The Prevalence of Non-classical Congenital Adrenal Hyperplasia Due to 21-hydroxylase Deficiency in Greek Women with Hirsutism and Polycystic Ovary Syndrome

EFTIHOS TRAKAKIS***, DEMETRIOS RIZOS***, CONSTANTINE LOGHIS***, ATHANASSIOS CHRYSSIKOPoulos*, MARILYN SPYROPOulos*, EMANUEL SALAMELEKIS***, GEORGE SIMEONIDES**, VASSILIS VAGOPoulos**, GEORGE SALAMELEKIS** AND DEMETRIOS KASSANOS***

Abstract. The study was aimed to find out the prevalence of non-classical congenital adrenal hyperplasia (NC-CAH) due to 21-hydroxylase deficiency (21-OHdef) among Greek women with hirsutism and polycystic ovary syndrome (PCOS) and to compare the results of ACTH stimulated 17-hydroxyprogesterone 60 min (17-OHP60) values, with human leukocyte antigens (HLA) phenotypes, in any patient diagnosed as having NC-CAH. One hundred and seven women with hirsutism and PCOS were included in the study. All were presented at the Reproductive Endocrinology Outpatient Clinic with hirsutism and PCOS. After ACTH stimulation test, 10 women were diagnosed as having NC-CAH because of high 17-OHP60 values ≥36 nmol/l, and 97 as having PCOS. Ten (10.3%) of the 97 women presented hormonal findings compatible with adrenal hyper-response due to ACTH testing, because of hyperstimulated 17-OHP60 values ≥21 nmol/l and <32 nmol/l. The HLA typing of 10 patients with NC-CAH revealed the phenotypes B14, DR1, B35, B7 and B44 which present positively genetic linkage disequilibrium with 21-OHdef, as reported in the literature. In conclusion: In Greek women with hirsutism and PCOS we have found that: a. The prevalence of NC-CAH among these women is relatively high and reaches at 10%. b. The HLA phenotypes B14, DR1, B35, B7 and B44 were found in high frequency in these NC-CAH patients. c. Adrenal NC-CAH due to 21-OHdef as well as adrenal hyperactivity, revealed after ACTH testing, constitutes an important reason of hirsutism and PCOS in these Greek women and both reach a rate of 20%.

Key words: Congenital adrenal hyperplasia, Prevalence, Polycystic ovary syndrome

CONGENITAL adrenal hyperplasia (CAH) caused by a deficiency of adrenal enzyme 21-hydroxylase (21-

Received: April 3, 2007
Accepted: September 10, 2007
Correspondence to: Eftihios TRAKAKIS M.D., Maikina 41 Zografos, GR 15772, Athens, Greece

Abbreviations: ACTH, adrenocorticotropic; CAH, congenital adrenal hyperplasia; C-CAH, classical congenital adrenal hyperplasia; 21-OH, 21-hydroxylase; 17-OHP, 17-hydroxyprogesterone; HLA; Human leukocytes antigens; NC-CAH, non-classical congenital adrenal hyperplasia; P, progesterone; F, cortisol; T, testosterone; DEHAS, dehydroepiandrosterone sulfate; FSH, follicle stimulating hormone; LH, luteinizing hormone; BMI, body mass index; 11b-OHdef, 11b-hydroxylase deficiency; 3b-HSDdef, 3b-hydroxysteroid-dehydrogenase deficiency.
CAH hyperandrogenism may be present, extending from virilization of external genitalia and salt wasting in C-CAH cases, to menstrual irregularity, obesity, short stature, infertility or subfertility and skin disorders (hirsutism, seborrhea and/or acne in peripubertal period) in NC-CAH cases. These clinical characteristics of NC-CAH cases do not differ from those shown in patients with polycystic ovary syndrome (PCOS), idiopathic hirsutism or hyperinsulinemia [8–13]. The incidence of 21-OHdef among hirsute women varies between 1.2–20% [8–12, 14]. The disparity in these findings could be due to the variability of the 21-OHdef gene in different populations, the variability in protocols of studies and the lack of similarity of the patient populations in different studies [7]. The accurate diagnosis of CAH can be confirmed by molecular gene analysis [12, 14], but even today, in clinical practice, molecular biology is not routinely available. ACTH stimulation test remains the principal diagnostic tool [15–19]. The reason for a quick diagnosis of this enzymatic disorder in clinical practice using the adrenal stimulation test is the simplicity of this test. In many studies simple diagnostic criteria were used [2, 3, 4, 6]. The positively genetic linkage disequilibrium between 21-OHdef and HLA phenotypes B14, DR1, Bw47, B35 and B5 has been reported in many studies [5, 7, 13, 15, 16].

The aim of this study was (a) to determine the prevalence of NC-CAH due to 21-OH def in a substantial sample of Greek women with hirsutism and PCOS, (b) to compare ACTH stimulation test results with HLA phenotypes in any person diagnosed as having NC-CAH due to 21-OHdef, (c) to compare the hormonal parameters between NC-CAH cases with hirsutism in relation to patients who presented with hirsutism due to PCOS.

**Materials and Methods**

**Patients**

One hundred and seven women were included in the study. All were presented at the Reproductive Endocrinology Outpatient Clinic of the Second and later of the Third Department of Obstetrics and Gynecology of Athens University, complaining of hirsutism with PCOS. All patients were evaluated for NC-CAH. Their age varied between 12 to 35 years (mean 22 years). The diagnosis of PCOS in these patients was defined by the presence of ovulatory dysfunction and clinical hyperandrogenism (Ferriman-Callwey modified score ≥8) and/or acne and/or hyperandrogenemia, in addition to the presence of polycystic ovaries on pelvic ultrasound (ovarian volume ≥10 cm³, multiple follicle (≥12) distributed peripherally, stroma increased), as described in the literature [20]. Patients who presented 2 or more of the previous criteria were considered as having PCOS. Patients with Cushing syndrome, adrenal or ovarian virilizing tumors, hyperprolactinemia and thyroid dysfunction were excluded from the study. None of the patients received hormonal therapy for at least 12 weeks before testing. All patients underwent an acute adrenal stimulation test to rule out or confirm 21-hydroxylase deficiency, as described below. Body mass index was calculated as weight/height² (kg/m²). The study was approved by the Institutional Review Board of Athens University.

**ACTH stimulation test**

All patients were informed about and accepted the diagnostic procedure. After overnight fasting, 0.25 mg of ACTH (1–24) (Synachten, Ciba-Geigy, Basel, Switzerland) were injected as intravenous bolus with the patient in the supine position, in the morning, between 08:00 and 10:00 hours. No dexamethasone was administered before testing. The test was performed in the early follicular phase (day 3–7 of the menstrual cycle), while in cases with secondary amenorrhea menstrual bleeding was produced after progesterone administration for 10 days. Blood samples were obtained before (0 min) and 60 min following ACTH administration. Serum was separated, aliquoted and stored at −20° until assay.

**Study design**

The patient population was separated into four groups, A, B, C and D, on the basis of serum 17-OHP60 values. Group A (17-OHP60 values ≥90.75 nmol/l) comprised of patients with severe form of 21-OHdef. Group B (17-OHP60 values ≥33.2 nmol/l and <90.75 nmol/l) included patients with a moderate form of 21-OH def. In groups A and B all patients were considered as having NC-CAH, as reported in the literature [2–7, 12–19]. Group C consisted of patients with 17-OHP60 values
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>15 nmol/l and <33.2 nmol/l. The members of the previously reported group included females unaffected from NC-CAH, who presented exaggerated adrenal or ovarian responses to ACTH testing and probably a small number of heterozygous carriers of 21-OHdef.

Group D with 17-OHP60 values <15 nmol/l was composed of unaffected females from NC-CAH and probably a very small number of heterozygous 21-OHdef carriers. In groups C and D all patients were considered as having PCOS, because they did not meet the criterion of 17-OHP60 values >33 nmol/l, of 21-OHdef, as reported in the literature [7, 13, 17, 18, 19, 21].

**Hormonal assays**

Hormone measurements of cortisol (F), follicle stimulating hormone (FSH) and luteinizing hormone (LH) were performed by polarization fluoroimmunoassay and micro-particle enzyme immunoassays, using commercially available kits: Medgenix, Fleurus, Belgium; TDX Abbott Laboratories, IL 60064, USA; and IMX, Abbott Lab, respectively. Sensitivities, intra and inter assays coefficients of variation (CV %) were found to be 12.41 nmol/l, 5.1 and 7.0 for cortisol. Testosterone and DHEAS measurements were performed with the analysis of Elecsyl 1010/2020 and Modstar analytics E 170 by Roche, with CV 5.6% and 6%, respectively. 17-OHP measurements were performed with RIA kits provided by the Diagnostic Systems International Inc Corporate Headquarters and Medical Center Blvd, Webster, TX USA, with CV 6.3%. Progesterone measurements were performed by fluoroimmunoassay and the CV was found to be 0.25 nmol/l, 5.0 and 7.0.

**Human leukocyte antigen (HLA) typing**

HLA A and B phenotypes were determined on peripheral blood leukocytes using the standard National Institutes of Health (NIH) two-stage microtoxicity test [22]. HLA DR typing was performed using PCR-SSP (sequence specific primer) technique [23, 24].

**Statistical analysis**

Statistical analysis was performed by the non-parametric Mann-Whitney U test for the comparison of hormonal parameters. A p value less than or equal to 0.05 was considered significant.

**Results**

The patient population in our study complained of hirsutism (100%), menstrual irregularities (72%) and acne (52%). The mean age of patients was 22.1 ± 4.56 (range 12–35). Four (3.74%) out of 107 patients, (group A) were diagnosed directly as having NC-CAH because of the high basal levels of 17-OHP. Six (5.60%) out of 107 patients, (group B) were diagnosed as having NC-CAH, because ACTH stimulated 17-OHP60 values were higher than 33.2 nmol/l, thus meeting the criterion of NC-CAH, as proposed in the literature. Finally 10 (9.34%) out of 107 patients suffered from NC-CAH due to 21-OHdef. Ninety-seven (90.65%) out of 107 patients, (22 of group C and 75 of group D), did not meet the diagnostic criterion for NC-CAH (because ACTH stimulated 17-OHP values were <33.2 nmol/l) and thus they were considered as having PCOS (Table 1). It is useful to point out that 10 patients out of 22 (45.45) of group C presented with 17-OHP60 values ≥21 nmol/l ≤32 nmol/l. It was considered that in these patients with PCOS, adrenal hyperstimulation was pronounced due to ACTH testing.

The HLA typing of 10 patients diagnosed as having NC-CAH, revealed the phenotypes B14, DR1, B35, B7 and B44 which positively present genetic linkage disequilibrium with CAH, as proposed in the literature.

None of the patients with CAH had ambiguous genitalia (the external genitalia were absolutely normal, without signs of virilization such as rugated and partially fused labia majora and/or penile enlargement of the clitoris. The vagina and urethra were also normal, without common urogenital sinus in place of a separate urethra and vagina). Two patients with 21-OHdef (pa-

<table>
<thead>
<tr>
<th>Patients</th>
<th>17-OHP 0</th>
<th>17-OHP 60</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>353</td>
<td>545.6</td>
</tr>
<tr>
<td>2</td>
<td>372</td>
<td>554.9</td>
</tr>
<tr>
<td>3</td>
<td>189</td>
<td>368.9</td>
</tr>
<tr>
<td>4</td>
<td>127.2</td>
<td>263.5</td>
</tr>
<tr>
<td>5</td>
<td>7.53</td>
<td>44.3</td>
</tr>
<tr>
<td>6</td>
<td>6.57</td>
<td>36.58</td>
</tr>
<tr>
<td>7</td>
<td>2.93</td>
<td>62</td>
</tr>
<tr>
<td>8</td>
<td>9.44</td>
<td>39</td>
</tr>
<tr>
<td>9</td>
<td>2.3</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>36.2</td>
</tr>
</tbody>
</table>
patients 1 and 3 of Table 1) had muscular body with short neck, muscular hands and feet and mild enlargement of the clitoris. In general, the members of group A presented severe post-pubertal hirsutism (100%), primary amenorrhea (patients 1 and 3 of Table 1), secondary amenorrhea (patients 2 and 4 of Table 1) and moderate acne (100%). The members of group B presented post-pubertal hirsutism (100%, with Lorenzo score ≥10), menstrual irregularities (100%) and acne (66%). Patient 6 of Table 1 was subfertile.

The hormonal profile of patients with NC-CAH and those with PCOS are shown in Table 3. Regarding hormonal parameters, statistically significant differences were found in 17-OHP60 values among group A in relation to groups B, C and D (p<0.00001), group B in relation to group C and D (p<0.00001), and C in relation to D (p<0.0001). Basal 17-OHP of group A in relation to groups B, C and D (p<0.00001) respectively and basal 17-OHP of groups B and C in relation to D (p<0.031). Also in progesterone (P) among group A in relation to groups B, C and D (p<0.001), testosterone (T) in group A in relation to groups B, C and D (p<0.001), as well as of cortisol (F) in group D, in relation to group A (p<0.039).

**Discussion**

Congenital adrenal hyperplasia due to 21-OHdef is a well recognized disorder among women with hirsutism and PCOS [25, 26]. The prevalence of this enzymatic deficiency in women with hirsutism and PCOS varies between 1%–33%, reflecting the different criteria in the methodology of patient collection and diagnosis of the disease [9–11, 15, 17]. It appears to be particularly high in ethnic groups around the Mediterranean, in the Jewish Ashkenazi, in Hispanics, Italians, Yugoslavians, Turks and in the Arabic populations [5, 27–31]. The high basic levels of 17-OHP (>15 nmol/l) and the values of 17-OHP 60 min (after ACTH stimulation test) higher than 33 nmol/l, are diagnostic for this enzymatic disorder [2–4, 6, 7, 12–15, 17, 19]. In this study we used the results of ACTH stimulated 17-OHP60 values, in a substantial sample of Greek women with hirsutism and PCOS, in order to estimate the prevalence of this enzymatic disorder. Evaluating the basic values of 17-OHP, 4 women out of 107 (3.73%) were

### Table 2

<table>
<thead>
<tr>
<th>Patients</th>
<th>HLA typing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A2, A33, B22 (55), B35, CW3, CW4, DR11, DRX, DQ3, DRW52</td>
</tr>
<tr>
<td>2</td>
<td>A11, A28, Bw44, B35, CW3, CW4, DR1, DR2, DR6,</td>
</tr>
<tr>
<td>3</td>
<td>A2, A10, B14, B18, CD4, CD7, DR1, DR5, DQ3</td>
</tr>
<tr>
<td>4</td>
<td>A11, A28, B22 (55), B35, CW3, CW4, DR2, DR6, DR1</td>
</tr>
<tr>
<td>5</td>
<td>A9, A33, B14, B18, BW6, CW7, DR1, DR5, DQ1, DQ3</td>
</tr>
<tr>
<td>6</td>
<td>A10, AX, B7, B70, BW6, CW7, DR2, DR10, DQ1</td>
</tr>
<tr>
<td>7</td>
<td>A2, A9, B14, B22 (55), CD4, DR1, DR6, DQ3</td>
</tr>
<tr>
<td>8</td>
<td>A11, A28, B12 (44), B35, BW4, BW6, CW4, DR3, DR5, DQ5, DQ2, DQ3 DRW52</td>
</tr>
<tr>
<td>9</td>
<td>A2, A10, B14, B22 (55), CW3, CW4, DR1, DQ3</td>
</tr>
<tr>
<td>10</td>
<td>A2, A10, B7, B70, BW6, CW4, DR10, DQ3</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>P (nmol/l)</th>
<th>F (nmol/l)</th>
<th>DHEAS (nmol/l)</th>
<th>T (nmol/l)</th>
<th>LH/FSH</th>
<th>BMI</th>
<th>17-OHP0 (nmol/l)</th>
<th>17-OHP60 (nmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n=4)</td>
<td>16.25 ± 11.74</td>
<td>313 ± 48.55</td>
<td>4328 ± 2599</td>
<td>8.2 ± 4.43</td>
<td>2.07 ± 0.27</td>
<td>21.9 ± 0.34</td>
<td>260.25 ± 121</td>
<td>432.85 ± 141.45</td>
</tr>
<tr>
<td>B (n=6)</td>
<td>0.432 ± 0.12</td>
<td>416.8 ± 131.3</td>
<td>5764.2 ± 1410</td>
<td>2.286 ± 1.118</td>
<td>2.0 ± 1.6</td>
<td>24 ± 2.4</td>
<td>6.74 ± 3.66</td>
<td>46.65 ± 13.5</td>
</tr>
<tr>
<td>C (n=22)</td>
<td>1.37 ± 1.1</td>
<td>447 ± 171</td>
<td>5718 ± 2890</td>
<td>2.6 ± 1.14</td>
<td>1.3 ± 0.9</td>
<td>22.3 ± 2.9</td>
<td>7.8 ± 4.23</td>
<td>22.5 ± 4.9</td>
</tr>
<tr>
<td>D (n=75)</td>
<td>1.33 ± 0.89</td>
<td>524 ± 199.5</td>
<td>5765 ± 2988</td>
<td>2.47 ± 1.16</td>
<td>1.65 ± 4.58</td>
<td>23.6 ± 4.5</td>
<td>4.66 ± 2.05</td>
<td>11.18 ± 2.81</td>
</tr>
</tbody>
</table>

P, progesterone; F, cortisol; DHEAS, dehydroepiandrosterone sulfate; T, testosterone; 17-OHP0 and 17-OHP60, 17-hydroxyprogesterone values at 0 and 60 minutes after ACTH stimulation, respectively, LH, luteinizing hormone, FSH, follicle stimulating hormone, BMI, body mass index
previously reported, the prevalence of 21-OHdef varies with those reported in the literature [5–12, 14–19]. As also concerning the HLA phenotypes, are compatible to the prevalence of NC-CAH due to 21-OHdef but persons unaffected of 21-OHdef. The severe form of NC-CAH (group A), in relation to reflecting a difficulty in F secretion in the group with B, C, D. Finally in F in group D, in relation to group A, Also in P and T values in group A, in relation to groups B, C, D. Finally in F in group D, in relation to group A, reflecting a difficulty in F secretion in the group with the severe form of NC-CAH (group A), in relation to persons unaffected of 21-OHdef.

The determination of HLA phenotypes of these 10 women with NC-CAH revealed the phenotypes B35, DR1, B35, B7, and B44 in high frequency, in agreement with the literature. Regarding hormonal parameters, the statistically significant differences in basal and 17-OHP 60 values, which were found in groups A, B, C, D, were in agreement with the severity of 21-OHdef. Also in P and T values in group A, in relation to groups B, C, D. Finally in F in group D, in relation to group A, reflecting a difficulty in F secretion in the group with the severe form of NC-CAH (group A), in relation to persons unaffected of 21-OHdef.

The previously reported findings not only in relation to the prevalence of NC-CAH- due to 21-OHdef but also concerning the HLA phenotypes, are compatible with those reported in the literature [5–12, 14–19]. As previously reported, the prevalence of 21-OHdef varies from 1% to 20%–30% and it is particularly high around the Mediterranean, in Italians, in the Jewish Ashkenazi, in the Hispanics, in the Turks and in the Arabs [34–38]. Recently published papers in Turkey, reported a high prevalence of NC-CAH due to 21-OHdef in women suffering from hirsutism and PCOS [11, 34, 35]. Kaml and Yarman reported a prevalence of 9.52% and 33%, respectively, in these women [34, 35]. A high prevalence was also reported by Italians, Hispanics, Yugoslavians and Arabs. In our previous two papers in Greek hyperandrogenic women, it was estimated that the frequency of NC-CAH was about 5%; however, a great number of women that reached the 11% had hormonal levels compatible with a possible heterozygosity of 21-OHdef [38, 39]. Dacou Voutetakis et al. in a substantial sample of children from Greece with premature adrenarche reported a high incidence of molecular defects of CYP 21 gene that reached to 33% [40]. The genetic linkage disequilibrium between 21-OHdef and HLA phenotypes B14, DR1, B35, B7, and B44 has been reported in many studies in the Caucasian populations and the Jewish Ashkenazi as well as in the populations around the Mediterranean. The same HLA phenotypes were also found in high frequency in the women of our population diagnosed as having NC-CAH.

These findings are in agreement with our previous paper on the high frequency and the positively genetic linkage disequilibrium of HLA phenotypes B14, DR1, B35, B47, B22 and B7 and 21-OHdef in Greece [41]. In group C (that, is the group of women with PCOS) there are a considerably great number of women with 17-OHP 60 values between 21 nmol/l and 32 nmol/l (10 out of 22) (45%). They were considered as suffering from adrenal hyperactivity revealed after ACTH testing (adrenal hyperstimulation due to ACTH testing) without excluding the probable presence of heterozygous forms of 21-OHdef (molecular biology gene analysis is needed for diagnosing heterozygosity) [42, 43]. These findings on the prevalence of NC-CAH and the frequency of HLA phenotypes B14, DR1, B35, B7, and B44 as well as the high percentage of women with adrenal hyperactivity revealed after ACTH testing, are reported for the first time in Greek population. The results of this study may be of epidemiological interest not only for the Mediterranean, but also for Caucasian populations in general.

In conclusion, in a substantial sample of Greek women with hirsutism and PCOS we have found that a. The prevalence of NC-CAH due to 21-OHdef among Greek women with hirsutism and PCOS, is relatively high and reaches at 10%. b. The HLA phenotypes B14, DR1, B35, B7, and B44 were found in high frequency in these NC-CAH patients. c. The enzymatic defects of adrenal and especially NC-CAH due to 21-OHdef as well as adrenal hyperactivity, revealed after ACTH testing, constitute an important reason of hirsutism and PCOS in these Greek women, reaching a rate of 20% (10% of 21-OHdef and 10% of adrenal hyperactivity, respectively).
References

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