European Journal of Endocrinology (2011) **165** 307–314 ISSN 0804-4643

CLINICAL STUDY

Relationship of *CYP21A2* genotype and serum 17-hydroxyprogesterone and cortisol levels in a large cohort of Italian children with premature pubarche

Lucia Ghizzoni, Marco Cappa[1], Alessandra Vottero[2], Graziamaria Ubertini[1], Daniela Carta[4], Natascia Di Iorgi[3], Valentina Gasco, Maddalena Marchesi[2], Vera Raggi[2], Anastasia Ibba[4], Flavia Napoli[3], Arianna Massimi[5], Mohamad Maghnie[3], Sandro Loche[4] and Ottavia Porzio[5]

Division of Endocrinology, Diabetology, and Metabolism, Department of Internal Medicine, University of Turin, Corso Dogliotti 14, 10126 Turin, Italy, [1]Ospedale Pediatrico Bambino Gesu', Rome, Italy, [2]Department of Pediatrics, University of Parma, Parma, Italy, [3]Department of Pediatrics, IRCCS G. Gaslini Institute, University of Genova, Genova, Italy, [4]Servizio di Endocrinologia Pediatrica, Ospedale Regionale per le Microcitemie, Cagliari, Italy and [5]Department of Internal Medicine, University of Rome "Tor Vergata", Rome, Italy

(Correspondence should be addressed to L Ghizzoni; Email: lucia.ghizzoni@unito.it)

Abstract

Objective: Premature pubarche (PP) is the most frequent sign of nonclassic congenital adrenal hyperplasia (NCCAH) due to 21-hydroxylase deficiency in childhood. The aim of this study was to assess the relationship between the *CYP21A2* genotype and baseline and ACTH-stimulated 17-hydroxyprogesterone (17-OHP) and cortisol serum levels in patients presenting with PP.

Patients and methods: A total of 152 Italian children with PP were studied. Baseline and ACTH-stimulated 17-OHP and cortisol serum levels were measured and *CYP21A2* gene was genotyped in all subjects.

Results: Baseline and ACTH-stimulated serum 17-OHP levels were significantly higher in NCCAH patients than in both heterozygotes and children with idiopathic PP (IPP). Of the patient population, four NCCAH patients (7.3%) exhibited baseline 17-OHP values <2 ng/ml (6 nmol/l). An ACTH-stimulated 17-OHP cutoff level of 14 ng/ml (42 nmol/l) identified by the receiver-operating characteristics curves showed the best sensitivity (90.9%) and specificity (100%) in distinguishing NCCAH patients. This value, while correctly identifying all unaffected children, missed 9% of affected individuals. Cortisol response to ACTH stimulation was <18.2 μg/dl (500 nmol/l) in 14 NCCAH patients (28%) and none of the heterozygotes or IPP children. Among the 55 NCCAH patients, 54.5% were homozygous for mild *CYP21A2* mutations, 41.8% were compound heterozygotes for one mild and one severe *CYP21A2* gene mutations, and 3.6% had two severe *CYP21A2* gene mutations.

Conclusion: In children with PP, baseline 17-OHP levels are not useful to rule out the diagnosis of NCCAH, which is accomplished by means of ACTH testing only. The different percentages of severe and mild *CYP21A2* gene mutations found in PP children compared with adult NCCAH patients is an indirect evidence that the enzyme defect is under-diagnosed in childhood, and it might not lead to the development of hyperandrogenic symptoms in adulthood. Stress-dose glucocorticoids should be considered in patients with suboptimal cortisol response to ACTH stimulation.

European Journal of Endocrinology **165** 307–314

Introduction

Premature pubarche (PP) refers to the appearance of pubic hair before the age of 8 years in girls and 9 years in boys, without other signs of puberty or virilization (1). Its precise etiology is not known. Generally, it has been attributed to the early maturation of the zona reticularis, which leads to an increase in adrenal androgens to levels normally seen in early puberty (2, 3). A hypersensitivity of the hair follicle to steroid hormones has also been proposed in those children in whom the premature development of pubic hair is not accompanied by raised adrenal androgen levels (4). A number of cases of PP are also associated with nonclassic congenital adrenal hyperplasia (NCCAH) due to 21-hydroxylase (21-OH) deficiency, with an incidence extremely variable, ranging from 0% in some reports to 40% in others, probably due to varying ethnic background and the use of different diagnostic criteria (5).

NCCAH due to 21-OH deficiency is one of the most frequent autosomal recessive diseases (6). The clinical manifestations of the enzyme deficiency may develop anytime during childhood or adult life and include hirsutism, acne, irregular menstrual cycles, and infertility, besides PP (7). The diagnosis is based on elevated baseline and ACTH-stimulated 17-hydroxyprogesterone

DOI: 10.1530/EJE-11-0119
Online version via www.eje-online.org

(17-OHP) levels. Baseline 17-OHP levels >5 ng/ml (>15 nmol/l) and ACTH-stimulated 17-OHP values >10 ng/ml (>30 nmol/l) are the cutoffs established for the diagnosis of the disease (8, 9). In studies performed before the identification of mutations in *CYP21A2* gene, heterozygous CYP21A2 mutation carriers presented variable peak responses of 17-OHP levels to ACTH stimulation, ranging from normal values to 10 ng/ml (30 nmol/l) (10).

The *CYP21A2* gene is located in the HLA gene cluster on the short arm of chromosome 6 (6p21.3) (11). Both the functional gene and a nonfunctional, highly homologous pseudogene (*CYP21P*) consist of 10 exons and are located in close proximity (12). In most cases, the mutations causing 21-OH deficiency are generated by unequal crossing over or gene conversion events. Commonly, the CYP21-inactivating point mutations are transferred by microconversions from the *CYP21P* to the *CYP21A2* gene (13). Approximately 65–75% of the CAH patients are compound heterozygotes, and the clinical expression correlates with the less severely mutated allele, and, consequently, with the residual activity of 21-OH (14). Generally, a good correlation is observed between *CYP21A2* genotype and clinical phenotype, although some discrepancies have been reported especially within mutation groups of intermediate severity, making phenotype prediction unreliable (14).

This study reports the molecular and biochemical features of the largest cohort of Italian patients with PP genotypically screened for nonclassic 21-OH deficiency described to date. The aim of this study was to assess the relationship between genotype and biochemical phenotype in patients exhibiting the same clinical phenotype, by analyzing the molecular spectrum of *CYP21A2* gene, and the baseline and ACTH-simulated 17-OHP levels in a large cohort of patients with PP.

Materials and methods

Subjects

The study group comprised of 152 unrelated children of Italian ancestry, 114 females and 38 males, who presented at four different Institutions for early growth of pubic or axillary hair (before 8 years in girls and 9 years in boys). All children were routinely genotyped and then randomly and retrospectively selected to obtain similar numbers in the three subgroups studied. The three different subgroups included i) children with PP and no detectable *CYP21A2* gene defects, defined as affected by idiopathic PP (IPP; $n=47$); ii) children with mutations in the *CYP21A2* gene detected in one allele, designated as heterozygotes ($n=50$); iii) children with *CYP21A2* gene mutations detected in two alleles, defined as affected by NCCAH due to 21-OH deficiency ($n=55$). None of the girls had signs of virilization or breast development, and none of the boys had testicular volume >3 ml. Bone age (BA) was evaluated using the standards of Greulich & Pyle (15). Height SDS was calculated as follows: actual height minus mean height for chronologic age (CA) divided by 1 S.D. of the height for age, using Tanner's growth data (16). An acute ACTH stimulation test was performed between 0700 and 0900 h. An i.v. catheter was placed in the forearm, and the subjects were allowed to rest for 30 min. Blood samples were obtained before and 60 min after 250 μg ACTH (1–24) i.v. injection to measure 17-OHP levels and cortisol. A 60 min stimulated cortisol ≤500 nmol/l (≤18.2 μg/dl) was used to define biochemical adrenal insufficiency. Serum 17-OHP concentrations were measured by RIA, using commercially available reagents from Siemens Healthcare Diagnostics, Inc., Los Angeles, CA, USA (intra- and inter-assay coefficients of variation (CVs) 1.9 and 5% respectively). Serum cortisol levels were measured by RIA using commercially available reagents from Radim, Pomezia, Italy (mean intraassay and interassay CVs were 4.1 and 6.5% respectively).

Molecular analysis of CYP21A2 *gene*

Informed consent for molecular analyses was obtained. Genomic DNA was prepared from peripheral blood leukocytes by standard procedures (17). The common and novel mutations were detected by direct sequencing of the entire *CYP21A2* gene and promoter as described previously (18). Gene conversion and deletions were detected by Southern blot analysis. Duplications of *CYP21A2* gene were detected by Multiplex ligation-dependent probe amplification assay (SALSA MLPA Kit P050B CAH, MRC Holland, Amsterdam, The Netherlands). Mutations and polymorphism were analyzed in both parents to verify the segregation of mutations and to confirm the presence of large gene rearrangements.

Statistical analysis

Data are presented as mean±S.D., unless otherwise indicated. Differences between baseline and stimulated 17-OHP levels were analyzed by two-tailed Student's *t*-test and among the groups by one-way ANOVA. When the ANOVA showed a significant difference, *post hoc* analysis was performed using the Bonferroni correction for multiple comparisons. The discriminating power of baseline and ACTH-stimulated 17-OHP values was evaluated by comparing the areas under receiver-operating characteristics (ROC) curves according to the standard method described by Hanley & McNeil (19). The concordance analysis between baseline and ACTH-stimulated 17-OHP values was assessed by the kappa index. A P value >80% was considered optimal. Data were analyzed with the program STATISTICA (StataSoft Inc., Tulsa, OK, USA), version 6.0 (1998). $P<0.05$ was considered statistically significant.

Results

Molecular analysis

Among the 152 subjects studied, 160 *CYP21A2*-mutated alleles were identified. The V2181L, P453S, P30L, and P482S mutations, defined as mild, were identified on 118 of the mutated alleles (73.7%). The predominant mutation was V218L, which was found on 67.5% of mutated alleles and in 77.1% of children carrying at least one mutated allele. Severe mutations accounted for 25.6% of mutated alleles. Among them, deletions and I172N were equally present on 5% of mutated alleles, each being the second most frequent abnormality in this study (Intr. 2 (A/C 656 to G): 3.12%; Q318X 3.75% (2.5%); conversion 3.12%; V281L+F306 (+1nt)+Q318X+R356W 2.5%; R356W 1.87% (0.19%); CL6 0.625%, Δ8bp 0.625%, P30L+intron2 (A/C 656 to G) 0.625%).

Among the 55 children affected by NCCAH, 52.7% had two mild *CYP21A2* gene mutations, 41.9% were compound heterozygotes for one mild and one severe *CYP21A2* gene mutations, and 3.6% had two severe *CYP21A2* gene mutations (Table 1). Of the patients with two mild mutations, 27 were homozygous for the V181L mutation, which accounts for 93.1% of the patients with two mild mutations, and 49.1% of all 55 patients, making it the most common genotype in the patient group (Table 1). In the compound heterozygote group (41.9% of patients), the most common genotypes were V281L/conversion and V281L/deletion (five patients in each group, 9.2%), followed in frequency by V281L/I172N (three patients, 5.5%) and V281L/R356W (two patients, 3.6%). Two siblings, male and female, were compound heterozygotes for V281L/V281L+F306 (+1nt)+Q318X+R356W (Table 1). There were two male patients in whom a severe mutation was detected on both alleles: one was homozygous for the I172N mutation and the other was a compound heterozygote for an I172L mutation and a large deletion. One patient had the V281L mutation on the maternal allele and the V281L+R366H mutations on the paternal allele: the functional characterization of the R366H new mutation is under study. When we compared the clinical and biochemical features of patients with mild/mild and mild/severe mutations, there were no differences between the groups in either baseline or stimulated 17-OHP levels (Fig. 1). The clinical and biochemical characteristics of the two patients with the severe/severe genotype are reported in Table 2.

Table 1 Genotype distribution in 55 children with premature pubarche and *CYP21A2* mutations on both alleles.

Severity of mutation	Genotypes	Probands		
		n	%	Total %
Mild/mild (*n*=29)	V281L/V281L	27	49.1	52.7
	P453S/P453S	2	3.6	
mild/severe (*n*=23)	V281L/conversion	5	9.2	41.9
	V281L/deletion	5	9.2	
	V281L/I172N	3	5.5	
	V281L/R356W	2	3.6	
	V281L/V281L+F306 (+1nt)+Q318X+R356W	2	3.6	
	V281L/intron 2 (A/C 656 to G)	2	3.6	
	V281L/CL6	1	1.8	
	Intron 2 (A/C 656 to G) /P453S	1	1.8	
	V281L/P30L+intron 2 (A/C 656 to G)	1	1.8	
	P30L/Δ8bp	1	1.8	
Severe/severe (*n*=2)	I172N/I172N	1	1.8	3.6
	I172N/deletion	1	1.8	
Novel genotype (*n*=1)	V281L/V281L+R366H	1	1.8	1.8

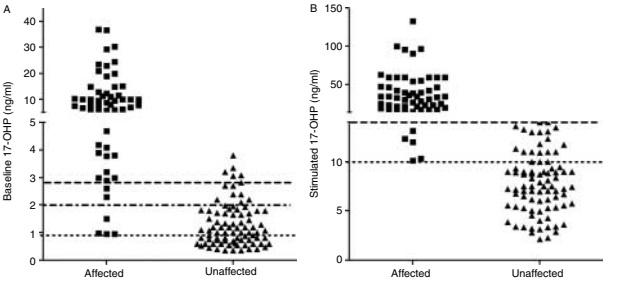

Figure 1 Concordance between baseline (panel A) and ACTH-stimulated (panel B) 17-OHP values based on the cutoffs identified by the ROC analysis (2.82 ng/ml (8.5 nmol/l): kappa index 0.81; 14 ng/ml (42 nmol/l): kappa index 0.89) and genotype. The concordance between the baseline and ACTH-stimulated 17-OHP values showing the best sensitivity for the diagnosis of NCCAH (baseline 0.89 ng/ml (2.7 nmol/l), kappa index: 0.33; ACTH-stimulated 10 ng/ml (30 nmol/l), kappa index: 0.71) and genotype is also shown. In addition, the concordance between the baseline 17-OHP value of 2 ng/ml (6 nmol/l), derived from the literature, and genotype is also shown (kappa index: 0.75).

Among the 50 heterozygous subjects, 36 carried a mild mutation (72%) and 13 (26%) a severe mutation. One child carried a new mutation (M150R), the study of the functional activity of which is in progress. The most frequent mutations were V281L (32 subjects, 64% of heterozygotes) and Q318X (four subjects, 8%; Table 3).

Table 2 Clinical and biological characteristics of the two nonclassic congenital adrenal hyperplasia patients carrying two severe *CYP21A2* gene mutations.

	Patient 1	Patient 2
Sex	Male	Male
Mutation	I172N/deletion	I172N/I172N
Age at diagnosis (years)	1	5.9
Bone age at diagnosis	NA	7.5
Height SDS	1.0	1.2
Symptoms at diagnosis	PP	PP
Baseline 17-OHP (ng/ml)	21	10.2
Baseline cortisol (μg/dl)	8.3	13.6
ACTH-stimulated 17-OHP (ng/ml)	48	47
ACTH-stimulated cortisol (μg/dl)	12	17.1

NA, not available; PP, premature pubarche.

Table 3 Genotype distribution in 50 children with premature pubarche and heterozygous *CYP21A2* mutations.

Severity of mutations	Genotypes	Subjects		
		n	%	Total %
Mild mutations	V281L	32	64	72
	P453S	2	4	
	P482S	1	2	
	P30L	1	2	
Severe mutations	Q318X	4	8	26
	Intron 2(A/C 656 to G)	2	4	
	Deletion	2	4	
	I172N	2	4	
	R356W	1	2	
	V281L+F306 (+1nt) +Q318X+R356W	2	4	
Novel mutation	M150R	1	2	2

Clinical and biochemical profile of patients

Children with PP and no detectable molecular defects were defined as affected by IPP, those with mutations in the *CYP21A2* gene detected in one allele were designated heterozygotes, and those with *CYP21A2* gene mutations detected in two alleles were defined as affected by NCCAH due to 21-OH deficiency. The clinical and biochemical features of the three distinct groups are reported in Table 4. CA at initiation of symptoms and at first evaluation, BA at first evaluation, BA to CA ratio (BA/CA), and height SDS at first evaluation were similar in the three groups of subjects. Baseline and ACTH-stimulated serum 17-OHP levels were significantly higher in NCCAH patients than in both heterozygotes and IPP children. There were four NCCAH patients (7.3%) with baseline 17-OHP values <2 ng/ml (<6 nmol/l) and eight children with IPP (16%) and five heterozygotes (10.6%) with baseline serum 17-OHP levels >2 ng/ml (>6 nmol/l). None of the NCCAH patients exhibited ACTH-stimulated 17-OHP levels below 10 ng/ml (30 nmol/l). ACTH-stimulated 17-OHP levels were between 10 and 15 ng/ml (30 and 45 nmol/l) in six patients, between 15 and 25 ng/ml (45 and 76 nmol/l) in 15 patients, and above 25 ng/ml (76 nmol/l) in the remaining 34 NCCAH patients. Six IPP and 12 heterozygotes exhibited ACTH-stimulated 17-OHP levels between 10 and 14 ng/ml (30 and 42 nmol/l). ACTH-stimulated 17-OHP levels <10 ng/ml (<30 nmol/l) were shown in 41 IPP and 38 heterozygotes. The analysis of baseline and post-ACTH 17-OHP levels according to the presence and/or severity of mutations showed significantly higher levels in NCCAH patients irrespective of the mutation, in comparison with subjects heterozygous for either a mild or a severe mutation and IPP children (Fig. 1).

Evaluation of the discriminating power of baseline and ACTH-stimulated 17-OHP values to make the diagnosis of NCCAH showed the best cutoffs, i.e. those with the best sensitivity and specificity, to be 2.82 ng/ml (8.5 nmol/l; sensitivity 84.1%, specificity 94.9%) and 14 ng/ml (42 nmol/l; sensitivity 90.9%, specificity 100%), respectively, in the whole population with PP. Areas under the ROC curves were 0.959% (95% confidence interval (CI)=0.896–0.989) and 0.990% (95% CI=0.938–0.998) for baseline and stimulated 17-OHP levels respectively. The cutoffs able to identify 100% of affected children were 0.89 ng/ml (2.7 nmol/l) and 10 ng/ml (30 nmol/l) for baseline and stimulated 17-OHP levels, with a specificity of 46.2 and 86.2% respectively. The concordance between baseline and ACTH-stimulated 17-OHP values based on the cutoffs identified by the ROC analysis (2.82 ng/ml (8.5 nmol/l), kappa index: 0.81; 14 ng/ml (42 nmol/l), kappa index: 0.89) and genotype is shown in Fig. 2. Overall, the baseline and stimulated 17-OHP cutoff values identified by the ROC analysis failed to recognize six and five patients respectively (false negative), while the false positive subjects detected were six and two respectively (kappa index). The concordance between genotype and baseline and ACTH-stimulated 17-OHP values based on the cutoffs of 0.89 ng/ml (2.7 nmol/l; kappa index: 0.33) and 10 ng/ml (30 nmol/l; kappa index: 0.71) is also shown in Fig. 2. With such cutoffs, all patients were detected, while 51 unaffected subjects with baseline 17-OHP values >0.89 (>2.7 nmol/l) and 17 with stimulated 17-OHP values >10 (>30 nmol/l) were identified as affected (false positive; kappa index).

When the post-ACTH 17-OHP levels of heterozygous subjects with severe mutations were compared with those of subjects heterozygous for mild mutations, no significant differences were detected (Fig. 1). The baseline and ACTH-stimulated 17-OHP levels of the 47 unaffected children were not significantly different from those observed in heterozygous subjects. The highest

Table 4 Clinical and hormonal characteristics of NCCAH patients, heterozygous subjects, and IPP children.

	Baseline 17-OHP ng/ml (nmol/l)	Peak 17-OHP ng/ml (nmol/l)	CA at initiation of symptoms	CA at 1st evaluation	BA at 1st evaluation	Height SDS at 1st evaluation	BA/CA at 1st evaluation
NCCAH patients (n=55)	10.7±8.7 (32.4±26.5)*	39.3±25.9 (119.1±78.5)*	6.72±1.06	8.02±1.57	9.65±1.95	1.12±1.14	1.38±0.32
HZ (n=50)	1.3±0.8 (3.9±2.4)	8.6±2.6 (26.0±7.8)	7.34±1.05	7.28±0.85	9.45±1.96	0.83±1.11	1.24±0.22
IPP (n=47)	1.2±0.8 (3.6±2.4)	6.74±3.2 (20.3±9.7)	6.72±1.36	7.08±1.64	8.56±1.40	1.20±1.30	1.27±0.18

HZ, heterozygous subjects; IPP, idiopathic premature pubarche. *$P<0.05$ vs IPP and HZ subjects.

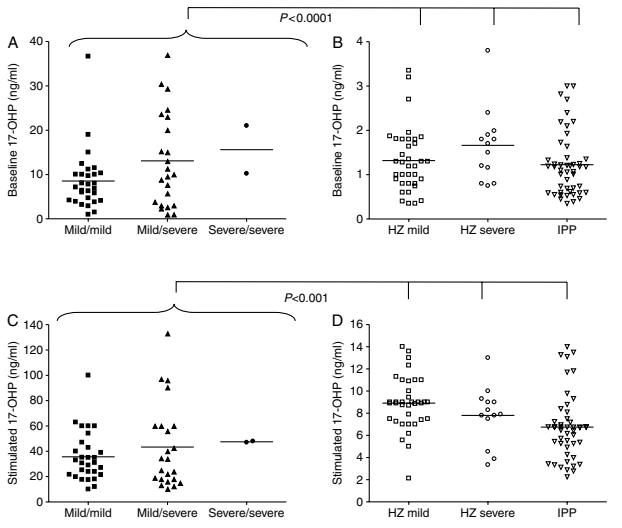

Figure 2 Baseline and ACTH-stimulated serum 17-OHP levels in NCCAH patients (panel A and C) according to the severity of mutations (mild/mild, patients homozygous for mild mutations; mild/severe, patients compound heterozygous (HZ), severe/severe, patients homozygous for severe mutations). In panel B and D, the baseline and ACTH-stimulated serum 17-OHP levels of heterozygous subjects according to the severity of mutations (HZ mild, heterozygous subjects carrying mild mutations; HZ severe, heterozygous subjects carrying severe mutations), and in IPP, are depicted. $P<0.0001$ indicates that groups mild/mild, mild/severe, and severe/severe are each significantly different from HZ mild, HZ severe, and IPP groups. $P>0.001$ indicates that groups mild/mild, mild/severe, and severe/severe are each significantly different from HZ mild, HZ severe, and IPP groups.

baseline and post-ACTH 17-OHP levels in these unaffected subjects were 3 ng/ml (9 nmol/l) and 14 ng/ml (42 nmol/l) respectively.

Baseline serum cortisol concentrations were not different among the three groups analyzed, whereas post-ACTH hormone levels were significantly lower in NCCAH patients compared with the other groups (NCCAH: 22.03 ± 7.63 μg/dl (607 ± 210 nmol/l); heterozygotes: 37.26 ± 7.68 μg/dl (1028 ± 212 nmol/l); IPP: 34.54 ± 6.35 μg/dl (953 ± 175 nmol/l, $P<0.0001$ ANOVA)). Two NCCAH patients, three heterozygous subjects, and six IPP children had baseline serum cortisol levels <5 μg/dl (138 nmol/l). None of the IPP children or heterozygous subjects showed serum cortisol concentrations in response to ACTH stimulation <18.12 μg/dl (500 nmol/l), whereas 14 NCCAH patients exhibited post-ACTH hormone levels <18.12 μg/dl (500 nmol/l). The analysis of baseline and post-ACTH cortisol values of NCCAH patients, heterozygous subject, and IPP according to the presence and/or severity of mutation, showed no differences among the distinct groups (Fig. 3, panel A and B). Post-ACTH cortisol values were significantly higher in NCCAH patients irrespective of the mutation in comparison with subjects heterozygous for either a mild or a severe mutation and IPP children (Fig. 3, panel C and D). None of the NCCAH patients with suboptimal cortisol response to ACTH ever reported illnesses consistent with adrenal insufficiency or had undergone either minor or major surgery.

Discussion

We herein describe the largest cohort of PP children genotypically screened for 21-OH deficiency. CA, BA, and the BA/CA ratio at diagnosis were not significantly different between the three subgroups analyzed (IPP, NCCAH, and heterozygous subjects), indicating that among PP patients, NCCAH-deficient patients do not have any distinct clinical characteristic. This is in contrast with previous studies reporting advanced BA maturation in children with PP due to NCCAH (20, 21). However, the results of these previous studies were derived from either a limited number or nongenetically characterized patients. Therefore, it can conceivably be concluded that children with PP due to NCCAH cannot be distinguished from those with IPP based on clinical grounds solely, and the decision whether to perform hormonal and molecular genetic testing to rule out the enzyme defect should not rely on patients' clinical features (BA or height).

Baseline 17-OHP blood concentrations are often reported to be elevated in patients with NCCAH and have been proposed as predictors of the disease. Azziz

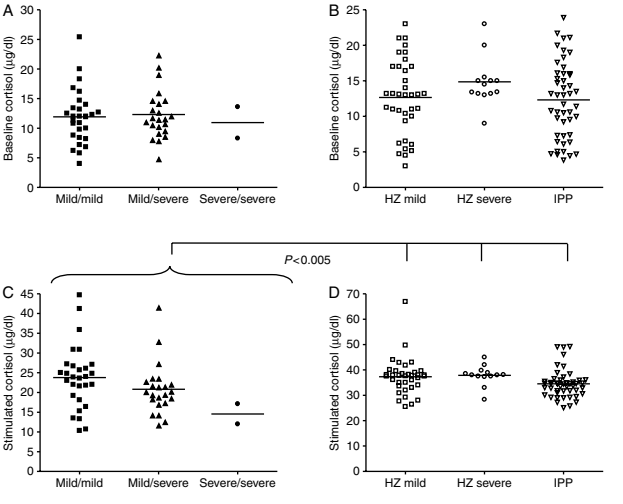

Figure 3 Baseline and ACTH-stimulated serum cortisol levels in NCCAH patients (panel A and C) according to the severity of mutations (mild/mild, patients homozygous for mild mutations; mild/severe, patients compound heterozygous; severe/severe, patients homozygous for severe mutations). In panel B and D, the baseline and ACTH-stimulated serum cortisol levels in heterozygous subjects according to the severity of mutations (HZ mild, heterozygous subjects carrying mild mutations; HZ severe, heterozygous subjects carrying severe mutations), and in IPP, are depicted. $P<0.005$ indicates that groups mild/mild, mild/severe, and severe/severe are each significantly different from HZ/mild, HZ/severe, and IPP groups.

et al. (10) reported that in a population of unselected hyperandrogenic women, only 20 of whom were genotyped for the most common *CYP21A2* gene mutations, baseline serum 17-OHP levels <2 ng/ml (<6 nmol/l) effectively ruled out NCAH with 100% sensitivity and 99% specificity. Similar figures were recently described in a French cohort of children with PP in whom the threshold level of 2 ng/ml (6 nmol/l) corresponded to the 94th percentile of the distribution of the hormone levels in the group of patients studied (22). In this large French cohort, only ten PP children were genetically documented to be affected by NCCAH. The results of this study are at variance with these previous reports. In our cohort, baseline 17-OHP serum levels >2 ng/ml (6 nmol/l), corresponding to the 80th percentile for the unaffected children, identified NCCAH with 92.7% sensitivity and 82.1% specificity (kappa index 0.75; Fig. 1). This means that among the 55 affected children of our cohort of PP subjects, four (7.3%) presented baseline 17-OHP levels <2 ng/ml (<6 nmol/l), indicating that this threshold value cannot rule out the diagnosis of NCCAH in our cohort of children with PP. When we tried to optimize the sensitivity (100%) by using a baseline 17-OHP threshold level of 0.89 (2.7 nmol/l), the specificity was greatly reduced (46.2%), and thus not useful in clinical practice. Therefore baseline 17-OHP levels are not useful to rule out the diagnosis of NCCAH in children with PP. The discrepancy between the results of this study and those suggesting baseline 17-OHP values >2 ng/ml (>6 nmol/l) as predictor of the disease may rely on the limited number of affected individuals analyzed in the latter studies (10, 22), that might have underestimated the percentage of patients with values below the threshold.

The cutoff level for ACTH-stimulated 17-OHP of 10 ng/ml (30 nmol/l) has been currently used for the diagnosis of NCCAH, although it has been the subject of discussion in the literature, with cutoffs of 20 (60 nmol/l) and even 30 ng/ml (90 nmol/l) being instead proposed by some authors (23–25). In our cohort of patients, a cutoff of 14 ng/ml (42 nmol/l) corresponding to a percentile of the distribution of the hormone levels in the group of unaffected children studied >97th showed the best sensitivity (90.9%) and specificity (100%). However, this value, while correctly identifying all unaffected children, missed five patients, equal to 9.1% of affected individuals. As we aim at correctly identifying all affected children and therefore value more the sensitivity than the specificity of the test, the value of 10 ng/ml (30 nmol/l) seems to be the most adequate in our population study (sensitivity 100%, specificity 86.2%). The use of such a cutoff would lead to the inappropriate testing of 9.9% of unaffected and heterozygous subjects, a percentage similar to the one of affected children who would be missed with the use of a higher cutoff. As the identification of patients is important both for the risk of potential adrenal insufficiency under stressful conditions and for genetic counseling, the cost-effectiveness of using a low cutoff for ACTH-stimulated 17-OHP levels is unquestionable.

Suboptimal cortisol responses to ACTH stimulation was found in 28% of our NCCAH patient, in agreement with recent studies showing low cortisol responses in a subset of patients with NCCAH (26–28). No signs or symptoms consistent with adrenal insufficiency were either reported by any of our patients or those included in the previous studies (28). The finding of abnormal biochemical data in the absence of remarkable clinical history is difficult to interpret, and the need in these patients for cortisol during stressful conditions is unknown. While we do not recommend routine stress dosing, we suggest our patients to wear or carry medical identification indicating that stress-dose glucocorticoids is needed in case of extreme trauma or major surgery only.

The *CYP21A2* genotyping of the 55 children affected by NCCAH showed that, as in other series, the most frequently observed mutation is V281L (23, 29, 30). Homozygosity for mild mutations was found in 52.7% of our patients, a percentage strikingly higher than that recently reported in a large series of French adult patients (26) and in previous studies in adult Brazilian patients (23), but similar to the one detected in pediatric Spanish (29) and Jewish patients (30), mostly comprised of children with PP. These different figures cannot be related to the different ethnicity of the populations studied, as the majority of the patients are of Caucasian origin. A plausible reason for the discrepant results might be that pediatric patients with PP and 21-OH deficiency may be under-diagnosed in childhood and do not develop signs of the disease requiring hormonal and genetic testing, in adulthood. Thus, this finding may represent indirect evidence that children with PP due to 21-OH deficiency are not at risk of developing hyperandrogenism, with the associated clinical features characteristic of NCCAH in adulthood.

The comparison of the clinical and hormonal phenotype of the different genotypes revealed no differences between the mild/mild and mild/severe genotypes, except for baseline 17-OHP levels that were significantly higher in patents with mild/severe mutations. When we analyzed the hormone levels within the group of patients homozygous for the V281L mutation, they appeared quite heterogeneous (data not shown). Paradoxically, the two siblings compound heterozygous for V281L/V281L+F306 (+1nt)+Q318X+R356W were among the patients with the lowest baseline and stimulated 17-OHP levels (0.94 ng/ml (2.8 nmol/l) and 0.96 ng/ml (2.9 nmol/l) for baseline and 10.3 ng/ml (31 nmol/l) and 18 ng/ml (54 nmol/l) for stimulated levels). This indicates that the phenotype cannot be always predicted from the genotype and suggests a minor influence of the allele carrying the severe mutation on both the biological and

the clinical expression of the disease, which is probably more pronounced in our cohort because of the more homogeneous clinical phenotype than in previously reported series. The failure of strict correspondence of phenotype and genotype with the possibility of the former to be even milder than expected is also suggested by the two patients in our cohort carrying two severe mutations. The first child was homozygous for the I172N mutation, which is usually associated with the classic simple virilizing form of the disease. Such a genotype was previously reported in a girl diagnosed at the age of 1 year because of secondary virilization (31) and in a 15 years old girl with hirsutism and primary amenorrhea (26). The other patient was a compound heterozygote for I172N and a large deletion, a combination previously described as responsible for severe salt-wasting disease (32). Our observation confirms that the I172N mutation in combination with an additional severe mutation of the *CYP21A2* gene may be associated with a nonclassic phenotype and clearly indicates that genetic studies are mandatory, besides hormonal ones, to define the precise status of the patient.

The description of the present large cohort of children with the same clinical phenotype characterized by PP but a variety of genotypes and biological phenotypes suggests the implication of other genetic and/or environmental factors modulating the biological and clinical expression of the disease. Such factors may include modifying genes, individual differences in adrenal and/or extra adrenal steroid metabolic pathways, and different cortisol requirements. Individual skin sensitivity to androgens may also play a role involving epigenetic modulation of the androgen receptor gene, as recently described in children with IPP (4). In the latter, a decreased methylation of the androgen receptor gene in an androgen target tissue such as hair follicles was shown to produce an increased gene expression, and in turn, more active androgen receptors and increased sensitivity to androgens. Such a mechanism could also play a role in the different sensitivity to androgens in PP patients with nonclassic 21-OH deficiency.

In conclusion, the results of this study indicate that a distinct phenotype such as PP can be the result of different molecular genetic defects and variable hormone levels, suggesting that modifier factors may modulate the expression of the disease. In children with PP, non-classic 21-OH deficiency can be ruled out only by means of ACTH testing. The different percentages of severe and mild *CYP21A2* gene mutations found in PP children compared to adult patients with NCCAH may indicate that the enzyme defect is under-diagnosed in childhood and may not lead to the development of hyperandrogenic symptoms in adulthood. Stress-dose glucocorticoids should be considered in patients with suboptimal cortisol response to ACTH stimulation.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

References

1 Saenger P & Reiter EO. Premature adrenarche: a normal variant of puberty? *Journal of Clinical Endocrinology and Metabolism* 1992 **74** 236–238. (doi:10.1210/jc.74.2.236)

2 Riddick LM, Garibaldi LR, Wang ME, Senne AR, Kilmah PE, Clark AT, Levine LS, Oberfield SE & Pang SY. 3α-Androstenediol glucoronide in premature and normal pubarche. *Journal of Clinical Endocrinology and Metabolism* 1991 **72** 46–50. (doi:10.1210/jcem-72-1-46)

3 Voutilainen R, Perheentupa J & Apter D. Benign premature adrenarche: clinical features and serum steroid levels. *Acta Paediatrica Scandinavica* 1983 **72** 707–711. (doi:10.1111/j.1651-2227.1983.tb09798.x)

4 Vottero A, Capelletti M, Giuliodori S, Viani I, Ziveri M, Neri TM, Bernasconi S & Ghizzoni L. Decreased androgen receptor gene methylation in premature pubarche: a novel pathogenetic mechanism? *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 968–972. (doi:10.1210/jc.2005-2354)

5 Ghizzoni L, Mastorakos G & Vottero A. Adrenal hyperandrogenism in children. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 4431–4435. (doi:10.1210/jc.84.12.4431)

6 Speiser PW, Dupont B, Rubinstein P, Piazza A, Kastelan A & New MI. High frequency of nonclassical steroid 21-hydroxylase deficiency. *American Journal of Human Genetics* 1985 **37** 650–667.

7 Speiser PW. Nonclassic adrenal hyperplasia. *Reviews in Endocrine & Metabolic Disorders* 2009 **10** 77–82. (doi:10.1007/s11154-008-9097-x)

8 New MI, Lorenzen F, Lerner AJ, Kohn B, Oberfield SE, Pollack MS, Dupont B, Stoner E, Levy DJ, Pang S & Levine LS. Genotyping steroid 21-hydroxylase deficiency: hormonal reference data. *Journal of Clinical Endocrinology and Metabolism* 1983 **57** 320–326. (doi:10.1210/jcem-57-2-320)

9 Dewailly D, Vantyghem-Haudiquet MC, Sainsard C, Buvat J, Cappoen JP, Ardaens K, Racadot A, Lefebvre J & Fossati P. Clinical and biological phenotypes in late-onset 21-hydroxylase deficiency. *Journal of Clinical Endocrinology and Metabolism* 1986 **63** 418–423. (doi:10.1210/jcem-63-2-418)

10 Azziz R, Hincapie LA, Knochenhauer ES, Dewailly D, Fox L & Boots LR. Screening for 21-hydroxylase-deficient nonclassic adrenal hyperplasia among hyperandrogenic women: a prospective study. *Fertility and Sterility* 1999 **72** 915–925. (doi:10.1016/S0015-0282(99)00383-0)

11 White PC, New MI & Dupont B. Structure of human steroid 21-hydroxylase genes. *PNAS* 1986 **83** 5111–5115. (doi:10.1073/pnas.83.14.5111)

12 Higashi Y, Yoshioka H, Yamane M, Gotoh O & Fujii-Kuriyama Y. Complete nucleotide sequence of two steroid 21-hydroxylase genes tandemly arranged in human chromosome: a pseudogene and a genuine gene. *PNAS* 1986 **83** 2841–2845. (doi:10.1073/pnas.83.9.2841)

13 Harada F, Kimura A, Iwanaga T, Shimozawa K, Yata J & Sasazuki T. Gene conversion-like events cause steroid 21-hydroxylase deficiency in congenital adrenal hyperplasia. *PNAS* 1987 **84** 8091–8094. (doi:10.1073/pnas.84.22.8091)

14 Wilson RC, Mercado AB, Cheng KC & New MI. Steroid 21-hydroxylase deficiency: genotype may not predict phenotype. *Journal of Clinical Endocrinology and Metabolism* 1995 **80** 2322–2329. (doi:10.1210/jc.80.8.2322)
15 Greulich WW & Pyle SI. Radiographic atlas of skeletal development of the hand and wrist. 2nd edn. Stanford, CA, USA: Stanford University Press, 1959.
16 Tanner JM, Whitehouse RH & Takaishi M. Standards from birth to maturity for height, weight, height velocity, and weight velocity: British children, 1965. I. *Archives of Disease in Childhood* 1966 **41** 454–471. (doi:10.1136/adc.41.219.454)
17 Miller SA, Dynes DD & Polesky H. A simple salting out procedures for extracting DNA from human nucleoted cells. *Nucleic Acids Research* 1988 **16** 1215. (doi:10.1093/nar/16.3.1215)
18 Porzio O, Cunsolo V, Malaponti M, De Nisco E, Acquafredda A, Cavallo L, Andreani M, Giardina E, Testi M, Cappa M & Federici G. Divergent phenotype of two siblings human leukocyte antigen identical, affected by nonclassical and classical congenital adrenal hyperplasia caused by 21-hydroxylase deficiency. *Journal of Clinical Endocrinology and Metabolism* 2006 **91** 4510–4513. (doi:10.1210/jc.2006-0779)
19 Hanley JA & McNeil BJ. A method of comparing the areas under receiver operating characteristic curves derived from the same cases. *Radiology* 1983 **148** 839–843.
20 Dacou-Voutetakis C & Dracopoulou M. High incidence of molecular defects of the CYP21 gene in patients with premature adrenarche. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 1570–1574. (doi:10.1210/jc.84.5.1570)
21 Siegel SF, Finegold DN, Urban MD, McVie R & Lee PA. Premature pubarche: etiological heterogeneity. *Journal of Clinical Endocrinology and Metabolism* 1992 **74** 239–247. (doi:10.1210/jc.74.2.239)
22 Armengaud JB, Charkaluk ML, Trivin C, Tardy V, Breart G, Brauner R & Chalumeau M. Precocious pubarche: distinguishing late-onset congenital adrenal hyperplasia from premature adrenarche. *Journal of Clinical Endocrinology and Metabolism* 2009 **94** 2835–2840. (doi:10.1210/jc.2009-0314)
23 Bachega TA, Billerbeck AE, Marcondes JA, Madureira G, Arnhold IJ & Mendonca BB. Influence of different genotypes on 17-hydroxyprogesterone levels in patients with nonclassical congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Clinical Endocrinology* 2000 **52** 601–607. (doi:10.1046/j.1365-2265.2000.00995.x)
24 Moran C, Azziz R, Carmina E, Dewailly D, Fruzzetti F, Ibanez L, Knochenhauer ES, Marcondes JA, Mendonca BB, Pignatelli D, Pugeat M, Rohmer V, Speiser PW & Witchel SF. 21-Hydroxylase-deficient nonclassic adrenal hyperplasia is a progressive disorder: a multicenter study. *American Journal of Obstetrics and Gynecology* 2000 **183** 1468–1474. (doi:10.1067/mob.2000.108020)
25 Azziz R, Dewailly D & Owerbach D. Clinical review 56: nonclassic adrenal hyperplasia: current concepts. *Journal of Clinical Endocrinology and Metabolism* 1994 **78** 810–815. (doi:10.1210/jc.78.4.810)
26 Bidet M, Bellanne-Chantelot C, Galand-Portier MB, Tardy V, Billaud L, Laborde K, Coussieu C, Morel Y, Vaury C, Golmard JL, Claustre A, Mornet E, Chakhtoura Z, Mowszowicz I, Bachelot A, Touraine P & Kuttenn F. Clinical and molecular characterization of a cohort of 161 unrelated women with nonclassical congenital adrenal hyperplasia due to 21-hydroxylase deficiency and 330 family members. *Journal of Clinical Endocrinology and Metabolism* 2009 **94** 1570–1578. (doi:10.1210/jc.2008-1582)
27 Verma S, Green-Golan L, Vanryzin C, Drinkard B, Mehta SP, Weise M, Eisenhofer G & Merke DP. Adrenomedullary function in patients with nonclassic congenital adrenal hyperplasia. *Hormone and Metabolic Research* 2010 **42** 607–612. (doi:10.1055/s-0030-1253385)
28 Nandagopal R, Sinaii N, Avila NA, Van Ryzin C, Chen W, Finkielstain GP, Mehta SP, McDonnell NB & Merke DP. Phenotypic profiling of parents with cryptic nonclassic congenital adrenal hyperplasia: findings in 145 unrelated families. *European Journal of Endocrinology* 2011 **164** 977–984. (doi:10.1530/EJE-11-0019)
29 Ezquieta B, Cueva E, Varela J, Oliver A, Fernandez J & Jariego C. Non-classical 21-hydroxylase deficiency in children: association of adrenocorticotropic hormone-stimulated 17-hydroxyprogesterone with the risk of compound heterozygosity with severe mutations. *Acta Paediatrica* 2002 **91** 892–898. (doi:10.1111/j.1651-2227.2002.tb02851.x)
30 Weintrob N, Brautbar C, Pertzelan A, Josefsberg Z, Dickerman Z, Kauschansky A, Lilos P, Peled D, Phillip M & Israel S. Genotype–phenotype associations in non-classical steroid 21-hydroxylase deficiency. *European Journal of Endocrinology* 2000 **143** 397–403. (doi:10.1530/eje.0.1430397)
31 Wedell A, Ritzen EM, Haglund-Stengler B & Luthman H. Steroid 21-hydroxylase deficiency: three additional mutated alleles and establishment of phenotype–genotype relationships of common mutations. *PNAS* 1992 **89** 7232–7236. (doi:10.1073/pnas.89.15.7232)
32 Speiser PW, Dupont J, Zhu D, Serrat J, Buegeleisen M, Tusie-Luna MT, Lesser M, New MI & White PC. Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Journal of Clinical Investigation* 1992 **90** 584–595. (doi:10.1172/JCI115897)

Received 11 May 2011
Accepted 6 June 2011