CLINICS

CLINICAL SCIENCE

Association of glucocorticoid receptor polymorphisms with clinical and metabolic profiles in polycystic ovary syndrome

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OBJECTIVES: We aimed to investigate whether glucocorticoid receptor gene polymorphisms are associated with clinical and metabolic profiles in patients with polycystic ovary syndrome. Polycystic ovary syndrome is a complex endocrine disease that affects 5-8% of women and may be associated with metabolic syndrome, which is a risk factor for cardiovascular disease. Cortisol action and dysregulation account for metabolic syndrome development in the general population. As glucocorticoid receptor gene (*NR3C1*) polymorphisms regulate cortisol sensitivity, we hypothesized that variants of this gene may be involved in the adverse metabolic profiles of patients with polycystic ovary syndrome.

METHOD: Clinical, metabolic and hormonal profiles were evaluated in 97 patients with polycystic ovary syndrome who were diagnosed according to the Rotterdam criteria. The alleles of the glucocorticoid gene were genotyped. Association analyses were performed using the appropriate statistical tests.

RESULTS: Obesity and metabolic syndrome were observed in 42.3% and 26.8% of patients, respectively. Body mass index was positively correlated with blood pressure, triglyceride, LDL-c, total cholesterol, glucose and insulin levels as well as HOMA-IR values and inversely correlated with HDL-c and SHBG levels. The *Bcl*I and A3669G variants were found in 24.7% and 13.4% of alleles, respectively. *Bcl*I carriers presented a lower frequency of insulin resistance compared with wild-type subjects.

CONCLUSION: The *Bcl*I variant is associated with a lower frequency of insulin resistance in women with polycystic ovary syndrome. Glucocorticoid gene polymorphism screening during treatment of the syndrome may be useful for identifying subgroups of at-risk patients who would benefit the most from personalized treatment.

KEYWORDS: Polycystic Ovary Syndrome; Glucocorticoid Receptor; Glucocorticoid Receptor Gene Polymorphisms; Metabolic Profile; NR3C1 Protein.

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INTRODUCTION

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies in women of reproductive age (1). The syndrome's presentation is highly heterogeneous and is characterized by menstrual dysfunction, hyperandrogenism and/or hyperandrogenemia and polycystic ovary morphology (1). Women with PCOS are frequently insulin resistant, regardless of the presence of obesity (1), and have an adverse metabolic profile.

Previous studies have suggested that cortisol dynamics are dysregulated in PCOS, primarily through increased hypothalamic-pituitary-adrenal (HPA) axis activity and enhanced cortisol sensitivity (1). Steroid hormones play a central role in the regulation of body composition. Glucocorticoids (GCs) have numerous effects, including regulation of fat distribution, lipid metabolism and insulin sensitivity (2); therefore, GC dysregulation may play a role in several PCOS manifestations.

The glucocorticoid receptor (GR) is crucial for the effects of GCs, and several GR gene (*NR3C1*) polymorphisms have been associated with altered GC sensitivity, suggesting that

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these polymorphisms can modulate the development of metabolic disorders (2,3). In the general population, several *NR3C1* polymorphisms are associated with differences in body composition, metabolic parameters and cardiovascular disease. For example, the *Bcl*I *NR3C1* polymorphism, located in intron 2, has been associated with increased GC sensitivity and an adverse metabolic profile (4-6). Similarly, the N363S polymorphism was reported to be associated with enhanced GC sensitivity and was linked to an increased body mass index (BMI), elevated LDL cholesterol levels and an increased risk of cardiovascular disease (7-9). In contrast, the A3669G and ER22/23EK polymorphisms are associated with decreased GC sensitivity and relative GC resistance, and GC resistance has been associated with a favorable metabolic profile and body composition in addition to a smaller decrease in cortisol levels after the dexamethasone suppression test (DST) (2). Furthermore, *NR3C1* polymorphisms are associated with the classical form of 21-hydroxylase deficiency, another hyperandrogenic condition (10).

Given the influence of GCs on body composition and metabolic control, we hypothesized that genetic variations might be linked to PCOS features. Thus, the aim of this study was to evaluate the influence of *NR3C1* polymorphisms (*Bcl*I, A3669G and ER22/23EK) on the phenotypic expression of PCOS.

MATERIALS AND METHODS

The procedures used were in accordance with the guidelines of the Helsinki Declaration on human experimentation. The study protocol was approved by the Ethics Committee of the Hospital das Clínicas da Universidade de São Paulo, and written informed consent was obtained from all the women before the start of the study.

Subjects

In total, 97 PCOS patients were selected for this study; their mean age was 24.9±5.1 years old. The patients' mean BMI was 29.6±6.9 kg/m^2, and their mean waist circumference was 90.1±15.2 cm. All subjects were referred for evaluation of hirsutism and/or menstrual abnormalities.

Eumenorrhea was defined as the presence of menstrual cycles between 25 and 34 days. At least two consecutive cycles with low progesterone levels (<3 ng/mL) were required for a diagnosis of anovulation. Oligomenorrhea was defined as the presence of three or more cycles of >35 days in the previous 6 months and amenorrhea due to a lack of vaginal bleeding for 3 months. Hypermenorrhea was defined as the presence of vaginal bleeding at intervals of <21 days.

PCOS diagnosis was performed according to the Rotterdam Consensus (11). Cushing's syndrome, late-onset 21-hydroxylase deficiency, thyroid dysfunction, hyperprolactinemia and androgen-secreting tumors were ruled out using the appropriate tests. None of the women had any other diseases or had taken any medications for at least 6 months prior to the study.

Abdominal or pelvic ultrasonography was performed on all subjects. The presence of polycystic ovaries was established by the presence of 12 or more follicles in each ovary measuring 2-9 mm in diameter and/or an ovarian volume greater than 10 mL.

Study protocol

BMI and waist circumference were measured in all subjects. The presence of hirsutism was quantified using the modified Ferriman-Gallwey (mFG) score, and the presence of acne was also evaluated.

All blood samples were collected between 7:30 and 8:30 A.M. after an overnight fast. Blood samples were collected from days 1-3 of the cycle in women with regular menstrual cycles. For those patients with menstrual abnormalities, sampling was performed regardless of the time of the last menstrual bleeding, and serum progesterone (P) was assayed to confirm the absence of ovulation at the time of the assessment.

For the oral glucose tolerance test (OGTT), a basal sample was obtained for the determination of glucose, insulin, triglycerides (TGs) and total cholesterol (TC) as well as TC fractions (LDL-c and HDL-c). A glucose load of 75 g was then given, and blood was drawn after 30, 60, 90 and 120 minutes for the determination of glucose and insulin levels. Insulin sensitivity was estimated using the homeostasis model assessment for insulin resistance (HOMA-IR), which was calculated as follows: insulin (μU/mL) × glucose (mg/dL)/22.5×18.

Metabolic syndrome was defined according to the Adult Treatment Panel III (NCEP-ATP III) criteria of the National Cholesterol Education Program as the presence of three or more of the following abnormalities: waist circumference >88 cm, fasting glucose ≥110 mg/dL and/or glycemia (glucose≥140 mg/dL) 120 minutes after the OGTT, fasting serum TGs≥150 mg/dL, serum HDL-c<50 mg/dL and blood pressure≥130/85 mmHg (12). High blood pressure was defined as values≥135/85 mmHg.

METHODS

Glucose, lipid and lipoprotein levels were measured in plasma. For the hormone assays, blood samples were processed by centrifugation, and the serum was stored at -20℃ until the assay. Progesterone, testosterone, androstenedione, insulin, LH and FSH were measured by an immunofluorometric assay (Wallac, Finland), and DHEAS levels were measured by a radioimmunoassay (Cisbio International, France and DSL, TX, USA). SHBG levels were measured by immunofluorometric assay. All the assays were performed in duplicate, and the intra-assay and inter-assay coefficients of variation did not exceed 10% and 15%, respectively.

Genetic analysis

DNA samples were obtained from peripheral blood leukocytes by salting-out procedures. PCR amplification of the GR gene regions was performed using the primer sequences and amplification conditions described previously (13,14).

The A3669G polymorphism is located in the 3′ untranslated region of exon 9b at nucleotide position 3669 (an A-to-G alteration), and this polymorphism was genotyped by sequencing. PCR products were sequenced using the BigDye Terminator Sequencing Kit™ (Applied Biosystems, Inc., Foster City, CA, USA), and capillary electrophoresis was performed using an ABI PRISM 3100 sequencer (Applied Biosystems, Inc.).

The ER22/23EK polymorphism comprises two linked single-nucleotide variations that are separated by one base pair in exon 2. The first substitution at nucleotide position

198 is silent, changing codon 22 from GAG to GAA. The second mutation changes codon 23 at nucleotide position 200 from AGG to AAG. The N363S polymorphism changes codon 363 of exon 2 at nucleotide position 1220 from AAT to AGT. Sequence traces were analyzed using Sequencher (version 4.5, build 1416).

The *Bcl*I polymorphism results in an intronic change (C to G) that occurs 646 nucleotides downstream from exon 2. This polymorphism was screened using allele-specific PCR, as previously described (14). The results of the allele-specific PCR were confirmed by direct sequencing in 20 patients.

Statistical analysis

Categorical or ordinal variables were represented as proportions and compared using the Chi-squared test. Continuous variables were tested for normality using the Shapiro-Wilk test with subsequent observation of the histograms to determine the distribution of the data. The normally distributed data are represented as means with standard deviations and compared using a Student's t test; non-parametric data are represented as the median and compared using the Mann-Whitney test. Multivariate analysis was performed using a logistic regression model for categorical variables. $P<0.05$ indicated a significant difference.

Hardy-Weinberg equilibrium was calculated for the *Bcl*I and A3669G variants. Statistical analysis was performed using SigmaStat software (version 3.5 for Windows; Systat Software, Point Richmond, CA).

RESULTS

Clinical profile of the PCOS patients

The clinical, hormonal and anthropometric data of the PCOS patients are described in Table 1. Obesity was observed in 41 patients (42.3%), and 26 patients were overweight (26.8%). Hypertension was observed in 17 patients (17.5%, Table 1). Metabolic syndrome, increased TG levels (≥150 mg/dL) and low HDL-c levels were observed in 26 (26.8%), 22 (22.7%) and 51 patients (52.6% of cases), respectively.

Decreased serum HDL-c levels were the most frequent component of metabolic syndrome (52.6%), followed by an increased waist circumference (47.4%), high serum TG levels (22.7%), high blood pressure (17.5%) and high fasting plasma glucose levels (4.1%). As expected, the frequencies of the metabolic syndrome components were higher in the obese patients compared with the non-obese patients (data not shown, $p<0.05$), independent of androgen levels.

Influence of GR polymorphisms on the metabolic profile of the PCOS patients

The allelic frequencies of the *Bcl*I and A3669G *NR3C1* polymorphisms were in Hardy-Weinberg equilibrium. The *Bcl*I polymorphism was found in 24.7% of the alleles: eight homozygous patients and 32 heterozygous patients. The A3669G polymorphism was found in 13.4% of the alleles: two homozygous patients and 22 heterozygous patients. The N363S and ER22/23EK alleles were identified in one patient who was heterozygous for each polymorphism. Considering that these alleles were present at a lower frequency and that few patients were homozygous, we considered the homozygous and heterozygous patients as a single group of patients defined as 'carriers'. For association analysis, we selected only polymorphisms with a frequency ≥10% (*Bcl*I and A3669G). Comparisons of clinical, hormonal and laboratory data between carriers and non-carriers of the *Bcl*I polymorphism are shown in Table 2.

Table 1 - Clinical, laboratory and anthropometric characteristics of the 97 patients with polycystic ovary syndrome.

Variables	Patients with polycystic ovary syndrome
Age, mean [SD], years	24.9 (5.1)
Menstrual pattern, n (%)	
Prolonged menstrual cycles	45.8
Amenorrhea	62.5
Eumenorrhea	6.2
Hypermenorrhea	6.2
BMI, mean [SD], kg/m^2	29.6 (6.9)
Waist circumference, mean [SD], cm	90.1 (15.2)
Fasting glucose, mean [SD], mg/dL	89.1 (10.3)
Insulin, mean [SD], mU/L	16.6 (12.7)
HOMA-IR index, mean [SD]	3.8 (3.3)
Total cholesterol, mean [SD], mg/dL	171 (31.6)
HDL-c, mean [SD], mg/dL	50.4 (14.1)
LDL-c, mean [SD], mg/dL	98.6 (26.1)
Triglycerides, mean [SD], mg/dL	115.2 (62.1)
Obesity, n (%)	41 (42.3)
Hypertension, n (%)	17 (17.5)
Diabetes, n (%)	4 (4.1)
Metabolic syndrome, n (%)	26 (26.8)
Acne, n (%)	47 (48.5)
Insulin resistance, n (%)	50 (51.5)
Testosterone, mean [SD], ng/dL	93.4 (39.4)
Free testosterone, mean [SD], pmol/L	68.4 (39.9)
SHBG, mean [SD], nmol/L	34.3 (19)
Androstenedione, mean [SD], ng/mL	3.8 (1.4)
Ferriman-Gallwey score, mean [SD]	12.5 (6.3)
*Bcl*I carriers, n (%)	40 (41.2)
A3669G carriers, n (%)	24 (24.7)

BMI, body mass index.

Table 2 - Influence of the *Bcl*I polymorphism on the clinical, hormonal and metabolic profiles of patients with polycystic ovary syndrome.

Variable	*Bcl*I (N = 40)	Wild-type (N = 57)	*p*-value
BMI, mean [SD], kg/m^2	28.5 (6.8)	30.5 (7)	0.160
Waist circumference, mean [SD], cm	87.6 (14.5)	91.7 (15.6)	0.225
Obesity, n, (%)	13 (31.7)	28 (68.3)	0.155
Hypertension, n, (%)	6 (35.3)	11 (64.7)	0.782
Metabolic syndrome, n, (%)	8 (30.8)	18 (69.2)	0.301
Insulin resistance, n, (%)	15 (30)	35 (70)	*0.035*
Fasting glucose, mean [SD], mg/dL	88.7 (9.9)	89.4 (10.7)	0.733
Insulin, mean [SD], mU/L	13.3 (9.2)	18.8 (14.3)	0.06
HOMA-IR index, mean [SD]	2.98 (2.19)	4.35 (3.68)	0.076
Total cholesterol, mean [SD], mg/dL	176 (32.2)	167.4 (30.9)	0.192
HDL-c, mean [SD], mg/dL	53 (15.5)	48.6 (13)	0.193
LDL-c, mean [SD], mg/dL	102 (26.3)	96.4 (26)	0.312
Triglycerides, mean [SD], mg/dL	108 (47.7)	120 (70.5)	0.748
Testosterone, mean [SD], ng/dL	94.5 (33.4)	92.7 (43.6)	0.821
SHBG, mean [SD], nmol/L	32.7 (16.4)	35.6 (20.9)	0.761
DHEAS, mean [SD], ng/dL	2,518 (1140)	2,276 (1208)	0.332
Androstenedione, mean [SD], ng/mL	4.1 (1.4)	3.7 (1.5)	0.231
Ferriman-Gallwey score, mean [SD]	11.9 (6.5)	12.9 (6.3)	0.423

BMI: body mass index.

There were no significant differences observed in BMI values; lipid, glucose and insulin levels; mFG scores; or MetS frequencies between carriers and non-carriers of the *Bcl*I polymorphism (Table 2). *Bcl*I carriers presented a lower frequency of insulin resistance (IR) compared with the non-carrier subjects (30% *vs*. 61.4%, $p = 0.03$; Table 2). There were no significant differences in the frequencies of the *Bcl*I polymorphism between the obese and non-obese patients (31.7% and 48.2%, respectively) or between patients with and without metabolic syndrome (30.8% and 45.1%, respectively).

Table 3 shows the clinical and laboratory data of A3669G and wild-type carriers. No differences were identified in cardiovascular risk factors such as BMI, waist circumference, blood pressure, HOMA-IR and IR, and differences were not found between the hormonal and lipid profiles. No significant differences were found in the frequencies of the A3669G polymorphism between the obese and non-obese patients (34.1% *vs*. 17.9%, respectively) or between patients with and without metabolic syndrome (26.9% *vs*. 23.9%, respectively).

The presence of *NR3C1* polymorphisms was not associated with either androgen (testosterone, DHEA and DHEAS) or gonadotropin (LH and FSH) levels or with menstrual cycle patterns (data not shown).

DISCUSSION

In this study, we investigated the influence of several GR polymorphisms on the metabolic profile of PCOS patients and found that the *BclI* variant is associated with lower IR in PCOS patients.

As expected, several features of metabolic syndrome, including IR, obesity and dyslipidemia, were present in PCOS patients, suggesting an increased risk of cardiovascular disease in this group of patients (15).

Table 3 - Influence of the A3669G polymorphism on the clinical, hormonal and metabolic profiles of patients with polycystic ovary syndrome.

Variable	A3669G (N = 24)	Wild-type (N = 73)	*p*-value
BMI, mean [SD], kg/m^2	31.9 (6.9)	28.9 (6.9)	0.071
Waist circumference, mean [SD], cm	91.5 (15.9)	89.6 (15.1)	0.618
Obesity, n, (%)	14 (34.1)	27 (65.9)	0.110
Hypertension, n, (%)	5 (29.4)	12 (70.6)	0.856
Metabolic syndrome, n, (%)	7 (26.9)	19 (73.1)	0.972
Insulin resistance, n, (%)	15 (30)	35 (70)	0.316
Fasting glucose, mean [SD], mg/dL	88.9 (12.5)	89.1 (9.6)	0.794
Insulin, mean [SD], mU/L	19.1 (14.7)	15.9 (12.1)	0.401
HOMA-IR index, mean [SD]	4.5 (3.6)	3.6 (3.1)	0.338
Total cholesterol, mean [SD], mg/dL	173.7 (29.4)	170.1 (32.4)	0.631
HDL-c, mean [SD], mg/dL	48.2 (12.4)	51.1 (14.7)	0.532
LDL-c, mean [SD], mg/dL	99.7 (30.3)	98.3 (24.8)	0.829
Triglycerides, mean [SD], mg/dL	124.7 (69.3)	112.1 (59.7)	0.543
Testosterone, mean [SD], ng/dL	96 (40.7)	92.7 (39.3)	0.730
SHBG, mean [SD], nmol/L	28.2 (12.1)	36.5 (20.6)	0.170
DHEAS, mean [SD], ng/dL	2,408 (1,227)	2,362 (1,174)	0.874
Androstenedione, mean [SD], ng/mL	4.2 (1.8)	3.7 (1.3)	0.209
Ferriman-Gallwey score, mean [SD]	12.8 (5.5)	12.4 (6.6)	0.882

BMI, body mass index.

In addition to the aforementioned factors, familial and/or genetic predisposition may be involved in the increased cardiovascular risk in PCOS patients. The influence of genetic polymorphisms on the metabolic profile of PCOS patients has been studied, and positive associations have been found with lipid profiles (16), insulin and homocysteine levels (17,18), high body weight and abdominal adiposity (19).

The influence of *NR3C1* polymorphisms on cardiovascular risk in the general population has been analyzed, and several are associated with changes in GC sensitivity, which influence the metabolic profile of their carriers (2-4,20-23). In PCOS patients, two studies have investigated the association between *NR3C1* polymorphisms and hormonal profiles. The first study evaluated a rare *NR3C1* gene allele, N363S, and its impact on the frequency of androgen excess. However, this polymorphism was found in only 3.3% of patients, and no significant difference was found between the frequencies of PCOS patients with and without androgen excess (24). The second study found a positive association between the A3669G and ER22/23EK polymorphisms, which decrease GC sensitivity, and changes in luteinizing hormone levels (25). For the first time, we have analyzed the impact of *NR3C1* polymorphisms on the metabolic profile of PCOS patients.

GC actions are mediated through the functional isoform of the GR, GR-α. An alternative isoform, GR-β, behaves as a dominant negative inhibitor of GR-α and is implicated in GC resistance. The A3669G polymorphism, located in exon 9β, increases GR-β protein expression, resulting in greater inhibition of GR-α and a subsequent increase in relative GC resistance. This carrier status could result in a phenotype that protects against the undesirable effects of GCs on fat distribution and glucose metabolism (2). However, we did not identify any differences between the metabolic profiles of carriers and non-carriers.

The *Bcl*I restriction fragment length polymorphism has been linked to a cluster of cardiovascular risk factors in men, including hypertension, obesity and IR (4,5,20). However, these associations were not found in our study. In contrast to what has been described in the literature, we found a positive association between the *Bcl*I variant and lower IR (Table 2), which may be due to the fact that our study group was exclusively composed of women. Indeed, gender-specific effects of *NR3C1* gene polymorphisms on anthropometric and metabolic variables have been observed in several studies (2,23,26,27). For example, in a recent study, *NR3C1* polymorphisms were associated with reduced first-phase glucose-stimulated insulin secretion and the disposition index in women, but not in men (28). Additionally, gender-related hormonal factors are known to affect β-cell function, as women receiving estrogen replacement therapy display a reduced prevalence of diabetes, which has been associated with the β-cell-protective effects of estrogens (29). Furthermore, the male sex hormone testosterone may also affect β-cell function (30). Therefore, it has been speculated that *NR3C1* gene polymorphisms could interact differently with sex hormones to affect β-cell function and consequently present different phenotypes.

The main weakness of our study is the small number of PCOS patients analyzed, which prevented us from drawing definite conclusions about the relationship between *NR3C1* polymorphisms and other clinical features; however, the findings of our study suggest that the *Bcl*I and A3669G polymorphisms may play a protective role. Multicentric studies are needed to further explore these early findings.

In conclusion, this is the first report revealing the influence of the *Bcl*I and A3669G polymorphisms on the metabolic profile of PCOS patients. In the future, polymorphism screening during PCOS treatment may potentially be used to improve the quality of treatment by identifying subgroups of at-risk patients who would benefit the most from personalized treatment.

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■ AUTHOR CONTRIBUTIONS

Maciel GA and Bachega TA conceived and designed the study, interpreted the data and wrote the manuscript. Moreira RP performed all the molecular experiments and discussed the findings. Bugano DD performed the statistical analysis, discussed the findings and assisted with writing the manuscript. Marcondes JA performed the ACTH and dynamic tests and assisted with data interpretation. Hayashida SA saw the patients and was responsible for the clinical data analysis. Gomes LG saw several of the patients and assisted with the writing. Mendonca BB and Baracat EC were responsible for mentorship and institutional support for the laboratories and the clinical setting.

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