FLAVIA AMANDA COSTA BARBOSA

SUPERIORIDADE DO 21-DEOXICORTISOL APÓS ESTÍMULO COM ACTH EM RELAÇÃO A 17-HIDROXIPROGESTERONA NA DETECÇÃO DE HETEROZIGOTOS PARA A DEFICIÊNCIA DE 21-HIDROXILASE COM O USO DA ESPECTROMETRIA DE MASSA EM *TANDEM* PRECEDIDA DE CROMATOGRAFIA LÍQUIDA

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina, para obtenção do Título de Doutor em Ciências.

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Superioridade do 21-deoxicortisol após estímulo com acth em relação a 17-hidroxiprogesterona na detecção de heterozigotos para a deficiência de 21-hidroxilase com o uso da espectrometria de massa em *tandem* precedida de cromatografia líquida. Flávia Amanda Costa Barbosa -- São Paulo, 2009.

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Esta tese é dedicada

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SUMÁRIO

1. Introdução e Objetivos

Introdução

A hiperplasia adrenal congênita (HAC) engloba um grupo de doenças de herança autossômica recessiva e que se caracteriza pelo comprometimento da esteroidogênese adrenocortical [(1)]. Os mecanismos fisiopatológicos desta doença foram melhores esclarecidos quando a sua natureza genética foi desvendada e as anormalidades hormonais caracterizadas na década de 1950 [(1,2)]. Mais de 90% dos casos de HAC decorrem de mutações do gene que codifica a enzima 21-hidroxilase *(CYP21A2)*.

Como o defeito fundamental na HAC por deficiência de 21-hidroxilase (D21OH) ocorre na síntese de cortisol, a elevação subsequente do ACTH torna as adrenais hiperplásicas e hiperestimuladas, produzindo um excesso de precursores esteróides e hormônios sexuais que não requerem 21-hidroxilação para sua síntese. Como conseqüência, a manifestação clínica principal da D21OH é a virilização desde o período intra-uterino com desenvolvimento de genitália ambígua em meninas e macrogenitossomia em meninos, além de crescimento somático acelerado e fusão epifisária prematura em ambos os sexos.

Aproximadamente 75% dos pacientes também são incapazes de sintetizar aldosterona em níveis suficientes para manutenção do balanço hidro-eletrolítico, predispondo-os ao desenvolvimento de desidratação e choque hipovolêmico e óbito. Essas são formas mais graves denominadas de “perdedoras de sal” (PS). Àqueles pacientes com virilização pré-natal e níveis elevados de precursores esteróides, mas com produção preservada de aldosterona, dá-se o nome de forma “virilizante simples” (VS). Tanto a PS com a VS são reconhecidas como “formas clássicas” da doença [(2,3)].

A forma não clássica (NC) manifesta-se mais tardiamente e de maneira mais leve, com sinais e sintomas mais discretos de hiperandrogenismo desde a infância até a vida adulta. A forma NC pode ser também assintomática, sendo comumente detectada apenas durante investigação bioquímica e ou molecular de familiares de indivíduos afetados [(2-5)].

Por fim, os heterozigotos são indivíduos carreadores de mutações do gene da *CYP21* presentes em apenas um dos alelos. Apesar de assintomáticos [(6,7)], alguns estudos mostram uma prevalência maior de hirsutismo ou acne em mulheres dessa população [(8-10)].

A atividade residual da enzima 21-hidroxilase (21OH) reflete as mutações do gene da *CYP21A2*, existindo uma correlação genótipo-fenótipo significativa nos casos de D21OH [11]. Os genótipos do grupo A, que resultam em atividade enzimática nula, estão relacionados com o fenótipo PS. Nas mutações moderadas, ou com genótipo do grupo B, a atividade residual da 21OH é de cerca de 3-7%, resultando nas formas VS. Já nas mutações consideradas leves, ou genótipo do grupo C, a atividade enzimática é de 20-50%, sendo relacionadas às formas NC [2,4,11].

Como uma grande parcela dos indivíduos afetados é de heterozigotos compostos (mutações diversas nos diferentes alelos), seu fenótipo reflete o alelo com mutação mais leve [2,11]. Por isso, apesar da D21OH ter padrão de herança autossômico recessivo, não obedece a um "padrão mendeliano" exato. Deste modo, o emprego do termo "heterozigoto" para indivíduos que carreiam apenas um alelo mutante, é inadequado. Mesmo assim, por já estar consagrada em textos médicos, manteremos essa terminologia, até por que o emprego dos termos "carreadores" ou "portadores" pode causar até maior confusão.

Estudos de rastreamento neonatal mostram que a incidência das formas clássicas (PS e VS) pode variar de 1:10.000 a 1:18.000 nascimentos vivos [2]. Recente estudo estimou a incidência da forma clássica no Brasil em 1:10.325 nascidos vivos [12]. No entanto, a forma NC é muito mais prevalente, e atinge aproximadamente 0,1% da população geral [2], com maior incidência entre os judeus Askenazi (1:27 indivíduos). Outros grupos com alta freqüência incluem os hispânicos (1:40), eslavos (1:50) e ítalo-americanos (1:300) [4, 13]. Esses dados comprovam que a forma NC é a doença de padrão autossômico recessiva mais comum no homem [2, 4]. A frequência de heterozigotos para a forma clássica da D21OH é de 1:60 indivíduos, enquanto para a forma NC estima-se que seja de 1:16 indivíduos, podendo chegar a até 1:3 em judeus do leste europeu [4,13-15].

O marcador bioquímico da D21OH é a 17-hidroxiprogesterona (17OHP), precursor imediato da 21OH; assim, o diagnóstico da D21OH fundamenta-se na elevação de seus níveis séricos [1-3]. Níveis basais elevados da 17OHP são diagnósticos para as formas clássicas da D21OH; porém, geralmente são insuficientes para o diagnóstico da forma NC. Para isso, a dosagem de 17OHP após 60 minutos da administração intravenosa de ACTH sintético (cosintropina) mostra-se essencial para caracterização desse grupo de pacientes.

O valor de corte tradicionalmente utilizado para os níveis de 17OHP após estímulo com ACTH é de 1.000 ng/dL (instituído num período prévio à avaliação do gene da CYP21). Valores acima desse limite de corte são compatíveis com a forma NC [4,16], enquanto valores abaixo não discriminariam entre heterozigotos e indivíduos da população normal. No entanto, com a introdução da genotipagem da CYP21A2, Azziz *et al* [6] e Bachega *et al* [17], propuseram valores diagnósticos mínimos de 17OHP após estímulo com ACTH respectivamente de 1.500 e 1.700 ng/dL para a forma NC. Assim, valores limites precisos de 17OHP para diagnóstico da forma NC da D21OH ainda não foram definitivamente estabelecidos.

Estudos prévios mostraram correlação significativa entre genótipo e níveis de 17OHP nas diferentes formas de D21OH, com valores mais elevados nas formas clássicas, seguidos da forma NC e heterozigotos. Dentre a forma NC, alelos com mutações graves (genótipo A/C) apresentam níveis mais elevados de 17OHP após-ACTH, do que aqueles com mutações mais leves (genótipo C/C) [17,18]. Em heterozigotos, não há correlação entre os níveis de resposta da 17OHP após-ACTH com mutações do gene *CYP21* no alelo afetado [19].

Como a D21OH é uma doença de morbidade importante e de apresentação clínica variável (desde assintomática até evolução para óbito), a detecção de heterozigotos e o aconselhamento genético aos familiares de risco, principalmente em populações de maior incidência, torna-se crítico.

Apesar de a biologia molecular permitir a identificação dos heterozigotos, a genotipagem é pouco disponível e de custo elevado; por isso, o emprego de marcador bioquímico poderia facilitar a triagem diagnóstica dessa população. O emprego da dosagem sérica da 17OHP para detecção de heterozigotos não apresenta boa precisão diagnóstica pela grande sobreposição de valores basais e estimulados (20-70%) com a população normal [20-24].

Por outro lado, o 21-deoxicortisol (21DF) é um esteróide originado de uma via alternativa resultante da11β-hidroxilação da 17OHP no córtex adrenal. Essa via é praticamente inativa em indivíduos normais, porém assume importância nos pacientes com D21OH [25]. Louriax *et al* [26] foram os primeiros a reportar níveis elevados de 21DF em pacientes com D21OH, e estudos posteriores confirmaram o papel da dosagem sérica do

21DF na detecção de "heterozigotos" para a D21OH [27]. Fiet *et al* [28-30] documentaram que os níveis de 21DF em resposta ao estímulo com ACTH mostram sensibilidade próxima de 100% na detecção dessa população. Portanto, o 21DF após-ACTH poderia ser utilizado como um "marcador" bioquímico eficiente na detecção de heterozigotos para a D21OH, com valores de corte entre (60-70 ng/dL). Foram estabelecidos também por Fiet *et al* [31] valores diagnósticos para a forma NC de 21DF após-ACTH: 400 ng/dL. No entanto, estudos mais recentes conjugando a genética molecular para confirmação das diferentes populações, evidenciaram que muitos indivíduos aparentemente normais (sem mutação do gene *CYP21*) apresentavam níveis acima de 60-70 ng/dL, atenuando a sensibilidade do 21DF previamente descrita [32]. Entretanto, valores falso-positivos poderiam também ser devidos à imprecisão metodológica dos radioimunoensaios empregados (apesar dos procedimentos de prévios de extração) [33].

Apesar de pouco empregados no diagnóstico da D21OH, a caracterização dos esteróides distais ao bloqueio enzimático, como o 11-deoxicortisol (S) e o cortisol (F), produtos da via 17-hidroxilada, e a deoxicorticosterona (DOC) e a corticosterona (B), produtos da via não 17-hidroxilada da zona fasciculada (ZF), poderiam ser complementares na caracterização das diferentes formas de D21OH [34,35]. Mais especificamente, as relações envolvendo precursores e produtos da 21OH, como a 17OHP e o 21DF sobre S, DOC e B, poderiam ser importantes instrumentos diagnósticos para a detecção de heterozigotos. Algumas dessas relações, como a 17OHP/DOC e a 17OHP/18OHDOC, foram estudadas anteriormente [36,37], sem, entretanto, a devida comprovação molecular das populações.

Na última década, a espectrometria de massa em *tandem* precedida da cromatografia líquida (LC-MS/MS), tornou-se o método ideal para a quantificação de esteróides [38], além de sua vantagem em dosar vários esteróides simultaneamente em pequeno intervalo de tempo (minutos). Enquanto a cromatografia prévia possibilita a separação de esteróides de peso molecular semelhante (isômeros), o MS/MS utiliza a relação massa/carga elétrica gerada pela fragmentação dos diversos esteróides, permitindo sua caracterização com excelente especificidade [39].

Objetivos

Com base no emprego da metodologia de LC-MS/MS para a dosagem de esteróides séricos, e na caracterização precisa dos grupos estudados pelo emprego da genotipagem da CYP21A2, o presente estudo se propos a:

(a) examinar a possível vantagem da dosagem sérica basal e após estímulo com ACTH do 21DF em relação à 17OHP na diferenciação entre heterozigotos para a D21OH e indivíduos normais e estabelecer valores de corte (*cutoffs*) desses esteróides nessas populações;

(b) avaliar o comportamento dos esteróides distais ao bloqueio da 21-hidroxilase (S, DOC e B), tanto individualmente como através da relação com seus precursores - 17OHP e 21DF -, nos diferentes subtipos de D21OH (pacientes com a forma clássica e não clássica), heterozigotos e indivíduos normais;

(c) realizar o estudo de frequência das mutações detectadas nos pacientes com a D21OH, e correlacionar os níveis de 21DF e 17OHP nos diversos grupos genotípicos de pacientes e de heterozigotos para a D21OH.

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2. Artigo 1

Superior discriminating value of ACTH-stimulated serum 21-deoxycortisol in identifying heterozygote carriers for 21-hydroxylase deficiency.

Costa-Barbosa FA, Tonetto-Fernandes VF, Carvalho VM, Nakamura OH, Moura V, Bachega TA, Vieira JGH, Kater CE.

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Superior discriminating value of ACTH-stimulated serum 21-deoxycortisol in identifying heterozygote carriers for 21-hydroxylase deficiency

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Abbreviated title: 21DF for detection of 21OHD carriers

Key words: 21-deoxycortisol (21DF); 17-hydroxyprogesterone (17OHP); ACTH stimulation; 21-hydroxylase deficiency (21OHD); *CYP21A2*; 21OHD carriers

Precis: ACTH-stimulated serum levels of 21DF (by LC-MS/MS) are superior to 17OHP in identifying heterozygote carriers for 21-hydroxylase deficiency, and could be used to complement molecular analysis in assisting genetic counseling among relatives in families with 21OHD.

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Abstract

Background: Congenital adrenal hyperplasia due to classic 21-hydroxylase deficiency (21OHD) is an autosomal recessive disorder with a high prevalence of asymptomatic heterozygote carriers in the general population, making case detection desirable by routine methodology. Carriers for classic and nonclassic (NC) forms have basal and ACTH-stimulated values of 17-hydroxyprogesterone (17OHP) that fail to discriminate them from the general population. 21-Deoxycortisol (21DF), an 11-hydroxylated derivative of 17OHP, could be an alternative approach to identify carriers for 21OHD. **Objective and Methods:** To determine the discriminating value of basal and ACTH-stimulated serum levels of 21DF in comparison to 17OHP in a population of genotypically-confirmed carriers for 21OHD (n=62), as well as in affected patients (31 classic; 12 NC) and in 26 genotypically normal control subjects (CS), using tandem mass spectrometry after HPLC separation (LC-MS/MS). **Results:** Basal 21DF levels were not different between carriers and CS, but stimulated values were significantly increased in the former and virtually non-responsive in CS. Only 17.7 % of the ACTH-stimulated 21DF levels overlapped with CS, as compared to 46.8 % for 17OHP. For 100 % specificity, the sensitivities achieved for ACTH-stimulated 21DF and 17OHP were 82.3 % and 53.2 %, using cutoffs of 40 ng/dL and 298 ng/dL, respectively. A positive and highly significant correlation ($r= 0.785$; $p<0.0001$) was observed between 21DF and 17OHP. **Conclusion:** This study confirms the superiority of ACTH-stimulated 21DF, as compared to 17OHP, measured by LC-MS/MS, in identifying carriers for 21OHD. Serum 21DF could be useful in genetic counseling to screen carriers among relatives in families with affected subjects, supporting molecular results.

Introduction

21-Hydroxylase deficiency (21OHD) is an autosomal recessive disorder that results in the most frequent form of congenital adrenal hyperplasia (CAH). According to the mutation affecting the *CYP21A2* gene, 21OHD will manifest in two clinical forms: classic and nonclassic (NC). The former, which is further subdivided into two types – with or without sodium loss (or simple virilizing) – manifests early in life, whereas the latter is either asymptomatic or diagnosed around puberty, especially in girls [1,2].

The prevalence of asymptomatic carriers for the disease in the general population has been estimated from 1:50 (classic form) to as high as 1:16 (NC form) [3]; in Ashkenazi Jews the carrier frequency is even higher, at 1:3 [4].

Elevated plasma 17-hydroxyprogesterone (17OHP) is the hallmark of 21OHD: whereas basal levels are extremely high in classic 21OHD, NC patients may have normal to slightly elevated basal levels, but a marked increase following ACTH stimulation [5-7]. Carriers for both classic and NC forms have basal and ACTH-stimulated values of 17OHP that fail to discriminate them from the general population, although the response to ACTH tends to be higher in carriers [8]. Thus, molecular analysis of the *CYP21A2* gene is critical to distinguish between the several forms of 21OHD, including the carrier status; however, this methodology is time consuming and not widely available. 21-Deoxycortisol (21DF) is an 11-hydroxylated derivative of 17OHP produced solely in the adrenal cortex. Because this pathway is negligible in normal subjects, serum 21DF seems useful as an alternative approach to screen carriers for 21OHD [9]. Previous studies suggested that ACTH-stimulated serum levels of 21DF are more sensitive than 17OHP in identifying 21OHD carriers among the general population, using immunoassay methods [10,11].

In the present study, we employed tandem mass spectrometry after HPLC separation (LC-MS/MS) to determine the discriminatory value of basal and ACTH-stimulated serum levels of 21DF in comparison to 17OHP in a cohort of genotypically-confirmed heterozygote carriers for classic and NC 21OHD, as well as in affected patients and in genotypically normal control subjects (CS).

Subjects and Methods

Subjects

The study encompassed 132 individuals: (a) 61 obligatory carriers (32F/29M; 23-62y; median: 39y) who were parents of affected patients with 21OHD followed in our institution (53

had children with the classic and 8 with the NC form); (b) 41 affected patients (probands) with 21OHD: 31 with the classic (21F/10M; 1-23y; median: 11y) and 10 with the NC forms (10F; 4-42y; median: 17.5y); and (c) 30 normal control subjects (CS; 19F/11M; 23-62y; median: 37y).

All carriers and CS denied the use of steroids or other medications that could potentially interfere with the study protocol, except for three female carriers who were on oral contraceptives. All adult women were pre-menopausal, except for three female carriers who were into menopause for at least 3y with no replacement therapy. Clinical hyperandrogenism was an exclusion criterion for normal control women. All 21OHD patients who were followed on regular replacement therapy had their medications withheld for 48h before the study.

The protocol was previously approved by the Ethics Committee of all institutions involved in the study and informed written consent was obtained from all participants and/or their responsible.

Methods

All subjects were submitted to an ACTH stimulation test (Synacthen®, Novartis, 250 µg IV bolus) performed in the morning, after overnight fasting, with blood samples drawn at 0 (between 0800 h and 0900 h) and 60 min after injection, except for nine 21OHD classic patients who had only baseline blood drawn at the time of diagnosis. An extra sample was also drawn at time 0 for molecular studies. All pre-menopausal women were studied in the early follicular phase of the menstrual cycle. Serum was separated within 2 h from collection and kept frozen at -20 °C until assayed.

Hormonal Assays

Serum 21DF, 17OHP, and cortisol were simultaneously determined in each sample by LC-MS/MS after on-line extraction, according to a recently described method [(12)]. The intra- and inter-assay coefficients of variation for 21DF, 17OHP, and cortisol were 10 % and 13.3%, 7.5% and 7.9%, and 3.0% and 8.7%, respectively. Quantification limits were respectively: 24 ng/dL, 15 ng/dL and 0.45 µg/dL. To convert metric (ng/dL) to SI units (nmol/L) multiply 21DF by 0.0289 and 17OHP by 0.0303; to convert µg/dL to nmol/L multiply cortisol by 27.6.

Molecular Analysis

DNA samples were extracted from peripheral blood leukocytes by standard procedures. DNA of the parents were screened for the point mutations found in the index case by allelic specific PCR [13,14]. Complete *CYP21A2* gene sequencing [14] was performed: (a) in all CS; (b) whenever stimulated 17OHP was higher than 1,000 ng/dL [15], and/or (c) if no mutations were detected in the carriers.

Subjects Relocation

After genotyping, and regardless of hormonal results, several relocations were mandatory among the participants: (a) two former male carriers (classic form) were found to be affected NC 21OHD patients; (b) four of the former CS were found to be carriers for 21OHD: two for the NC and one for the classic form; the fourth presents a new intronic substitution that is presently under investigation; thus, this patient was excluded awaiting further definition. All others were properly relocated.

After regrouping, the final numbers used for statistical analysis were: (a) 62 carriers, 52 of whom with the classic (27F/25M; 23-62y) and 10 with the NC form (7F/3M; 29-45y), (2) 31 classic (21F/10M; 1-23y) and 12 NC patients (10F/2M; 9-58y), and (3) 26 CS (15F/11M; 23-65y).

Statistical Analysis

Statistical Package for the Social Sciences for Windows, Version 13.0 (SPSS Inc. Chicago, IL, USA) was used for calculations. Receiver operator characteristics (ROC) curves were used to define the cutoff points for ACTH-stimulated serum 21DF and 17OHP in the genotypically-confirmed populations of 21OHD carriers and CS. The one-way non-parametric ANOVA Mann-Whitney U test was used for comparisons between groups. For statistical purposes, all values below the quantification limit for the particular steroid were arbitrarily considered equal to the quantification level divided by the square root of 2 (therefore defining an average number for all undetectable values).

All results are presented in the text and tables as the mean ± SD and the median (and range) for all three steroids in the several groups. All statistical differences <0.05 (5%) were considered significant.

Results

Any comparison with the 21OHD patients would have to consider that their replacement therapy was withheld for only 48 h (for ethical reasons), a period not necessarily sufficient to permit a full reemergence of the pre-treatment steroid profile. This consideration is particularly true when analyzing the overlap of serum 21DF values that occurred between 21OHD patients and carriers in this study.

Because there were no statistical differences between classic and NC carriers for all three steroids (table 1), both groups were subsequently combined for statistical purposes and named simply 21OHD carriers.

21DF (table 1; figure 1)

Although stimulated 21DF values were similarly elevated in classic and NC 21OHD patients, basal levels were significantly higher in the former ($p<0.0001$). Basal 21DF levels were higher in carriers than CS ($p<0.04$), and stimulated values were significantly increased in the former ($p<0.0001$) and virtually non-responsive in CS. While basal 21DF was undetectable in all but 9 carriers (14.5 %) and all CS, post-ACTH values remained undetectable in only 6 carriers (9.7 %), but in all CS, except two in whom values were barely above detection (29.5 and 39 ng/dL) (figure 1).

Only 11 out of the 62 carriers (17.7%) overlapped with CS regarding stimulated 21DF. Thus, 82.3% of the carriers had 21DF above 39 ng/dL, the highest normal post-ACTH value.

In the conditions of the study (short term glucocorticoid withdrawal), six out of the forty-three 21OHD patients (5 classic and 1 NC), still had ACTH-stimulated 21DF values that overlapped with carriers. Nevertheless, the lowest basal and post-ACTH 21DF levels observed in the six untreated NC patients (164 and 1,319 ng/dL) were above the upper limits observed in carriers (76 and 412 ng/dL, respectively).

17OHP (table 1, figure 1)

Classic 21OHD patients had both basal and stimulated 17OHP levels that were significantly increased as compared to NC patients ($p<0.0001$). Although basal 17OHP levels were not different between carriers and CS, stimulated levels were significantly higher in the former ($p<0.0001$); however, individual post-ACTH 17OHP values disclosed an important overlap with

CS (29 out of 62 carriers; 46.8 %) (figure 1). Thus, only 53.2 % of the carriers had values above the upper normal limit (298 ng/dL).

Correlation between 21DF and 17OHP (figure 2)

A positive and highly significant correlation (r= 0.7854; p<0.0001) was observed between all 21DF and 17OHP pairs of values (basal and post-ACTH; n= 201) obtained from all carriers and classic and NC 21OHD patients.

Cortisol (table 1)

Classic 21OHD patients had low basal and ACTH-unresponsive cortisol levels, whereas NC patients had normal basal but impaired responses. 21OHD carriers had both basal and post-ACTH cortisol values that were virtually identical to CS.

ROC Curve

Basal levels of both 21DF and 17OHP were not accurate enough to separate 21OHD carriers from CS, but when ACTH-stimulated values were used, the area-under-the-curve (AUC) for 21DF was significantly larger than that for 17OHP (0.944 and 0.858, respectively; p<0.0001). Also, the cutoff point defined for ACTH-stimulated 21DF – 32.7 ng/dL -, provided 87.1 % sensitivity and 96.2 % specificity for distinguishing carriers from CS, whereas that for 17OHP – 200 ng/dL -, conferred only 82.3 % sensitivity and 84.6 % specificity. For 100 % specificity, the sensitivities achieved for ACTH-stimulated 21DF and 17OHP were 82.3 % (40 ng/dL) and 53.2 % (298 ng/dL), respectively.

Discussion

In its classic form, 21OHD is a serious and even fatal disease. Because the prevalence of heterozygote carriers for 21OHD in the general population is high, and obviously higher among populations at risk (e.g.: relatives in families with an affected patient) [(3)], their screening and case detection using routine methodology is greatly desirable. In the present study we demonstrate the advantage of ACTH-stimulated serum levels of 21DF in comparison to 17OHP (both determined by LC-MS/MS) in distinguishing genotypically-confirmed carriers for 21OHD from normal control individuals (“wild-types” for *CYP21A2* gene mutations).

Serum/plasma 17OHP has been generally considered the best biochemical marker for the diagnosis of 21OHD. However, it is also known that ACTH-stimulated 17OHP fails to identify the carrier state in 20 % to 71 % of the cases [16-19]. Considerable overlap is also observed between carriers and normal controls using the incremental rate of 17OHP (in ng/dL/min) following ACTH infusion [20]. The same difficulty was shown when ACTH stimulation was preceded by adrenal suppression with overnight dexamethasone [21]. Somewhat better results were reported with the use of substrate/product steroid ratios after ACTH stimulation: 17OHP:18-hydroxy-deoxycorticosterone (18OHDOC) ratio disclosed 94 % separation [22], whereas 17OHP:DOC ratio was reported in one study to result in 100 % separation [23].

Moreover, the methodology for steroid quantification has advanced from classic radioimmunoassay [24,25] to up-to-date highly specific tandem mass spectrometry preceded by liquid or gas chromatography [26,27], in special for steroids whose determinations are not widely available, as 21DF [28]. Because 21DF is an 11β-hydroxy-derivative of 17OHP and its production is limited to the adrenal cortex, its serum levels are significantly and concurrently elevated in the affected patient with 21OHD, whereas its production is virtually nil in normal subjects [29]. Thus, 21DF response to ACTH stimulation might be a distinctive feature of the carrier state, as has been proposed in previous studies [10,18,30]. The strong positive correlation observed between 21DF and 17OHP in our carrier population, as well as in 21OHD classic and NC patients, indicates the functionality of this otherwise inactive pathway.

Using a cutoff point of 40 ng/dL for ACTH-stimulated 21DF we could discriminate carriers from CS with greater accuracy than using 298 ng/dL for ACTH-stimulated 17OHP (82.3% *vs* 53.2% sensitivity, for 100% specificity). Also, different from Tardy *et al* [11], who detected "some normal genotyped subjects with post-ACTH 21DF higher than 50 ng/dL", and Bidet *et al* [31], who found 3 out of 37 (8.1%) "unaffected subjects" with values higher than 53 ng/dL, none of our genotypically-confirmed CS had ACTH-stimulated 21DF values above 40 ng/dL. Thus, the LC-MS/MS methodology employed in the present study to measure 21DF seems to warrant enough consistency to recommend its use towards the detection of carriers for 21OHD.

In our series, one female carrier had exaggerated 21DF and cortisol responses to ACTH (412 ng/dL and 37.9 μg/dL, respectively, - the highest values among carriers), in face of moderately elevated 17OHP (960 ng/dL). Subsequently, she admitted the use of a contraceptive pill for 21 days before the study. Similar but less apparent values were observed for the other two carriers who were also on birth control pills (21DF: 187 and 171 ng/dL; cortisol: 32.7 and 23.1 μg/dL). Pregnancy and use of estrogen/progesterone products are known to be associated with elevated cortisol and 21DF levels due to the increased concentration of cortisol binding globulin (18). Thus, caution must be exerted whenever ACTH-stimulated cortisol levels are above 30

μg/dL, since they could be related to falsely elevated 21DF and 17OHP (and false-positive case detection of carriers) [7]. On the other hand, six carriers (4 classic and 2 NC) had unresponsive 21DF after ACTH (< 17 ng/dL), similar to most CS. Although only one admitted the use of corticosteroid medications, we could not exclude the unintentional use of corticosteroid-containing creams and nasal sprays that could impair 21DF response to ACTH. In fact, several affected patients in our study had low ACTH-stimulated 21DF levels that overlapped with carriers, different from their respective 17OHP levels that clearly segregate. In view of the short corticosteroid withdrawal period in these patients, reemergence of 17OHP seems to precede that of 21DF, suggesting that the latter is more sensitive to ACTH suppression.

These data validate the superiority of ACTH-stimulated serum 21DF, as compared to 17OHP - both measured by LC-MS/MS -, in identifying carriers for 21OHD. Even though molecular analysis is the "gold standard" in diagnosing subtypes of 21OHD, serum 21DF seems to be a useful tool in genetic counseling to screen for carriers among relatives in families with affected subjects, supporting molecular results. A further gain in assay sensitivity for 21DF will hopefully permit a better separation in the near future.

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Table 1: Mean (±SD) and median [range] of basal and post-ACTH cortisol, 17OH-progesterone (17OHP), and 21-deoxycortisol (21DF), in patients with 21OHD (22 classic; 12 NC), carriers (52 classic; 10 NC), and 26 control subjects. (despite being presented in separate, classic and NC carriers were combined for statistical analysis).

	21DF (ng/dL)		**17OHP (ng/dL)**		**Cortisol (µg/dL)**	
	Basal	**Post-ACTH**	**Basal**	**Post-ACTH**	**Basal**	**Post-ACTH**
Classic 21OHD Patients	1,238±1,692* 463 [154-7,079]	1,438±1,656[&] 748 [143-6,326]	12,841±8,855* 11,346 [816-34,911]	15,329±10,108* 14,651 [1,185-40,090]	1.1±1.2* 0.6 [0.2-4.4]	1.1±1.1* 0.7 [0.2-3.3]
Nonclassic 21OHD patients	300±395† 161 [47-1,423]	1,577±1,094† 1,260 [302-3,619]	2,113±3,535† 1,068 [574-13,293]	7,095±3,339† 6,649 [3,008-13,256]	11.3±5.3 9.7 [6.1-25.0]	16.5±6.4† 14.8 [6.4-32.5]
Classic 21OHD Carriers	19.9±10.3 17.0 [17.0-76.0]	98.5±73.4 81.5 [17.0-412.3]	88±75 71 [20- 445]	419±308 314 [60-1,347]	10.5±4.3 9.9 [3.0-22.8]	24.3±5.4 23.8 [13.5-37.9]
Nonclassic 21OHD Carriers	30.5±18.1[£] 17.0 [17.0-60.0]	109.3±87.7[££] 89.5 [17.0-295.9]	79.2±75.9 45.2 [23-256]	487±507[££] 265 [143-1,704]	10.4±5.8 7.7 [5.9-24.0]	24.4±3.6 25.3 [17.1-29.0]
Normal control subjects	17.0±0.1 17.0 [17.0-17.0]	18.3±4.9 17.0 [17.0-39.0]	60.6±40.6 54.8 [10.6-175]	162.9±57.2 162.7 [63.2-296.4]	10.3±3.6 10.0 [5.4-17.4]	24.1±4.3 24.0 [16.3-31.7]

[*]$p<0.0001$ *vs* NC patients, carriers, and CS; [&]$p<0.0001$ *vs* carriers and CS; [†]$p<0.0001$ *vs* carriers and CS; [£]$p<0.04$ *vs* CS; [££] $p<0.0001$ *vs* CS.

Figure 1: Individual ACTH-stimulated serum 21DF and 17OHP levels in 21OHD subtypes: classic and nonclassic (NC) patients, combined classic and NC carriers and control subjects.

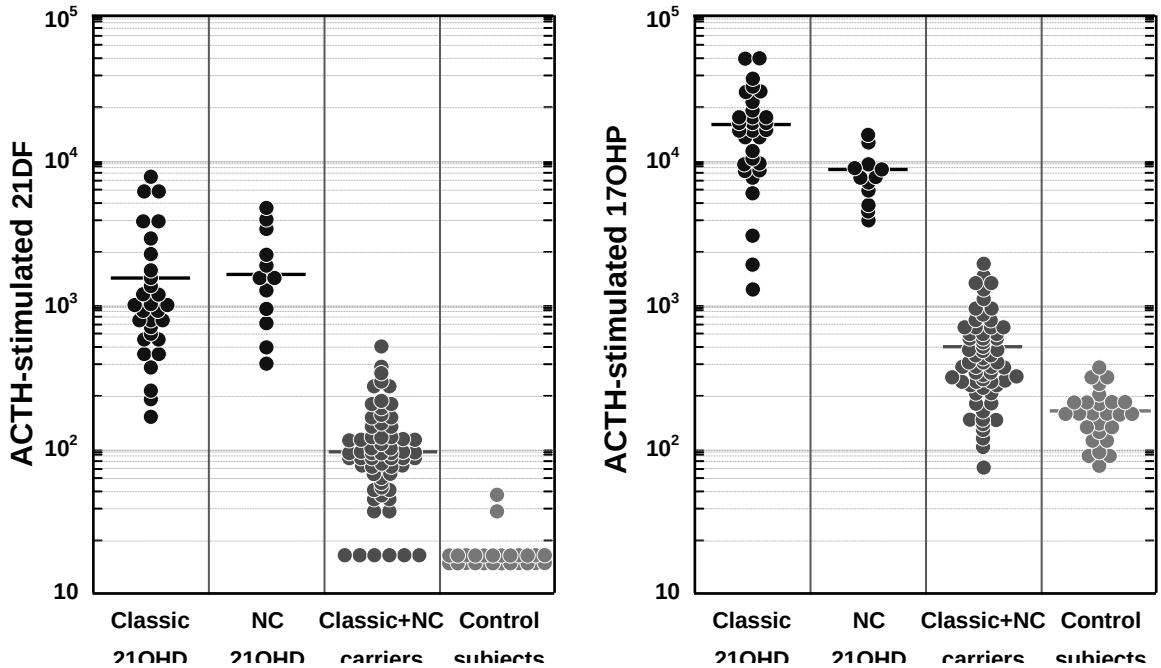
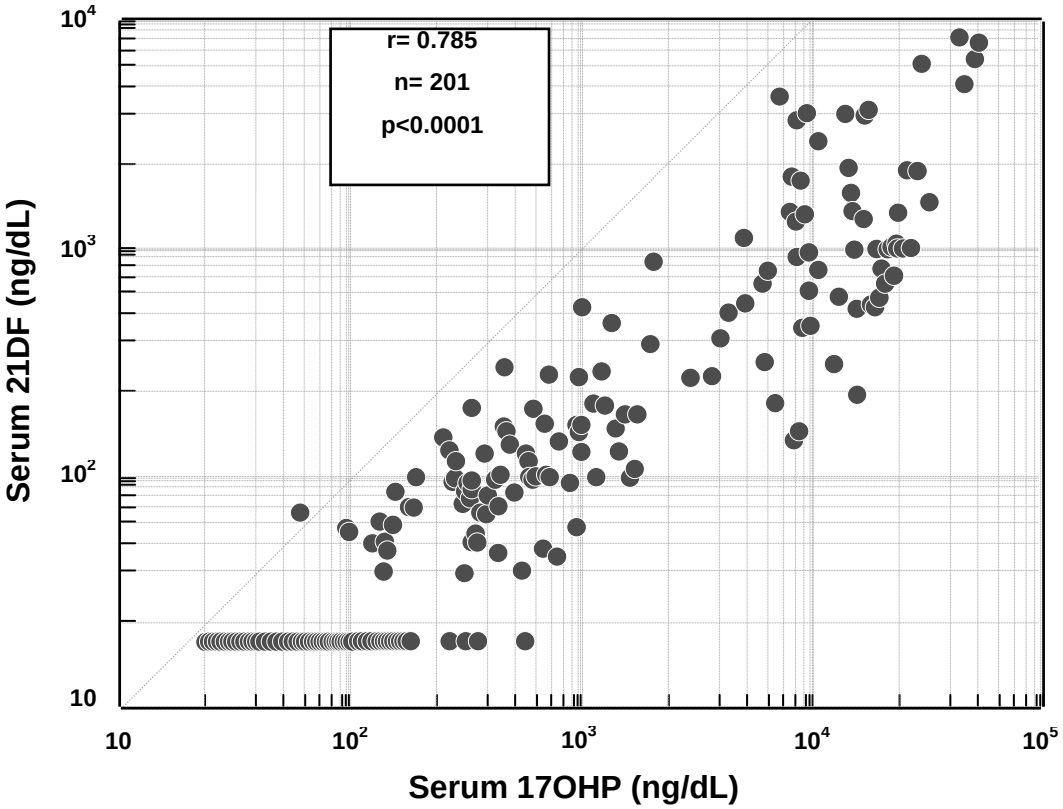


Figure 2: Correlation between serum 21DF and 17OHP (basal and ACTH-stimulated levels) in 21OHD patients and carriers.



3. Artigo 2

Genotype correlations with 21-deoxycortisol and 17-hydroxyprogesterone in classic and nonclassic patients and heterozygote carriers for 21-hydroxylase deficiency validated by CYP21A2 molecular analysis

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Genotype correlations with 21-deoxycortisol and 17-hydroxyprogesterone in classic and nonclassic patients and heterozygote carriers for 21-hydroxylase deficiency validated by *CYP21A2* molecular analysis

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Abbreviated title: Screening CYP21 gene mutations and correlation with 21DF

Key words: 21-hydroxylase deficiency; *CYP21A2* genotype; 21OHD carriers; 21-deoxycortisol; 17-hydroxyprogesterone;

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Abstract

Introduction: In 21-hydroxylase deficiency (21OHD) there is a good correlation between genotype and basal 17-hydroxyprogesterone (17OHP) levels in the classic, and also with post-ACTH 17OHP levels in the nonclassic (NC) form, but not in carriers. 21-Deoxycortisol (21DF) is a good biochemical marker of 21OHD, in special to identify the "carriers", but there are no studies correlating 21DF levels with genotypes. **Objective:** Compare 21DF and 17OHP levels - determined by LC-MS/MS -, with genotype in 29 21OHD patients and in 62 heterozygote carriers. CYP21A2 from all subjects were sequenced and the genotypes classified according to residual enzymatic activity: Group A (mutations abolishing the enzymatic activity), B (3-7% of residual activity) and C (20%-50% residual activity). **Results:** Mutations were identified in 85/88 (96.5%) of alleles from the patients; the 3 unidentified alleles were from NC patients. Basal but not post-ACTH 21DF was significantly higher in the A group compared to the C group, whereas both basal and post-ACTH 17OHP were significantly higher in the former group. In carriers, there were no differences in basal and post-ACTH 17OHP and 21DF levels between A, B, and C groups. Post-ACTH 21DF and 17OHP levels higher than 300ng/dL and 1,700/dL, respectively, could discriminate NC patients from carriers. **Conclusion:** Basal 21DF levels differentiated A to C genotypes in the patient groups, demonstrating its utility for diagnostic purposes, such as in boys in the neonatal period. Additionally, difficulties in differentiating NC form from carriers with 17OHP profiling could be determined with the use of 21DF.

Introduction

Congenital adrenal hyperplasia owed to 21-hydroxylase deficiency (21OHD) is one of the commonest autosomal recessive disorder in humans [1,2]. The clinical spectrum of the disease encompasses classic and nonclassic (NC) forms reflecting the residual 21-hydroxylase activity, resulting from the wide range of *CYP21A2* gene mutations. In the most severe salt wasting (SW) form, the enzymatic activity is null (its genotype is termed type A), whereas in the simple virilizing form (SV), less than 5% of residual adrenal 21-hydroxylation appears sufficient to prevent salt loss, but not virilization manifestations in the neonatal and/or post-natal periods (type B genotype). In the milder NC form, in which remaining enzymatic activity varies from 20% to 50% (type C genotype), patients can be asymptomatic or manifest hyperandrogenic signs during childhood or early adult life [3].

Until recently, the human gene mutation database (www.hgmd.cf.ac.uk) depicted more than 150 different *CYP21A2* gene mutations, two-thirds of which represented by point mutations. Among the ethnic heterogeneous Brazilian population, as it occurs in other populations, most of the affected alleles carry mutations derived from gene conversion events involving the active (*CYP21A2*) and its highly homologous pseudogene (*CYP21A1P*), and a high frequency of alleles with multiple mutations are observed [4-9].

Because most patients are compound heterozygotes, their phenotypes result from the less impaired allele [1-3]. A good correlation between genotype and 17-hydroxyprogesterone (17OHP) levels is observed in classic and NC 21OHD [10,11], but not in carriers for the disease [12-15].

21-deoxycortisol (21DF) is an additional biochemical marker of 21OHD that has been shown to be superior to 17OHP in: (a) the follow-up of adolescent and adult patients with 21OHD during treatment [16] and, (b) identifying the “heterozygote carrier state” [17,18]. However, there are no studies correlating 21OHD genotypes and 21DF [19]. Considering, that *CYP21A2* molecular analysis is not an easy and widely available methodology, the 21DF measurements could be a useful tool in identifying the different phenotypes of 21-hydroxylase deficiency, which present important implications in the treatment decisions and genetic counseling.

In the present study, we analyzed the basal and pos-ACTH 21DF and 17OHP levels, both determined by liquid chromatography coupled to mass spectrometry (LC-MS/MS), among the different 21OHD phenotypes for diagnostic purposes. In addition, we evaluated whether the different genotype severities, in patients and carriers, could be predicted by the basal and pos-ACTH 21DF and 17OHP levels.

Subjects and Methods

The study encompassed a cohort of 45 Brazilian patients with classic and NC 21OHD and 62 heterozygote carriers for the disease. The patients were: 43 unrelated index cases, being 33 with the classic (25 SW; 18F/7M, and 8 SV; 6F/2M) and 10 with the NC form (10F), and two parents (2M), who were identified as affected by NC form during genotyping. The carriers were 31F/28M obligatory heterozygotes, parents from index cases and another three (3F) detected among the general population by entire *CYP21A2* sequencing. All but, three female carriers admitted the use of oral contraceptives. 21OHD patients who were followed on regular replacement therapy had their medications withheld for 48h before the study [(16)].

Diagnosis of 21OHD was confirmed in the newborn period or early infancy in all classic patients by hormonal profiling (basal 17OHP > 5,000 ng/dL). The NC female patients were diagnosed in the adolescence or early adult life during investigation for hirsutism and/or menstrual irregularities, except for three who presented with precocious pubarche and advanced bone age; all had ACTH-stimulated 17OHP above 3,000 ng/dL. The two NC male patients mentioned above were asymptomatic and presented normal fertility.

Analysis of frequency of the *CYP21A2* gene mutations was carried out exclusively in the 43 index cases, plus two unrelated alleles from the affected parents, summing up 88 nonrelated alleles. We also determined the frequency of mutations in the 62 carriers.

All subjects enrolled in the study gave their informed written consent after being told of the purposes of the present protocol that have been previously approved by the Ethic Committees in Human Research of both institutions.

Molecular Analysis

DNA samples were extracted from peripheral blood leukocytes by standard procedures. Patients were screened by allele-specific PCR for the 15 most frequent point mutations [(6,14)]. If no mutations were found, the entire gene was sequenced. The mutations found in the index cases were segregated in DNA samples from all obligatory heterozygotes, Complete *CYP21A2* gene sequencing, including the promoter region, was performed whenever stimulated 17OHP was higher than 1,000ng/dL in the obligatory heterozygotes [(20)].

When a large rearrangement was suspected, as suggested by point mutations in homozygous state, a *MLPA*-multiplex ligation-dependent probe amplification (SALSA MLPA kit P050B CAH, MCR-Holland, Amsterdam, The Netherlands*)* was employed to differentiate *CYP21A2* deletions from large gene conversions [(21)]. The SALSA MLPA kit contains 5 specific

probes for the active 21-hydroxylase gene, 3 for the pseudogene, one for the *C4B* and one for the *C4A* genes.

Genotype Categories

CYP21A2 genotypes were grouped according to the predicted impairment of enzymatic activity observed in *in vitro* studies [(3)]: type A genotype (carry mutations resulting in total or near total impairment of 21OH activity), this group comprised patients homozygotes or compound heterozygotes for mutations I2 splice, Q318X, *CYP21A2* deletion and large gene conversion, etc); type B genotype (resulting in severe impairment or less than 7% of residual activity), comprised patients homozygotes for the I172N mutation, or compound heterozygosis with any type A mutation; and type C genotype (moderate impairment or 20%-50% of residual activity), comprised patients homozygotes for V281L, P30L, P453S, R339H, and V304M, or compound heterozygosis with any group A or B mutations.

Since carriers have one normal or non-affected allele that presumably expresses full enzymatic activity, the frequency analysis and genotype/phenotype correlations in this group were done only for academic purposes, employing the single mutated allele and the same classification.

Hormonal Assays

Thirty of the 45 patients (12 SW, 6 SV, and all 12 NC - including the two asymptomatic fathers) and all 62 carriers were submitted to an ACTH stimulation test (Synacthen®, Novartis, 250µg IV bolus) performed in the morning, after overnight fasting and 30' recumbence; blood samples were drawn at 0 (around 08:30h) and 60 min after injection. All premenopausal women were studied in the early follicular phase of the menstrual cycle. Serum 21DF and 17OHP were simultaneously determined in each sample by LC-MS/MS, according to a previously described method [(22)]. The intra- and inter-assay coefficients of variations for 21DF were 10% and 13.3%, and for 17OHP, 7.5% and 7.9%. To convert metric (ng/dL) to SI units (nmol/L) multiply 21DF by 0.0289 and 17OHP by 0.0303.

Statistical Analysis

The one-way non-parametric test of Kruskal-Wallis was used for comparisons between groups. For statistical purposes, all values below the quantification limit for the particular steroid were arbitrarily considered equal to the quantification level divided by the square root of 2

(therefore defining an average number for all undetectable values). A P value <0.05 was considered statistically significant.

Results

21OHD Patients

A total of 17 different mutations were identified in 85 of the 88 (96.6%) alleles investigated, 88.2% of which being point mutations. A single mutation was present in 77 of the 85 (90.6%) identified alleles, whereas two (7/85; 8.3%) and three (1/85; 1.1 %) mutations per allele were documented in the remaining eight. I2 splice was the most frequently observed mutation (23.5%), followed by V281L (22.3%), R356W (14%), and I172N (11.8%), accounting for 71.6% of all mutations. In the NC form, V281L was present in 16 out of the 22 alleles (72.7%), whereas in SV, I172N was present in 62.5% and in SW form; I2 splice was present in 36% of the alleles (Table 1).

Twenty patients were homozygotes and 25 were compound heterozygotes (Table 2). One SV (type B) female patient disclosed a rare mutation - V304M (23) - in cis with the I172N mutation, confirmed by segregation analysis in the parent DNA samples.

In two NC female patients, despite they presented serum steroid levels compatible with the NC form diagnosis (basal 17OHP: 1,431 and 1,223 ng/dL, post-ACTH 17OHP: 8,839 and 8,056ng/dL, and post-ACTH 21DF: 842 and 1,319ng/dL, respectively), mutations were identified in only one allele (V281L) from the first patient and in none from the second one. The entire *CYP21A2* sequencing identified a new substitution A>G in the splice acceptor site of intron 5 in the former patient, disclosing the genotype V281L/IVS 5 – 2 A>G. However, this patient was excluded from data analysis until functional studies will be concluded. In the other patient, no mutations were identified despite exhaustive sequencing. Both patients are identified with question marks in Table 2.

We also included as NC patients two asymptomatic parents who had elevated 17OHP (basal: 856 and 1,087ng/dL, post-ACTH: 6,566 and 12,492ng/dL) and 21DF levels (basal: 203 and 233ng/dL, post-ACTH: 1,885 and 2,964ng/dL). Their molecular analysis disclosed the genotypes V281L/I2 splice and V281L/R356W, which belong to group C (Table 2).

When 21OHD patients were separated according to the genotype category, basal 21DF and 17OHP levels were significantly higher in group A as compared to group C. However, post-ACTH levels were significantly higher in group A vs C only for 17OHP (Table 3). There were no

statistical differences between groups A vs B, or B vs C, for both basal and post-ACTH 21DF and 17OHP levels.

Among the 10 completely genotyped NC patients six were homozygous for the V281L mutation (C/C), whereas four were compound heterozygous for mutations from group A (A/C). The latter group, harboring a severe (A) mutation had higher post-ACTH 17OHP levels than the former, whereas no differences were observed for 21DF levels.

21OHD Carriers

The most frequent mutations detected in the heterozygote carriers were: I2 splice, V281L, R356W, large gene conversion/*CYP21A2* deletion, and Q318X, accounting for 72.7% of all mutations (Table 1). Seven carriers (11.3%) had two or three point mutations in the same allele. Although asymptomatic, carriers were also grouped according to the mutated allele in: group A (n=46; 74.2%), group B (n=6; 9.7%) and group C genotypes (n=10; 16.1%).

Since basal and ACTH-stimulated 21DF and 17OHP levels were almost identical in genotype groups A, B, and C, their values were combined in Table 3.

Five genotypically confirmed carriers had stimulated 17OHP values between 1,000-1,500ng/dL. Three of them, carrying mutations from group A, had high ACTH-stimulated 17OHP levels (1,347; 1,289; and 1,256 ng/dL), with somewhat different stimulated 21DF (91; 267; 217ng/dL, respectively). The two remaining carriers had similar post-ACTH 17OHP and 21DF values (post-ACTH 17OHP and 21DF of 1,103 and 1,044ng/dL, and 182 and 184ng/dL, respectively) despite they carried mutations from groups B and C, respectively.

Another asymptomatic female carrier had the highest post-ACTH 17OHP and 21DF levels at 1,704 and 296ng/dL, respectively. However, she was confirmed as a group C carrier since only one V281L mutation was detected after exhausting sequencing.

Discussion

Molecular analysis is the gold standard for the diagnosis and classification of 21OHD phenotypes, including the carrier state. In this paper, we report the frequencies of *CYP21A2* gene mutations in a cohort of Brazilian patients and carriers for the 21OHD form of CAH. The four most prevalent *CYP21A2* gene mutations from the 17 found in our study (I2 splice, R356W, V281L, and I172N) accounted for more than two-thirds of all detected mutations; I2 splice was the most frequent one (23.3%). Even in the presence of the ample miscegenation of the Brazilian population,

the frequency of *CYP21A2* gene mutations in this series was similar to other worldwide screening studies [1, 24-29].

At present, as also observed in this series, all mutated alleles could be identified in classic 21OHD applying entire *CYP21A2* gene sequencing [29]. However, in spite of the advances in molecular genotyping for 21OHD, up to 20% of the alleles from NC form remain unidentified [19,29-31]. In our series, we were not able to identified mutations in 13% of the NC alleles.

Earlier studies with classic 21OHD demonstrated that genotype correlates better with clinical (SW and SV) than hormonal parameters (17OHP, plasma renin), whereas in the NC form, these correlations are less evident [1,10,32,33]. In our data, there was a fair correlation between genotype categories A, B, and C with basal and stimulated 17OHP. Because 21DF is a product of 17OHP, its basal levels were also higher in group A than in group C patients. Surprisingly, stimulated 21DF levels were not significantly different among these groups; in fact, there was a trend towards higher values in group C than in group A and B patients. It may be possible that the short-term glucocorticoid washout (only two days) in classic patients was not sufficient for this susceptible steroid to recover from ACTH suppression. Additional studies in untreated subjects may be necessary to clarify this discrepancy.

Regarding NC patients, post-ACTH levels of 17OHP were higher in A/C than in C/C compound mutations, as previously reported [10,11,19]; however, this was not observed with 21DF levels.

Even though the unaffected allele is able to code normal 21-hydroxylase activity in carriers, ACTH-stimulated 17OHP levels are known to be variably increased in these subjects, suggesting that the mutated allele may interfere with the normal response [12-15,33]. In several studies with 21OHD carriers, ACTH-stimulated 17OHP levels correlated inversely with the severity of the single mutated allele, suggesting a dominant-negative effect of the V281L mutation in carriers with high 17OHP levels [12,15,34]. However, we and others [13,14] were not able to confirm these findings with either 17OHP or 21DF.

According to several authors, carriers may reach ACTH-stimulated 17OHP levels up to 1,500 ng/dL [1,31,32], or 1,700 ng/dL [10], different from a previous threshold value of 1,000 ng/dL [35,36]. Actually, five of our carriers had post-ACTH 17OHP levels between 1,044 and 1,347 ng/dL and also had very high 21DF levels (183-267 ng/dL). In addition, another carrier had elevated post-ACTH levels of 17OHP and 21DF even higher: 1,704 and 296 ng/dL, respectively; however, after repeated gene sequencing, a second mutation was not detected in this asymptomatic

female. On the other hand, the lowest ACTH-stimulated 17OHP level observed in our NC patients was 3.008 ng/dL.

Despite the small sample of NC patients, and to the extent of the limitations imposed by the possible use of interfering medications (oral contraceptives, glucocorticoids) , the lowest ACTH-stimulated 21DF level in a genotypically confirmed NC patient in our series was 302 ng/dL, just above the highest stimulated 21DF level documented in carriers (296 ng/dL). Thus, in addition to the present upper limit for ACTH-stimulated 17OHP proposed by several authors [1,10,32] to separate carriers from NC 21OHD patients (1,500-1,700 ng/dL), we suggest a cutoff level of 300 ng/dL for post-ACTH 21DF, lower than that of 400 ng/dL recommended by Fiet et al [16,37], in order to increase sensitivity.

On the other hand, molecular studies may not be absolutely conclusive. For example, two of our alleged NC patients (noted with question marks in Table 2) disclosed post-ACTH values of 17OHP and 21DF well into the NC range [1,10,32,35], but could not be genotypically characterized as affected NC, despite repeated *CYP21A2* gene sequencing, including the promoter region. Thus, in an attempt to identify new mutations in the so-far "unaffected" alleles in unidentified cases, strict genetic scrutiny is presently underway focusing on *POR* (P450oxido-reductase) gene variations that could modulate 21OHD phenotypes [29,38], and the search for an yet unrevealed factor(s) that could interfere with 17OHP and 21DF responses to ACTH.

Therefore, even in the light of an appropriate molecular definition for 21OHD, establishment of distinct 17OHP and/or 21DF cutoff levels to separate NC 21OHD patients from carriers of the disease and those from normal controls (wild-types) may still be a matter of debate.In summary, genotype-phenotype correlation in patients with 21OHD was fair with serum 17OHP, but only modest with 21DF levels. In carriers, post-ACTH 21DF and 17OHP did not correlate with the severity of the single mutated allele. Using LC-MS/MS methodology, post-ACTH 21DF levels higher than 300ng/dL concurrently with post-ACTH 17OHP levels higher than 1,700 ng/dL, were typical of NC 21OHD. Additionally, because routine *CYP21A2* gene sequencing is not an easy and widely available methodology, difficulties in diagnosing 21ODH subtypes, particularly NC form and carriers with 17OHP profiling, could be better ascertained with the use of 21DF.

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Table 1. Frequency of *CYP21A2* gene mutations found in unrelated alleles of 45 patients with the SW, SV and NC forms of 21OHD, and in the affected allele of 62 obligatory carriers.

CYP21A2	Affected: Phenotype / Clinical forms					Carrier state	
Mutations	SW	SV	NC	Total	(%)	Total	(%)
I2 splice	17	1	0	18	20.5	12	19.4
V281L	0	0	16	16	18.2	10	16.1
R356W	10	0	0	10	11.4	9	14.5
I172N	0	8	0	8	9.1	4	6.5
Q318X	5	0	0	5	5.7	5	8.1
Conv	5	0	0	5	5.7	4	6.5
Deletion	4	1	0	5	5.7	5	8.1
Cluster	2	1	0	3	3.4	3	4.8
1003^1004 insA	1	0	0	1	1.1	1	1.6
C168 lΔ1nt	1	0	0	1	1.1	0	0.0
F306+1nt	1	0	0	1	1.1	0	0.0
InsT	0	1	0	1	1.1	1	1.6
R408C	0	1	0	1	1.1	1	1.6
IVS2-2 A>G	0	0	1	1	1.1	0	0.0
P30L	0	0	1	1	1.1	0	0.0
IVS2+5 G>A, V281L	1	0	0	1	1.1	1	1.6
R356W, Q318X, InsT	1	0	0	1	1.1	1	1.6
I2 splice, V281L	1	0	0	1	1.1	1	1.6
R356W, Q318X	1	0	0	1	1.1	1	1.6
I172N, V304M	0	1	0	1	1.1	1	1.6
I2 splice, InsT	0	1	0	1	1.1	1	1.6
I172N, V281L	0	1	0	1	1.1	1	1.6
P30L, Cluster	0	0	1	1	1.1	0	0
None found	0	0	3	3	3.4	0	0
Total	50	16	22	88	100.0	62	100.0

Table 2: Genotype categories according to the residual enzymatic activity in classic and NC 21OHD patients (see text for details).

Group A (n= 25)			Group B (n= 8)		
sex	clin.form	Genotype	Sex	clin.form	Genotype
F	SW	Conv / Conv	F	SV	I172N / Cluster
M	SW	Conv / Conv	F	SV	I172N / Del
F	SW	Conv / Cluster	F	SV	I172N / I172N
F	SW	Del / Del	M	SV	I172N / I172N
F	SW	I2 splice / 1003^1004 insA	F	SV	I172N / I2 splice, InsT
M	SW	I2 splice / C168 delΔ1+I4nt	F	SV	I172N / R408C
M	SW	I2 splice / Del	M	SV	I172N, V281L / I2 splice
F	SW	I2 splice / F306+1nt	F	SV	I172N, V304M / InsT
F	SW	I2 splice / I2 splice			
F	SW	I2 splice / I2 splice			
F	SW	I2 splice / I2 splice			
M	SW	I2 splice / I2 splice			
M	SW	I2 splice / IVS2+5 G>A, V281L			
M	SW	I2 splice / Q318X			
F	SW	I2 splice / R356W			
M	SW	I2 splice / R356W			
F	SW	I2 splice / R356W, Q318X, InsT			
F	SW	Q318X / Q318X			
F	SW	Q318X / Q318X			
F	SW	R356W / Deletion			
F	SW	R356W / I2 splice, V281L			
F	SW	R356W / R356W			
F	SW	R356W / R356W			
F	SW	R356W / R356W			
F	SW	R356W, Q318X / Cluster			

Group C (n= 12)		
Sex	clin.form	Genotype
F	NC	P30L / P30L, Cluster
F	NC	V281L / IVS2-2 A>G
M*	NC	V281L / I2 splice
M*	NC	V281L / R356W
F	NC	V281L / V281L
F	NC	V281L / V281L
F	NC	V281L / V281L
F	NC	V281L / V281L
F	NC	V281L / V281L
F	NC	V281L / V281L
F	NC	V281L / (IVS5-2 A>G)??
F	NC	?? / ??

* Fathers of affected patients found to be asymptomatic NC during molecular evaluation.
?? No mutations found or functional studies not completed.

Table 3. Basal and post-ACTH serum levels of 21DF and 17OHP (median and interquartile interval) in 21OHD patients and carriers grouped according to the *CYP21A2* gene mutation (see text for details).

	21-DF (ng/dL)		**17OHP (ng/dL)**	
Genotype	**Basal**	**Post-ACTH**	**Basal**	**Post-ACTH**
21OHD Affected Patients				
Group A (12)	739*	876	15-661*	15,182*
	373-1,500	642-1,548	5,314-18,346	9,413-17,585
Group B (6)	329	725	8,827	13,348
	180-619	380-1,719	6,866-10,933	8,111-15,303
Group C (11)	158	1,200	910	6,566
	99-203	737-2,425	853-1,259	4,415-7,630
21 OHD Carriers (62)	17	82	65	303
	17-17	51-126	33-115	216-497

*$p<0.05$ vs Group C patients

4. Artigo 3

Zona fasciculata 21-hydroxysteroids and precursor-to-product ratios in 21-hydroxylase deficiency: further characterization of classic and nonclassic patients and heterozygote carriers

Costa-Barbosa FA, Carvalho VM, Nakamura OH, Bachega TA, Vieira JGH, Kater CE.

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Zona fasciculata 21-hydroxysteroids and precursor-to-product ratios in 21-hydroxylase deficiency: further characterization of classic and nonclassic patients and heterozygote carriers

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Running title: S, DOC, and B profile in 21OHD

Key words: 11-deoxycortisol (S), 11-deoxycorticosterone (DOC), corticosterone (B), 21-deoxycortisol (21DF), 21-hydroxylase deficiency (21OHD).

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Abstract

Introduction: Although much is known about the increased levels of the 21-hydroxylase substrates 17-hydroxyprogesterone (17OHP) and 21-deoxycortisol (21DF) - the biochemical markers of all forms of 21-hydroxylase deficiency (21OHD), only limited information is available on the zona fasciculata (ZF) 21-hydroxysteroids: 11-deoxycortisol (S), 11-deoxycorticosterone (DOC) and corticosterone (B), products distal to the enzymatic block. **Objective**: To investigate whether basal and post-ACTH levels of S, DOC, and B and the 21-hydroxylase precursor-to-product ratios determined by LC-MS/MS could disclose distinct profiles in genotypically confirmed classic (n=14) and nonclassic (NC; n=12) patients, heterozygote carriers (n=62) and wild-types (WT; n=26) for 21OHD. **Results**: Salt wasting (SW) and simple virilizing (SV) classic 21OHD patients had high basal levels of DOC with no further increase in response to ACTH. Stimulated DOC was similar in 21OHD patients and carriers, but reduced as compared to WT. ACTH-stimulated B increased gradually from SW and SV through WT. The post-ACTH 21DF/B ratio was able to identify 92% of the carriers. All NC patients could be identified by post-ACTH 17OHP/DOC and 21DF/B, with no overlap with carriers. **Conclusion**: Although 21-hydroxylase is a key step in both 17-hydroxy and 17-deoxy pathways of ZF, the reaction is mostly affected in the latter pathway, leading to a significant impairment of B production, which may further characterize the 21OHD subtypes. Basal DOC levels are elevated in SW patients probably as a result of extraadrenal production from increased progesterone. Finally, the use of specific precursor-to-product ratios are informative and may additionally characterize 21OHD patients and carriers.

Introduction

21-hydroxylase deficiency (21OHD) is the most frequent autosomal recessive disorder in humans. If not rapidly diagnosed, the salt-wasting form (SW) is a life-threatening condition that manifests several weeks after birth by acute adrenal insufficiency (lack of cortisol and aldosterone), whereas in the simple-virilizing (SV) form sufficient aldosterone is produced to prevent salt loss. In both classic forms, prenatal exposure to increased androgen levels result in ambiguous genitalia in the female and macrogenitosomia in the male newborn. In the milder nonclassic (NC) form, cortisol production is normal at the expense of increased ACTH levels, which in turn result in clinical hyperandrogenism (acne, hirsutism, menstrual abnormalities, infertility), manifested from childhood through early adult life [1,2].

Although much is known about the increased levels of the 21-hydroxylase steroid substrates, progesterone, 17-hydroxyprogesterone (17OHP) and 21-deoxycortisol (21DF) - biochemical markers of 21OHD [3,4] - few detailed information is available on the zona fasciculata (ZF) 21-hydroxylated products distal to the enzymatic block: 11-deoxycortisol (S), deoxycorticosterone (DOC) and corticosterone (B) [5,6].

A host of *CYP21A2* gene mutations determine the severity of the enzymatic activity in both ZF and zona glomerulosa (ZG) of the adrenal cortex, resulting in characteristic steroid profiles for all forms of 21OHD. Residual enzymatic activity from the less impaired allele may be associated with virtually normal mineralocorticoid production in mild and moderate forms [1,7]. In addition, previous studies in these patients have shown decreased levels of cortisol, S, and B (21-hydroxylated steroids from both the 17-hydroxy and 17-deoxypathways of ZF) [3,5-8].

Use of the ACTH-stimulated 17OHP levels to discriminate the heterozygote carrier population (referred herein simply as “carriers”) from NC 21OHD and from normal subjects can be challenging. In fact, the several cutoff levels proposed to differentiate carriers from NC and normal subjects show significant overlaps between these groups [8-11]. Therefore, ACTH-stimulated 21DF levels and/or specific precursor-to-product ratios (17OHP:S and 17OHP:18OHDOC) have been proposed as diagnostic tools to identify carriers for 21OHD [12,13].

In this study, we employed tandem mass spectrometry after HPLC separation to examine baseline and ACTH-stimulated serum levels of the steroids distal to the 21-hydroxylase block - S, DOC, and B -, in classic (SW, SV) and NC patients with 21OHD, carriers and wild-types (WT) for the disease. We also investigated whether specific precursor-to-product ratios could be a useful diagnostic tool in distinguishing 21OHD carriers from normal subject (WT), and NC patients from carriers.

Subjects and Methods

The study group included: (a) 26 affected patients with 21OHD: 9 SW (4M/5F; 1-23y), 5 SV (1M/4F; 8-28y), and 12 NC (2M/10F; 4-42y), whose phenotypes were ascertained by clinical and biochemical data, and confirmed by genotyping; (b) 62 genotypically confirmed obligatory carriers of classic (n= 52; 25M/27F; 23-62y) and NC 21OHD (n= 10; 3M/7F; 29-45y), and (c) 26 genotypically normal control subjects (21-hydroxylase "wild-types" [WT]; 11M/15F; 23-65y).

Patients who were on replacement therapy had their medications withheld for 48h before the study. None of the subjects reported the recent use of any medications that could potentially interfere with the study parameters. All subjects were submitted to an ACTH stimulation test (Synacthen®, Novartis, 250 μg IV bolus) after overnight fasting and 30' recumbence, with blood samples drawn at 0' (between 0800h and 0900h) and 60' after injection. Premenopausal women were studied in the early follicular phase (days 2 to 5) of the menstrual cycle.

The study protocol was previously approved by the Committees on Human Research of all involved institutions and informed written consent was obtained from all subjects and/or legal responsible.

Hormonal assays

Serum S, DOC, B, 17OHP, and 21DF were simultaneously determined in each sample by tandem mass spectrometry preceded by HPLC separation (LC-MS/MS), according to a previously described method [(14)]. 17OHP and 21DF values are to be reported on a separate publication. The intra- and inter-assay coefficients of variation for S, DOC, and B were respectively: 10.4% and 12.1%, 8.4% and 12.5%, and 7.8% and 7.8%. Quantification limits for these steroids were: 15 ng/dL, 10 ng/dL, and 95 ng/dL, respectively. To convert metric (ng/dL) to SI units (nmol/L) multiply S or B by 0.0289 and DOC by 0.0303.

Precursor-to-product ratios

Individual values of specific precursor-to-product ratios were calculated by dividing basal and post-ACTH 17OHP and 21DF by S, DOC, and B. 17OHP/B and 21DF/B were multiplied by 10 and 10^2, respectively. The ratios are unitless since all steroids are reported in ng/dL.

Molecular analysis

All 21OHD patients, carriers and WT have been previously genotyped and the results will be reported elsewhere.

Statistical analysis

The "R Project of Statistical Computing" version 2.8.1 (hosted by Department of Statistics and Mathematics, Vienna University, Austria -www.r-project.org) was used for calculations. The nonparametric multiple comparison test of Kruskal-Wallis, was used for comparisons between groups. Receiver operator characteristics (ROC) curves were used to define cutoff points for ACTH-stimulated 21DF and 17OHP over S, DOC and B ratios in order to separate 21OHD carriers, NC, and WT. For statistical purposes all values below the quantification limit for any of the steroids were arbitrarily considered equal to the quantification level divided by the square root of 2 (therefore defining an average number for all undetectable values). The level of statistical significance was set up at 5% ($p<0.05$).

Results

Table 1 depicts the mean±SE and the median plus the interquartile interval (IQI) of serum S, DOC and B for classic (SW and SV) and NC 21OHD patients, carriers and WT.

11-Deoxycortisol

Basal S levels were reduced and ACTH-unresponsive in SW 21OHD; both values were significantly lower than in the other groups. Although higher than in SW, basal S levels were also ACTH-unresponsive in SV patients. Except for higher basal values in NC patients, basal and post-ACTH S levels were similar among NC patients, carriers and WT.

11-Deoxycorticosterone

Basal DOC levels were higher in SW patients than in all the other groups (but significantly only against carriers). However, DOC responses to ACTH stimulation were abolished in both SW and SV patients and significantly impaired in NC and carriers, as compared to WT.

Corticosterone

Compared to WT, basal B levels were slightly reduced in all other groups (but significantly only for carriers). However, B responses to ACTH were virtually abolished in classic and reduced in NC patients and carriers, as compared to WT.

The significant overlap (75% to >90%) observed for the individual basal and post-ACTH levels of S, DOC, and B among 21OHD NC, carriers and WT precluded discrimination between these groups.

Ratios of 17OHP and 21DF over S, DOC, and B (figures 1 and 2)

Because basal levels of 17OHP and 21DF are already markedly increased in classic patients with 21OHD, the precursor-to-product ratios are of little additional value for diagnostic purposes. Therefore, these ratios (in particular the ACTH-stimulated ones) were applied mainly to differentiate 21OHD carriers from WT and NC patients from carriers.

Separating 21OHD carriers from WT

Table 2 shows the cutoff points, areas under the curve (AUC), sensitivity, specificity, and overlap for the 17OHP and 21DF over S, DOC, and B ratios, for carriers *versus* WT. For 100% specificity, the sensitivities of the 17OHP ratios were: 61% (for a cutoff ratio of 2.67), 16% (cutoff ratio of 37.2), and 35% (cutoff ratio of 0.24), respectively, whereas those of the 21DF ratios were 76% (cutoff ratio of 0.44), 79% (cutoff ratio of 24.4), 92% (cutoff ratio of 1.53), respectively. Overall the ratios that employed the 21DF as the numerator offered a better discrimination, and among them, the 21DF/B ratio was the best to separate carriers from WT, with 91.9% sensitivity and 100% specificity.

Separating NC patients from carriers

Table 2 also shows the cutoff points, areas under the curve (AUC), sensitivity, specificity, and overlap for the 17OHP and 21DF over S, DOC, and B ratios, for NC patients *versus* carriers. As anticipated, the separation of NC patients from carriers is simpler, and although all gave a good performance, the 21DF/B and the 17OHP/DOC ratios had both 100% sensitivity and specificity.

Discussion

Different from precursor products, steroids distal to the enzymatic block are seldom investigated in congenital adrenal hyperplasia. They may, however, be additional diagnostic tools by themselves or when analyzed in combination with the respective precursor steroid, as precursor-to-product ratios. In 21OHD, it is expected that synthesis of B, DOC, S, and F are all impaired in the adrenal ZF due to the enzymatic blockade, which in turn results in increased production of 17OHP and 21DF. In this paper we employed LC-MS/MS to examine basal and post-ACTH serum levels of S, DOC, and B, and their quotient (ratio) under 17OHP and 21DF levels in classic and NC patients with 21OHD, carriers and normal subjects (WT for the disease) in an attempt to further improve the discrimination of these groups.

Aside from the classic SW patients, all other groups are unremarkable regarding basal levels of S and B that were not significantly different from WT. However, their responses to additional ACTH stimulation were invariably blunted in SW and SV patients, and otherwise normal to slightly impaired in NC and carriers. The normal to slightly increased basal values of S in SV and NC, and especially DOC in SW and SV, could be justified in several ways: (a) as a result from reduced efficiency of 11β-hydroxylation due to increased androgen levels acting as pseudosubstrates, as previously suggested [15,16]; (b) due to residual 21-hydroxylase activity in the ZF biosynthetic pathways, associated to milder *CYP21* gene mutations; (c) due to extraadrenal 21-hydroxylation of progesterone [17-19].

Teleologically, the increased levels of DOC observed in the SW patient is a metabolic effort to conserve salt in the newborn period, a mechanism that is usually inefficient; interestingly, several of these patients lead a normal adult life without the need of mineralocorticoid replacement [20]. Past studies have suggested the adrenal ZG as the origin of DOC in classic 21OHD since its production is renin-angiotensin responsive (increase in response to upright posture and suppress following salt loading), in contrast to its normal ACTH-dependence in the ZF [6,21-23]. Later on, several tissues were shown to perform *CYP21*- independent 21-hydroxylation [20,24,25], and recently it has been demonstrated that hepatic CYP2C19 and CYP3A4 are able to convert progesterone to DOC, although less efficiently (10-17%) than 21-hydroxylase [26,27].

Our results demonstrate that unlike S and DOC, B unveils a spectrum of ACTH responses in the study groups, from slight impairment in carriers throughout virtual obliteration in SW patients (table 1), indicating the progressive damage to 21-hydroxylation in the ZF 17-deoxypathway [5,21]. Overall, the basal and post-ACTH levels of the steroids distal to the 21-hydroxylase block are not sufficient per se to be used as additional diagnostic markers of the

disease, mostly because of their significant overlaps. In this respect, B seems the best distal-to-the block steroid to identify 21OHD subgroups.

Because S, DOC, and B could not precisely characterize 21OHD subgroups, we used precursor-to-product ratios in order to improve diagnostic accuracy, particularly for distinguishing carriers from WT and NC patients from carriers.

21DF is an 11β-hydroxy-derivative of 17OHP that was shown to be an important additional marker of 21OHD, both for patients and carriers [(3,28)]. Thus, we contemplate that precursor-to-product ratios employing 21DF instead of 17OHP could be of better diagnostic value. Our data demonstrate that the post-ACTH 21DF ratios (particularly 21DF/B) surpass those using 17OHP in order to detect carriers among genotypically normal subjects (WT), confirming previous results that ACTH-stimulated 21DF is superior to 17OHP in differentiating these groups [(28-30)].

Also, *in vitro* studies indicate that the 17-hydroxylation of progesterone proceeds three times faster than its 21-hydroxylation, providing more substrate to the 17-hydroxy- than to the 17-deoxy-pathway of ZF [(31)]. Therefore, the 21DF/DOC and 21DF/B ratios would be more robust diagnostic tools than 21DF/S to separate 21OHD subgroups.

Although previous studies have shown a clear-cut separation of carriers from "normal controls" using post-ACTH 17OHP/DOC ratio [(13)], our data disclose an important overlap of 84% between carriers and WT. In a similar study, Pardini et al detected 94% of 21OHD carriers using the ratio of 17OHP to a more distal-to-the block product, 18-hydroxydeoxycorticosterone (18OHDOC) [(12)]. However, the availability to measure this steroid is limited. But since 18-hydroxylation and 11β-hydroxylation of DOC are performed by a single enzyme - *CYP11B1* -, B would be equivalent to 18OHDOC for this purpose; yet, our data show that the 17OHP/B ratio was as weak as 17OHP/DOC (65% and 84% overlap, respectively), even when outliers are excluded (remaining 24% and 26% overlap, respectively).

Another diagnostic dilemma is the distinction of NC patients from 21OHD carriers, especially in the male. However, the higher basal and post-ACTH levels of 17OHP and 21DF appear sufficient to characterize them [(1-3)]. Nevertheless, values of post-ACTH precursor-to-product ratios (in especial the 21DF/B ratio) are unambiguous in identifying all NC patients from carriers. This may be particularly useful in individuals with mild increases in 17OHP levels [(8-11)].

In summary, the ZF 21-hydroxysteroids S, DOC, and B disclose an interesting profile in the 21OHD subtypes ranging from reduced to increased basal levels but with a systematic impaired response to ACTH stimulation. The increased basal levels of DOC and S may result from (a) inefficient 11β-hydroxylation due to androgen excess, (b) residual 21-hydroxylase activity due to specific *CYP21* gene mutations, and/or (c) extraadrenal 21-hydroxylation of progesterone by

hepatic CYP enzymes. The selective gradual response of B to ACTH and the precursor-to-product ratios, particularly 21DF/B, are distinctive characteristics of 21OHD subtypes that can be used to assist in the separation of carriers from normal subjects and of NC from carriers.

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Table 1. Basal and post-ACTH values of S, DOC, and B (mean±SE, and median plus interquartile interval) in classic (SW and SV) and nonclassic 21OHD patients, carriers and wild-types (WT) for the disease.

	S (ng/dL)		**DOC (ng/dL)**		**B (ng/dl)**	
	Basal	**pACTH**	**Basal**	**pACTH**	**Basal**	**pACTH**
SW 21OHD	12.8±1.5*	13.2±1.7[£]	17.1±2.5**	17.8±4.2[&]	180±56	203±58[†]
(n= 9)	10.6 [10.6-10.6]	10.6 [10.6-10.6]	14.6 [10.5-23.0]	12.6 [10.5-22.0]	104 [67-185]	146 [107-176]
SV 21OHD	47.8±8.2	46.4±7.5[†]	14.7±2.0	13.7±2.5[&]	267±127	454±188†
(n= 5)	48.5 [34.1-63.1]	43.9 [34.8-46.0]	13.7 [12.0-14.0]	11.7 [11.6-16.0]	97 [67-390]	316 [157-624]
NC 21OHD	67.2±8.2**	125.6±15.9	10.4±1.4	19.4±4.2[&]	175±38	1,214±426[&]
(n= 12)	71.5 [39.2-84.2]	117.0 [86.9-144.3]	7.1 [7.1-12.5]	14.5 [7.1-27.0]	142 [67-211]	753 [530-1,174]
Carriers	30.4±4.0	127.5±7.6	9.0±0.4	19.6±1.4[&]	225±37[&]	1,893±84[&]
(n= 62)	18.0 [10.6-34.9]	112.5 [83.7-156.5]	7.1 [7.1-10.4]	17.1 [12.1-25.1]	105 [67-233]	1,756 [1,420-2,372]
WT	33.1±4.6	141.6±16.5	11.1±0.8	31.2±2.9	336±73	3,247±274
(n= 26)	25.9 [19.3-37.8]	116.2 [90.2-163.8]	11.2 [7.1-13.7]	29.0 [18.7-41.3]	226 [137-377]	3,153 [2,283-3,821]

*$p<0.05$ *vs* SV, NC, carriers, and WT; [£]$p<0.05$ vs NC, carriers, and WT; †$p<0.05$ *vs* carriers, and WT; **$p<0.05$ *vs* carriers; [&]$p<0.05$ vs WT

Table 2. Cutoff points, areas-under-the-curves (AUC), sensitivities, specificities, and overlaps of the post-ACTH precursor (17OHP and 21DF)-to-product (S, DOC, and B) ratios between carriers versus wild-types (WT) and between nonclassic (NC) patients and carriers for 21OHD.

	17OHP/S	**17OHP/DOC**	**17OHP/B (x10)**	**21DF/S**	**21DF/DOC**	**21DF/B (x100)**
Carriers *vs* WT						
Cutoff value	2.0	9.67	0.96	0.25	1.47	1.53
AUC	0.937	0.935	0.911	0.927	0.951	0.975
Sensitivity	82.3%	83.9%	80.6%	85.5%	85.5%	91.9%
Specificity	96.2%	92.3%	92.3%	88.5%	92.3%	100%
Overlap	39%	84%*	65%*	24%	21%	8%
NC *vs* Carriers						
Cutoff value	11.2	75	10.5	1.21	23.2	30
AUC	0.999	1.000	0.999	0.983	0.999	1.000
Sensitivity	100%	100%	100%	100%	100%	100%
Specificity	98.4%	100%	98.4%	82.3%	98.4%	100%
Overlap	2%	0%	2%	5%	2%	0%

* Overlap drops to 26% and 24%, respectively, after removing one outlier from each analysis.

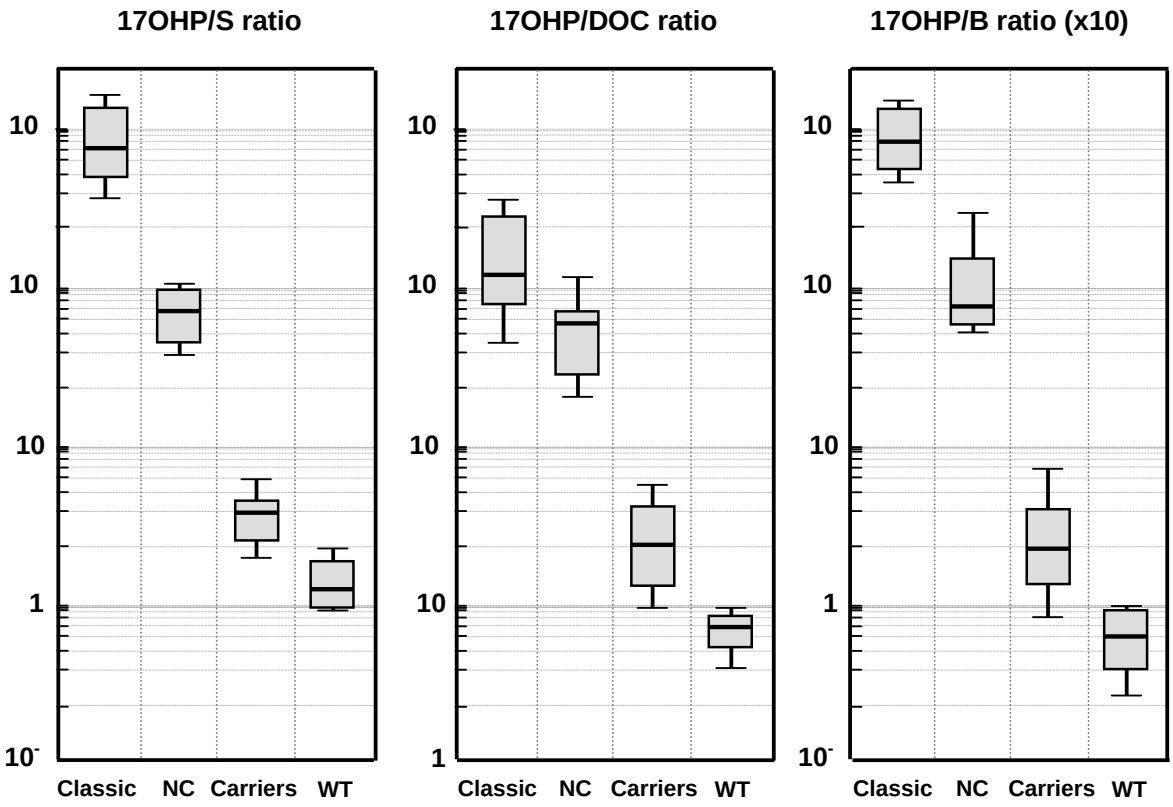

Figure 1: The box-plots represent post-ACTH 17OHP over S, DOC, and B ratios in 21OHD subtypes: classic and nonclassic (NC) patients, carriers and WT. Bars across the box represent the median; the edges of the box, the 25th and the 75th percentiles (P25 and P75) and the error bars, the 10th and 90th percentiles (P10 and P90), respectively.

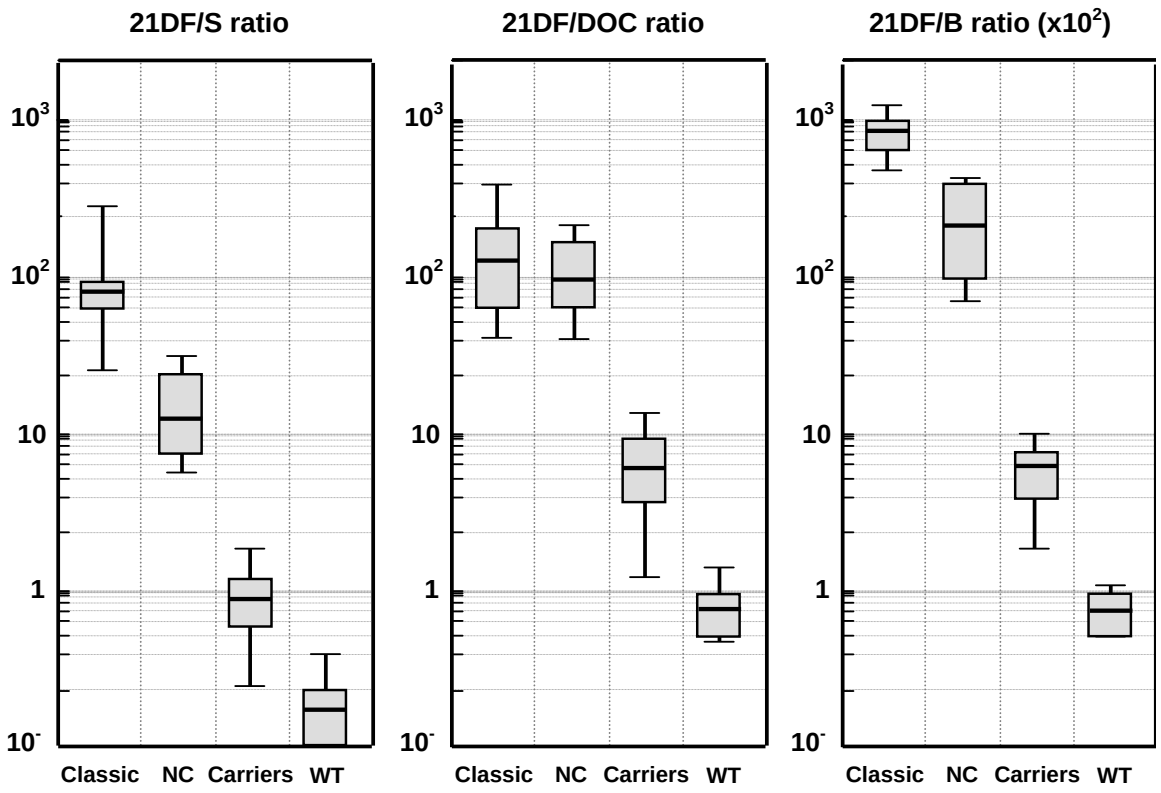

Figure 2: The box-plots represent post-ACTH 21DF over S, DOC, and B ratios in 21OHD subtypes: classic and nonclassic (NC) patients, carriers and WT. Bars across the box represent the median; the edges of the box, the 25th and the 75th percentiles (P25 and P75) and the error bars, the 10th and 90th percentiles (P10 and P90), respectively.

5. Principais Achados, Conclusões e Novas Direções

Principais Achados

- Os dados desse conjunto de estudos confirmaram que o 21DF após estímulo pelo ACTH é superior à 17OHP na identificação de heterozigotos para a D21OH entre uma população de indivíduos normais. Para uma especificidade de 100%, níveis de 21DF após ACTH maiores do que 40 ng/dL mostraram sensibilidade de 82% na detecção de heterozigotos para a D21OH, enquanto que níveis de 17OHP após ACTH maiores do que 298 ng/dL tiveram sensibilidade significativamente menor (53%). Por outro lado, níveis de 21DF após ACTH maiores do que 300 ng/dL - em conjunto com 17OHP após ACTH maiores do que 1.700 ng/dL - permitiram melhor discriminação de pacientes com a forma NC da 21OH dentre os heterozigotos para a doença.

- No estudo de frequência de mutações do gene da CYP21, foram identificadas mutações em 85 (96,6%) dos 88 alelos não relacionados estudados. Os únicos três alelos não identificados eram de pacientes com a forma NC (cujos níveis de 17OHP e 21DF basais e estimulados eram característicos de D21OH).

- Diferentemente da 17OHP, os níveis de 21DF após ACTH não se correlacionaram com os diferentes grupos genotípicos nos pacientes com a D21OH. Nesta situação, a ausência de correlação pode ser devida à pequena população de pacientes estudados e à dosagem dos esteróides após curto período de suspensão do tratamento clínico.

- Dentre os esteróides da zona fasciculada distais ao bloqueio da 21-hidroxilase - S, DOC e B -, o melhor marcador na caracterização dos diferentes subtipos da D21OH foi B após ACTH, apesar de sobreposição importante entre essas populações.

- Níveis normais ou pouco elevados de DOC nos pacientes com a forma perdedora de sal podem denotar produção extra-adrenal desse esteróide.

- Dentre as relações envolvendo precursores (17OHP e 21DF) e produtos (S, DOC, B) da 21-hidroxilase, aquelas que envolviam o 21DF (sobretudo 21DF/B após ACTH), foram superiores à 17OHP na diferenciação de heterozigotos e indivíduos genotipicamente normais. Embora pacientes com a forma NC já tivessem sido identificados pela 17OHP, as relações 21DF/B e 17OHP/DOC após estímulo com ACTH separaram 100% desses pacientes dos heterozigotos.

Conclusões e Novas direções

- Com o uso da metodologia de LC-MS/MS, a dosagem do 21DF após ACTH confirmou-se superior na detecção de heterozigotos para a D21OH, podendo ser instrumento diagnóstico valioso durante o aconselhamento genético de familiares de pacientes afetados com esta condição. Sua dosagem também pode ser útil nos casos em que persiste dúvida diagnóstica na caracterização de formas NC entre heterozigotos. Além disso, a análise dos esteróides adrenais distais ao bloqueio da 21-hidroxilase apresenta-se como ferramenta adicional na propedêutica diagnóstica dos diversos subtipos de D21OH.

- A definição de valores de corte mais acurados para o 21DF, especialmente após estímulo pelo ACTH, poderá ser útil na identificação de indivíduos com risco de heterozigose (e mesmo indivíduos portadores da forma não clássica assintomáticos) durante investigação de familiares para D21OH e, eventualmente, na população geral.

6. *Anexos*

Anexo 1. Características clínicas, genotípicas e níveis séricos basais e após-ACTH dos vários esteróides estudados em carreadores da D21OH.

No.	Sexo	Idade	Família	Mutação no *CYP21A2*	Grupo mutacional	**Cortisol** ug/dL		**17OHP** ng/dL		**21-DF** ng/dL		**S** ng/dL		**DOC** ng/dL		**B** ng/dL	
						basal	**pACTH**	**basal**	**pACTH**	**basal**	**pACTH**	**basal**	**pACTH**	**basal**	**pACTH**	**basal**	**pACTH**
1	F	23	1	R356W	A	8,3	18,5	40,0	109,0	17,0	40,0	10,6	86,0	7,1	11,0	67,4	1400,0
2	M	25	1	R356W	A	5,2	20,3	77,3	225,2	17,0	28,9	10,6	130,2	10,4	30,7	67,4	1696,5
3	F	34	2	Q318X	A	8,9	22,1	33,8	154,7	17,0	17,0	10,6	91,4	14,3	22,3	161,6	2334,9
4	M	42	2	Q318X	A	6,8	24,5	108,5	650,0	17,0	35,9	18,8	214,8	14,5	17,2	67,4	969,6
5	M	48	3	R356W	A	4,9	17,0	55,4	277,8	17,0	55,8	21,0	79,9	12,9	20,0	67,4	1769,6
6	F	42	3	I2 splice, V281L	A	10,2	30,4	42,2	330,7	17,0	100,3	10,6	121,1	10,2	29,5	67,4	1706,2
7	F	41	4	Q318X	A	12,9	21,5	77,0	412,0	17,0	29,0	114,0	326,0	7,1	46,0	333,0	1550,0
8	M	36	4	Q318X	A	9,6	25,0	108,0	479,0	17,0	82,0	10,6	158,0	7,1	7,1	67,4	1402,0
9	M	28	5	InsT	A	9,2	25,6	110,8	296,1	17,0	111,1	10,6	92,4	10,3	25,3	153,2	2845,1
10	M	36	6	I2 splice	A	15,3	25,0	111,9	272,1	17,0	43,9	36,0	93,1	14,5	26,7	347,0	2810,4
11*	F	27	6	I2splice	A	22,8	37,9	51,3	960,7	17,0	412,3	15,8	70,1	10,2	15,9	177,9	1410,5
12	F	52	7	I2 splice	A	7,5	25,0	60,7	208,1	17,0	118,8	10,6	73,3	11,4	16,0	100,9	2324,5
13	M	52	7	1003^1004 ins A	A	16,5	33,0	209,7	446,5	17,0	104,3	78,6	146,9	17,8	27,5	700,2	2384,0
14	M	34	8	I2 splice	A	18,0	23,7	234,0	355,0	76,0	236,2	76,6	110,0	7,1	12,8	952,0	2594,0
15	F	25	8	R356W	A	4,8	19,9	27,6	358,7	17,0	140,1	10,6	100,6	7,1	12,3	67,4	2272,5
16	M	53	10	R408C	A	12,1	27,4	91,5	451,4	17,0	89,4	31,7	122,8	7,1	23,2	226,3	1677,7
17	M	50	11	cluster	A	6,2	22,5	62,0	307,0	17,0	81,0	10,6	128,0	7,1	18,0	67,4	1765,0
18	M	39	12	I 2 splice	A	19,3	25,8	134,3	213,1	48,8	88,3	56,9	97,9	7,1	7,1	612,2	1379,6
19*	F	27	12	I 2 splice	A	5,9	21,1	50,6	492,9	17,0	170,5	20,9	110,1	7,1	11,3	67,4	2393,8
20*	F	33	13	Del 21A2	A	11,8	32,7	20,0	243,0	17,0	187,5	10,6	82,0	7,1	22,0	67,4	3424,0
21	M	38	13	R356W	A	6,4	25,2	159,0	561,3	17,0	214,0	10,6	172,4	7,1	30,0	67,4	2539,0
22	M	31	14	Grande conversão	A	9,9	23,6	29,1	152,1	17,0	75,4	21,6	68,5	7,1	7,1	187,0	1785,4
23	F	24	14	Grande conversão	A	10,8	29,4	28,1	188,2	17,0	95,0	21,4	72,2	7,1	7,1	99,7	1167,6
24	M	57	15	R356W	A	13,3	35,5	83,9	780,2	17,0	73,8	10,6	249,0	15,5	38,7	113,5	3002,0
25	F	41	16	R356W,Q318X	A	5,9	13,5	37,0	298,0	17,0	54,0	22,0	111,0	7,1	11,0	67,4	908,0
26	M	52	16	Cluster	A	10,4	25,4	249,0	863,0	17,0	138,0	28,0	192,0	7,1	28,0	121,0	2565,0
27	M	43	17	Del 21A2	A	13,7	19,9	146,1	152,9	17,0	17,0	68,5	82,9	18,1	23,5	549,6	1657,7
28	F	33	17	Del 21A2	A	9,9	24,6	23,3	210,2	17,0	77,8	22,1	141,7	7,1	7,1	97,3	1236,7
29	F	33	18	I2 splice	A	4,9	19,7	20,0	203,4	17,0	151,3	10,6	47,1	7,1	7,1	67,4	1084,4
30[&]	F	25	19	IVS2+ 5G >A, V281L	A	13,7	18,8	134,0	279,0	17,0	17,0	125,0	228,0	11,0	30,0	299,0	900,0
31	M	47	20	I2 splice	A	15,4	31,7	137,7	663,8	17,0	128,4	20,8	156,7	16,8	20,1	239,5	2074,6

32	F	52	20	Del 21A2	A	7,6	31,1	25,2	1289,5	17,0	266,9	10,6	262,0	7,1	31,9	67,4	1771,1
33	F	47	21	R356W	A	8,0	29,5	58,0	545,9	17,0	37,9	10,6	229,3	15,4	36,9	67,4	1632,6
34	F	47	22	Ins T, R356 W, Q318X	A	9,2	29,0	23,0	320,0	17,0	36,0	24,0	156,0	7,1	13,0	67,4	1466,0
35	M	47	22	I 2 splice	A	7,9	26,8	64,0	388,0	17,0	72,0	10,6	139,0	7,1	11,0	67,4	1675,0
36	F	47	23	R356W	A	20,2	23,0	149,0	227,0	17,0	73,0	69,0	99,0	7,1	7,1	941,0	1405,0
37	M	54	23	R356W	A	11,4	22,5	130,0	299,0	17,0	70,0	17,0	103,0	7,1	7,1	176,0	2243,0
38	M	39	24	Del 21A2	A	10,6	14,1	444,9	1347,4	17,0	91,0	170,0	257,8	7,1	24,6	214,6	865,1
39	M	29	25	Cluster	A	14,0	20,2	88,0	115,0	46,0	50,0	46,0	63,0	7,1	13,0	881,0	2274,0
40	F	28	26	I2 splice	A	7,4	22,0	22,3	120,3	17,0	41,0	10,6	53,4	13,1	13,3	67,4	1449,1
41	F	41	27	Grande conversão	A	3,0	15,7	32,0	257,0	17,0	83,0	10,6	75,0	7,1	11,0	67,4	1115,0
42	M	39	27	Grande conversão	A	14,6	34,4	88,0	1256,0	17,0	217,0	52,0	226,0	7,1	63,0	234,0	3746,0
43	M	62	28	I 2 splice, InsT	A	13,5	27,9	119,4	378,5	29,2	120,3	20,0	126,8	7,1	30,7	319,7	2540,0
44	M	26	31	I 2 splice	A	8,3	22,8	116,0	220,0	17,0	62,0	16,0	66,0	7,1	7,1	67,4	1528,0
45	F	43	cont	Q318X	A	7,0	18,3	20,6	59,5	17,0	17,0	10,6	47,6	7,1	13,5	108,7	2446,5
46	F	25	31	I 2 splice	A	8,2	25,2	36,0	320,0	17,0	60,0	18,0	163,0	7,1	24,0	110,0	1743,0
47	F	49	10	I172N	B	10,6	21,0	87,5	498,7	17,0	91,1	15,3	115,8	11,6	32,4	167,7	2317,3
48	F	45	11	I172N	B	15,8	26,4	65,0	555,0	17,0	100,0	50,0	144,0	7,1	22,0	284,0	1774,0
49	M	33	18	I172N, V281L	B	13,0	17,1	121,0	239,1	36,7	65,4	54,8	113,9	7,1	7,1	365,8	1089,0
50	F	35	24	I172N	B	5,1	23,8	27,6	437,0	17,0	107,0	10,6	116,2	7,1	13,7	67,4	1730,8
51	F	32	5	I172N,V304X	B	8,2	20,9	24,3	222,6	17,0	63,5	10,6	74,7	10,7	25,1	67,4	1655,2
52	F	62	28	I172N	B	10,2	23,8	86,9	1102,9	17,0	182,8	42,1	174,4	7,1	15,4	145,5	1207,3
53	F	29	9	V281L	C	10,9	26,1	37,0	175,0	17,0	60,0	21,0	81,0	7,1	13,0	148,0	1783,0
54	F	41	30	V281L	C	24,0	28,0	167,0	214,0	60,0	104,0	85,0	95,0	7,1	16,0	1625,0	2997,0
55	M	41	9	V281L	C	5,9	24,8	87,0	276,0	17,0	40,0	27,0	135,0	7,1	17,0	67,4	1648,0
56	M	45	30	V281L	C	15,7	29,0	256,0	342,0	41,0	144,0	48,0	87,0	7,1	19,0	663,0	2675,0
57	F	39	32	V281L	C	7,5	21,5	47,0	554,0	55,0	156,0	18,0	154,0	7,1	25,0	67,4	1747,0
58	M	42	32	V281L	C	6,2	22,0	78,0	1044,0	47,0	184,0	17,0	169,0	7,1	24,0	67,4	1767,0
59	F	32	_	V281L	C	7,1	17,1	23,0	253,0	17,0	75,0	16,0	89,0	7,1	12,0	67,4	827,0
60	F	25	cont	V281L	C	7,8	25,8	28,3	163,7	17,0	17,0	10,6	102,9	7,1	16,7	67,4	2726,1
61	F	34	cont	V281L	C	12,9	23,2	25,3	143,4	17,0	17,0	16,6	66,4	12,9	17,0	229,6	2452,4
62	F	36	29	V281L	C	6,4	26,8	43,4	1704,4	17,0	295,9	10,6	166,7	7,1	23,1	67,4	2054,9

* Uso de anticoncepcional; [&] Uso de glicocorticóide;
cont- indivíduos que foram transferidos do grupo controle após genotipagem *CYP21 A2*
_ mãe de paciente NC de outro serviço

Anexo 2. Características clínicas, genotípicas e níveis séricos basais e após-ACTH dos vários esteróides estudados em indivíduos controle (WT para D21OH).

No.	Sexo	Idade	Família	Genótipo *CYP21A2*	**Cortisol** ug/dL		**17OHP** ng/dL		**21-DF** ng/dL		**S** ng/dL		**DOC** ng/dL		**B** ng/dL	
					basal	**pACTH**	**basal**	**pACTH**	**basal**	**pACTH**	**basal**	**pACTH**	**basal**	**pACTH**	**basal**	**pACTH**
1	F	24	1	nl/nl	11,2	20,8	31,0	63,2	17,0	17,0	29,0	71,4	14,0	34,4	394,0	2768,4
2	F	25	1	nl/nl	6,6	17,7	10,6	80,0	17,0	17,0	17,7	90,3	7,1	17,6	186,1	2520,8
3	F	23	2	nl/nl	17,4	25,5	41,2	85,4	17,0	17,0	24,0	74,3	12,1	15,7	566,2	3651,1
4	F	24	2	nl/nl	5,4	26,1	25,0	92,2	17,0	17,0	10,6	106,9	7,1	25,3	130,6	4727,8
5	F	28	3	nl/nl	14,6	25,2	42,6	105,2	17,0	17,0	32,7	88,8	14,2	21,2	580,0	4002,8
6	F	29	3	nl/nl	16,3	24,4	58,2	107,4	17,0	17,0	33,0	40,2	12,3	17,8	902,4	3072,1
7	F	48	4	nl/nl	14,5	22,2	54,0	132,0	17,0	17,0	107,0	164,0	7,1	44,0	1381,0	4755,0
8	M	39	4	nl/nl	7,2	22,8	95,0	139,0	17,0	17,0	10,6	91,0	7,1	17,0	155,8	1891,5
9	F	49	5	nl/nl	6,4	31,7	34,5	144,4	17,0	17,0	10,6	98,8	11,0	42,2	97,8	4999,2
10	M	65	6	nl/nl	12,1	27,7	75,3	151,8	17,0	17,0	25,7	148,7	17,2	33,6	324,0	3877,5
11*	F	27	6	nl/nl	13,0	30,9	22,0	153,7	17,0	29,5	10,6	78,2	7,1	16,1	255,7	3105,9
12	F	42	7	nl/nl	7,0	23,5	10,6	157,0	17,0	17,0	18,0	153,0	7,1	13,0	114,0	1893,0
13	M	40	7	nl/nl	5,7	18,7	93,3	161,7	17,0	17,0	35,1	465,5	7,1	65,4	67,4	1309,3
14	M	23	8	nl/nl	11,2	25,0	92,4	163,7	17,0	17,0	25,5	86,8	11,2	28,2	154,9	2203,8
15	F	39	8	nl/nl	10,8	30,4	30,0	167,0	17,0	17,0	23,0	125,0	7,1	23,0	210,9	3200,2
16	F	45	10	nl/nl	13,5	21,3	55,5	170,5	17,0	17,0	43,2	143,9	12,7	29,8	427,0	1786,2
17	F	36	11	nl/nl	9,3	30,1	18,7	170,5	17,0	17,0	10,6	90,1	12,1	25,8	105,6	3583,6
18	F	42	12	nl/nl	7,4	26,2	16,9	183,0	17,0	17,0	23,7	188,1	12,2	60,5	204,8	4023,9
19*	M	31	12	nl/nl	10,6	26,0	88,0	185,0	17,0	39,0	26,0	106,0	7,1	30,0	274,0	3235,0
20*	M	30	13	nl/nl	13,1	20,1	86,7	186,1	17,0	17,0	74,4	193,0	17,8	24,0	257,4	2118,6
21	M	35	13	nl/nl	8,6	20,3	88,9	194,3	17,0	17,0	38,7	107,4	18,0	47,1	241,9	7893,4
22	M	34	14	nl/nl	7,7	21,8	71,4	197,1	17,0	17,0	41,3	233,9	11,2	46,6	319,4	3528,5
23	M	41	14	nl/nl	15,1	20,2	137,0	220,4	17,0	17,0	88,3	163,1	18,1	38,6	1015,5	2534,0
24	M	59	15	nl/nl	7,0	16,3	175,0	260,0	17,0	17,0	34,0	137,0	7,1	7,1	108,0	1130,0
25	M	39	16	nl/nl	6,9	20,5	90,0	268,0	17,0	17,0	43,0	189,0	7,1	36,0	183,0	3007,0
26	F	43	16	nl/nl	8,4	30,7	31,5	296,4	17,0	17,0	24,6	247,7	15,2	50,0	67,4	3602,5
27	F	38	17	IVS2-4???	15,8	29,2	52,8	151,3	17,0	17,0	25,4	113,8	10,3	46,6	733,2	5555,7

* Em aguardo do estudo funcional, excluída da análise estatística

Anexo 3. Características clínicas, genotípicas e níveis séricos basais e após-ACTH dos vários esteróides estudados em pacientes com a forma clássica da D21OH.

No.	Sexo	Idade	Família	Mutação no *CYP21A2*	Grupo mutacional	Forma clínica	**Cortisol** ug/dL		**17OHP** ng/dL		**21-DF** ng/dL		**S** ng/dL		**DOC** ng/dL		**B** ng/dL		Artigo
							basal	pACTH	basal	pACTH	basal	pACTH	basal	pACTH	basal	pACTH	basal	pACTH	
1	F	14	22	I2 splice/ R356W, Q318X, InsT	A/A	PS	0,3	0,3	2.861	2.431	211	208	10,6	10,6	23,0	22,0	67,4	67,4	1,2,3
2	F	4	12	I2 splice / I2 splice	A/A	PS	0,3	0,3	3.849	4.749	443	555	10,6	10,6	24,4	12,5	67,4	67,4	1,2,3
3	F	8	is	I2 splice / I2 splice	A/A	PS	0,3	0,3	6.389	7.373	1.366	1.698	10,6	10,6	7,1	7,1	135,0	163,6	1,2,3
4	M	7	19	I2 splice/ IVS 2 + 5 G> A, V281L	A/A	PS	0,3	0,5	17.823	13.521	556	815	10,6	10,6	14,6	10,5	103,6	106,8	1,2,3
5	M	21	20	I2splice/Del	A/A	PS	0,6	0,5	16.003	15.166	922	1.149	21,4	22,2	13,7	12,6	67,4	124,4	1,2,3
6	M	18	is	I2 splice /C168 delΔ1+I4nt	A/A	PS	0,8	0,9	15.535	15.197	3.015	2.904	19,6	10,6	28,0	47,0	185,0	146,0	1,2,3
7	F	16	is	I2 splice/ F306+1nt	A/A	PS	0,3	0,4	15.786	16.984	412	682	10,6	10,6	23,0	26,0	104,0	176,0	1,2,3
8	F	5	25	cluster/conv	A/A	PS	0,8	0,7	32.448	40.090	7.079	6.326	10,6	10,6	10,5	12,8	574,4	586,7	1,2,3
9	M	1	31	I2 Splice/I2 Splice	A/A	PS	0,2	0,2	21.861	26.279	1.901	1.498	10,6	22,0	10,0	10,0	312,0	389,0	1,2,3
10	F	23	28	I 2 splice, InsT/I172N	A/B	VS	2,7	2,9	14.135	11.106	3.649	2.881	68,0	46,0	14,0	22,0	390,0	624,0	1,2,3
11	F	16	Is	I172N/ I172N	B/B	VS	1,4	1,7	14.472	18.161	407	1.015	25,2	43,9	11,3	7,1	67,4	156,9	1,2,3
12	M	13	Is	I172N/ I172N	B/B	VS	4,4	3,3	6.740	12.561	689	1.954	48,5	32,5	12,0	11,6	96,8	316,3	1,2,3
13	F	10	13	R356W/Del	A/A	PS	0,7	-	17.931	-	864	-	10,6	-	7,1	-	152,1	-	1,2
14	M	23	21	I2 splice/ R356W	A/A	PS	1,1	-	10.069	-	2.381	-	28,0	-	12,9	-	241,9	-	1,2
15	F	9	27	Conv/Conv	A/A	PS	0,3	-	14.780	-	1.528	-	29,0	-	10,0	-	203,0	-	1,2
16	F	16	3	R356W/I2 splice , V281L	A/A	PS	0,3	-	1.702	-	296	-	10,6	-	7,1	-	67,4	-	1,2
17	F	8	6	I2 splice/I2 splice	A/A	PS	-	-	4.981	10.093	255	671	30,0	104,0	-	-	-	-	1,2
18	F	4	26	I2 splice/Q318X	A/A	PS	-	-	5.425	16.701	190	458	81,0	139,0	-	-	-	-	1,2
19	M	10	14	conv/conv	A/A	PS	-	-	19.913	19.389	1.312	937	84,0	108,0	-	-	-	-	1,2
20	F	7	24	I172N/ Del	A/B	VS	-	-	816	1.185	154	362	32,0	73,0	-	-	-	-	1,2
21	F	8	5	I172N,V304X/InsT	A/B	VS	-	-	10.412	15.692	250	435	59,0	66,0	-	-	-	-	1,2
22	M	6	18	I2splice/ I172N,V281L	A/B	VS	-	-	7.242	6.627	156	143	49,0	45,0	-	-	-	-	1,2
*23	F	16	-	I172N/-	B/?	VS	2,1	2,8	34.911	39.424	4.051	5.123	63,1	74,7	22,6	16,0	713,0	1.103,0	1,3
24$	F	17	-	-		VS	1,1	1,2	7.842	8.790	323	512	34,1	34,8	13,7	11,7	67,4	67,4	1,3
25	F	16	-	-	-	PS	0,5	1,0	11.586	9.248	482	342	10,6	15,0	19,0	18,2	67,4	67,4	1
26	F	8	-	-	-	PS	2,3	-	24.420	-	5.102	-	18,4	-	7,1	-	708,1	-	1
27	F	4	-	-	-	PS	2,6	-	22.266	-	949	-	66,9	-	14,4	-	213,9	-	1

28	M	18	-	-	-	PS	0,3	-	7.143	-	2.913	-	21,0	-	7,1	-	342,5	-	1
29	F	16	-	-	-	PS	2,1	-	20.229	-	925	-	60,3	-	23,3	-	334,1	-	1
30	F	16	-	-	-	PS	0,9	-	18.466	-	601	-	33,1	-	7,1	-	67,4	-	1
31	F	11	-	-	-	PS	-	-	14.504	23.438	190	192	38,0	41,0	-	-	-	-	1
32	F	11	2	Q318X/Q318X	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
33	F	8	4	Q318X/Q318X	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
34	F	16	16	R356W,Q318X/cluster	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
35	F	9	1	R356W/ R356W	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
36	F	2	15	R356W/R356W	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
37	F	20	23	R356W/R356W	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
38	F	22	7	I2 splice/ 1003^1004 ins A	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
39	F	3	17	Del/ Del	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
40	F	3	8	I2 splice/ R356W	A/A	PS	-	-	-	-	-	-	-	-	-	-	-	-	2
41	F	9	11	I172N/ Cluster	A/B	VS	-	-	-	-	-	-	-	-	-	-	-	-	2
42	F	21	10	I172N /R408C	A/B	VS	-	-	-	-	-	-	-	-	-	-	-	-	2

Pacientes 1-22 e 32-42 incluídos no estudo de frequência de mutações do gene da CYP21.

* Paciente com diagnóstico clínico da forma VS, feita análise molecular em outro estudo.

$ irmã do paciente 12 (VS sem análise molecular)

is- casos isolados

Tabela 4. Características clínicas, genotípicas e níveis séricos basais e após-ACTH dos vários esteróides estudados em pacientes com a forma não clássica da D21OH.

No.	Sexo	Idade	Família	Mutação no *CYP21A2*	Grupo mutacional	Forma clínica	**Cortisol** ug/dL		**17OHP** ng/dL		**21-DF** ng/dL		**S** ng/dL		**DOC** ng/dL		**B** ng/dL		Artigo
							basal	pACTH	basal	pACTH	basal	pACTH	basal	pACTH	basal	pACTH	basal	pACTH	
1	M*	31	19	I2 splice / V281L	A/C	NC	9,4	13,3	856	6.566	203	1.885	31,0	77,0	11,0	12,0	67,4	5.553,0	1,2,3
2	M*	58	21	V281L / R356W	A/C	NC	9,0	15,3	1.087	12.492	233	2.964	39,6	123,9	14,1	17,8	67,4	875,3	1,2,3
3	F	23	is	P30L/ P30L, cluster	A/C	NC	6,1	6,4	13.293	13.256	1.423	1.691	83,0	85,0	20,7	25,6	335,0	546,0	1,2,3
4	F&	22	is	V281L/ IVS2-2A>G	A/C	NC	9,0	12,5	1.049	3.193	95	401	69,0	96,0	7,1	7,1	67,4	224,0	1,2,3
5	F&	39	is	V281L/ V281L	C/C	NC	25,0	32,5	574	3.831	92	1.060	74,0	132,0	7,1	7,1	108,0	631,0	1,2,3
6	F&	11	is	V281L/ V281L	C/C	NC	10,9	14,3	836	4.998	47	631	96,0	110,0	12,0	17,0	492,0	1.154,0	1,2,3
7	F	9	30	V281L /V281L	C/C	NC	17,1	22,5	1.746	5.641	722	3.619	38,0	55,0	7,1	31,0	215,0	1.232,0	1,2,3
8	F&	11	29	V281L /V281L	C/C	NC	10,0	18,2	904	6.732	158	1.200	62,0	185,0	7,1	38,8	210,0	1.081,0	1,2,3
9	F&	13	32	V281L/ V281L	C/C	NC	14,3	16,3	910	3.008	104	302	120,0	245,0	7,1	12,0	183,0	528,0	1,2,3
10	F	4	9	V281L /V281L	C/C	NC	7,3	19,4	1.452	8.527	164	3.006	74,0	178,0	17,0	50,0	147,0	1.940,0	1,2,3
11	F	23	is	V281L/ IVS5-2 A>G???	C/??	NC	10,0	13,3	1.223	8.056	253	1.319	33,3	87,5	7,1	7,1	67,4	530,0	1,2,3
12	F&	42	is	???/???	??/??	NC	7,8	14,0	1.431	8.839	103	842	87,0	133,0	7,1	7,1	136,0	279,0	1,2,3

Pacientes 3-12 incluídos no estudo de frequência de mutações do gene da CYP21

* Pais dos pacientes da D21OHclássica detectados na investigação bioquímica e segregação familiar (os alelos não doados foram incluídos no estudo de frequência

& Pacientes em tratamento prévio da D21OH

is- casos isolados

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