

Distinctive profile of the 17-hydroxylase and 17,20-lyase activities revealed by urinary steroid metabolomes of patients with CYP17 deficiency

Perfil característico das atividades 17-hidroxilase e 17,20-liase reveladas por meio do metaboloma de esteroides urinários de pacientes com deficiência de CYP17

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ABSTRACT

Objectives: (1) Characterize serum (S) and urinary (U) steroid metabolites in complete CYP17 deficiency (cCYP17D); (2) analyze the relative 17 α -hydroxylase (17OH) and 17,20-lyase (17,20L) activities *in vivo*; and (3) compare data from the two most prevalent mutations in Brazil. **Subjects and methods:** 20 genotyped cCYP17D patients from a previously reported cohort were homozygous for W406R or R362C; 11 controls were CYP17 wild types (WT). WT and cCYP17D patients had S and U samples drawn to measure: cortisol (F), corticosterone (B), deoxycorticosterone (DOC), 18OH-B, 18OH-DOC, and 17OHP; and tetrahydro (TH)-B, THA, THDOC, THF+5 α -THF, TH-cortisone, androsterone, etiocholanolone, 5-pregnenediol, 17OH-pregnenolone and pregnanetriol. **Results:** Compared to WT, cCYP17D patients had marked elevations of B, DOC, 18OH-B and 18OH-DOC, whereas 17OHP, F and adrenal androgens (AA) were reduced; U steroids parallel S findings. Metabolite ratios revealed that both 17OH and 17,20L activities were impaired in cCYP17D. There were no differences between W406R and R362C mutations. **Conclusions:** cCYP17D patients show parallel overproduction/overexcretion of 17-deoxysteroids, and marked reduction of F and AA. In addition to 17OH, 17,20-L activity was also impaired in cCYP17D. W406 and R362C mutations disclose similar S and U patterns. *Arq Bras Endocrinol Metab.* 2010;54(9):826-32

Keywords

Congenital adrenal hyperplasia; CYP17; 17-hydroxylase deficiency; 17,20-lyase deficiency; urinary steroid metabolome; corticosterone

RESUMO

Objetivos: (1) Caracterizar os esteroides séricos (S) e urinários (U) na deficiência completa da CYP17 (DcCYP17); (2) analisar as atividades da 17 α -hidroxilase (17OH) e 17,20-liase (17, 20 L) *in vivo*; e (3) comparar as duas mutações mais prevalentes no Brasil. **Sujeitos e métodos:** 20 pacientes genotipados para a DcCYP17, de uma coorte anterior, eram homocigotos para W406R ou R362C (8 cada); 11 controles eram CYP17 *wild types* (WT). Amostras de S e U foram colhidas dos WT e pacientes para dosagem de: cortisol (F), corticosterona (B), deoxicorticosterona (DOC), 18-OH-B, 18OH-DOC e 17OHP; e tetraidro(TH)-B, THA, TH-DOC, THF+5 α -THF, THE, androsterona, etiocolanolona, 5-pregnenediol, 17OH-pregnenolona e pregnanetriol. **Resultados:** Comparados aos WT, os pacientes com DcCYP17 revelaram elevações acentuadas de B, DOC, 18OHB e 18OHDOC, enquanto 17OHP, F e andrógenos adrenais (AA) estavam reduzidos. Os esteroides na U acompanham os achados no S. As relações de metabólitos mostraram que as atividades de 17OH e 17,20L estavam reduzidas em pacientes com DcCYP17. Não houve diferenças entre pacientes com as mutações W406R e R362C. **Conclusões:** Na DcCYP17, a produção e a excreção dos 17-deoxiesteroides estão aumentadas em paralelo, em contraste com a reduzida produção/excreção de F e AA. As atividades da 17OH e 17,20-L estão diminuídas na DcCYP17. As mutações W406 e R362C apresentam achados semelhantes em S e U. *Arq Bras Endocrinol Metab.* 2010;54(9):826-32

Descritores

Hiperplasia adrenal congênita; CYP17; deficiência de 17-hidroxilase; deficiência de 17,20-liase; metaboloma de esteroides urinários; corticosterona

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INTRODUCTION

17-Hydroxylase deficiency (17OHD) is generally considered a rare cause of congenital adrenal hyperplasia (CAH). However, it is not so uncommon in specific ethnic groups or geographical areas, as among the Mennonite descendants of Dutch Frieslanders (1) and in Brazil, where 17OHD is considered the second most frequent form of CAH, accounting for 5%-7% of all cases (2,3).

The disease is caused by mutations in the *CYP17A1* gene which encodes cytochrome P450c17, an enzyme expressed in the adrenal cortex and the gonads displaying both 17 α -hydroxylase and 17,20-lyase activities, among others (4-12). The former activity is carried out by converting pregnenolone and progesterone to 17 α -hydroxypregnenolone (17OHPreg) and 17 α -hydroxyprogesterone (17OHP), respectively, whereas the latter performs the C17-20 side-chain cleavage of 17OHPreg to yield DHEA, an androgen precursor in the adrenal zona reticulata, ovaries and testes. For maximal activities, the enzyme requires a functional steroid-binding site domain and a functional redox partner-binding site domain for interaction with the electron-transfer protein P450 oxidoreductase and the co-factor cytochrome b5 (7,13-15). Most of the 75 reported mutations in CYP17 (16) occurs in the steroid-binding domain resulting in combined or complete 17 α -hydroxylase/17,20-lyase deficiency. Thus, failure to produce sex steroids in these patients results in sexual infantilism and primary amenorrhea in the female (46,XX) and pseudohermaphroditism in the male (46,XY) (17-20). In addition, impaired production of cortisol – typical of complete 17OHD –, leads to increased ACTH secretion that stimulates overproduction of deoxycorticosterone (DOC) and corticosterone (B) – non-17-hydroxylated steroid intermediates with mineralocorticoid activity –, causing severe hypertension and hypokalemia (2,21-25). Although the serum steroid profile has been extensively studied in 17OHD, systematic evaluation of the full urinary steroid metabolite (metabolome) in these patients is limited.

In this paper we determined serum levels and the steroid metabolome in a large group of 17OHD patients and examined specific precursor-to-product metabolite ratios in order to: (1) define possible differences concerning both enzymatic activities, (2) compare metabolite ratios in patients with complete 17OHD

with those from previously reported patients with isolated 17,20-lyase deficiency (26), and also (3) compare the metabolic pattern between the two most prevalent CYP17 mutations documented in Brazil, the W406R and the R362C.

SUBJECTS AND METHODS

Twenty patients affected with the complete 17OHD form of CAH (twelve 46,XY, and eight 46,XX, all phenotypic female) were selected from a larger cohort of patients previously reported (2). At the time of the study, patients ranged in age from 14 to 42 years (median of 22 yr); in most, clinical diagnosis had been suspected by the absence of secondary sex characteristics and of menstrual periods at the time of puberty, mostly associated with the presence of hypertension and hypokalemia. Complete 17OHD was established by typical laboratory values, as reduced cortisol, aldosterone, and plasma renin activity and elevated LH, FSH, and progesterone, with low to absent sex steroids. All had been genotyped and specific CYP17 mutations identified (R362C, W406R, R362C+W406R, Y329D+splice, Y329X and W406R+MIT) (2).

Eleven normal subjects were also studied as controls (5 male; 6 female, 15 to 57 years of age; median of 26 yr). They were selected among the families of patients with 17OHD, but whose genotyping did not identify mutations in any of the alleles (“wild types”).

The study protocol was performed in all subjects and included urinary collections (mostly spot urine samples) to measure steroid metabolites and a morning blood sampling for serum steroids. All patients on ongoing glucocorticoid replacement therapy had their medications discontinued for 72h, in an attempt to reestablish their pre-treatment steroid profile. Five of them had never been treated before.

The study protocol was previously approved by the Committee on Human Research of our institution and informed written consent obtained from all participants.

Analytical methods

All patients and controls had a morning blood sample drawn and were instructed to collect a urinary sample (a few collected 24-hour urine specimens) and keep it under refrigeration until taken to the laboratory. The following serum steroids were measured in the Clinical

Research Center at San Francisco General Hospital by radioimmunoassay after HPLC separation: corticosterone (B), deoxycorticosterone (DOC), 18-hydroxyB (18OHB), 18OHDOC, cortisol, aldosterone, and 17OH-progesterone.

From each urinary specimen, a 20 mL aliquot was passed through a C-18 cartridge for steroid metabolite extraction. Cartridges were subsequently mailed to the Children's Hospital of Oakland Research Institute (CHLS) for gas chromatography-mass spectrometry analysis. The compounds of interest were identified and quantified (concentrations expressed as $\mu\text{g/L}$ of urine) and specific steroid ratios were calculated to express specific enzymatic activities (27).

The following urinary metabolites were measured: $\Delta 5$ -pregnenediol (5PD) and pregnenediol (PD) (metabolites of the non-17-hydroxylated precursors, pregnenolone and progesterone, respectively); tetrahydro-DOC (THDOC) (metabolite of DOC); tetrahydrocorticosterone (THB) and $5\alpha(\text{allo})$ -THB plus tetrahydro-11-dehydrocorticosterone or compound A (THA) and $5\alpha(\text{allo})$ -THA (metabolites of B); 17-hydroxypregnenolone (17HP) and pregnanetriol (PT) (metabolites of 17-hydroxyprogesterone); tetrahydrocortisol (THF) and $5\alpha(\text{allo})$ -THF plus tetrahydrocortisone (THE) (metabolites of cortisol), as well as the urinary metabolites of sex steroids: andros-

terone (AN) plus etiocholanolone (ET) (metabolites of androstenedione).

Enzymatic activities were inferred from calculation of the specific precursor-to-product metabolite ratios (Figure 1). For simplicity, the following groups of metabolites: (THB+ 5α THB), (THA+ 5α THA), (THF+ 5α THF), and THE, will be named herein as "Bs", "As", "Fs", and "E", respectively: (1) (5PD+PD)/(AN+ET) and (Bs+As)/(AN+ET) are used as expressions of global CYP17 activity (17-hydroxylase plus 17,20-lyase); (2) (5PD+PD)/(17HP+PT), PD/PT, and (Bs+As)/(Fs+E) stand for the efficiency of 17-hydroxylation solely; and, (3) (17HP+PT)/(AN+ET), PT/(AN+ET), and (Fs+E)/(AN+ET) indicate specifically 17,20-lyase activity.

The underlined ratios shown above were employed to compare data from our patients with those from a previous publication that studied patients with "isolated 17,20-lyase deficiency" (26).

RESULTS

Patients with 17OHD showed marked elevations (20- to > 100-fold) of serum B, DOC, 18OHB, 18OHDOC and progesterone, as compared to WT (Table 1). Conversely, cortisol and 17OHP were virtually undetectable with normal to reduced aldosterone levels. Adrenal androgens were also typically low (data not shown).

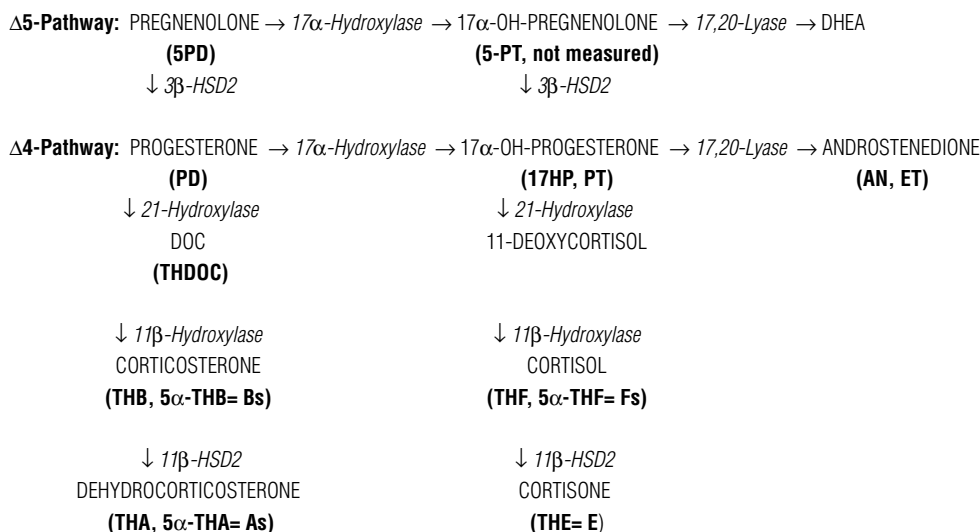


Figure 1. Schematic illustration of the adrenocortical steroid biosynthesis, identifying steroids produced in the delta-5 and in the delta-4-pathways, the sites where 17-hydroxylase and 17,20-lyase operate, the additional hydroxylation steps (in italic), and the respective urinary steroid metabolites (in bold and parenthesis). 5PD: 5-pregnenediol; PD: pregnenediol; THDOC: tetrahydro-11-deoxycorticosterone; THB: tetrahydrocorticosterone; 5α -THB: allo-tetrahydrocorticosterone; THA: tetrahydro-11-dehydrocorticosterone; 5α -THA: allo-tetrahydro-11-dehydrocorticosterone; 5-PT: 5-pregnenetriol; 17HP: 17-hydroxypregnenolone; PT: pregnanetriol; THF: tetrahydrocortisol; 5α -THF: allo-tetrahydrocortisol; THE: tetrahydrocortisone; AN: androsterone; ET: etiocholanolone.

Table 1. Basal serum steroid levels (mean \pm SE and median plus interquartile interval) in patients with 17OHD and “wild types” (WT) for the disease (all given in ng/dL, except for cortisol, in μ g/dL)

Steroid	17OHD (n = 20)	WT (n = 11)	p
B	16,299 \pm 1,488 14,800 (4,749-32,900)	358 \pm 150 129 (55-1,557)	< 0.001
DOC	323 \pm 42 325 (120-921)	4.3 \pm 1.0 4.4 (0.4-10.9)	< 0.001
180HB	312 \pm 40 278 (169-635)	23.5 \pm 2.4* 22.1 (10.0-39.7)	< 0.001
180HDOC	306 \pm 36 276 (126-704)	10.9 \pm 5.2 3.1 (2.0-49.4)	< 0.001
Progesterone	905 \pm 94 865 (590-1,330)	(30-150 / 30-70)**	
Cortisol	0.3 \pm 0.0 0.2 (0.2-0.5)	11.6 \pm 1.8 10.4 (4.3-25.7)	< 0.001
170HP	2.3 \pm 0.3 2.5 (1.0-3.0)	90 \pm 23 84 (6-215)	< 0.001
Aldosterone	3.8 \pm 0.8 2.6 (0.2-11.0)	4.6 \pm 0.8 5.1 (2.0-10.6)	NS

* Reference values obtained from ref. 5.

** Normal ranges for male and female controls (at the follicular phase) obtained from ref. 28.

In 17OHD patients, the urinary steroid metabolomes were concordant with their serum findings. Excretion of B metabolites: THA, 5 α THA, THB, and specially 5 α THB were significantly increased, in parallel with THDOC (Figure 2). Accordingly, metabolites of the 17-hydroxylase substrates, pregnenolone and progesterone (5PD and PD) were also significantly increased (Figure 2). In contrast, cortisol metabolites, THF,

5 α THF, and THE, were decreased, as well as 17HP plus PT and AN plus ET (Figure 3).

Ratios

The (5PD+PD)/(AN+ET) and (Bs+As)/(AN+ET) ratios were significantly elevated (4,500- to 8,500-fold) in 17OHD patients as compared to WT, as a result of virtually total CYP17 deficiency (combined 17 α -hydroxylase/17,20-lyase activities) (Table 2). The values observed for the latter ratio in patients with “isolated 17,20-lyase deficiency” were intermediate (around 400-fold higher than WT) (26).

When examined in separate, 17 α -hydroxylation was predominantly affected over 17,20-lyase activity, as revealed by the disproportionate increase in the (5PD+PD)/(17HP+PT) and (Bs+As)/Fs+E ratios (> 400-fold), as compared to (17HP+PT)/(AN+ET) and (Fs+E)/(AN+ET) (4- to 10.5-fold) (Table 2). However, 17-hydroxylase activity was slightly (PD/PT) to moderately (Bs+As)/Fs+E affected in patients with “isolated 17,20-lyase deficiency” (1.7-fold and 37-fold higher than WT, respectively). When the PT/(AN+ET) ratio is considered, 17,20-lyase activity was significantly less impaired in our patients with 17OHD than in those with “isolated 17,20-lyase deficiency” (0.84 [0.64-1.23] vs. 2.25 [1.72-4.30]), whereas it was similar when the (Fs+E)/(AN+ET) ratio is analyzed (8.37[3.44-13.38] vs. 6.63[3.88-14.94]).

When homozygote 17OHD patients for the W406R and R362C mutations were compared, all individual metabolite values and their respective ratios were similar and not statistically different.

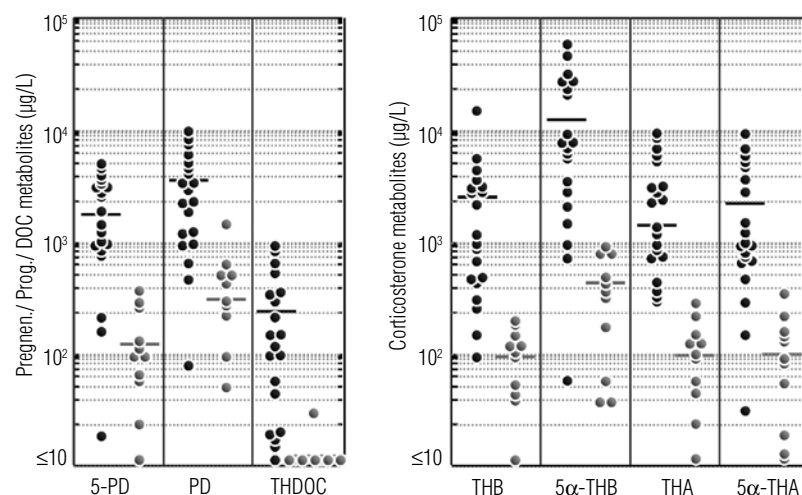


Figure 2. Urinary steroid levels from 20 patients with 17OHD (dark circles) and 11 normal control individuals (light circles). Left-hand panel – steroid metabolites of pregnenolone (Pregnen), progesterone (Prog) and deoxycorticosterone (DOC): 5-PD, PD, and THDOC. Right-hand panel – steroid metabolites of corticosterone: THB, 5 α -THB, THA, 5 α -THA.

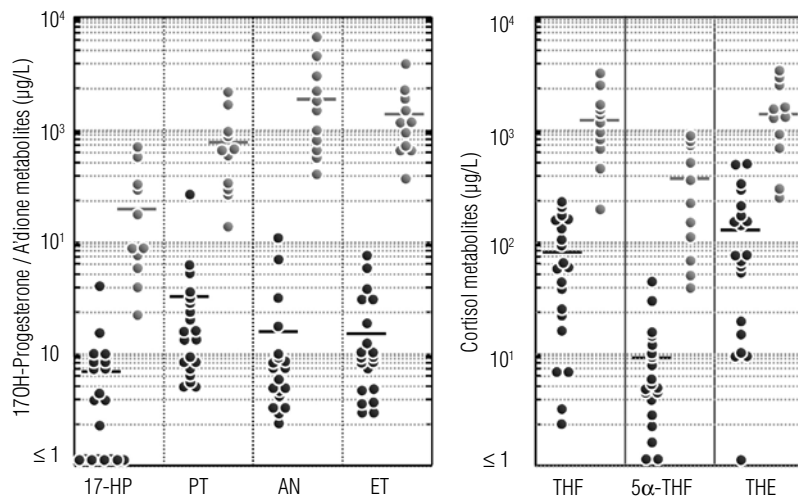


Figure 3. Urinary steroid levels from 20 patients with 17OHD (dark circles) and 11 normal control individuals (light circles). Left hand panel - steroid metabolites of 17-OH-progesterone (17HP and PT) and androstenedione (AN and ET). Right hand panel – steroid metabolites of cortisol: THF, 5 α -THF and THE.

Table 2. Relative enzymatic activities of CYP17 as determined by diagnostic ratios of urinary steroid metabolites (median plus interquartile interval) in patients with 17OHD, “isolated 17,20-lyase deficiency” (17,20-LD*) and “wild types” (WT) for the disease

Urinary steroid metabolite ratios	17OHD (n = 20)	17,20-LD* (n = 6)	WT (n = 11)
CYP17 (17-hydroxylase+17,20-Lyase)			
(5PD+PD)/(AN+ET)	243.6 (130.3-315.8)	–	0.14 (0.10-0.22)
(Bs+As)/(AN+ET)	714.5 (348.3-1,136.8)	58.4 (29.6-67.4)	0.15 (0.11-0.28)
17-hydroxylase activity			
(5PD+PD)/(17HP+PT)	219.3 (139.0-296.8)	–	0.53 (0.42-0.74)
PD/PT	149.2 (107.8-226.4)	0.74 (0.57-1.15)	0.43 (0.35-0.64)
(Bs+As)/(Fs+E)	94.7 (63.7-114.4)	8.07 (5.04-9.66)	0.22 (0.14-0.24)
17,20-lyase activity			
(17HP+PT)/(AN+ET)	1.04 (0.77-1.48)	–	0.27 (0.21-0.29)
PT/(AN+ET)	0.84 (0.64-1.23)	2.25 (1.72-4.30)	0.20 (0.17-0.23)
(Fs+E)/(AN+ET)	8.37 (3.44-13.38)	6.63 (3.88-14.94)	0.80 (0.62-1.26)

5PD: 5-pregnenediol; PD: pregnenediol; AN: androsterone; ET: etiocholanolone; Bs: THB (tetrahydrocorticosterone) + 5 α (allo)-THB; As: THA (tetrahydro-11-dehydrocorticosterone) + 5 α (allo)-THA; 17HP: 17-hydroxypregnenolone; PT: pregnanetriol; Fs: THF (tetrahydrocortisol) + 5 α (allo)-THF; E: THE (tetrahydrocortisone).

* Ratios derived from data reported in ref. 26.

DISCUSSION

In this study we examined the serum steroid levels and the urinary steroid metabolomes from 20 patients with 17OHD, in whom enzymatic activity was proven to be

completely abolished by the specific *CYP17A1* gene mutations W406R and R362C, as reported elsewhere (2).

The serum steroid profile was comparable to those previously published (8,20,24,25,28) and disclosed marked increases of B, DOC and their 18-hydroxy-derivatives – 18OHB and 18OHDOC –, the so-called 17-deoxysteroids (from the “mineralocorticoid pathway”) of the zona fasciculata.

Also confirmed in this study is the dissociation between the raised levels of 18OHB (aldosterone precursor in the zona glomerulosa) in the presence of reduced aldosterone, a unique finding explained by the production of 18OHB in the zona fasciculata from its abundant precursor corticosterone, stimulated by increased ACTH production (22,23). Since elevated DOC and B play a major role in the pathogenesis of mineralocorticoid hypertension with hypokalemia and chronic renin suppression, zona glomerulosa aldosterone is characteristically reduced in 17OHD (together with a negligible 18OHB contribution from this zone).

Progesterone is an earlier steroid biosynthetic precursor and the major delta-4 substrate for CYP17. It is invariably increased in patients with 17OHD regardless of gender, and also frequently elevated in other adrenal enzymatic deficiencies. The marked progesterone increase found in our patients corroborate data reported by Martin and cols. (28) who suggested that elevated progesterone in the presence of hypergonadotrophic hypogonadism is a simple yet strong evidence for the diagnosis of 17OHD. In isolated 17,20-lyase deficiency progesterone is also elevated, mostly after ACTH stimulation, but suspicion is

best raised when the 17OHP to androstenedione ratio is increased, as suggested by Auchus and Miller (6).

The urinary steroid metabolomes evaluated in this study were in agreement with serum findings, implying an appropriate hepatic, renal and peripheral metabolism of the steroid mixture. Urinary metabolites of early biosynthetic steroids, such as pregnenolone and progesterone (5PD and PD), are substantially increased in 17OHD patients. Also, both B metabolite isomers, the reduced (THB, 5 α THB) and the dehydrogenated (THA, 5 α THA) forms, are distinctly elevated, as well as THDOC. The magnitude of metabolite excretion of these steroid is somewhat disproportional and less expressive than that seen with serum levels (25-fold vs. 65-fold higher than WT, respectively), possibly reflecting the broad dispersion into several other metabolites and/or an uneven excretory processing. Nevertheless, the preferential 5 α -reduction catabolic pathway remains functionally active, if not increased, in these patients.

On the other hand, both cortisol and androgen metabolites are appreciably reduced in patients with complete 17OHD, reflecting their virtually absent production. In addition, the 17-hydroxylated intermediate metabolites, especially 17OH-progesterone (17HP and PT), are also substantially reduced.

The relative enzymatic activities of CYP17 (17-hydroxylase and 17,20-lyase) were estimated by analyzing specific precursor-to-product metabolite ratios. This *in vivo* assessment has proven useful for the diagnosis of several biosynthetic defects (26,27).

The precursor-to-product metabolite ratios that estimate global enzymatic activity in patients with 17OHD showed very high and unequivocal values – in the range of 4,000- to 8,000-fold higher than WT –, typical of a complete loss of CYP17 function. A separate evaluation of 17 α -hydroxylase and 17,20-lyase activities in the same patients, also demonstrates expressive enzymatic deficiencies (increases of proper metabolite ratios); however, they were at least one and two orders of magnitude less (400- to 900-fold and 3- to 10-fold, respectively) than complete 17OHD, denoting a predominant 17-hydroxylase obliteration. The lower yet significant increased ratios for the latter is not surprising since the amount of substrate for this second reaction (17,20-lyase) would be reduced as a result of deficient 17-hydroxylation.

For comparison with the report by Tiosano and cols. (26) who studied patients initially assumed to have isolated 17,20-lyase deficiency, we examined slightly different metabolite ratios which, nevertheless, express similar enzymatic functions. Using this set of

data, global enzymatic activity was much less affected in Tiosano's patients than in ours: although nearly 400-fold higher than in WT, the Bs+As/AN+ET ratio in our patients was 12-fold higher than in theirs.

Analysis of the 17-hydroxylase activity alone also confirms the previous findings observed for 17OHD patients as compared to WT (~400-fold higher in the former). However, in the six patients reported by Tiosano and cols. (26), these ratios reach values 1.7- to 37-fold higher than WT, indicating a partial 17-hydroxylase deficiency, an observation already made by the authors.

Concerning 17,20-lyase activity, the PT/(AN+ET) ratio was the only one that discloses higher values in Tiosano's patients than in ours (11-fold vs 4-fold in comparison with WT, respectively), indicating the predominant impairment of 17,20-lyase over 17-hydroxylase, or in other words, a greater availability of 17-hydroxylated products in Tiosano's than in our 17OHD patients. Similar but less evident results could be observed with the Fs+E/AN+ET ratio.

In this regard, it is tempting to speculate that some patients with "complete 17OHD" may in fact have "isolated 17-hydroxylase deficiency", in analogy to those with "isolated 17,20-lyase deficiency" reported by Tiosano in whom the E305G mutation in the *CYP17A1* gene was shown *in vivo* to affect also 17-hydroxylation, although *in vitro* studies indicate normal activity (29). Indeed, both activities may have an impairment spectrum as a result of different *CYP17A1* mutations, since the rarity of "true" isolated 17,20-lyase deficiency (14,15) precludes systematic metabolic studies. Also, in at least one family previously diagnosed as having 17,20-lyase deficiency, reinvestigation showed a homozygous mutation G539R in the gene of P450 oxidoreductase (30).

The urinary metabolome and the metabolite ratios studied in this paper confirm previous *in vitro* findings of absent 17-hydroxylase and 17,20-lyase activities in 17OHD patients. However, as observed by Tiosano and cols. (26), data derived from these *in vivo* studies seem more precise than those obtained from enzymatic expression *in vitro* (26). This observation allows the assumption that many enzymatic processes are under the influence of multiple modulators or modifying factors and characterize the variability of the individual phenotype sometimes associated to the same mutations.

In spite of these observations, data analysis from the two more prevalent mutations in Brazilian patients with 17OHD, both known to abolish CYP17 enzymatic activity, did not evidence any significant differences between groups.

In conclusion, patients with 17OHD disclose parallel overproduction and overexcretion of the adrenocortical 17-deoxysteroids (“the mineralocorticoid pathway of zona fasciculata”), in contrast with a marked decrease in the production and excretion of cortisol and adrenal androgens. In addition to 17-hydroxylation, 17,20-lyase activity is also impaired in complete 17OHD. Homozygote patients for the CYP17 mutations W406 and R362C, both associated with absent 17OH activity, disclosed a similarly abnormal pattern of serum steroids and urinary metabolites.

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