Genotype–phenotype associations in non-classical steroid 21-hydroxylase deficiency

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Abstract

Objective: To evaluate whether genotype differences can explain the clinical variability of non-classical steroid 21-hydroxylase deficiency (NC21-OHD) and to determine if genotype is related to ethnic origin.

Design: Genotyping for mutations in the steroid 21-hydroxylase (CYP21) gene was performed in 45 unrelated Israeli Jewish patients (nine males) with NC21-OHD (60 min 17-hydroxyprogesterone (17-OHP), 45–386 nmol/l) who were referred for evaluation of postnatal virilization or true precocious/early puberty. Eleven siblings diagnosed through family screening were genotyped as well.

Methods: Patients were divided by genotype into three groups: (A) homozygous or compound heterozygous for the mild mutations (V281L or P30L) (n=29; eight males); (B) compound heterozygous for one mild and one severe mutation (Q318X, I2 splice, I172N) (n=12; no males); (C) mild mutation detected on one allele only (n=4; one male; peak 17-OHP 58–151 nmol/l). We then related the genotype to the ethnic origin, clinical phenotype and hormone level. Since group C was very small, comparisons were made between groups A and B only.

Results: At diagnosis, group B tended to be younger (5.8±3.0 vs 8.1±4.3 years, P<0.09), had greater height SDS adjusted for mid-parental height SDS (1.6±1.1 vs 0.7±1.4, P=0.034), tended to have more advanced bone age SDS (2.9±1.5 vs 1.7±2.1, P=0.10) and had a higher peak 17-OHP level in response to ACTH stimulation (226±92 vs 126±62 nmol/l, P<0.01). Group B also had pubarche and gonadarche at an earlier age (5.1±2.4 vs 7.4±2.2 years, P<0.01 and 7.4±1.8 vs 9.9±1.4 years, P<0.001, respectively) and a higher rate of precocious puberty (50 vs 17%, P=0.04). Stepwise logistic regression analysis (excluding males) yielded age at gonadarche as the most significant variable differentiating the two groups, with a positive predictive value of 86% for a cut-off of 7.5 years.

Conclusions: The findings suggest that genotype might explain some of the variability in the phenotypic expression of NC21-OHD. Compound heterozygotes for one mild and one severe mutation have a higher peak 17-OHP associated with pubarche and gonadarche at an earlier age and more frequent precocious puberty. Hence, the severity of the enzymatic defect might determine the timing and pattern of puberty.

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mutations have been classified according to the degree of the resultant enzymatic inactivation, so that the genotype can usually predict the clinical phenotype (3, 4). Previous studies have correlated the genotype with the three main forms of the disease, although data on the correlation of the genotype with the various clinical presentations of the non-classical form are lacking.

The prevalence of NC21-OHD among Ashkenazi Jews is remarkably high (3.2–3.7%) (5); about 80% carry the mild mutation V281L (6). Screening for CYP21 gene mutations in NC21-OHD in other Jewish ethnic groups has not been done.

In this study we screened for CYP21 gene mutations in Jewish patients with NC21-OHD to evaluate whether genotype differences might explain the clinical variability of this disorder and to determine whether there is an association between genotype and ethnic origin.

Subjects and methods

The genetic study was carried out between 1995 and 1998 in patients who had been followed in our institute since the late 1970s.

The study population included 45 unrelated Jewish patients (nine males) with NC21-OHD who were referred for evaluation of postnatal virilization or true precocious/early puberty. The clinical diagnosis of NC21-OHD was based upon presenting signs of postnatal hyperandrogenism and the characteristic 60-min response of 17-OHP to 0.25 mg adrenocorticotropin (ACTH) stimulation (45±386 nmol/l) (7) with normal levels of 11-deoxycortisol. Age at diagnosis ranged from 0.1 to 17.5 years (median, 7.25). Ethnic origin and clinical signs at presentation are shown in Table 1. For the second part of the study, the genotype and clinical characteristics of 11 affected siblings (six males) detected by family screening were compared with the index case. The study protocol was approved by the Ethics Committee of Rabin Medical Center. We obtained informed consent from the patients and also from the parents of the children who were less than 18 years old.

Height of the patients and parents was measured with the Harpenden–Holtain stadiometer. Auxological results were expressed in terms of standard deviation scores (SDS) for age, based on the standards of Tanner & Whitehouse (8). Bone age (BA) was determined according to Greulich & Pyle (9). Height was accepted as the final height when the increment was < 1 cm over 1 year or when BA was ≥ 15 years in girls and ≥ 17 years in boys. Clitoris and penile size was measured by sliding caliper. Clitoromegaly and macrogenitosomia were considered when measurements were > 2 S.D. for age and Tanner stage (10, 11). We transformed the measurements of clitoral size made from 1980 to 1992 to the clitoral index published in 1992 (10).

Puberty was assessed according to Marshall & Tanner (12, 13). Pubarche was defined as Tanner 2 for pubic hair (12, 13). The criterion of true puberty was gonadarche: breast buds in girls and a testicular volume reaching 4 ml in boys. True precocious puberty was diagnosed when gonadarche appeared before the age of 8 years in girls and 9 years in boys. When gonadarche was present, a gonadotropin-releasing hormone (GnRH) stimulation test was performed concomitantly with the ACTH test.

Plasma 17-OHP and cortisol levels were measured in the morning after an overnight fast before and 60 min after an i.v. bolus of a fixed dose of 0.25 mg ACTH (7) (Synacthen, Ciba-Geigy, Basel, Switzerland). Levels of plasma luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured before and 30 and 60 min after an i.v. bolus of 50 μg/m² GnRH (Relisorm; Serono, Aubonne, Switzerland). Pubertal LH response was defined as ≥ 5 IU/l (14). Also determined were basal levels of androstenedione, testosterone, 11-deoxycortisol and estradiol. Measurements were performed by radioimmunoassay (RIA) with commercial kits (17-OHP, cortisol, testosterone, estradiol: DPC, Los Angeles, CA, USA; androstenedione: Buhlman, Basel, Switzerland; 11 deoxycortisol: Sigma, St Louis, MO, USA; LH and FSH: chemiluminescent enzyme immunoassay: DPC). The plasma was separated and stored at −20°C until assayed. It is noteworthy that we have been using the DPC RIA for determination of 17-OHP and androgen levels since the mid-1980s. Before that, homemade kits were used and findings were evaluated against norms established in our laboratory for each Tanner stage. Measurements done from the mid-1980s were compared with the norms evaluated by Lashansky et al. (15). For each method, elevated androgen level

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**Table 1** Demographic and clinical characteristics of 45 Jewish patients with NC21-OHD.

<table>
<thead>
<tr>
<th>Ethnicity*</th>
<th>No. (%n = 45)</th>
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<tbody>
<tr>
<td>Ashkenazi</td>
<td>24 (53)</td>
</tr>
<tr>
<td>Sephardic</td>
<td>8 (18)</td>
</tr>
<tr>
<td>Asian</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Combined</td>
<td>12 (27)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Presenting sign†</th>
<th>No. (%n = 45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precocious puberty</td>
<td>20 (44)</td>
</tr>
<tr>
<td>True precocious puberty at diagnosis</td>
<td>12 (27)</td>
</tr>
<tr>
<td>Clitoromegaly/macrogenitosomia</td>
<td>19 (42)‡</td>
</tr>
<tr>
<td>PCO-like syndrome</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Growth acceleration</td>
<td>3 (7)</td>
</tr>
<tr>
<td>Short final height + S/P fast puberty</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Hypertrichosis</td>
<td>1 (2)</td>
</tr>
</tbody>
</table>

* By country of origin: Ashkenazi – Eastern Europe and former USSR; Sephardic – North Africa, Spain and the Balkans; Asian – Yemen, Iraq and Iran.
† Most patients had more than one presenting sign.‡ 13/36 females (36%) and 6/9 males (66%).
was defined as > 2 s.d. from the mean. Since the androgen levels were determined by different methods, we did not compare the absolute levels between groups, but rather the frequency of the patients with elevated androgen levels.

Oral hydrocortisone (HC) therapy was instituted when there was evidence of progressive virilization, acceleration of growth, true precocious puberty, advanced BA at diagnosis (≥1 s.d. for age) or an increase in ABA/Δage of at least 6 months during follow-up. The dose (7.5–15 mg/m², divided into two to three doses) was adjusted at least 6 months during follow-up. The dose (7.5±4 mg/m²) was adjusted over several years, and therefore this regimen was introduced to only three girls of group B, four girls of group A and one girl of group C; age at start of therapy was 6.7–12.5 years, and duration of therapy was 1.9–3.5 years.

Genetic analysis

The following mutations were sought in the patients, their parents and affected siblings: deletion of 8 bp in exon 3, five point mutations: P30L exon 1, A/C655G intron 2 (I2 splice), I172N exon 4, V281L exon 7, Q318X exon 8 and a cluster of three point mutations in exon 6 (I236N+V237E+M239K). Blood samples were collected into EDTA and genomic DNA was isolated by the salting out method (16). Identification of mutations was performed by the PCR sequence-specific oligonucleotide probe method. In brief, two sets of primers were used according to Wedell & Luthman (17) with some modification. Set one – CYP11: 5'-GGAGCATAAACAGGAGAAACTGACCTTCCTAGC-3' and CYP48A: 5'-GGAGCACTTCCCCGTCTCTG-3'. Set two – CYP1: 5'-TTCCAGGATTCAGGAAGGC-3' and CYP48: 5'-CAGAGCAGGAGGAGCT-3'.

The two PCR products were blotted onto nylon membranes and hybridized with sequence specific oligonucleotide probes which were end-labeled with digoxigenin ddUTP (Boehringer Mannheim Biochemicals, Mannheim, Germany). The membranes were then washed with 3 mol/l tetramethylammonium chloride solution at increasing temperatures ranging between 48°C and 57°C. Alkaline phosphatase-bound antidigoxigenin was used with the chemiluminescent substrate for alkaline phosphate (CDP star; Boehringer Mannheim) for chemiluminescence detection. The oligonucleotide probes used for detection for wild-type sequence (W) (including two wild-type polymorphic forms, Wa and Wc, for the I2 splice mutations) and mutations (M) were as follows:
P30L, W: 5'-CCACCTCCGCCCTCTTTG-3', P30L, M: 5'-CCACCTCCGCCCTCTTTG-3', I2 splice, Wa: 5'-AGCCCCCAACCTCCCTGCA-3', I2 splice, Wc: 5'-AGCCCCCAACCTCCCTGCA-3', I2 splice, M: 5'-AGCCCCCAACCTCCCTGCA-3'

The data were analyzed with the BMPD program (18). Data are presented as means ± s.d. unless otherwise indicated. Between-group differences were analyzed by ANOVA or ANCOVA, as appropriate. The associations between the clinical and laboratory characteristics and the genotype were analyzed with Pearson’s chi-square test or Fisher’s exact test, as appropriate. Stepwise logistic regression was applied to estimate the best predictor for differentiating between groups A and B (see below).

Results

The 90 alleles of the 45 unrelated Jewish subjects with NC21-OHD contained the following disease-causing mutations: 72 (80%) V281L, six (6.7%) I2 splice, four (4.4%) Q318X, two (2.2%) P30L and two (2.2%) I172N; in four alleles (4.4%), none of the above mutations was detected. Figure 1 shows the genotype distribution among the study patients.

Previous studies relating mutations to level of enzymatic activity (19, 20) have shown that the mild mutations (V281L and P30L) result in an enzyme with 20–50% of normal activity, and the severe mutations (I2 splice, Q318X and I172N) in an enzyme with 0–2% of normal activity. Based on these findings, we divided the patients into three groups: (A) homozgyous or compound heterozygous for the mild mutations (n=29; eight males); (B) compound heterozygous for one mild and one severe mutation (n=12; no males); and (C) mild mutation detected on one allele only (n=4; one male; peak 17-OHP 58–151 nmol/l).
Table 2 Clinical and laboratory characteristics of the study population by genotype (means ± S.D.).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A (n = 29)</th>
<th>Group B (n = 12)</th>
<th>Group C (n = 4)</th>
<th>Significance of difference (P&lt;sup&gt;a&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>At diagnosis</td>
<td></td>
<td></td>
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<tr>
<td>Age (years)</td>
<td>8.1 ± 4.3</td>
<td>5.8 ± 3.0</td>
<td>5.9 ± 3.4</td>
<td>0.09</td>
</tr>
<tr>
<td>Bone age SDS</td>
<td>1.7 ± 2.1</td>
<td>2.0 ± 1.5</td>
<td>0.7 ± 0.7</td>
<td>0.10</td>
</tr>
<tr>
<td>Height SDS</td>
<td>0.7 ± 1.4</td>
<td>1.6 ± 1.1</td>
<td>1.3 ± 0.6</td>
<td>0.034&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Basal 17-OHP (nmol/l)</td>
<td>40 ± 39</td>
<td>46 ± 31</td>
<td>9 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Peak* 17-OHP (nmol/l)</td>
<td>126 ± 62</td>
<td>226 ± 92</td>
<td>105 ± 42</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starting age (years)</td>
<td>8.7 ± 3.8</td>
<td>6.4 ± 2.8</td>
<td>8.9 ± 3.6</td>
<td>0.07</td>
</tr>
<tr>
<td>HC dose (mg/m&lt;sup&gt;2&lt;/sup&gt; per day)</td>
<td>14.9 ± 4.2</td>
<td>14.9 ± 3.8</td>
<td>9.7 ± 3.5</td>
<td>NS</td>
</tr>
<tr>
<td>Puberty</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at pubarche (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole group</td>
<td>7.4 ± 2.2</td>
<td>5.1 ± 2.4</td>
<td>7.7 ± 2.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Females only</td>
<td>7.2 ± 2.9</td>
<td>5.1 ± 2.4</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Age at gonadarche (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole group</td>
<td>9.9 ± 1.4</td>
<td>7.4 ± 1.8</td>
<td>8.0 ± 2.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Females only</td>
<td>9.7 ± 1.5</td>
<td>7.4 ± 1.8</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Menarche&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.5 ± 1.4</td>
<td>12.2 ± 1.0</td>
<td>11.2</td>
<td>NS&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Final height SDS&lt;sup&gt;d&lt;/sup&gt;</td>
<td>−0.53 ± 0.9</td>
<td>−1.3 ± 0.9</td>
<td>0.5 ± 0.2</td>
<td>NS&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mid-parental height SDS</td>
<td>−0.2 ± 0.6</td>
<td>−0.3 ± 1.3</td>
<td>0.1 ± 0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Group A = homozygous or compound heterozygous for the mild mutations, group B = compound heterozygous for one mild and one severe mutation and group C = mild mutation (V281L) detected on one allele only.

<sup>a</sup> Between A and B only.

<sup>b</sup> Adjusted for mid-parental height.

<sup>c</sup> No. of girls: 17, 7 and 1 for groups A, B and C respectively.

<sup>d</sup> No. of subjects: 22, 5 and 2 for groups A, B and C respectively.

<sup>e</sup> 60 min after ACTH stimulation.

NS, not significant.

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The characteristics of the study population by genotype group are shown in Tables 2 and 3. The correlation of the 17-OHP level to group yielded similar basal levels in groups A and B and a significantly lower level in group C ($P < 0.05$, Bonferroni correction for multiple comparisons) (Table 2); however, mean peak 17-OHP was similar in groups A and C, and significantly higher in group B ($P < 0.01$ for B vs A and B vs C).

Because group C was very small, it was excluded from the statistical analysis of the other clinical characteristics. We found that at diagnosis, compared with group A, group B tended to be younger ($P = 0.09$), had a greater height SDS adjusted for mid-parental height SDS ($P = 0.034$) and tended to have a more mature BA SDS ($P = 0.10$). The number of patients with clitoromegaly or macrogenitosomia detected at diagnosis was similar in the two groups, as was the number of patients with elevated androgen levels, the number of treated patients (Table 3) and the dose of HC/m² (Table 2).

Group B had pubarche and gonadarche (Table 2) at an earlier age than group A ($P < 0.01$ and $P < 0.001$ respectively) and a higher rate of precocious puberty ($P = 0.04$) (Table 3). Since there were no males in group B, comparisons of age at pubarche and gonadarche were also done separately for girls, and yielded similar results (Table 2).

Final height SDS, adjusted for mid-parental height SDS (Table 2), was similar between the two groups, but only 22 patients of group A and five of group B had reached final height by the time of the study.

Application of stepwise logistic regression analysis (excluding males) using the variables that showed significance revealed that age at gonadarche was the most significant variable differentiating between the two groups, with a positive predictive value of 86% for a cutoff of 7.5 years.

### Ethnicity
Among the whole cohort, 44 patients (98%), including all patients of Ashkenazi origin, carried at least one allele with the V281L mutation (Fig. 1). Two patients, one of Yemenite origin and the other of mixed Yemenite–Ashkenazi origin, carried the P30L mutation (21). In both cases, the mutation was carried by parents of Yemenite origin.

There was no significant difference in the ethnic group distribution between group A and group B (Table 3). In group A patients the non-Ashkenazi comprised only 10% compared with 59% of Ashkenazi but the difference did not reach statistical significance.

### Family screening
Eleven siblings (six males, five females) of ten families were diagnosed as having NC21-OHD according to 17-OHP response to ACTH stimulation (basal 17-OHP $> 8.5$ nmol/l, peak 17-OHP $> 58$ nmol/l). All were diagnosed at an earlier age than their index case owing to the family screening. Eight displayed similar degrees of disease manifestations and three are still asymptomatic at the age of 6.5 (V281L/I2 splice), 7.0 and 9.0 (V281L/V281L) years; we do not yet know if they have the cryptic form or will develop symptoms in the coming years. All siblings shared the same genotype. Six siblings (two of the same family) were homozygous for V281L, three were compound heterozygous for V281L/I2 splice, and two were heterozygous for V281L.

A search for the mutations of the index cases among their parents yielded 83 carriers of one mutation (in four parents, no mutation was detected) and three parents (two fathers and one mother) of group 1 patients who were homozygous for V281L; all were Ashkenazi. One father had a history of short stature, and his height was 162 cm. His treated daughter and
son with NC21-OHD are 162 and 175 cm respectively, and both are homozygous for V281L. The other father is 167 cm tall and first shaved at 15 years of age. The mother is 160 cm tall and hirsute. She had menarche at age 13 and three spontaneous pregnancies.

Discussion
In agreement with previous studies, the majority of our Jewish NC21-OHD patients were of Ashkenazi origin (5, 22) and almost all carried at least one allele with the V281L mutation (4, 6, 23). The P30L mutation (21) was found only in two patients, both of Yemenite origin, probably secondary to founder effect. The distinct genetic background of the Yemenite Jews has been probably secondary to founder effect. The distinct genetic background of the Yemenite Jews has been shown in studies of many other inherited disease (24).

Our results suggest that some of the variability in the clinical spectrum of NC21-OHD can be explained by genotype differences. The patients with compound heterozygosity for one mild (either V281L or P30L) and one severe (Q318X, I2 splice, I172N) mutation (group B) had a more severe clinical picture than those who carried two mild mutations (group A). At presentation, they were younger (all were diagnosed in the first decade) and had a greater height SDS adjusted for mid-parental height SDS and slightly more mature BA. They also had earlier age at pubarche and gonadarche, a higher incidence of true central precocious puberty, and a higher peak 17-OHP in response to ACTH stimulation. Thus, even though compound heterozygotes typically exhibit phenotypes corresponding to the less severely affected allele (20, 25), their clinical course seems to be more severe than that of patients carrying two mild mutations. Furthermore, while all the patients of group B presented in the first decade with either premature/early pubarche, precocious/early puberty, clitoromegaly or growth acceleration, the presentation of group A subjects was much more variable: all the postpubertal patients belonged to this group, as well as the three ‘asymptomatic’ parents who were detected by family screening. The explanations for the phenotypic heterogeneity among individuals carrying the same mutations are still hypothetical and include the presence of other still unidentified mutations, the activity of other genes encoding proteins with extra-adrenal 21-hydroxylase activity (26), individual variation in the amount of protein produced (27), differences in intra-adrenal concentrations of progesterone (2), and differences in sensitivity to androgens (28).

Our results are in agreement with Rumsby et al. (23) who studied mainly adult females with NC21-OHD and found more frequent oligomenorrhea/amenorrhea and infertility in those with one mild and one severe mutation, and mostly regular menses in those homozygous for the mild mutation. Among our patients were two females, both homozygous for V281L, who presented during adolescence with severe polycystic ovary syndrome with hirsutism and oligomenorrhea. Both resumed regular menses on treatment with low-dose dexamethasone, and one who sought pregnancy conceived on dexamethasone without any additional therapy.

Of note is the high frequency (50%) of true central precocious puberty (CPP) among patients with compound heterozygosity for one mild and one severe mutation (group B). True CPP has been described previously in classical 21-OHD (29), NC21-OHD (22), and virilizing adrenal tumors (30), either at presentation or following institution of therapy when the hyperandrogenism resolved. The presentation of CPP associated with hyperandrogenism usually correlates with advanced BA to the range of the pubertal years (29, 30). Our group B patients tended to have more advanced BA SDS at diagnosis than group A (Table 2), but only three out of six had a BA of > 8 years at gonadarche, which appeared before initiation of therapy, i.e. before androgen suppression. Therefore, it is probably the mildly elevated androgen levels that initiate physiologic adrenarche and prematurely prime the hypothalamic–pituitary–gonadal axis. Since CPP is quite frequently a presenting sign of NC21-OHD (27% in our group), we suggest that the ACTH stimulation test should be added to its evaluation.

In four patients with peak 17-OHP levels within the accepted range for NC21-OHD (105±42 nmol/l), a mild mutation (V281L) was detected on one allele only (group C). Their mean basal 17-OHP level was, indeed, significantly lower than that of groups A and B (Table 2), but their mean peak 17-OHP level was compatible with NC21-OHD (7) and was similar to that of group A. We did not perform screening for more mutations (R356W, P453S), or direct sequencing to detect rare alleles, owing to technical limitations. In other large studies screening for mutations in patients with clinical and laboratory evidence of 21-OHD (3, 4), mutations were not observed in 5–10% of chromosomes in which they were expected according to the clinical status of the index case. However, sequencing of the CYP21 gene was not done and, therefore, rare mutations might have been missed. Since genotyping for all the mutations described so far, or direct sequencing, is expensive, laborious and restricted to only a few research centers, the diagnosis of 21-OHD deficiency should probably still rely on peak 17-OHP level in response to ACTH stimulation (7, 25).

We believe that genotyping is justified in patients with NC21-OHD owing to the existence of a subgroup with compound heterozygosity for one mild and one severe mutation. These findings have important implications for future genetic and prenatal counseling, since prenatal treatment with dexamethasone for fetuses carrying two severe mutations is now feasible (31).

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