CLINICAL STUDY

Adrenal 21-hydroxylase gene mutations in Slovenian hyperandrogenic women: evaluation of corticotrophin stimulation and HLA polymorphisms in screening for carrier status

Vita Dolžan¹, Janez Preželj², Blanka Vidan-Jeras³ and Katja Breskvar¹¹Institute of Biochemistry, Faculty of Medicine, Ljubljana, Slovenia, ²Department of Endocrinology and Metabolism, University Medical Centre, Ljubljana, Slovenia and ³Tissue Typing Centre, Blood Transfusion Centre of Slovenia, Ljubljana, Slovenia

(Correspondence should be addressed to V Dolžan, Institute of Biochemistry, Faculty of Medicine, Vrazov trg 2, 1000 Ljubljana, Slovenia; Email: dolzan@ibmi.mf.uni-lj.si)

Abstract

Objective: To study the incidence of 21-hydroxylase deficiency in Slovenian hyperandrogenic women, at the gene level. Previous endocrine studies indicated large differences in the incidence of 21-hydroxylase deficiency in hyperandrogenic women. The predictive values of the 17-hydroxyprogesterone (17-OHP) response to ACTH stimulation and of HLA typing in screening for carrier status were re-evaluated.

Design: Molecular analysis of *CYP21* gene, ACTH stimulation and human leucocyte antigen (HLA) typing were performed in 83 consecutive Slovenian hyperandrogenic women.

Measurements: Cortisol and 17-OHP concentrations were measured at baseline and 60 min after ACTH stimulation. Basal adrenal androgen concentrations were also measured.

Results: None of 83 hyperandrogenic patients was affected with non-classical 21-hydroxylase deficiency, but 12 of 81 patients (14.8%) had high concentrations of 17-OHP after stimulation, indicative of carrier status. The increase in 17-OHP concentrations could be explained by a carrier status for *CYP21* gene mutations in only three of 12 patients (25%), whereas seven of 69 patients (10.1%) with normal concentrations of 17-OHP after stimulation were found to be carriers of *CYP21* gene mutations, indicating low positive predictive values of ACTH stimulation as a screening test for carriers of 21-hydroxylase deficiency. In total, 11 carriers were identified among 83 patients: seven *CYP21* gene deletions/conversions, two Gln³¹⁸Stop and one Val²⁸¹Leu mutation and one gene conversion extending from exon 4 to exon 7 were found. The association between Val²⁸¹Leu mutation and HLA-B14 antigen was confirmed in this Slovenian population.

Conclusions: Basal or ACTH-stimulated 17-OHP concentrations are not a good indicator of the carrier status for 21-hydroxylase deficiency among Slovenian hyperandrogenic patients. Reliable screening for carriers of 21-hydroxylase deficiency is possible only by molecular analysis of the *CYP21* gene.

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Introduction

Non-tumoral hyperandrogenism is one of the most common endocrinological disorders in women. It is manifested by one or more of the following clinical signs and symptoms: oligo/amenorrhoea, decreased fertility, hirsutism, acne resistant to treatment or androgenic alopecia. Increased plasma concentrations of one or more adrenal or ovarian androgens are often found (1). There is no specific marker on the basis of which hyperandrogenism could be distinguished from the polycystic ovary syndrome (PCOS), especially as polycystic ovaries are also detected by ultrasound in healthy women without clinical signs of hyperandrogenism (2). Heterogeneity of clinical symptoms and biochemical features in hyperandrogenism complicates the search

for its possible genetic causes (3). Mutations in the 21-hydroxylase gene, the 11 β -hydroxylase gene, the 3 β -hydroxysteroid dehydrogenase gene and the insulin receptor gene have been found to be associated with hyperandrogenism (4).

Non-classical congenital adrenal hyperplasia (NC-CAH) caused by 21-hydroxylase deficiency is one of the most common genetic defects. Its incidence is 1/1000 in white populations, in general but is much higher in some selected populations: 1/27 in Ashkenazi Jews, 1/53 in Spaniards, 1/63 in Croats and 1/330 in Italians (5, 6). The incidence of NC-CAH in hyperandrogenic women has been reported to be 1.2–20% (7–9). This variability may be due to a different frequency of NC-CAH in studied populations of different ethnic origin or differences in selection criteria, but may also reflect

difficulty in distinguishing between NC-CAH and non-tumoral hyperandrogenism on the basis only of clinical symptoms and hormonal findings. The corticotrophin (ACTH) test is used to identify NC-CAH, but it is difficult to distinguish between the conditions when the 17-hydroxyprogesterone (17-OHP) response to ACTH stimulation is above normal (9.6 nmol/l) but below values characteristic of NC-CAH (36 nmol/l) (7). It has been suggested that hyperandrogenic women with borderline increases in 17-OHP after ACTH stimulation are heterozygous carriers of mutations in the 21-hydroxylase gene (*CYP21*) (10). In agreement with this, it was reported that 55 of 69 French hyperandrogenic women carried mutations in the *CYP21* gene (11). It has also been suggested that heterozygosity for 21-hydroxylase deficiency may be associated with adolescent hyperandrogenism (12). In contrast, it seems that obligate heterozygous carriers of *CYP21* gene mutations are not at increased risk for hyperandrogenism (13).

CYP21 genes located in the central region of the major histocompatibility complex (MHC) on chromosome 6 are in close genetic linkage with human leucocyte antigens (HLA) (14). Characteristic HLA haplotypes are associated with CAH, but this association differs among populations. In some populations, up to 70% of NC-CAH alleles are characterised by Val²⁸¹Leu mutation of *CYP21* genes and are associated with ancestral haplotype 65.1 characterised by HLA-B14, -C4A2, -C4B1+2, -DR1 (5, 15); however, these markers seem to be absent from Croatian patients with NC-CAH (6). In populations with known associations between HLA haplotypes and disease, HLA typing can help to identify carriers.

The aims of our study were: to determine the incidence of NC-CAH in Slovenian hyperandrogenic women, to find out if HLA associations with CAH described in the literature are also valid for the Slovenian population and could be used for screening purposes, and to define if the carrier status for *CYP21* gene mutations is reflected in the basal plasma androgen concentrations or in the 17-OHP response to ACTH stimulation, or both.

Study participants and methods

Study participants

The study population consisted of 83 Slovenian female hyperandrogenic patients, aged 17–32 years (median 24 years), referred to the outpatient endocrinological unit in Ljubljana. They were complaining of one or more of the following clinical symptoms: oligo/amenorrhoea (12 of the patients were oligomenorrhoeic, the others eumenorrhoeic), hirsutism, acne resistant to treatment, or androgenic alopecia. The presence of hirsutism was assessed by the examining endocrinologist, but was not formally scored. The presence of polycystic ovaries as determined

by ultrasound was not a selection criterion. Androgen-secreting tumours and thyroid dysfunction were excluded by clinical and hormonal findings.

Family members of patients who had *CYP21* gene mutations or increased 17-OHP after an ACTH test were asked to participate in the study. Eleven family members of four patients agreed to take part in HLA typing and molecular analysis, but were not hormonally tested.

Two control groups were analysed for point mutations in the *CYP21* gene. The first control group of 18 young women aged 20–29 years (median 21 years) served also to provide information on the normal basal and stimulated 17-OHP and cortisol concentrations in the healthy Slovenian population. All were eumenorrhoeic, without clinically evident signs of hirsutism or acne, and none was receiving hormonal medication. The second control group consisted of 38 DNA samples randomly chosen from Slovenian female blood donors previously typed for HLA (16).

The study design was approved by the Slovene Ethical Committee for Research in Medicine. Written informed consent was obtained from all participants in the study.

ACTH testing and hormonal analysis

The ACTH test was performed with 0.25 mg ACTH i.m. (Synacthen; Ciba-Geigy, Basel, Switzerland) between 0800 and 0900 h. Healthy controls and eumenorrhoeic hyperandrogenic women were tested in the early follicular phase. Blood samples were obtained at 0 min and 60 min after the administration of ACTH and were assayed for 17-OHP and cortisol. Normal upper basal and stimulated concentrations of 17-OHP were determined as mean + 2s.d. of those in healthy controls and were less than 4.1 nmol/l for basal 17-OHP and less than 9.8 nmol/l for stimulated 17-OHP. In patients, basal blood samples were also assayed for free testosterone (normal value <7.7 pmol/l), androstenedione (normal value <11.6 nmol/l) and dehydroepiandrosterone sulphate (DHEAS; normal value <8.8 nmol/l). The following RIA kits were used: free testosterone, Diagnostic Products Corporation (DPC; Los Angeles, CA, USA); androstenedione, Immunotech International (Marseille, France); DHEAS, Diagnostic System Laboratories (Webster, TX, USA); 17-OHP, DPC. A fluoroimmunoassay kit from Wallac (Turku, Finland) was used to determine cortisol concentration. The mean intra- and interassay coefficients of variation (CV) were less than 6%.

HLA typing

Standard microlymphocytotoxicity testing (17) was used to determine class I HLA phenotypes in 77 female hyperandrogenic patients. A local set of serostatically defined polyclonal alloantisera and polyclonal alloantisera from Biotest (Dreieich, Germany) were used. *HLA-DRB1* alleles were analysed by oligonucleotide hybridisation of PCR-amplified second exons of the *HLA-DRB1* gene (18) or by sequence-specific PCR

Table 1 Primers used for direct sequencing of the *CYP21* gene.

Exon	Forward primer*	Reverse primer*
1	(-42) 5'-GAGCTATAAGTGGCACCTCAG-3'	(238) 5'-CTCCTATGGTGAGGGCCAGA-3'
2	(255) 5'-AGAGGGTCTCTCTCCGCT-3'	(464) 5'-CAGCCTGGGCAACATAGCA-3'
4 + 5	(899) 5'-GCCTGCTGAACCTCACTG-3'	(1267) 5'-CAGCCTCTCCCCTACAACC-3'
6	(1282) 5'-CTCCTTCCCACAGCTGCATT-3'	(1462) 5'-CGGTAGCATCACTGGCTGT-3'
7	(1511) 5'-GCCTGTTGCCACTCTGTACTC-3'	(1811) 5'-GGAAGGAGCCTTTTGCTTGT-3'
8	(2000) 5'-CACTGGGTTGCTGAGGGAG-3'	(2194) 5'-GGGCTTTCCTCACTCATCC-3'
9	(2172) 5'-TTGGGGATGAGTGAGGAAAG-3'	(2388) 5'-AGCCTCCACCACATTTTGAC-3'
10	(2384) 5'-TGCCGTGAAAATGTGGTGGA-3'	(2754) 5'-GCAATAAAGGAGAACTGAGGT-3'

* Primers were designed using the Primer3 program by S Rozen & HJ Skaletsky, 1996 available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html

(Dynal; Oslo, Norway) (19). The data obtained from the group of 38 healthy controls previously typed for HLA alleles (16) were used to compare HLA frequencies.

Molecular analysis

DNA was isolated from peripheral blood leucocytes (20). Ten micrograms genomic DNA were digested with TaqI restriction endonuclease. Fragments were separated on 0.7% agarose gels and blotted on the Hybond N⁺ membrane (21). Two cDNA probes, *CYP21* probe (22) and *C4* probe (23), were labelled with ³²P-dCTP and used to determine *CYP21* and *C4* gene deletions, duplications and large gene conversions in 64 hyperandrogenic patients. Both probes were obtained through the courtesy of M C Carroll (Harvard University, Boston, MA, USA). Because only TaqI digests were analysed, gene conversions involving the TaqI restriction site were not distinguishable from deletions of the *CYP21* gene. Deletions/conversions of *CYP21* were identified by decreased intensity of the 3.7 kb TaqI fragment compared with that of the 3.2 kb TaqI fragment (24).

To screen for point mutations, exons 1–3 and exons 3–8 of the *CYP21* gene were selectively amplified with two sets of primers (25) in all patients and controls. Dot blot hybridisation analysis of the PCR product with 3'DIG-ddUTP end-labelled oligonucleotides was used to screen for the presence of normal or mutated sequences at the following eight sites of the *CYP21* gene: Pro³⁰Leu (exon 1), A/C⁶⁵⁶G (-13 intron 2), Ile¹⁷²Asn (exon 4), Ile-Val-Glu-Met^{236–239}Asn-Glu-Glu-Lys (exon 6), Val²⁸¹Leu (exon 7), insT³⁰⁷ (exon 7), Gln³¹⁸Stop (exon 8) and Arg³⁵⁶Trp (exon 8). Oligonucleotide sequences were essentially as previously described (25). Allele-specific PCR was used to analyse for an 8 bp deletion in exon 3 (26).

For sequence analysis, the *CYP21* gene and its proximal promoter region were first specifically amplified in two large fragments, the first extending from -264 in the proximal promoter region to the *CYP21*-specific site in exon 3 (986 bp, forward primer 5'-AGCTGACTCTGGATGCAGGA-3', reverse primer

5'-AGCAGGGAGTAGTCTCCCAAG -3') and the second comprising exons 3 to 10 (2095 bp, forward primer 5'-TTGTCCTTGGGAGACTACTCC-3', reverse primer 5'-ACTGTGTTTACAGGGGGAG-3'). Amplified fragments were purified on QIAquick PCR purification columns (Quiagen, Hilden, Germany) and sequenced by α-³⁵S-dATP incorporation with the forward and reverse primers shown in Table 1, using an AmpliCycle sequencing kit (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ, USA).

Statistical analysis

Hormonal data are expressed as means±s.d. Student's *t*-test was used for statistical analysis of hormonal data. χ^2 and Fisher's exact test were used to compare HLA phenotype frequencies between patients and controls. Statistical significance was assigned a value of *P*<0.05.

Results

17-OHP responses to ACTH stimulation

The individual values of basal and stimulated 17-OHP concentrations in Slovenian hyperandrogenic patients are shown in Fig. 1. All patients were subjected to an ACTH test, but 17-OHP was determined only in 81 patients, among whom 20 (24.7%) demonstrated basal 17-OHP values greater than 4.1 nmol/l. At 60 min, increased 17-OHP concentrations (range 9.8–21.5 nmol/l) were found in 12 of 81 patients (14.8%). No patient demonstrated stimulated 17-OHP greater than 36.6 nmol/l that would be consistent with 21-hydroxylase deficient NC-CAH (7). Basal 17-OHP concentrations were significantly greater in hyperandrogenic women than in healthy controls (3.3±2.0 compared with 2.5±0.8 nmol/l; *P*=0.006). However, stimulated 17-OHP concentrations were similar in both groups (7.0±3.1 compared with 6.6±2.0 nmol/l). Basal cortisol (397±152 nmol/l and 396±116 nmol/l) and stimulated cortisol concentrations (727±147 nmol/l and 716±72 nmol/l) were not significantly different between the groups.

Figure 1 17-OHP concentrations before and 60 min after ACTH stimulation in Slovenian female hyperandrogenic patients. ○, no mutations detected; ●, Val²⁸¹Leu normal/mutation heterozygous carriers (N/M); ■, CYP21 deletion/conversion N/M; ▲, Gln³¹⁸Stop N/M; ▼, conversion in exon 4 to exon 7 region N/M. Horizontal line represents the mean+2s.d. of stimulated 17-OHP values in the control group.

CYP21 gene mutations and HLA associations

Eleven heterozygous carriers of CYP21 mutations were identified among 83 hyperandrogenic patients analysed (13.3%). Basal and stimulated 17-OHP concentrations, HLA haplotypes and specificity of mutations in hyperandrogenic carriers of CYP21 gene mutations are shown in Table 2.

CYP21 gene deletion/conversion carriers were identified among seven of 64 hyperandrogenic patients (10.9%), of whom two had high and four had normal stimulated 17-OHP values. In one patient, 17-OHP was

not determined. CYP21 gene deletions/conversions were not associated with HLA-B47 typical for CAH in Northern European populations. HLA-B40, but not HLA-DR1, was present in two patients with CYP21 gene deletion/conversion.

Three heterozygous carriers of CYP21 point mutations and one gene conversion encompassing four exons were identified among 83 hyperandrogenic patients (4.8%). Point mutations identified were Val²⁸¹Leu in one and Glu³¹⁸Stop in two patients. Similar to the findings described by Weddel *et al.* (27), in one patient, four consecutive mutations were found – Ile¹⁷²Asn,

Table 2 17-OHP concentrations at baseline and 60 min after ACTH stimulation, and HLA haplotypes, in female hyperandrogenic carriers of CYP21 gene mutations.

Patient	17-OHP (nmol/l)		HLA haplotype	CYP21 mutations
	0 min	60 min		
E36	6.1	9.9	A28 A24 B62 BX DR1 DR13	conv/del N/M
E46	6.8	9.6	A3 A24 B18 B40 DR11 DR13	conv/del N/M
E2	1.3	5.0	A28 A3 B51 B44 DR16 DR7	conv/del N/M
E12	5.0	8.9	A24 A26 B51 B56 DR3 DR11	conv/del N/M
E21	ND	ND	A2 A11 B38 B35 DR1 DR16	conv/del N/M
E33	1.9	4.1	A2 A24 B21 B40 DR11 DR14	conv/del N/M
E89	2.1	3.8	A3 A34 B44 B35 DR7 DR14	conv/del N/M
E83	3.6	12.8	A3 A32 B14 BX DR13 DR13	²⁸¹ N/M
E54	5.0	6.5	A3 A26 B51 B35 DR4 DR8	conv Ex4-7 N/M
E77	2.5	8.4	A1 A2 B8 B50 DR3 DR7	³¹⁸ N/M
E72	2.9	7.0	A2 A23 B62 B50 DR1 DR3	³¹⁸ N/M

ND, not done; conv, conversion; del, deletion; N/M, heterozygous carrier; Ex. exon. Polymorphisms and neutral mutations are not shown.

A Family E77

B Family E2

Figure 2 Pedigrees of two families affected by the Gln³¹⁸Stop mutation. a, b, c, d, HLA haplotypes of family members; haplotypes associated with mutation are underlined. Open symbols represent Gln³¹⁸Stop mutation; half-solid symbols represent heterozygous carriers of the Gln³¹⁸Stop mutation. Arrows indicate female hyperandrogenic patients.

cluster of mutations in exon 6, Val²⁸¹Leu and insertion of T³⁰⁷ – and a gene conversion extending from exon 4 to exon 7 was suspected, because of the normal response to ACTH stimulation. The carriers of Glu³¹⁸Stop mutation, a mutation frequently found in patients with classical 21-hydroxylase deficiency, had normal 17-OHP values after stimulation. Gln³¹⁸Stop mutation was associated with HLA-A2 and -B50 in both patients; in one, this association was confirmed by a family study (Fig. 2A).

The carrier of the Val²⁸¹Leu mutation on the HLA-B14 DR13 extended haplotype showed an elevated 17-OHP response to ACTH stimulation. To investigate further the association between HLA-B14 and Val²⁸¹Leu mutation in the Slovenian population, five additional DNA samples of HLA-B14 carriers were selected from the registry of the Tissue Typing Centre in Ljubljana and analysed for point mutations in the *CYP21* gene. Three of them (60%) were found to be heterozygous carriers of the Val²⁸¹Leu mutation (Table 3).

No *CYP21* point mutations were found in 56 healthy

Table 3 HLA phenotypes of healthy Slovenian HLA-B14 carriers analysed for Val²⁸¹Leu mutation.

Carrier	Val ²⁸¹ Leu	HLA phenotype
CTT1	N/M	A3 A33 B14 B7 DR1 DR13
CTT2	N	A1 A2 B14 B44 DR7 DR16
CTT3	N	A1 A33 B14 B7 DR10 DR8
CTT4	N/M	A28 A33 B14 B18 DR16 DRX
CTT5	N/M	A11 A33 B14 B49 DR3 DR11

Val²⁸¹Leu, codon 281; N, normal; M, mutation; N/M, heterozygous carrier.

controls. However, a heterozygous Gln³¹⁸Stop mutation associated with the HLA-A2 B21 DR4 haplotype (Fig. 2B) was found in the mother and the sister of one hyperandrogenic patient without this mutation.

All mutations detected by oligonucleotide hybridisation were confirmed by sequencing. In addition, sequence analysis of the proximal promoter region, exons and exon–intron junctions of the *CYP21* gene was used to screen those hyperandrogenic patients who had high 17-OHP values after stimulation for other mutations or polymorphisms that could explain their exaggerated response to the ACTH test. Sequencing analysis revealed 10 neutral polymorphic sites (mostly in exons 1, 7 and 10) in 11 patients, but no additional mutations were found. In the carrier of a gene conversion extending from exon 4 to exon 7, polymorphisms characteristic of the pseudogene were also present in a heterozygous form.

Adrenal function and serum androgen concentrations in hyperandrogenic carriers of *CYP21* gene mutations

In 27 of 82 hyperandrogenic patients (33%), one androgen (mostly free testosterone or DHEAS) was increased in serum, and in 20 of the 82 patients (24%), two androgens were increased. Androstenedione was increased only in two of the 82 (2.4%). To evaluate the influence of *CYP21* gene mutations on adrenal function, 17-OHP, cortisol and adrenal androgen concentrations were compared between hyperandrogenic carriers of *CYP21* gene mutations and patients without mutations (Table 4). Basal cortisol concentrations were significantly lower in mutation carriers. Concentrations of free testosterone were significantly greater in mutation carriers and the difference in androstenedione concentrations reached borderline statistical significance ($0.1 > P > 0.05$). No significant differences in basal or ACTH-stimulated 17-OHP concentrations or in 17-OHP increments were observed between the groups.

Discussion

The present study represents the first systematic screening for 21-hydroxylase deficiency in Slovenian

Table 4 17-OHP, cortisol and androgen concentrations in Slovenian female hyperandrogenic patients with and without *CYP21* gene mutations. Values are expressed as mean $\pm$ s.d.

	Carriers of <i>CYP21</i> mutations	Patients without <i>CYP21</i> mutations	<i>P</i>
17-OHP ₀ (nmol/l)	3.7 $\pm$ 1.9	3.2 $\pm$ 2.0	0.473
17-OHP ₆₀ (nmol/l)	7.6 $\pm$ 2.8	7.0 $\pm$ 3.1	0.540
17-OHP _{increment} (nmol/l)	3.9 $\pm$ 2.3	3.8 $\pm$ 2.9	0.874
Cortisol (nmol/l)	308 $\pm$ 57	409 $\pm$ 157	0.048
Free testosterone (pmol/l)	11.5 $\pm$ 9.4	7.9 $\pm$ 4.0	0.035
Androstenedione (nmol/l)	8.0 $\pm$ 3.6	5.9 $\pm$ 2.9	0.059
DHEAS (mmol/l)	10.1 $\pm$ 4.1	8.1 $\pm$ 3.8	0.119

17-OHP₀, 17-OHP₆₀, 17-OHP_{increment}, concentrations at baseline and 60 min after stimulation with ACTH, and the difference between these values.

hyperandrogenic women. ACTH stimulation, HLA typing and molecular analysis of the *CYP21* gene region were used to define the carrier status for 21-hydroxylase deficiency.

High 17-OHP concentrations after stimulation, indicative of heterozygous carriers of 21-hydroxylase deficiency (10), were found in 12 of 81 hyperandrogenic patients (14.8%). As no patient demonstrated a stimulated 17-OHP concentration indicative of non-classical 21-hydroxylase deficiency, we cannot draw any conclusions as to the incidence of non-classical 21-hydroxylase deficiency in Slovenian hyperandrogenic women; it may be lower than or consistent with the reported 1–2% frequency in white hyperandrogenic women in general (7, 8). However, high frequencies of non-classical 21-hydroxylase deficiency that are found in some other selected populations (5) are practically excluded in the Slovenian population. Therefore routine screening for 21-hydroxylase deficiency is not recommended in Slovenian hyperandrogenic women.

Molecular analysis of the *CYP21* gene revealed that 11 of 83 hyperandrogenic patients (13.3%) were heterozygous carriers of 21-hydroxylase deficiency. These results differ from those of Azziz & Owerbach (10), who found carriers of inherited defects in *CYP21* in the majority of patients with an exaggerated 17-OHP response to ACTH stimulation. No point mutations were identified in 56 healthy Slovenian controls.

Carriers of *CYP21* gene mutations could not be distinguished from other hyperandrogenic patients on the basis of their response to ACTH stimulation; in fact, the increase in 17-OHP concentrations after ACTH stimulation could be explained by a carrier state for *CYP21* gene mutations only in three of 12 patients analysed (25%). In addition, *CYP21* gene mutations were found in 7 of 69 hyperandrogenic patients (10.1%) with normal 17-OHP values after stimulation. The response to the ACTH test was normal also in the carrier of a gene conversion extending from exon 4 to exon 7. Our results indicate that 7 of 11 carriers (72.7%) would be missed if ACTH stimulation alone was used as a screening test, and that carriers of severe mutations

characteristic of classical 21-hydroxylase deficiency alleles (five of seven deletions/conversions, two of two Gln³¹⁸Stop mutations) would be at risk of being given inadequate genetic advice. *CYP21* gene mutations that contributed to increased 17-OHP concentrations after ACTH stimulation were two gene deletions/conversions and one Val²⁸¹Leu mutation. The Val²⁸¹Leu mutation resulting in an enzyme with decreased activity (28) seems to compromise the adrenal function of the carrier to a greater extent than does the complete absence of the product of one allele.

Our study confirmed the association between Val²⁸¹Leu mutation and HLA-B14, a marker characteristic of NC-CAH in several populations (15, 29) also in the Slovenian population. Further studies of HLA haplotypes in Slovenian patients with CAH and their families are needed to ascertain associations between HLA haplotypes and *CYP21* gene mutations before HLA typing can be used to screen for carriers.

Heterozygous carriers of *CYP21* mutations had higher concentrations of serum free testosterone, androstenedione and DHEAS than patients without *CYP21* gene defects. However, the two groups did not differ in basal or ACTH-stimulated 17-OHP concentrations. This is in agreement with observations that heterozygosity for *CYP21* mutations may result in mild abnormalities of androgen biosynthesis reflected by higher mean free testosterone (13, 30) or by higher mean DHEAS concentrations (10). Our results suggest that, in contrast with findings in patients with NC-CAH, among hyperandrogenic women, basal or ACTH-stimulated 17-OHP concentrations are not a good indicator of the carrier status for inherited defects of *CYP21*, as observed also by other investigators (13, 31, 32) who defined the carrier status by molecular analysis of *CYP21* gene, the most accurate method to define carriers for inherited defects of *CYP21* (10). Older studies reported that the majority of heterozygotes for 21-hydroxylase deficiency can be detected by measuring the 17-OHP concentration 60 min after ACTH stimulation; however, the diagnosis of 21-hydroxylase deficiency was based on HLA genotyping (33). Perhaps

measuring 21-deoxycortisol (34) and the ratio of 17-OHP/21-deoxycortisol (35), not done in our study, might improve prediction of heterozygous carriers of 21-hydroxylase deficiency by hormonal testing.

It remains unclear what was the cause of borderline elevations in ACTH-stimulated 17-OHP concentrations in nine of 12 hyperandrogenic patients (75%), in whom even extensive sequencing of the CYP21 gene and its proximal promoter region revealed no mutations. Falsely positive ACTH tests may be due to high 17-OHP concentrations reflecting adrenal and non-adrenal secretion of this steroid precursor (30). Also, other adrenal and ovarian androgens may interfere with adrenal steroidogenesis, mimicking steroid profiles of patients with 21-hydroxylase deficiency (36). Finally, high 17-OHP concentrations may be the consequence of generalised hyperreactivity of the adrenal cortex in hyperandrogenic women (30). Factors other than a mild impairment of 21-hydroxylase activity may also contribute to biochemical findings in these patients (29). Studies of the 17 α -hydroxylase/17-20-lyase gene showed that mutations in this gene do not contribute to increased stimulated 17-OHP concentrations in Slovenian hyperandrogenic women (37). Further studies may show which additional factors influence the phenotypic traits of 21-hydroxylase deficiency in heterozygous carriers.

In conclusion, our results demonstrate the difficulty in choosing appropriate cut-offs in biochemical tests and the low sensitivity of ACTH stimulation for detection of carriers of 21-hydroxylase deficiency. Reliable detection of carriers is possible only by molecular analysis of the CYP21 gene.

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