Serum anti-carbonic anhydrase I and II antibodies and polycystic ovary syndrome

ABSTRACT

Aim: The aim of this study is to investigate anti-carbonic anhydrase antibodies (anti-CA I and CA II antibodies) in the sera of women with polycystic ovary syndrome (PCOS).

Methods: In this study serum anti-CA I and II antibody levels of age and BMI matching fifty women with PCOS and fifty women without PCOS on day three of menstrual cycle were assessed with an ELISA method previously developed by Hosoda and modified by Alver et al.

Results: The mean serum anti-CA I antibody levels were significantly higher in women with PCOS and anti-CA II antibody levels were not significantly different in women with PCOS compared with control subjects. For serum anti-CA I antibody, the absorbance higher than 0.484 (mean + 3SD of control subjects) was taken as positive, Anti-CA I antibody was detected in 13 of 50 patients with PCOS (26%). Considering serum anti-CA II antibody, the absorbance higher than 0.654 (mean + 3SD of control subjects) was taken as positive. Anti-CA II antibody was detected in 2 of 50 patients with PCOS (4%). All patients with positive anti-CA II antibody also had positive anti-CA I antibody. Autoantibodies specifically reactive to CA I were found to be present at a higher frequency than CA II in the serum of subjects with PCOS in the present study.

Conclusion: The results may suggest that autoimmune responses against CA I may be involved in the pathogenesis of PCOS.

Keywords: autoantibody, carbonic anhydrase I, carbonic anhydrase II, polycystic ovary syndrome.

Conflict of Interest

The authors had not personal relationships with other individuals or organizations that might inappropriately influence their work during the submission process and last twenty four months.

ÖZET

Amaç: Bu çalışmanın amacı polikistik over sendromlu (PCOS) kadınlarının serumlarında anti karbonik anhidraz antikorlarının (CA I ve CA II otoantikor) düzeylerini araştırmaktır.

Yöntem: Bu çalışmada yaş ve BMI (beden-kütle indeksi) uyumlu 50 PCOS’lu ve 50 PCOS olmayan kadının menstrual sikluslarının üçüncü gününde alınan serum örneklerinde anti CA I ve CA II seyriyeleri, daha önce Hosoda tarafından geliştirilmiş, Alver ve arkadaşları tarafından değiştirilmiş olan ELISA yöntemi kullanılarak ölçüldü. Anti-CA I antikor düzeyi için 0,484'ten (kontrollerin ortalama + 3SD) yüksek değerler pozitif olarak kabul edildi ve 50 PCOS’lu hastanın 13’ü pozitif olarak tespit edildi (% 26). CA II antikor düzeyleri ise 0,654'ten (kontrollerin ortalama + 3SD) yüksek değerler pozitif olarak değerlendirildi. Anti-CA II PCOS’lu bireylerden 2 tanesinde pozitif olarak bulundu (% 2). CA II antikoru pozitif bulunan tüm bireylerin CA I otoantikorları da pozitif değerlerde bulundu. PCOS’lu hastalarında CA II otoantikorunun bulunma sıklığı CA I’ye göre daha yüksek bulundu.

Sonuç: Sonuç olarak CA I proteinine karşı olan otoimmün yanıtın, PCOS’un patogenezinde rolü olabileceği kanısına varıldı.

Anahtar Kelimeler: otoantikor, karbonik anhidraz I ve II, polikistik over sendromu
Introduction

Polycystic ovary syndrome (PCOS) is currently one of the most frequent endocrine/metabolic disorders, occurring in 6-8% of women of fertile age and a cause of infertility. PCOS is now thought to be a complex genetic trait, similar to cardiovascular disease, type 2 diabetes mellitus, and the metabolic syndrome, where multiple genetic variants and environmental factors interact to foster the development of the disorder [1]. The Androgen Excess Society proposed that PCOS should be diagnosed by the presence of three features: androgen excess (clinical and/or biochemical hyperandrogenism), ovarian dysfunction (oligo-anovulation and/or polycystic ovarian morphology), and exclusion of other androgen excess or ovulatory disorders [2]. Although exact pathophysiological mechanisms are not clear, premature adrenarche, obesity, insulin resistance, type 2 diabetes mellitus, endometrial carcinoma, ovulatory dysfunction, androgen excess, polycystic ovaries, complex genetic trait, cardiovascular disease and metabolic syndrome were associated with PCOS [3-6].

Several autoimmune diseases have been demonstrated in women with PCOS and in sera of those, various systemic and organ-specific autoantibodies have been recognized [6-8]. Some of these autoantibodies are antinuclear antibodies, anti-histone, anti-nucleosome, anti-dsDNA antibodies, smooth muscle cells, liver-kidney microsome, thyroid microsome, gastric parietal cell, reticulin, mithocondrial antibodies, autoimmune thyroiditis, thyroperoxidase or thyroglobulin antibodies and thyrotropin [6].

Carbonic anhydrases (EC 4.2.1.1) (CAs) are zinc-containing enzymes and sixteen CA isoenzymes (CA I-XVI) have been described up to now and shown that their localization and tissue distribution are very different in mammals. These enzymes catalyze a very simple physiological reaction, the inter conversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of CO₂/bicarbonate between metabolizing tissues and lungs, pH and CO₂ homeostasis, electrolyte secretion in a variety of tissues/organs, and biosynthetic reactions and many other physiologic or pathologic processes including reproductive tract [9,10]. Anti CA I and/or CA II autoantibodies were determined in the sera of subjects with recurrent pregnancy loss, pre-eclampsia, metabolic syndrome, endometriosis and Graves’ disease [11-16]. However, the role of anti CA I and/or CA II autoantibodies on PCOS patients has not been reported previously.

The aim of this study is to investigate CA I and CA II antibodies in the sera of women with PCOS based on the information and considerations of autoimmune relation of PCOS and bring a new insight to autoimmune base of PCOS.

Materials and Methods

This study was conducted with patients with PCOS and women without PCOS hospitalized at the Department of Obstetrics and Gynecology of the Karadeniz Technical University, Faculty of Medicine, Turkey. The study was approved by the local ethical committee.

Patient selection

Following the allocation of women with PCOS to the study group (n = 50), women who were age- and BMI-matched with the study group were selected for the control group. The control group comprised of healthy, regularly menstruating women (n = 50) attending the gynaecology unit for routine gynaecological examination. The women in control group had no history of autoimmune diseases and had no abnormalities in serum hepatic, renal function tests.

PCOS was diagnosed according to Androgen Excess Society criteria (the presence of three features: androgen excess (clinical and/or biochemical hyperandrogenism), ovarian dysfunction (oligo-anovulation and/or polycystic ovarian morphology), and exclusion of other androgen excess or ovulatory disorders) [2]. All women had normal renal, hepatic and thyroid function. Androgen excess was quantified by one examiner using the Modified Ferriman–Gallwey scoring system, in which a score >8 indicates hyperandrogenism.

Exclusion criteria were as follows: (i) endocrinopathies (including diabetes mellitus, hyperprolactinaemia, Cushing’s disease and congenital adrenal hyperplasia), a systemic disease (e.g., asthma), a collagen disorder, hypercholesterolaemia, sickle cell anaemia or a history of neoplasm; (ii) patients with hypertension, a family history of coronary artery disease and any known vascular, infectious or inflammatory diseases; (iii) use of any medication (e.g., insulin-sensitizing drugs, oral contraceptives, antiandrogens, statins, aspirin, corticosteroids and gonadotropin-releasing hormone agonists and antagonists) in the preceding 3 months; (iv) current smoker; (v) abnormal renal, hepatic and thyroid function test results; and (vi) refusal to participate in the study.

A total of 5mL blood sample for each subject was collected in serum separator gel containing vacutainer tube. After clotting, blood samples were centrifuged at 3000 rpm for 10 minutes. Serum samples were stored at -80°C until anti-CA I and II antibodies measurements.

Enzyme-linked immunosorbent assay (ELISA) for serum autoantibody to CA I and CA II

Human CA I and II, electrophoretically purified from erythrocytes, were purchased from Sigma Chemical Co. (St. Louis, MO). Serum anti-CA I and CA II were detected by ELISA according to a previously described method [13]. Briefly, microtiter plates (high binding, flat-bottomed plates; Bioscience) were coated with 50 µL of
10 µg/mL CA I or CA II in carbonate buffer (0.05 mM, pH 9.6) and incubated overnight at 4°C. The wells were washed four times with phosphate buffer (pH=7.4) and blocked with 3% skim milk in phosphate buffer for 2 h at room temperature (RT). After being washed four times with phosphate buffer containing 0.05% Tween-20, wells were incubated with 100 µL of serum diluted with dilution buffer included 1% skim milk in phosphate buffer (1:200) for 2 h at RT. After washing, each well was incubated for 2 h at RT with 100 µL of 1:2000 dilution of peroxidase-conjugated anti-human IgG anti-serum (Sigma) in dilution buffer. Following five washes with phosphate buffer containing 0.05% Tween-20, wells were incubated with 100 µL substrate solution for 20 min at RT. The reaction was stopped by the addition of 100 µL of 2M H₂SO₄ to each well. The absorbance was read at 480 nm. Control wells that were not coated with CA I and II were also used for ELISA of each serum studied.

All assays were performed in duplicate, and the specific binding of serum antibody to CA II or CA I was calculated as follows: the average absorbance of the antigen coated wells minus the average absorbance of control wells (Specific binding=A coated -A control).

Analytical performance characteristics had been evaluated in a previous study in our laboratory [13]. According to the study the samples from a positive Rheumatoid arthritis (RA) patient (absorbance: 0.965) and a control subject (absorbance: 0.140) were used for determination of intra-assay coefficient of variation. For anti-CA II antibody assay, the intra-assay coefficient of variation was 8.2% (n=8) in RA patient, and 5.5% (n=8) in control subject. And also in an unpublished study in our laboratory on patients with endometriosis CV % values were calculated and found 3% for positive patients and 9% for negative controls.

Statistical analysis

The rates of demographic characteristics were compared using Fisher’s exact chi-squared test. Results for anti CA I and II are expressed as mean ± SD. Descriptive statistical analysis was performed for all the studied variables. Anti-CA antibodies concentrations in each group were tested for normal distribution using the Kolmogorov–Smirnov test. The differences between the PCOS and control groups were investigated with Mann-Whitney’s U-test or student-t test (for demographic characteristics). The relationship between serum levels of the anti-CA antibodies were assessed by using Pearson’s correlation coefficient analysis. The area beneath the receiver operating characteristic (ROC) curves was used to determine the discriminative power of the anti-CA antibodies in the diagnosis or exclusion of PCOS. Sensitivity, specificity, negative predictive values (NPV) and positive predictive values (PPV) were calculated according to ROC curves for PCOS. Statistical significance was assumed at a level of p<0.05.

Results

The mean ages of the women in PCOS and control groups were 23.82 ± 5.47 and 24.38 ± 3.73, respectively. There were no significant differences in demographic factors among the groups (Table 1).

The mean serum anti-CA I (0.311 ± 0.180 absorbance unit (ABSU) vs. 0.190 ± 0.098 ABSU, p<0.0001) antibody levels were significantly higher in women with PCOS and anti-CA II (0.332 ± 0.174 ABSU vs. 0.333 ± 0.107 ABSU, p>0.05) antibody levels were not significantly different in women with PCOS compared with control subjects, respectively.

Table 1. Demographic characteristic of women with PCOS and controls.

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>PCOS (n=50)</th>
<th>Controls (n=50)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year)</td>
<td>23.82 ± 5.47</td>
<td>24.38 ± 3.73</td>
<td>0.551&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Gravida (no.)</td>
<td>0.48 ± 0.89</td>
<td>1.98 ± 0.77</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Parity (no.)</td>
<td>0.32 ± 0.71</td>
<td>1.78 ± 0.65</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.51 ± 3.88</td>
<td>25.26 ± 3.77</td>
<td>0.746&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ferriman-Gallwey score (no.)</td>
<td>13.00 ± 4.14</td>
<td>4.06 ± 1.86</td>
<td>&lt;0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Patients with androgen excess (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50 (100%)</td>
<td>0</td>
<td>&lt;0.001&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Patients with ovarian dysfunction (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>50 (100%)</td>
<td>0</td>
<td>&lt;0.001&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Oligo-anovulation (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32 (64%)</td>
<td>0</td>
<td>.</td>
</tr>
<tr>
<td>Polycystic ovarian morphology (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36 (72%)</td>
<td>0</td>
<td>.</td>
</tr>
</tbody>
</table>

Values are given as mean and standard deviation or *number of cases and percentages in parentheses.
<sup>a</sup>Student-t test, <sup>b</sup>Mann-Whitney U test, <sup>c</sup>Fisher exact Chi-squared test, were used for comparison.
The anti-CA I antibody levels in patients with PCOS and controls were quantified by ELISA at serum dilution of 1:200 (Fig. 1a). The mean absorbance value for the control subjects was 0.190 ± 0.098 (n = 38) and the absorbance higher than 0.484 was taken as positive (mean + 3SD of control subjects). Anti-CA I antibody was detected in 13 of 50 patients with PCOS (26%, 0.311 ± 0.180).

The anti-CA II antibody levels in patients with PCOS diagnosis and control subjects were quantified by ELISA at serum dilution of 1:200 (Fig. 1b). The mean absorbance value for the control subjects was 0.333 ± 0.107 (n = 50) and the absorbance higher than 0.654 was taken as positive (mean + 3SD of control subjects). Anti-CA II antibody was detected in 2 of 50 patients with PCOS (4%, 0.332 ± 0.174).

All patients with positive anti-CA II antibody also had positive anti-CA I antibody. We found significant positive correlation between anti-CA I antibody and anti-CA II antibody titers in patients with PCOS (r = 0.474, p < 0.05) (Fig. 1c).

For evaluation of laboratory findings using the ROC curve method, the optimum diagnostic cut off points for certain parameters were; anti-CA I antibody: 0.190, anti-CAII antibody: 0.330. The specified cut off points and the sensitivity, specificity, PPV, NPV and the area underneath the ROC curve (AUC) belonging to those cut off points are shown in Table 2 and Figures 2a and 2b.

**Discussion**

In this, age and BMI matched study, we showed that serum anti CA I antibodies have a higher concentration in women with PCOS than in those with without PCOS. This is the first report describing significant increases in serum anti-CA I antibody concentrations in women with PCOS. These findings may support the autoimmune based pathophysiology of PCOS. PCOS is characterized by a chronic hyperandrogenic anovulatory state associated with a number of clinical symptoms. The main pathophysiological mechanisms of PCOS are inherent ovarian dysfunction, disturbances of the hypothalamic-pituitary-ovarian axis by external and internal factors and hyperinsulinaemia [17]. Besides these, nowadays, autoimmune disturbances have also been demonstrated in some cases [6, 8]. Although, many organ specific antibodies were studied in PCOS, anticarbonic anhydrase antibodies have not been well studied previously. In one study, only in nine patients with PCOS, endometriosis, and unexplained infertility, the presence of anti-CA I antibodies were demonstrated [8]. However the literature review failed to show any study reporting statistically significant difference in anti-CA antibodies’ levels between women with and without PCOS.

CAs have a wide tissue and organ distribution in human body. A monoclonal antibody (SP-1) raised against this protein was found to react with the ductal cells of several exocrine organs, and with human CA II [18]. Many studies

![Figure 1a](image1.png) **Figure 1a** Anti-CA I antibodies in sera from women with PCOS and healthy controls. The dotted line indicates the mean value plus 3SD of healthy control sera (A<sub>480</sub> = 0.484). *Significant difference in comparison of mean ± SD value between women with PCOS and healthy controls (p < 0.0001).

![Figure 1b](image2.png) **Figure 1b** Anti-CA II antibodies in sera from women with PCOS and healthy controls. The dotted line indicates the mean value plus 3SD of healthy control sera (A<sub>480</sub> = 0.654). *Significant difference in comparison of mean ± SD value between women with PCOS and healthy controls (p > 0.05).

![Figure 1c](image3.png) **Figure 1c** Correlation between anti-CA I antibody titer and anti-CA II antibody titer in patients with PCOS. Dotted lines indicate the mean value + 3 SD of healthy control sera absorbance (A<sub>480</sub>) in both antibodies as described in results and in legends to Fig. 1a Fig. 1b.
have reported that antibodies against CA II and CA I exist in sera of patients with autoimmune disease with endocrine organ originated [13, 14, 19-21]. As it is known, PCOS is also an endocrine organ originated disease, and it is highly suspected to be involved in autoimmune based pathogenesis. A recent case report about PCOS and hyperthyroid Graves’ disease, also supports this suggestions [22].

In this study, we revealed the association of PCOS with propensity for CA I and CA II autoantibodies. Although our findings showed that there is an increased immune response to CA I, no immune response is against to CA II in PCOS women. We found 13 (26%) anti-CA I and 2 (4%) anti-CA II antibody positive subjects in 50 PCOS women. It has been suggested that autoimmune mechanisms may be involved, and antibodies against different candidate auto antigens, including carbonic anhydrases as a group of metalloenzymes widely distributed in human tissues, have been demonstrated in subjects with some autoimmune diseases [8]. Although previously CA II autoantibodies have been observed in various autoimmune and idiopathic diseases, such as Systemic Lupus Erythematosus (SLE) (28.5%), Graves’ disease (25%), Sjögren syndrome (SjS) (62%), and primary biliary cirrhosis (25%), rheumatoid arthritis (27.8%), endometriosis (69.6%) [8, 19-21], according to our findings there were no significant differences in anti-CA II antibody frequencies between PCOS patients and controls (p>0.05).

Serum anti-CA I antibodies have been reported in SLE, SjS and primary biliary cirrhosis [8,19-21] . In contrast with CA II, CA I is expressed mainly in erythrocytes. So, it is not known why autoantibody reactivated with CA I is produced in patients with various autoimmune diseases. In the present study, significant differences for anti-CA I antibody levels were found between the PCOS and control groups. In PCOS patients, there was a significant correlation between anti CA I and anti CA II antibody titers. All patients with positive anti-CA I antibody also had positive anti-CA II antibody (Fig 1c). A probable reason of that may be a cross-reactivity because of homology between CA I and CA II.

Women with PCOS have an increased prevalence of several established cardiovascular risk factors. Based on recent studies’ results, this may be due to the oxidative stress and microvascular ischemic precondition of

<table>
<thead>
<tr>
<th></th>
<th>Cutoff Point</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
<th>PPV (%) (95% CI)</th>
<th>NPV (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CA I Antibody</td>
<td>&gt;0.190</td>
<td>84.0 (70.9-92.8)</td>
<td>65.8 (48.6-80.4)</td>
<td>76.4 (62.8-86.9)</td>
<td>75.8 (57.7-88.9)</td>
</tr>
<tr>
<td>Anti-CA II Antibody</td>
<td>&gt;0.330</td>
<td>70.0 (55.4-82.1)</td>
<td>60.5 (43.4-76.0)</td>
<td>70.0 (55.2-82.3)</td>
<td>60.5 (43.4-76.0)</td>
</tr>
</tbody>
</table>

Figure 2a Receiver operating characteristic curves for Anti-CA I antibody. AUC, area under the curve.

Figure 2b Receiver operating characteristic curves for Anti-CA II antibody. AUC, area under the curve.
PCOS [4, 23, 24]. Previously, studies reported an increased oxidative stress in serum of patients with PCOS [15]. Aliyazıcıoglu et al. demonstrated a positive correlation between anti-CA II bodies and oxidative stress parameters suggesting an association between autoimmunity and oxidative stress [12]. Based on our study results we suggest that increased oxidative stress in PCOS may cause antibody formation against CA I but not CA II.

In conclusion, serum anti CA I antibody titers were elevated in women with PCOS. This result supports the association between autoimmunity and PCOS. Elevation of CA I auto antibodies but not CA II requires further studies to understand the mechanism of autoantibody formation and the significance of anti CA antibody production in patients with PCOS. Further studies with large sample sizes are needed to elucidate the relationship between high anti-CA I antibodies and PCOS, and to support our results.

Acknowledgements

Author contribution: guarantor of integrity of the entire study: AM; study concepts and design: AM, SG; literature research: AM, AA; clinical studies: SG; experimental studies / data analysis: AM, AS, SD; statistical analysis: IT; manuscript preparation: AM; manuscript editing: SCK, AS, AA, SG.

Ethical approval

The study approved by judgment with 2012-93 reference number of Local Ethical Committee.

Conflict of Interest

The authors had not personal relationships with other individuals or organizations that might inappropriately influence their work during the submission process and last twenty four months.

References