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Original Article

Classic and non-classic 21-hydroxylase deficiency can be discriminated from P450 oxidoreductase deficiency in Japanese infants by urinary steroid metabolites

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Abstract. We previously reported a two-step biochemical diagnosis to discriminate classic 21-hydroxylase deficiency (C21OHD) from P450 oxidoreductase deficiency (PORD) by using urinary steroid metabolites: the pregnanetriolone/tetrahydrocortisone ratio (Ptl / the cortisol metabolites 5α- and 5β-tetrahydrocortisone (sum of these metabolites termed THEs), and 11β-hydroxyandrosterone (11OHAn). The objective of this study was to investigate whether both C21OHD and non-classic 21OHD (C+NC21OHD) could be biochemically differentiated from PORD. We recruited 55 infants with C21OHD, 8 with NC21OHD, 16 with PORD, 57 with transient hyper-17α-hydroxyprogesteronemia (TH17OHP), and 2,473 controls. All infants were Japanese with ages between 0–180 d. In addition to Ptl, THEs, and 11OHAn, we measured urinary tetrahydroaldosterone (THAldo) and pregnenediol (PD5). The first step: by Ptl with the age-specific cutoffs 0.06 mg/g creatinine (0–10 d of age) and 0.3 mg/g creatinine (11–180 d of age), we were able to differentiate C+NC21OHD and PORD from TH17OHP and controls (0–10 d of age: 0.065–31 vs. < 0.001–0.052, 11–180 d of age: 0.40–42 vs. < 0.001–0.086) with 100% sensitivity and specificity. The second step: by the 11OHAn/THAldo or 11OHAn/PD5 ratio with a cutoff of 0.80 or 1.0, we were able to discriminate between C+NC21OHD and PORD (1.0–720 vs. 0.021–0.61 or 1.8–160 vs. 0.005–0.32, respectively) with 100% sensitivity and specificity. Ptl, 11OHAn/THAldo, and 11OHAn/PD5 could differentiate between C+NC21OHD and PORD in Japanese infants.

Key words: urinary steroid metabolites, non-classical 21-hydroxylase deficiency, cytochrome P450 oxidoreductase deficiency

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Introduction

The clinical differential diagnoses of 21-hydroxylase deficiency (21OHD) and cytochrome P450 oxidoreductase deficiency (PORD) are sometimes difficult since both deficiencies can have similar phenotypes and high levels of 17α-hydroxyprogesterone (17OHP) in the blood. We previously reported specific cutoff(s) to discriminate between classic 21OHD (C21OHD) and PORD by using urinary steroid metabolites, i.e. the pregnanetriolone (Ptl)/ the cortisol metabolites 5α- and 5ß-tetrahydrocortisone (sum of these metabolites termed THEs) ratio and 11ß-hydroxyandrosterone (11OHAn), by using gas chromatography/mass spectrometry (GC/MS) (1). However, we did not investigate whether the cutoffs were able to discriminate between non-classic 21OHD (NC21OHD) and PORD. The prevalence of NC21OHD is estimated at 1 case out of 2 million individuals in Japan (2), whereas it is reported to be 1 out of 1,000 in Caucasians (3, 4), and is considered to be the most common form of congenital adrenal hyperplasia. Patients with NC21OHD have mildly impaired 21-hydroxylase activity leading to various symptoms from childhood to adulthood, such as precocious pubarche, acne, hirsutism, infertility, etc. (5, 6). Biochemical diagnosis of NC21OHD is challenging because of the relatively mild glucocorticoid deficiency seen in patients. We previously reported that clinically diagnosed 21OHD, including classic and non-classic 21OHD (C+NC21OHD), can be distinguished from transient hyper-17α-hydroxyprogesteronemia (TH17OHP) and controls by Ptl measurements in GC/MS (7). Additionally, we reported in the same study that C+NC21OHD could be differentiated from PORD by the ratio between 11OHAn and pregnanediol, which is a metabolite of progesterone, in three infants between the ages of 1 and 3 months (7).

The objective of this study was to investigate whether C+NC21OHD could be biochemically differentiated from PORD in Japanese infants. In addition to Ptl, THEs, and 11OHAn, we focused on the pregnenolone (P5) metabolite pregnenediol (PD5), and the aldosterone metabolite tetrahydroaldosterone (THAldo). We focused on these metabolites because in PORD, (i) blood P5 was shown to be higher (8, 9), and (ii) blood aldosterone and urinary THAldo were shown to be normal or slightly higher, respectively, compared to that in normal subjects (7, 9, 10).

Materials and Methods

All legal guardian(s) gave written informed consent and the study was approved by the Institutional Review Boards at Keio University Hospital and Keio University School of Medicine. We recruited 55 infants with C21OHD (gestational age, 35–41 wk; birth weight, 1,658–4,174 g), 8 infants with NC21OHD (37–40 wk; 2,704–3,408 g), 16 infants with PORD (34–41 wk; 1,018–3,418 g), 57 infants with TH17OHP (37–41 wk, 2,062–4,980 g), and 2,473 controls (34–41 wk, 770–4,610 g). All infants were Japanese with ages between 0–180 d, the period during which most patients with C21OHD or PORD are diagnosed (7, 11). The diagnosis of 21OHD and PORD was confirmed by *CYP21A2* and *POR* gene analyses, respectively. Notably, all patients with NC21OHD were positive in newborn mass-screening in Japan. Patients with 21OHD having normal genitalia and elevated dried blood spot 17OHP (positive results in newborn mass-screening), but without any evidence of salt wasting (low serum sodium, high serum potassium, high plasma renin activity, etc.) were classified as NC21OHD. Any subjects with abnormal physical findings except for external genitalia were excluded. None of the subjects received antenatal or perinatal glucocorticoid before urine sampling.

We measured urinary steroid metabolites by GC/MS (12). The 21-deoxycortisol metabolite Ptl, and the cortisol metabolites 5α-tetrahydrocortisone and 5ß-tetrahydrocortisone (hereafter referred

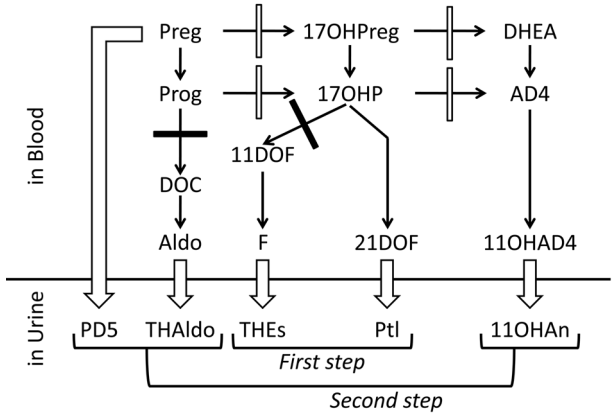

Fig. 1. A steroid metabolic map. Solid arrow, steroid synthesis; open arrow, steroid metabolism; solid line, impaired 21-hydroxylase activity; open line, impaired 17-hydroxylase/17,20-lyase activity. First step, differentiation of C+NC21OHD and PORD from TH17OHP and the control. Second step, discrimination between C+NC21OHD and PORD. Both 21-hydroxylase and 17-hydroxylase/17,20-lyase activity are reduced in PORD, whereas only 21-hydroxylase is reduced in C+NC21OHD. Preg, pregnenolone; Prog, progesterone; DOC, deoxycorticosterone; Aldo, aldosterone; 17OHPreg, 17α-hydroxypregnenolone; 11DOF, 11-deoxycortisol; DHEA, dehydroepiandrosterone; AD4, androstendione.

to collectively as THEs) were measured and the ratio of Ptl to the cortisol metabolites (Ptl/THEs) (1, 13, 14) was calculated to differentiate C21OHD, NC21OHD, and PORD from TH17OHP and control (Fig. 1). Ptl was considered equal to 0.001 mg/g creatinine for calculations in infants whose Ptl was under the detection limit (< 5 pg/injection). In addition to the metabolite of 11β-hydroxyandrostenedione, 11OHAn, (1), we measured THAldo and PD5. These steroids and their metabolites were previously shown to be increased in PORD (8–10). We then calculated the 11OHAn/THAldo and 11OHAn/PD5 ratios to discriminate between C+NC21OHD and PORD (Fig. 1). Quantification and quality ions of each metabolite were as follows (*m*/*z*): Ptl 449, 359; 5αTHE 488, 578; 5βTHE 488, 578; 11OHAn 448, 358; THAldo 506 (quantified ion only); PD5 372, 462. Urinary creatinine was measured by IATRO-LQ CRE (A)II (LSI Medience Co., Tokyo, Japan). Urinary steroid concentration was expressed relative to urinary creatinine (mg/g creatinine).

Statistical analysis was performed using the Mann-Whitney U test. A p value of < 0.05 was considered statistically significant.

Results

Differentiation of C+NC21OHD and PORD from TH17OHP and controls

Results of Ptl and Ptl/THEs are shown in Table 1 and Fig. 2. Both Ptl and Ptl/THEs showed similar overlap between C+NC21OHD, PORD, TH17OHP, and control within 10 days of age by uniform cutoff through 0–180 d of age (Ptl 0.1 and Ptl/THEs 0.020). We then separately set the cutoff for 0–10 d of age and 11–180 d of age. Ptl differentiated C+NC21OHD and PORD from TH17OHP and control with 100% (95% confidence interval (CI): 97.6–100%) sensitivity and 100% (95% CI: 99.9–100%) specificity using the 0.06 mg/g creatinine (0–10 d of age) and 0.3 mg/g creatinine (11–180 d of age) cutoffs. Ptl/THEs differentiated with 100% (95% CI: 96.5–100%) sensitivity and 99.9% (95% CI: 99.8–99.9%) specificity using the 0.01 (0–10 d of age) and 0.02 (11–180 d of age) cutoffs.

Discrimination between C+NC21OHD and PORD

Table 2 and Fig. 3 show the results of urinary 11OHAn in C+NC21OHD and PORD. 11OHAn discriminated between C21OHD and PORD with 96.8% (95% CI: 93.3–96.8%) sensitivity and 100% (95% CI: 86.1–100%) specificity using the 0.35 mg/g creatinine cutoff. We then focused on the aldosterone and P5 metabolites, THAldo and PD5. Although both metabolites showed significantly higher distribution in PORD than in C+NC21OHD (THAldo $p < 0.001$, PD5 $p < 0.001$),

Table 1 Results of Ptl and Ptl/THEs in C+NC21OHD, PORD, TH17OHP, and controls

	Ptl (mg/g creatinine)		Ptl/THEs	
C+NC21OHD	8.7	(0.12–42)	2.8	(0.13–20)
PORD	1.0	(0.065–2.6)	0.13	(0.010–0.23)
TH17OHPnemia	0.007	(< 0.001–0.086)	< 0.001	(< 0.001–0.006)
Controls	< 0.001	(< 0.001–0.085)	< 0.001	(< 0.001–0.027)

mean (range).

Table 2 Results of 11OHAn, THAldo, and PD5 in C+NC21OHD and PORD

	11OHAn		THAldo		PD5	
C+NC21OHD	2.3	(0.16–22)	0.093	(0.005–0.80)	0.123	(0.012–1.3)
PORD	0.037	(0.007–0.22)	0.34	(0.074–0.94)	0.63	(0.32–2.7)

mean (range), mg/g creatinine.

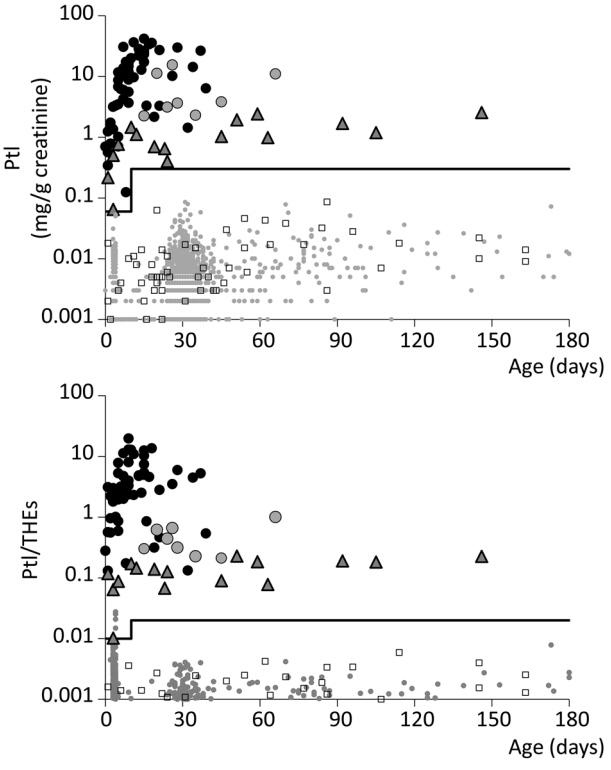

Fig. 2. Urinary Ptl and Ptl/THEs in infants with C+NC21OHD, PORD, TH17OHP, and controls. The upper graph is for Ptl and the lower one is for Ptl/THEs. Lines indicate cutoffs: Ptl 0.05 mg/g creatinine (0–10 d of age) and 0.1 mg/g creatinine (11–180 d of age), Ptl/THEs 0.01 (0–10 d of age) and 0.02 (11–180 d of age). Closed circle, C21OHD; gray circle, NC21OHD; triangle, PORD; open square, TH17OHP; dot, control.

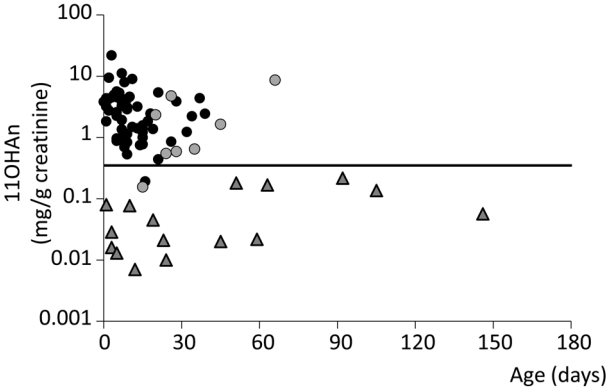

Fig. 3. Urinary 11OHAn in infants with C21OHD, NC21OHD, and PORD. Line indicates cutoff of 0.35 mg/g creatinine. Closed circle, C21OHD; gray circle, NC21OHD; triangle, PORD.

there was clear overlap between the two groups (Table 2). Table 3 and Fig. 4 show the results of urinary 11OHAn/THAldo and 11OHAn/PD5 ratio calculations. 11OHAn/THAldo discriminated with 100% (95% CI: 97.2–100%) sensitivity and 100% (95% CI: 88.9–100%) specificity using the 0.80 cutoff. 11OHAn/PD5 discriminated with 100% (95% CI: 97.2–100%) sensitivity and 100% (95% CI: 88.9–100%) specificity using the 1.0 cutoff.

Table 3 Results of 11OHAn/THAldo and 11OHAn/PD5 in C+NC21OHD and PORD

	11OHAn/THAldo		11OHAn/PD5	
C+NC21OHD	20	(1.0–720)	18	(1.8–160)
PORD	0.15	(0.021–0.61)	0.059	(0.005–0.32)

mean (range).

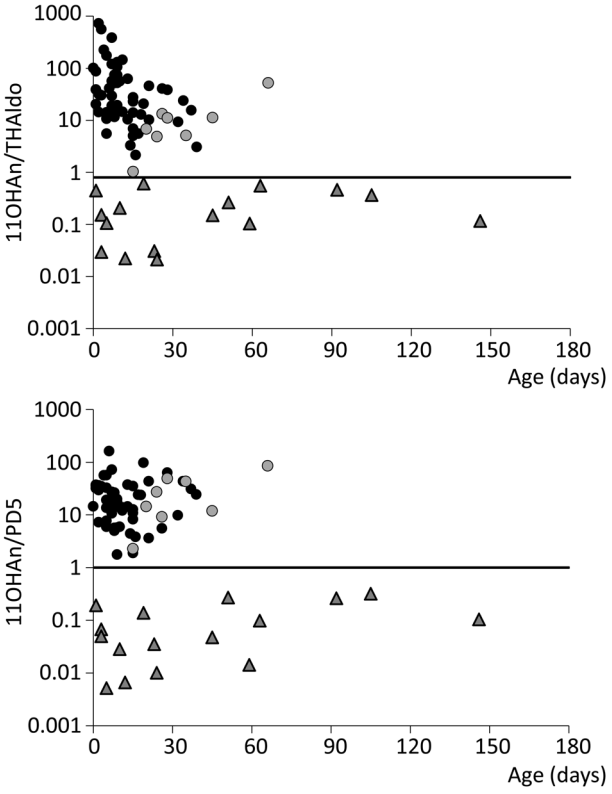

Fig. 4. Urinary 11OHAn/THAldo and 11OHAn/PD5 in infants with C21OHD, NC21OHD, and PORD. The upper graph is for 11OHAn/THAldo and the lower one is for 11OHAn/PD5. Lines indicate cutoffs: 11OHAn/THAldo 0.80, 11OHAn/PD5 1.0. Closed circle, C21OHD; gray circle, NC21OHD; triangle, PORD.

Discussion

We demonstrated that a two-step biochemical diagnosis using urinary steroid metabolites is useful for diagnosis of 21OHD and PORD when patients with NC21OHD were included among the subjects. We propose a two-step biochemical diagnosis using Ptl for the first step, and 11OHAn/THAldo or 11OHAn/PD5 for the second step, because these two markers showed no overlap in each step.

In the first step, we set the age-specific cutoff of Ptl to differentiate C+NC21OHD and PORD from TH17OHP and the control. We separately set the cutoff at 0–10 and 11–180 d of age because patients with 21OHD and PORD who were 0–10 d old showed lower Ptl values. Assuming that steroid metabolic enzyme activity in the liver was immature at early ages after birth, we could explain the imperfectness of the uniform cutoff through 0–180 d of age.

In the second step, a downstream/upstream metabolites ratio, 11OHAn/THAldo or 11OHAn/PD5, was more useful than the single metabolite, 11OHAn. Steroid downstream/upstream metabolite ratios have previously been used as markers of enzyme defects (15, 16). Indeed, as mentioned in the Introduction, we had used the ratio between 11OHAn and pregnanediol (PD), a metabolite of progesterone, for distinguishing PORD from 21OHD (7). As PD measurement is sometimes problematic in our GC/MS methods in newborns (our unpublished data), we used a single metabolite, 11OHAn, to discriminate between C21OHD and PORD (1). In this study, we chose metabolites of aldosterone and pregnenolone, THAldo, and PD5, for the following two reasons. First, aldosterone and pregnenolone are upstream of 17α-hydroxylase (Fig.1). Second, THAldo and PD5 can be measured in all newborn infants (our unpublished data).

In this study, we recruited 0–180 d old infants because most patients with C21OHD or PORD

are diagnosed in this period (7, 11). Although it was reported that patients with PORD who were above 180 d of age showed similar trends in urinary steroid metabolites (i.e., high Ptl and normal-range 11OHAn) (11), further analysis is required to determine whether our cutoffs can be applicable to infants over 180 d of age.

This method has two advantages compared to repeated 17OHP measurement: it is a single assay and offers the noninvasiveness of urine sampling. Thus, this method is a potential option for scrutiny of newborn mass-screened positive patients together with liquid chromatography/ tandem mass spectrometry (17, 18) and genetic analysis (19, 20).

Some limitations of this study should be discussed. First, we do not know whether our two-step method is applicable to all cases of NC21OHD. In this study, NC21OHD patients had positive results in a newborn mass-screening program in Japan. A few NC21OHD cases have been reported to be positive in newborn mass-screening programs (21), but most were negative because of the relatively low baseline levels of 17OHP (5). Positive NC21OHD may possess less 21-hydroxylase activity than negatives ones; i.e., their Ptl, 11OHAn, and PD5 may be higher or THEs and THAldo may be lower. Second, as we described in a previous study (1), our data in Japanese infants may not apply to other ethnicities because of differences in common POR mutations and their residual activities in PORD (9, 22, 23). Additional studies are required for non-Japanese individuals. Third, preterm infants were not included in this study whose gestational age was less than 34 wk. Those infants might have more immature steroid metabolism in the liver, theoretically leading to lower Ptl and 11OHAn compared with the subjects of this study.

In conclusion, we demonstrated a two-step biochemical diagnosis of C+NC21OHD and PORD by urinary steroid profiling using Ptl, THAldo, PD5, and 11OHAn.

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References

1. Koyama Y, Homma K, Fukami M, Miwa M, Ikeda K, Ogata T, *et al.* Two-step biochemical differential diagnosis of classic 21-hydroxylase deficiency and cytochrome P450 oxidoreductase deficiency in Japanese infants by GC-MS measurement of urinary pregnanetriolone/ tetrahydroxycortisone ratio and 11ß-hydroxyandrosterone. Clin Chem 2012;58: 741–7. [Medline] [CrossRef]
2. Kashimada K, Ishii T, Nagasaki K, Ono M, Tajima T, Yokota I, *et al*. Clinical, biochemical, and genetic features of non-classical 21-hydroxylase deficiency in Japanese children. Endocr J 2015;62: 277–82. [Medline] [CrossRef]
3. Azziz R, Hincapie LA, Knochenhauer ES, Dewailly D, Fox L, Boots LR. Screening for 21-hydroxylase-deficient nonclassic adrenal hyperplasia among hyperandrogenic women: a prospective study. Fertil Steril 1999;72: 915–25. [Medline] [CrossRef]
4. Speiser PW, Dupont B, Rubinstein P, Piazza A, Kastelan A, New MI. High frequency of nonclassical steroid 21-hydroxylase deficiency. Am J Hum Genet 1985;37: 650–67. [Medline]
5. New MI. Extensive clinical experience: nonclassical 21-hydroxylase deficiency. J Clin Endocrinol Metab 2006;91: 4205–14. [Medline] [CrossRef]
6. Kashimada K, Ono M, Onishi T, Koyama S,

Toyoura T, Imai K, *et al*. Clinical course of patients with nonclassical 21-hydroxylase deficiency (21-OHD) diagnosed in infancy and childhood. Endocr J 2008;55: 397–404. [Medline] [CrossRef]
7. Fukami M, Hasegawa T, Horikawa R, Ohashi T, Nishimura G, Homma K, *et al*. Cytochrome P450 oxidoreductase deficiency in three patients initially regarded as having 21-hydroxylase deficiency and/or aromatase deficiency: diagnostic value of urine steroid hormone analysis. Pediatr Res 2006;59: 276–80. [Medline] [CrossRef]
8. Shackleton C, Marcos J, Malunowicz EM, Szarras-Czapnik M, Jira P, Taylor NF, *et al*. Biochemical diagnosis of Antley-Bixler syndrome by steroid analysis. Am J Med Genet A 2004;128A: 223–31. [Medline] [CrossRef]
9. Fukami M, Horikawa R, Nagai T, Tanaka T, Naiki Y, Sato N, *et al*. Cytochrome P450 oxidoreductase gene mutations and Antley-Bixler syndrome with abnormal genitalia and/or impaired steroidogenesis: molecular and clinical studies in 10 patients. J Clin Endocrinol Metab 2005;90: 414–26. [Medline] [CrossRef]
10. Fukami M, Nishimura G, Homma K, Nagai T, Hanaki K, Uematsu A, *et al*. Cytochrome P450 oxidoreductase deficiency: identification and characterization of biallelic mutations and genotype-phenotype correlations in 35 Japanese patients. J Clin Endocrinol Metab 2009;94: 1723–31. [Medline] [CrossRef]
11. Homma K, Hasegawa T, Nagai T, Adachi M, Horikawa R, Fujiwara I, *et al*. Urine steroid hormone profile analysis in cytochrome P450 oxidoreductase deficiency: implication for the backdoor pathway to dihydrotestosterone. J Clin Endocrinol Metab 2006;91: 2643–9. [Medline] [CrossRef]
12. Homma K, Hasegawa T, Masumoto M, Takeshita E, Watanabe K, Chiba H, *et al*. Reference values for urinary steroids in Japanese newborn infants: gas chromatography/mass spectrometry in selected ion monitoring. Endocr J 2003;50: 783–92. [Medline] [CrossRef]
13. Caulfield MP, Lynn T, Gottschalk ME, Jones KL, Taylor NF, Malunowicz EM, *et al*. The diagnosis of congenital adrenal hyperplasia in the newborn by gas chromatography/mass spectrometry analysis of random urine specimens. J Clin Endocrinol Metab 2002;87: 3682–90. [Medline] [CrossRef]
14. Homma K, Hasegawa T, Takeshita E, Watanabe K, Anzo M, Toyoura T, *et al*. Elevated urine pregnanetriolone definitively establishes the diagnosis of classical 21-hydroxylase deficiency in term and preterm neonates. J Clin Endocrinol Metab 2004;89: 6087–91. [Medline] [CrossRef]
15. Koyama Y, Homma K, Hasegawa T. Urinary steroid profiling: a powerful method for the diagnosis of abnormal steroidogenesis. Expert Rev Endocrinol & Metab 2014;9: 273–82. [CrossRef]
16. Krone N, Hughes BA, Lavery GG, Stewart PM, Arlt W, Shackleton CH. Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS). J Steroid Biochem Mol Biol 2010;121: 496–504. [Medline] [CrossRef]
17. Lacey JM, Minutti CZ, Magera MJ, Tauscher AL, Casetta B, McCann M, *et al*. Improved specificity of newborn screening for congenital adrenal hyperplasia by second-tier steroid profiling using tandem mass spectrometry. Clin Chem 2004;50: 621–5. [Medline] [CrossRef]
18. Janzen N, Peter M, Sander S, Steuerwald U, Terhardt M, Holtkamp U, *et al*. Newborn screening for congenital adrenal hyperplasia: additional steroid profile using liquid chromatography-tandem mass spectrometry. J Clin Endocrinol Metab 2007;92: 2581–9. [Medline] [CrossRef]
19. Nordenström A, Thilén A, Hagenfeldt L, Larsson A, Wedell A. Genotyping is a valuable diagnostic complement to neonatal screening for congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency. J Clin Endocrinol Metab 1999;84: 1505–9. [Medline]
20. Kösel S, Burggraf S, Fingerhut R, Dörr HG, Roscher AA, Olgemöller B. Rapid second-tier molecular genetic analysis for congenital adrenal hyperplasia attributable to steroid 21-hydroxylase deficiency. Clin Chem 2005;51: 298–304. [Medline] [CrossRef]

21. Therrell BL Jr, Berenbaum SA, Manter-Kapanke V, Simmank J, Korman K, Prentice L, *et al*. Results of screening 1.9 million Texas newborns for 21-hydroxylase-deficient congenital adrenal hyperplasia. Pediatrics 1998;101: 583–90. [Medline] [CrossRef]
22. Miller WL, Huang N, Agrawal V, Giacomini KM. Genetic variation in human P450 oxidoreductase. Mol Cell Endocrinol 2009;300: 180–4. [Medline] [CrossRef]
23. Huang N, Pandey AV, Agrawal V, Reardon W, Lapunzina PD, Mowat D, *et al*. Diversity and function of mutations in p450 oxidoreductase in patients with Antley-Bixler syndrome and disordered steroidogenesis. Am J Hum Genet 2005;76: 729–49. [Medline] [CrossRef]