± 2.35	5.27 ± 2.55	0.636
AUC glucose	12.98 ± 1.93	12.27 ± 1.65	0.196
AUC insulin	157.80 ± 66.38	131.62 ± 51.61	0.160
FSH [U/L]	5.10 ± 1.38	5.56 ± 1.31	0.255
LH [U/L]	13.59 ± 4.34	4.97 ± 2.28	< 0.001
Testosterone [nmol/L]	2.06 ± 0.48	0.72 ± 0.28	< 0.001
SHBG [nmol/L]	21.56 ± 10.14	33.60 ± 14.40	0.001
FAI	12.43 ± 7.86	2.64 ± 1.57	< 0.001
Androstenedione [ng/mL]	1.92 ± 0.34	1.71 ± 0.43	0.068
DHEAS [μmol/L]	6.84 ± 2.64	4.69 ± 1.95	0.004
Progesterone [nmol/L]	2.52 ± 1.28	32.29 ± 9.86	< 0.001
Thiol groups [μmol/L]	10.25 ± 3.83	10.21 ± 2.21	0.967
Nitrotyrosine [nmol/L]	84.31 ± 43.02	63.79 ± 33.46	0.215
Uric acid [μmol/l]	253.70 ± 41.21	258.40 ± 20.51	0.600
GPX [U/L]	494.00 ± 140.36	505.76 ± 100.90	0.759
SOD [U/L]	106.96 ± 24.22	104.27 ± 19.44	0.711

Values are presented as mean ± standard deviation. Statistical differences in the variables between groups were assessed by the t-test for independent samples. A p-value < 0.05 was considered statistically significant
PCOS, polycystic ovary syndrome; n, number of samples; OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA β, homeostasis model assessment of β cell function index; IGI, insulinogenic index; $ISI_{(Matsuda)}$, insulin sensitivity index-Matsuda; FSH, follicle-stimulating hormone; LH, luteinising hormone; SHBG, sex hormone-binding globulin; FAI, free androgen index; DHEAS, dehydroepiandrosterone sulphate; GPX, glutathione peroxidase; SOD, superoxide dismutase

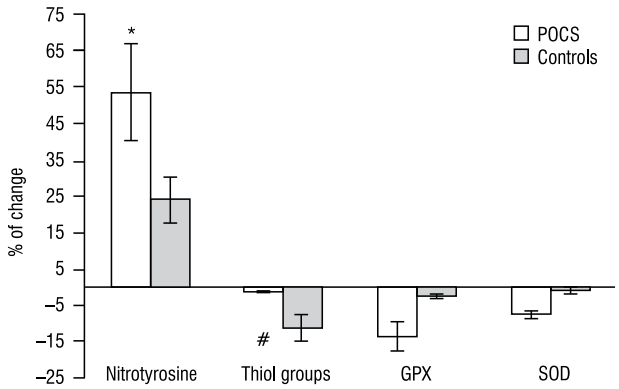

Figure 1. *The percentage change in plasma nitrotyrosine, thiol groups, GPX, and SOD activity when fasting samples were compared with the samples collected 2 h after glucose ingestion*
**the percentage change in nitrotyrosine concentrations of PCOS girls was greater, in comparison with that of lean controls, p < 0.05*
#the percentage change in thiol group concentrations of PCOS girls showed a more pronounced decrease in comparison with that of controls, p < 0.05

Rycina 1. *Zmiana procentowa nitrotyrozyny w osoczu, grup tiolowych, aktywności GPX i SOD — porównanie próbek na czczo z próbkami pobranymi dwie godziny po spożyciu glukozy*
**zmiana procentowa stężeń nitrotyrozyny u dziewcząt z PCOS była większa w porównaniu z osobami szczupłymi z grupy kontrolnej, p < 0,05*
#zmiana procentowa stężeń grup tiolowych dziewcząt z PCOS wykazywała wyraźniejszy spadek w porównaniu z osobami z grupy kontrolnej, p < 0,05

and activity of GPX enzyme in PCOS girls and controls. As depicted in Figure 1, in response to oral glucose challenge the percentage of change in both parameters of protein oxidative damage was more significantly changed in PCOS girls compared to weight-matched controls ($p < 0.05$). Namely, the percentage of change in nitrotyrosine was significantly higher, while change of thiol group content, as sacrificial protein antioxidants, was significantly lower in PCOS. However, change in activities of SOD and GPX did not reach statistical significance between groups ($p > 0.05$).

Correlations between oxidative stress parameters with hormone concentrations and OGTT indices

The observed alteration of oxidative stress parameters during hyperglycaemia correlated with parameters of hyperandrogenism present in PCOS. Specifically, testosterone (including total, free, and bioavailable testosterone), DHEAS, androstenedione, FAI, and LH were positively correlated with the percentage of change in nitrotyrosine concentrations, and DHEAS was correlated with the percentage change in thiol groups ($r = –0.425$, $p = 0.014$) (Table III). When analysing the association between oxidative stress markers and indices of insulin resistance, no significant correlations were obtained (data not shown). Interestingly, we found association of AUC for glucose with the percentage of change in thiol groups. However, considering the

Table III. ***Correlations between oxidative stress markers (percentage change) and hormones in non-obese PCOS***

Tabela III. ***Zależność między markerami stresu oksydacyjnego (zmiana procentowa) a hormonami u nieotyłych pacjentów z PCOS***

Percentage change[a]								
	Nitrotyrosine		Thiol groups		GPX		SOD	
	r	p	r	p	r	p	r	p
FSH [U/L]	–0.069	0.759	0.080	0.659	–0.207	0.232	0.072	0.762
LH [U/L]	0.496	0.036	0.068	0.697	–0.180	0.300	0.731	< 0.001
Testosterone [nnmol/L]	0.493	0.020	–0.024	0.890	–0.020	0.908	0.016	0.947
SHBG [nnmol/L]	–0.365	0.095	0.172	0.338	0.065	0.711	0.067	0.752
FAI	0.491	0.020	0.020	0.913	0.085	0.626	0.078	0.743
Androstenedione [nmol/L]	0.568	0.006	–0.041	0.819	–0.304	0.076	–0.029	0.902
DHEAS [nnmol/L]	0.469	0.028	–0.425	0.014	–0.318	0.062	0.123	0.604
AUC glucose	0.083	0.713	–0.344	0.043	–0.100	0.566	–0.063	0.764
AUC insulin	0.285	0.198	0.110	0.531	–0.338	0.047	0.373	0.106

[a]the percent change in oxidative stress markers was determined when fasting samples were compared with the samples collected 2 h after OGTT

Pearson's correlation coefficient (r) was used for all variables; significance of correlation is indicated by the p-values. A p-value < 0.05 was considered statistically significant

FSH, follicle-stimulating hormone; LH, luteinising hormone; SHBG, sex hormone-binding globulin; FAI, free androgen index; DHEAS, dehydroepiandrosterone sulphate; GPX, glutathione peroxidase; SOD, superoxide dismutase.

Table IV. *Correlations between indices of insulin resistance and hormones in non-obese PCOS*
Tabela IV. *Zależność między wskaźnikami insulinooporności a hormonami u nieotyłych pacjentów z PCOS*

	Testosterone [nnmol/L]		FSH [IU/L]		LH [IU/L]		DHEAS [nnmol/L]		SHBG [nnmol/L]	
	r	p	r	p	r	p	r	p	r	p
HOMA-IR	0.122	0.485	0.157	0.368	0.012	0.947	–0.072	0.680	–0.167	0.336
HOMA β	0.032	0.855	0.201	0.247	0.137	0.434	–0.054	0.757	0.051	0.772
IGI	0.132	0.450	0.150	0.389	0.118	0.501	–0.077	0.662	–0.276	0.109
ISI	–0.173	0.321	–0.176	0.311	–0.359	0.034	–0.095	0.588	0.197	0.256

Pearson's correlation coefficient (r) was used for all variables; Significance of correlation is indicated by the p-values. A p-value < 0.05 was considered statistically significant FSH, follicle-stimulating hormone; LH, luteinising hormone; SHBG, sex hormone-binding globulin; DHEAS, dehydroepiandrosterone sulphate; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA β, homeostasis model assessment of β cell function index; IGI, insulinogenic index; $ISI_{(Matsuda)}$, insulin sensitivity index-Matsuda

association between indices of insulin resistance and hormones of PCOS patients, significant inverse correlation was found for ISI and LH (r = –0.359, p = 0.034) (Table IV).

Evaluation of GST polymorphism in PCOS and control groups

The distribution of four common GST polymorphisms (*GSTA1, GSTM1, GSTP1,* and *GSTT1*) in PCOS adolescent girls and the control group are presented in Table V. Specifically, deletion polymorphisms of *GSTM1* and *GSTT1* consequently result in complete absence of specific enzyme activity in *GSTM1-null* or *GSTT1-null* individuals, while carriers of *GSTM1-active* or *GSTT1-active* genotype have fully active enzyme. Single-nucleotide polymorphisms of *GSTA1* and *GSTP1* can result in either lower expression of enzyme in the case of *GSTA1-variant* or altered enzyme activity in *GSTP1-variant* genotype carriers. As presented, the observed difference in frequency of various GST genotypes between study populations and controls was not significant.

Comparison of hormone concentrations according to the GST genotype in the PCOS and control groups

The data on the hormonal status of PCOS girls (testosterone, androstenedione, DHEAS, progesterone) stratified according to their GST genotype are presented in Figure 2A-D. Adolescent girls with PCOS, carriers of *GSTM1-null* genotype, presented significantly lower testosterone concentrations as compared to those with the GSTM1-active genotype ($p < 0.05$) (Figure 2A).

Table V. *Distribution of GSTA1, GSTM1, GSTP1, and GSTT1 genotypes in PCOS and controls*
Tabela V. *Rozkład genotypów GSTA1, GSTM1, GSTP1 i GSTT1 u pacjentów z PCOS i osób z grupy kontrolnej*

Variable	PCOS (n, %)	Controls (n, %)	P
GSTA1			
CC (active)	15 (44.1)	3 (17.6)	
CT + TT (low activity)	19 (55.9)	14 (82.4)	0.062
GSTM1			
Active[a]	15 (44.1)	10 (58.8)	
Null[b]	19 (55.9)	7 (41.2)	0.322
GSTP1			
Ilelle (wild type)	8 (23.5)	1 (6.2)	
IleVal + ValVal (variant)	26 (76.5)	15 (93.8)	0.138
GSTT1			
Active[a]	28 (82.4)	11 (64.7)	
Null[b]	6 (17.6)	6 (35.3)	0.161

Differences in investigated parameters were assessed by using χ^2 test for categorical variables. A p-value < 0.05 was considered statistically significant PCOS, polycystic ovary syndrome; n, number of samples; GSTA1, glutathione S-transferase alpha 1; GSTM1, glutathione S-transferase mu 1; GSTP1, glutathione S-transferase pi 1; GSTT1, glutathione S-transferase theta 1 [a]*active* (present), if at least one active allele present; [b]*null* (inactive) if no active alleles present; [c]*low activity*, if at least one T allele present; [d]*variant*, if at least one *Val* allele present

Discussion

In this study we showed that the hormonal status of non-obese adolescent girls, newly diagnosed with PCOS could promote increased production of free radicals in response to glucose challenge, based on significant correlations between alteration of oxidative stress markers and testosterone, DHEAS, androstenedione, and LH. Moreover, we found, for the first time, that PCOS girls, carriers of *GSTM1*-null genotype, have significantly lower plasma concentration of testosterone in comparison to PCOS carriers of *GSTM1*-active genotype, probably in relation to the ability of GSTM1 to serve as a steroid-binding protein of testosterone.

The interplay between oxidative stress and low-grade inflammation with hyperandrogenism and insulin resistance has been extensively studied in PCOS [7, 11, 21, 22]. Recent evidence suggest that the major cause of increased generation of ROS in PCOS might be

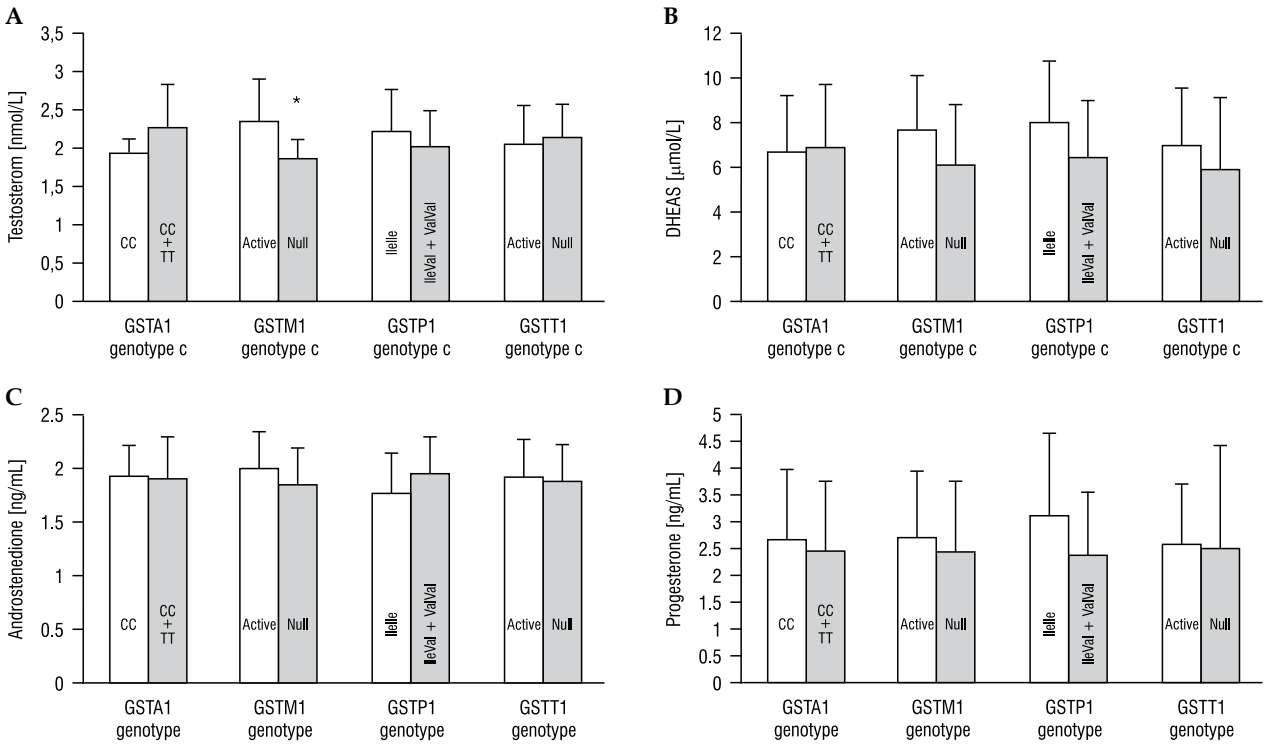

Figure 2. *The concentrations of testosterone (**A**), dehydroepiandrosterone sulphate (**B**), androstenedione (**C**), and progesterone (**D**) in PCOS girls according to* GSTA1, GSTM1, GSTP1, *and* GSTT1 *genotype*
**adolescent girls with* PCOS, *carriers of* GSTM1-null *genotype, presented significantly lower testosterone concentrations as compared to those with the* GSTM1-active *genotype ($p < 0.05$)*
GSTA1 *genotype: low activity, if at least one T allele present (CT + TT);* GSTM1 *genotype: active (present) if at least one active allele present, null (inactive) if no active alleles present;* GSTP1 *genotype: wild type, if both Ile alleles are present, variant, if at least one Val allele present (IleVal + ValVal);* GSTT1 *genotype: active (present) if at least one active allele present, null (inactive) if no active alleles present*

Rycina 2. *Stężenia testosteronu (**A**), siarczanu dehydroepiandrosteronu (**B**), androstenedionu (**C**) i progesteronu (**D**) u dziewcząt z PCOS zgodnie z genotypami* GSTA1, GSTM1, GSTP1 *i* GSTT1. *Dorastające dziewczęta z PCOS, nosicielki genotypu* GSTM1-null, *wykazywały znacząco niższe stężenia testosteronu w porównaniu z dziewczętami z genotypem* GSTM1-active *($p < 0,05$)*
Genotyp GSTA1: *„o niskiej aktywności" (low activity), jeśli obecny jest co najmniej jeden allel T (CT + TT);*
Genotyp GSTM1: *aktywny (active), jeśli obecny jest co najmniej jeden aktywny allel; zerowy (ang. null) (nieaktywny), jeśli brak jest aktywnych alleli;*
Genotyp GSTP1: *typ dziki (wild type), jeśli obecne są oba allele Ile; wariant (ang. variant), jeśli co najmniej jeden allel Val jest obecny (IleVal + ValVal);*
Genotyp GSTT1: *aktywny (active), jeśli obecny jest co najmniej jeden aktywny allel; zerowy (null) (nieaktywny), jeśli brak jest aktywnych alleli*

related to the impairment of the mitochondrial electron transport chain [23], as well as increased enzymatic activities of NADPH oxidase and xanthine oxidase [22, 24]. After oral glucose challenge, a more pronounced increase in proinflammatory cytokine TNFα, activity of NADPH-oxidase, and different oxidative stress by-products was demonstrated in PCOS in contrast to normal weight ovulatory women [11, 21]. It seems that hyperandrogenaemia *per se* induces increased production of free radicals, primarily by enhancing expression and activity of NADPH-oxidase [25]. In a manner similar to PCOS, acute androgen treatment of healthy women causes increased sensitivity of their leukocytes to glucose ingestion [21]. Moreover, a significant transient decrease in the activity of key antioxidant enzyme GPX during glucose challenge test seems to further disturb redox balance and contributes to pro-oxidant state of PCOS women [11]. In this study, as a response to glucose challenge, we found the more pronounced change of oxidative stress by-products, nitrotyrosine, and thiol groups in PCOS girls in comparison to controls, which is in agreement with our previous findings [11, 22]. Finally, data on higher concentrations of plasma oxidative stress parameters, together with unchanged activities of both key antioxidant enzymes GPX and SOD in PCOS adolescent girls suggests subtle disturbances in redox balance in this early phase of PCOS clinical presentation.

Based on the similar values of basal insulin and surrogate indexes of insulin resistance, PCOS adolescents

in the present study were not considered as insulin resistant. Therefore, the increased production of ROS in the PCOS group is probably related to hyperandrogenism. Still, significant inverse correlation between ISI and LH, as well as the association of AUC for glucose with oxidative stress alteration during OGTT (thiol groups) obtained in our study could provide important information towards predicting insulin resistance in newly diagnosed PCOS adolescents. Further examination of potential link between insulin resistance and oxidative stress would also include the investigation of these parameters after euglycaemic-hyperinsulinaemic clamp, especially bearing in mind the recent study on the limited value of surrogate indices in estimation of insulin resistance in PCOS [26]. Nevertheless, the clear associations between plasma testosterone levels, DHEAS, androstenedione and LH, and alternated concentrations of oxidative stress parameters during acute hyperglycaemia support the assumption that hyperandrogenism might be the progenitor of diet-induced oxidative stress in PCOS. For further confirmation on the link between the hormonal status and oxidative stress, especially in predicting early vascular damage, more focused analyses and consequent follow-up of the larger cohort of PCOS patients are needed.

It is important to note that transient decrease in GPX activity together with increasing oxidative stress by-products in healthy controls as a consequence of glucose challenge actually represents physiological response, necessary for proper insulin signalling. Regarding insulin signalling cascade, binding of insulin to its receptor results in enhanced ROS production, by the activation of NADPH oxidases and inhibition of membrane antioxidant enzyme peroxiredoxin I [27]. In that way, oxidative redox environment favours the propagation of the insulin signalling cascade by enhancing kinase activity with simultaneous inhibition of protein tyrosine phosphatase [27, 28]. On the other hand, the excessive load of ROS might affect the activity of redox-sensitive PI_3K–PDK1–Akt signalling axis, as demonstrated for free radical peroxynitrite, contributing to the development of insulin resistance [27]. These findings offer new insight into the role of free radicals and oxidative stress, especially according to new definition, in which the term "oxidative eustress" denotes physiological oxidative stress and serves in the distinction from excessive load, "oxidative distress", which causes oxidative damage [29].

In addition to detoxifying xenobiotics and free radicals, the GST superfamily is also involved in catalysing the conjugation of different endogenous substrates, including steroids and prostaglandins [30]. What is more, GSTs may also act as binding proteins for bile acids, steroid hormones, and neurotransmitters [30]. Almost all cytosolic members of the GSTs exhibit genetic polymorphism, resulting in complete lack or alteration in enzyme activity [30, 31]. Consequently, great inter-individual differences in GST genotype exist, affecting individual biotransformation ability and potential ligand-binding capacity. Specifically, GSTM1 serves as a steroid-binding protein with the ability to bind testosterone and estradiol. To our knowledge, polymorphisms in common cytosolic GSTs have not yet been analysed in such a context in PCOS. Interestingly, we found that in PCOS girls, in whom GSTM1 enzyme is absent as a consequence of GSTM1-deletion polymorphism (carriers of *GSTM1*-null genotype) circulating testosterone was significantly lower in comparison to ones with *GSTM1*-active genotype. Further analysis of the potential role of GSTs in hormonal disturbance in PCOS seems to be a new promising approach in PCOS treatment.

In conclusion, our adolescent girls with PCOS exhibited high production of free radicals together with unchanged antioxidant enzymatic capacity, independently from obesity and insulin resistance. This suggests that increased free radical production, probably as a consequence of hyperandrogenaemia happens in the early phase of PCOS and could contribute to further development of insulin resistance and other metabolic disturbances associated with this syndrome. Therefore, a primary prevention model of reducing oxidative stress by means of antioxidant supplementation and regular exercise training [32], may be beneficial in patients with polycystic ovary syndrome.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

The study was approved by the Ethics Committee of the Mother and Child Health Care Institute of Serbia, and the research was carried out in compliance with the Declaration of Helsinki.

Informed consent

Written, informed consent was obtained from all study participants or from their parents.

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