Adrenal steroid profiling as a diagnostic tool to differentiate polycystic ovary syndrome from nonclassic congenital adrenal hyperplasia: pinpointing easy screening possibilities and normal cutoff levels using liquid chromatography tandem mass spectrometry

Grethe Å. Ueland, Ph.D.,[a] Sandra R. Dahl, Ph.D.,[b] Paal Methlie, Ph.D.,[a,c] Saleh Hessen, M.D.,[d] Eystein S. Husebye, Professor,[a,c] and Per M. Thorsby, M.D., Ph.D.[b]

[a] Department of Medicine, Haukeland University Hospital, Bergen, Norway; [b] Hormone Laboratory, Department of Medical Biochemistry and Biochemical Endocrinology and Metabolism Research Group, Oslo University Hospital, Aker, Oslo, Norway; [c] Department of Clinical Science and K.G. Jebsen Center for Autoimmune Diseases, University of Bergen, Bergen, Norway; and [d] Innlandet Hospital, Department of Internal Medicine, Hamar, Norway

Objective: To define liquid chromatography tandem mass spectrometry (LC-MS/MS)–based cutoff levels and panels of steroid hormones, to improve diagnosis of nonclassic congenital adrenal hyperplasia (NCCAH) and other partial enzyme defects in the adrenals.
Design: Prospective cohort analysis.
Setting: University hospital-based tertiary endocrine center.
Patients: One hundred and twenty-one healthy adults and 65 patients evaluated for possible NCCAH (validation cohort).
Interventions: The LC-MS/MS–determined cutoffs for 11 steroids (basal and cosyntropin-stimulated) were defined by 2.5% and 97.5% percentile in healthy subjects. Validation cohort was used for comparison.
Main Outcome Measures: Percentage of patients diagnosed with NCCAH among patients with polycystic ovary syndrome (PCOS)-like symptomatology. Evaluation of the defined LC-MS/MS–based cutoff levels for steroid hormones among this patient group.
Results: Of the 65 PCOS-like patients evaluated for possible NCCAH, 8 (12.5%) were discovered and genetically verified, and 2 had classic congenital adrenal hyperplasia. Cosyntropin-stimulated 17-hydroxyprogesterone (17OHP) showed the best diagnostic accuracy for NCCAH with an area under the curve of 0.95 (0.89–1.0 with a sensitivity of 86% and a specificity of 88%. In homozygote patients, 21-deoxycortisol and 17OHP levels were elevated, in heterozygote patients only 17OHP (basal or stimulated) was raised. Four healthy patients in the validation cohort had 17OHP above the basal cutoff.
Conclusions: The NCCAH syndrome is frequent in patients with suspected PCOS, and should be considered as a routine screening when assessing infertility. We suggest the use of serum steroid profiling, including 21-deoxycortisol, together with the cosyntropin

Received February 7, 2022; revised and accepted May 6, 2022; published online June 18, 2022.
P.M.T. is employed as a consultant for AntiDoping Norway. E.S.H. has nothing to disclose. S.H. has nothing to disclose. G.Å.U. has nothing to disclose. S.R.D. has nothing to disclose. P.M.T. has nothing to disclose.
G.Å.U. and S.R.D. should be considered similar in author order and both are contributed equally to the study.
Supported by honoraria from NovoNordisk and Ferring (to P.M.T.).
Reprint request: Grethe Å. Ueland, Ph.D., Department of Medicine, Haukeland University Hospital, Bergen, Norway (E-mail: geas@helse-bergen.no).

Fertility and Sterility® Vol. 118, No. 2, August 2022 0015-0282

https://doi.org/10.1016/j.fertnstert.2022.05.012

stimulation test with 17OHP. Our data support a 17OHP cutoff of 8.5 nmol/L (2.8 ng/mL) 60 minutes after cosyntropin stimulation, when measured with LC-MS/MS, significantly lower than current European guidelines.
Clinical trials Number: NCT0218660. (Fertil Steril® 2022;118:384-91. ©2022 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Nonclassic congenital adrenal hyperplasia, infertility, NCCAH, PCOS, serum steroid profiling

DIALOG: You can discuss this article with its authors and other readers at https://www.fertstertdialog.com/posts/34731

Congenital adrenal hyperplasia (CAH) is caused by deficiency of adrenal enzymes, most often 21-hydroxylase (21OH), and is the most frequent autosomal recessive disorder in humans (1). In the milder nonclassic form (NCCAH), normal cortisol levels are produced at the expense of increased adrenocorticotropic hormone levels, which in turn results in clinical hyperandrogenism, manifested from childhood through early adult life (2–4). The symptoms and signs highly resemble what is found in polycystic ovary syndrome (PCOS), and NCCAH is an important differential diagnosis in women with acne, hirsutism, menstrual abnormalities, and infertility (5).

The diagnostics of NCCAH is based mainly on increased basal and cosyntropin-stimulated level of the 21OH substrate 17-hydroxyprogesterone (17OHP), and the "backdoor product" 21-deoxycortisol (21DF), both biochemical markers of 21OH deficiency (6, 7) (Supplemental Fig. 1, available online [8]). However, the current basal and cosyntropin-stimulated 17OHP cutoff levels are based on old, nonstandardized immunologic methods that are no longer in use (9). The basal blood sample ideally should be drawn in the early menstrual follicular phase, but irregular and infrequent menstruations make it difficult to time sample correctly.

In recent years, liquid chromatography tandem mass spectrometry (LC-MS/MS) for quantitative determination of steroid hormones has been implemented increasingly, and is now recommended as the gold standard (10, 11). Liquid chromatography tandem mass spectrometry offers superior analytic specificity compared to immunoassays and is traceable to one accuracy basis (i.e., international reference materials), enabling comparison of results across methods, time, and location. Its multiplexing capabilities enabling assessment of several steroid hormones in one analytic run to create a "steroid fingerprint" is another major advantage. As recognized by the Endocrine Society (12, 13), new reference ranges and clinical cutoff levels must be established and validated with the introduction of new analytic methods.

The cosyntropin test is used to diagnose partial enzyme defects in NCCAH. An increase in serum 17OHP above 30–45 nmol/L (10–15 ng/mL) 60 minutes after cosyntropin stimulation has been considered diagnostic for CAH/NCCAH (9) with immunologic assays. Applying LC-MS/MS measurements, we found cosyntropin-stimulated 17OHP level <9 nmol/L (3.0 ng/mL) as a valid cutoff to exclude the diagnosis of NCCAH (14), but information for basal or cosyntropin-stimulated 21DF levels in CAH/NCCAH is lacking.

The diagnosis of NCCAH should be verified by CYP 21A2 genotyping. The CAH/NCCAH syndrome is caused by deletions, macro conversions, micro conversions, or variants of a pseudogene for the region *CYP21A2* on chromosome 6. There are large differences in adrenal steroid profiling, even with the same genotype, emphasizing the difference between genotype and phenotype in these diseases (15). Heterozygote carriers also may show mild symptoms of hyperandrogenism, and as the disorder is common, there is a risk for carriers to pass the disease over to their offspring. The risks related to fertility, pregnancy, delivery, and offspring in heterozygote carriers are unknown.

Less common enzyme defects in the adrenal steroid synthesis may be suspected by increased levels of upstream precursors in the steroid pathway. Adrenal steroid profiling may uncover such rare abnormalities.

In this study, we aimed to estimate the prevalence of NCCAH among female patients with acne, hirsutism, menstrual abnormalities, and infertility. Further, to pave the way for serum steroid profiling as a diagnostic tool for NCCAH, we have established LC-MS/MS–based specific cutoff levels for basal and cosyntropin-stimulated steroid precursors in a large cohort of healthy controls. The findings are validated in a cohort of female patients with symptoms of PCOS and evaluated for possible NCCAH.

MATERIALS AND METHODS

Subjects and Study Design

From June 2016 to March 2021, we consecutively included patients referred for evaluation of NCCAH to the tertiary endocrine specialist center at Haukeland University Hospital, Bergen, Norway. Two patients diagnosed with classic CAH at Hamar Hospital in the same period were included for comparison. Simultaneously, between June 2016 and June 2017, healthy controls were engaged from hospital and university staff (66 women). None of the healthy controls was using any medications. The median age was 40 (23–68) years, and the median body mass index was 23 (15–33) kg/m^2. Only 5 individuals were daily smokers. The median creatinine was 70 (50–100) μmol/L. The systolic blood pressure at the day of examination was 128 (109–130) mmHg, and the diastolic blood pressure was 78 (58–99) mmHg. The testing was not timed according to the menstrual cycle of the healthy controls. A total of 63 patients with clinical suspected NCCAH, 2 with classic CAH, and 138 healthy controls were enrolled. Of the healthy controls, 17 were taking oral estrogens. None of the study subjects used glucocorticoids.

All study subjects performed a standard short cosyntropin test between 8 AM and 12 PM in the morning. Serum samples

TABLE 1

Overall median, 2.5%, and 97.5% levels including 90% confidence interval for steroid hormones basal and after 60 minutes, all numbers nmol/L.

	0 minutes			60 minutes		
	Median	**2.5%**	**97.5%**	**Median**	**2.5%**	**97.5%**
17OHP	1.6	0.33 (0.26–0.38)	4.6 (3.9–5.9)	3.2	1.2 (0.91–1.5)	8.5 (7.7–9.9)
Female	1.0	0.28	5.7			
Male	2.0	0.78	3.4			
11DF	0.53	0.26 (0.13–0.27)	1.7 (1.3–2.5)	1.9	0.78 (0.41–1.0)	5.1 (4.1–5.3)
21DF	[a]	[a]	[a]	0.45	0.26 (0.26–0.28)	1.7 (1.2–2.0)
17OHPreg	5.8	3.2 (3.1–3.3)	19.0 (14.0–20.0)	17.0	5.6 (4.8–8.1)	41.0 (33.0–54.0)
DHEA	13.0	3.5 (3.0–4.1)	75.0 (58.0–94.0)	35.0	8.6 (6.4–11)	115.0 (98.0–128.0)
DOC	0.13	0.071 (0.071–0.074)	0.38 (0.37–0.39)	0.54	0.21 (0.12–0.27)	1.7 (1.3–2.2)
Corticosterone	7.8	1.4 (1.3–2.2)	44.0 (23.0–75.0)	79.0	41.0 (17.0–45.0)	145.0 (132.0–161.0)
A4						
Female						
18–49 y	3.3	1.2	8.5	4.6	1.6	11.0
>50 y	1.5	1.1	[b]	2.7	1.3	-[b]
Male						
18–49 y	2.5	1.2	4.9	4.1	1.3	7.3
>50 y	1.9	1.1	[b]	2.7	1.8	-[b]
T						
Female						
18–49 y	0.8	0.25	1.8			
>50 y	0.65	0.45	[b]			
Male						
18–49 y	14	5.9	27.0			
>50 y	16	8.5	[b]			
Cortisone	7.8	25.0 (12.0–27.0)	91.0 (74.0–105.0)	44.0	18.0 (16.0–27.0)	82.0 (62.0–87.0)

Note: For conversion of nmol/L to ng/mL conversion factors, see section liquid chromatography tandem mass spectrometry assay of steroids. A4 = androstenedione; DF = deoxycortisol; DHEA = dehydroepiandrosterone; DOC = deoxycorticosterone; T = testosterone; 17OHP = cosyntropin-stimulated 17-hydroxyprogesterone; 17OHPreg = 17-hydroxypregnenolone.
[a] All results <0.25 nmol/L.
[b] Not possible to calculate because of low number.

Ueland. Adrenal steroid profiling. Fertil Steril 2022.

were obtained before (0 minutes) and 60 minutes after intravenous administration of 250 μg cosyntropin with the patient placed in the recumbent position. The test was performed nonfasting. Abnormal test results were evaluated further according to European guidelines for NCCAH (9), depending on the pretest probability for these diseases. The study subjects were categorized as having NCCAH (heterozygote or homozygote), CAH or healthy. All samples were centrifuged immediately and stored at −80°C.

The LC-MS/MS Assay of Steroids

The samples were analyzed by LC-MS/MS at the Hormone Laboratory, Oslo University Hospital, Norway. The assay measures endogenous and synthetic steroids. A detailed description of the method and performance metrics is provided in the Supplementary material (available online) (8). For the purpose of this study, cortisol, 17OHP, 11-deoxycortisol (11DF), 21DF, 17-hydroxypregnenolone (17OHPreg), dehydroepiandrosterone (DHEA), deoxycorticosterone (DOC), corticosterone, androstenedione (A4), and testosterone (T), and cortisone were analyzed. The limits of quantifications for cortisol, 17OHP, 11DF, 21DF, 17OHPreg, DHEA, DOC, corticosterone, cortisone, A4, and T were 0.5, 0.2, 0.20, 0.25, 3.0, 3.0, 0.071, 0.2, 1.0, 0.2, and 0.1 nmol/L (0.18, 0.066, 0.069, 0.087, 1.0, 0.87, 0.023, 0.069, 0.036, 0.057, 0.029 ng/mL), respectively. The analytic coefficient of variation % ranged from 6%–24%, and the accuracy ranged between 90% and 110% for all steroid hormones. The Hormone Laboratory is accredited according to NS-EN ISO/IEC 17025:2017.

Our laboratory is participating in the UK NEQAS for steroid hormones (Birmingham Quality; 17OHP, A4, T), the SKML hormones in serum (Dutch Foundation for Quality Assessment in Medical Laboratories; for 11DF, 21DF, and corticosterone), Instand adrenal gland pituitary (Society for Promotion of quality assurance in medical laboratories; for DHEA), and Labquality (for cortisone).

To convert nmol/L to ng/mL, use the following factors; 0.362 (cortisol), 0.330 (17OHP and DOC), 0.345 (11DF, 21DF, and corticosterone), 0.333 (17OHPreg), 0.288 (DHEA and T), 0.360 (cortisone) and 0.286 (A4).

Statistical Analyses

Categorical data are reported as numbers (percent) and continuous data as median (range). The data were not normally distributed and, therefore, nonparametric statistics were applied. The Mann-Whitney *U* test was used to compare groups. The significance level was set to 0.05. Spearman correlation was used to evaluate the degree of correlation when appropriate. Dixons criteria was used to detect outliers (16). The upper cutoff levels for the steroids analyzed were defined as the 97.5th percentile with 90% confidence interval (CI) in

TABLE 2

Characteristics of patients in the validation cohort included from the endocrine outpatient clinic, suspected of having NCCAH.

	Validation cohort not diagnosed with NCCAH (n = 55)	NCCAH Total (n = 8)	CAH (n = 2)
Age median yrs (range)	28.0 (19.0–66.0)	50.0 (19.0–66.0)	26.0 (25.0–27.0)
Sex women, n (%)	57.0 (98.0)	6.0 (100.0)	2.0 (100.0)
Body mass index median kg/m^2 (range)	27.5 (19.9–46.7)	24.8 (19.5–39.4)	24.5 (24.0–25.5)
Basal samples given in median (range)			
ACTH, pmol/L	4.1 (1.6–136)	3.4 (1.7–7.7)	8.1 (4.6–11.7)
cortisol, nmol/L	324.8 (108.4–623))	346.0 (140.0–771.0)	413.0 (378.0–448.0)
17OHP, nmol/L	1.69 (0.21–6.7)	4.95 (1.0–6.9)	130.0 (127.0–133.0)
11DF, nmol/L	0.72 (0.25–5.27)	1.7 (0.25–3.0)	3.5 (3.1–3.9)
21DF, nmol/L	0.25 (0.25–0.25)	0.25 (0.25–0.76)	18.5 (15–22)
17OHPreg, nmol/L	6.64 (2.5–34.78)	4.9 (3.0–19.9)	
DHEA, nmol/L	21.4 (4.0–86.3)	15.7 (7.6–32.2)	
DOC, nmol/L	0.10 (0.01–1.75)	0.21 (0.07–1.05)	0.09 (0.07–0.10)
Corticosterone, nmol/L	7.9 (1.6–48.78)	7.3 (2.1–39.6)	10.2 (8.4–12.0)
A4, nmol/L	5.2 (1.1–14.3)	4.19 (1.4–6.6)	
T, nmol/L	1.23 (0.41–10.9)	1.15 (0.35–1.61)	
Cortisone, nmol/L	47.4 (21.9–83.0)	39.0 (22.9–85.0)	77.0 (75.0–79.0)
Samples 60 minutes after synachten, given in median and range			
cortisol, nmol/L	678.9 (485.2–949.0)	683.0 (283.0–852.0)	447.0
17OHP, nmol/L	4.5 (1.08–21.5)	10.4 (8.0–30.1)	31
11DF, nmol/L	2.68 (0.59–13.1)	4.2 (3.3–7.7)	5.1
21DF, nmol/L	0.30 (0.25–1.2)	0.61 (0.25–5.6)	37
17OHPreg, nmol/L	33.1 (15.5–94.9)	27.2 (5.3–60.2)	
DHEA, nmol/L	44.0 (10.6–130.4)	38.4 (28.5–61.1)	
DOC, nmol/L	0.6 (0.18–7.73)	0.9 (0.5–5.3)	0.25
Corticosterone, nmol/L	83.8 (31.0–156.4)	78.1 (34.4–170.9)	34.0
A4,nmol/L	6.8 (2.59–13.4)	5.8 (1.9–10.3)	
T, nmol/L	1.29 (0.42–11.1)	1.17 (0.4–1.6)	
Cortisone, nmol/L	51.4 (27.6–74.8)	48.3 (27.6–69.8)	63.0

Note: For conversion of nmol/l to ng/mL conversion factors, see section liquid chromatography tandem mass spectrometry assay of steroids. A4 = androstenedione; ACTH = adrenocorticotropic hormone; CAH = congenital adrenal hyperplasia; DF = 21-deoxycortisol; DHEA = dehydroepiandrosterone; DOC = deoxycorticosterone; NCCAH = nonclassic congenital adrenal hyperplasia; T = testosterone; 17OHP = cosyntropin-stimulated 17-hydroxyprogesterone; 17OHPreg = 17-hydroxypregnenolone.

Ueland. Adrenal steroid profiling. Fertil Steril 2022.

healthy controls. The lower cutoff levels for the steroids were defined as the 2.5th percentile with 90% CI in healthy controls. The need for dividing reference data into subgroups (partitioning) for age and sex was tested according to Harris and Boyd's criteria (17).

Ethics

The study was approved by the local ethics committee (REK nr: 2016/174) and all participants signed the informed consent form.

RESULTS

Healthy Controls

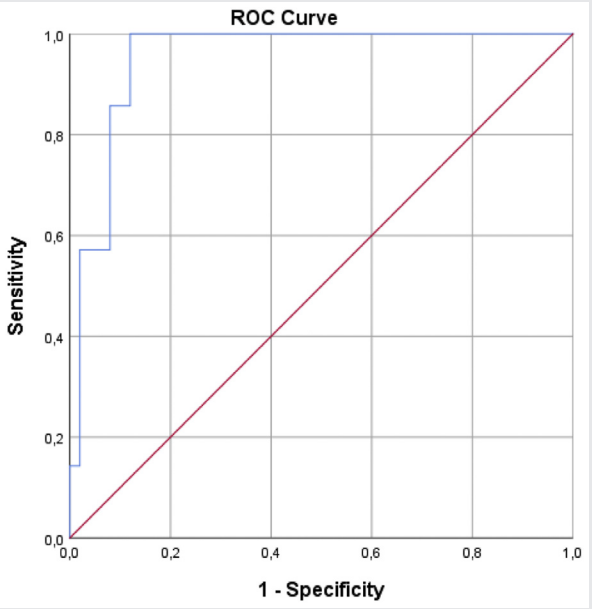
A total of 121 healthy controls (54 men, and 67 women) not using oral estrogens were included in the study. The median age was 40 (range 23–68). Table 1 reports basal and stimulated levels as median concentration and upper and lower cutoff levels (97.5% and 2.5% percentiles, respectively) for the 11 steroid hormones determined. Cortisol levels have been reported previously (14). Based on Dixons criteria, 2 outliers were identified and excluded (1 man with high basal 11DF, and 1 women with high basal 17OHPreg).

All 21DF levels were <0.25 nmol/L (0.09 ng/mL), and hence the 2.5/97.5 centile could not be determined. The 97.5th centile for 60 minutes 21DF in the total cohort was 1.7 (90% CI 1.2–2.0) nmol/L (0.60 [0.42–0.69] ng/mL).

There was a significant negative correlation between age and all steroid hormones at baseline, except for cortisol and corticosterone. Sixty minutes after stimulation there was a negative correlation between age vs. DHEA and 17OHPreg. The need for partitioning into different age groups was tested according to Harris and Boyd (17), and found necessary for T and A4 only. For these analytes the cohort was divided into age groups 18–49 years (n = 102; 56 women and 46 men) and >50 years (n =19; 11 women and 8 men). Age specific cutoff levels are given in Table 1.

For 3 analytes there was a statistically significant difference in concentration level between sexes. Females had significantly higher 17OHP levels at basal conditions (P=.0001), but not after cosyntropin stimulation (P=.1229). The 97.5th centile was 5.7 nmol/L (1.9 ng/mL) for women and 3.4 nmol/L (1.1 ng/mL) for men (0 minutes). The 97.5th centile for 60 minutes 17OHP in the total cohort was 8.5 (90% CI, 7.7–9.9) nmol/L (2.8 [2.5–3.3] ng/mL). For A4 the 97.5th centile at basal conditions was 8.5 nmol/L (2.4 ng/mL, women 18–49 years) and 4.9 nmol/L (1.4 ng/mL, men 18–49 years). The 97.5th centile 60 minutes after stimulation

FIGURE 1



Receiver operating characteristic curve of 17OHP 60 minutes after stimulation with cosyntropin.

Ueland. Adrenal steroid profiling. Fertil Steril 2022.

was 11 nmol/L (3.2 ng/mL, women 18–49 years) and 7.3 nmol/L (2.1 ng/mL, men 18–49 years), respectively. For the age group of >50 years, the 97.5th centile could not be calculated because of a low number of individuals. For T no change 30 or 60 minutes after stimulation with cosyntropin was observed (women and men). The 97.5th centile at basal conditions for women and men and at different ages are given in Table 1.

Healthy Controls Using Oral Estrogens

A total of 17 healthy controls were using oral estrogens. For the steroids 21DF, 11DF, DOC, and DHEA the median levels of persons with and without oral estrogens were not statistically different. The 17OHP levels in patients using oral estrogens showed significantly lower levels than healthy control persons not taking oral estrogens. The median cortisone and corticosterone levels in the patients using oral estrogens were significantly higher than in healthy control persons without oral estrogens (for Cohort only 60 minutes after stimulation).

Validation Cohort

A total of 63 patients, attending the outpatient endocrine clinic at Haukeland University Hospital, were enrolled in the study (98.3% women). In addition, 2 patients with classic CAH diagnosed at Hamar Hospital in the same period were included. Their median age was 29 (19–66) years (Table 2). Eight patients had genetically verified NCCAH, of which 3 were homozygote, 5 heterozygote carriers. In addition, 2 had classic CAH. The other 55 patients were not diagnosed with NCCAH.

The results of the steroid profiling in the verification cohort are presented in Table 2. Of the patients not diagnosed with NCCAH, 4 had elevated basal 17OHP above our suggested sex-specific cutoff of 5.7 nmol/L (1.9 ng/mL, women), and 2 of them also showed elevated 17OHP after 60 minutes. All 4 had normal genotyping for NCCAH. We also noticed that patients suspected of having NCCAH (but found to be healthy) showed significantly higher testosterone levels than healthy controls ($P<.01$), compared to healthy controls, 14 (25%) of the women showed a value above the suggested cutoff for women.

Eight of 63 patients (12.5%) tested for NCCAH were heterozygous or homozygous for the condition. Supplemental Table 1 (available online) shows the 17OHP and 21DF results for each patient diagnosed with NCCAH (all women) (8). The 2 patients with classic CAH had unstimulated and stimulated values above our suggested cutoff levels. The heterozygote carriers showed basal or stimulated 17OHP levels above our suggested cutoffs, but normal basal and stimulated 21DF. The patients homozygote for NCCAH also had elevated 17OHP levels above the cutoff (basal and/or stimulated), and 21DF was positive in 2 of 3 subjects.

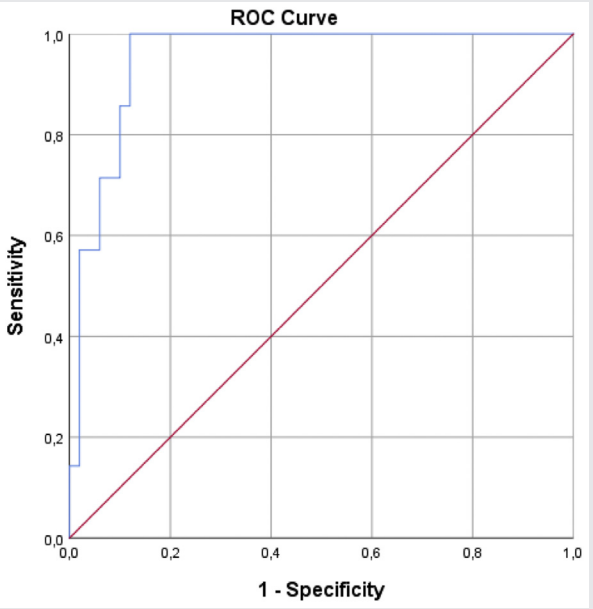
Receiver operating characteristic (ROC) curves for basal 17OHP and 21DF and stimulated 17OHP and 21DF yielded an area under the curve of 0.69 (0.48–0.90), 0.63 (0.39–0.86), 0.95 (0.89–1.0), and 0.75 (0.53–0.96), respectively. The 17OPH value after stimulation (Fig. 1) showed the best ability to discriminate healthy from diseased, and at our suggested cutoff of 8.5 nmol/L (2.8 ng/mL) the sensitivity was 71% and the specificity 98%. The optimal cutoff for stimulated 17OHP according to the ROC calculations were 8.0 nmol/L (2.6 ng/mL) with a sensitivity of 86% and specificity of 88%. The 2 patients with classic CAH were excluded from the ROC calculations. A combined ROC for stimulated 17OHP and stimulated 21DF showed an even better diagnostic accuracy than 17OHP stimulated alone, with an area under the curve of 0.95 (0.90–1.0; $P<.01$; Fig. 2).

DISCUSSION

From the 63 patients evaluated for possible NCCAH because of hirsutism, infertility, and/or an atypical presence of PCOS, 8 patients (12.6%) were diagnosed with homozygote or heterozygote NCCAH. This is important information as NCCAH may affect fertility, and some of these patients may improve fertility by a period of steroid treatment (18). We suggest screening for NCCAH to be of more concern in the assessment of infertility.

We report basal and cosyntropin-stimulated normal ranges for 17OHP and 21DF determined by highly specific LC-MS/MS methodology, and apply these in the diagnostics of NCCAH. In addition, cutoff levels for 10 other steroids that can be used for diagnostics of other more uncommon variants of NCCAH than the ones affecting 21OHD activity. The use of serum steroid profiling in the workup of NCCAH is attractive because it is easy to perform, and adrenal steroid precursors will accumulate or decrease depending on the

FIGURE 2



Receiver operating characteristic curve of the combination of 17OHP and 21DF 60 minutes after stimulation with cosyntropin.

Ueland. Adrenal steroid profiling. Fertil Steril 2022.

pathway position of the defect enzyme. All biomarkers showed consistency with only few outliers in healthy subjects. The data support that age and sex-specific cutoff levels are not required for 7 of the 8 steroids given in Table 1. Sex-specific cutoff levels were presented for 17OHP, A4, and T and in addition the cutoff levels for A4 and T were partitioned by age.

With reduced 21OH enzyme activity, 17OHP and 21DF will increase in basal and stimulated samples, and potentially also 17OHPreg, T, A4, and DHEA. The 11DF, DOC, corticosterone, and cortisol levels may be lower as a result of decreased function of the enzyme. However, the levels depend on the degree of enzyme function. The respective cutoff levels for basal and 60 minutes are presented in Table 1. Stimulated levels of cortisol >485 nmol/L (176 ng/mL, 60 minutes), 17OHP <8.5 nmol/L (2.8 ng/mL, 60 minutes), and 21DF <1.7 nmol/L (0.60 ng/mL), as well as basal 21DF <0.25 nmol/L (0.09 ng/mL) indicate normal 21OH activity.

The cutoff levels were validated in an independently collected cohort of patients referred to the hospital's endocrine outpatient clinic because of hirsutism and/or fertility problems, and clinical suspicion of NCCAH. We found that stimulated 17OHP with a cutoff of 8.5 nmol/L (2.8 ng/mL) was the best marker to discriminate healthy from the diseased (homozygote and/or heterozygote). The ROC analysis suggested an even lower cutoff level of 8.0 nmol/L (2.6 ng/mL) as the optimal cutoff. We previously presented similar cutoff levels for stimulated 17OHP measured by LC-MS/MS; however, this work was done using an LC-MS/MS method at a different laboratory, and the difference in cutoff of 0.5 nmol/L (0.17 ng/mL) is in the range of what is expected based on method variation. Nevertheless, the stimulated 17OHP cutoff level is considerably lower than that of Merke and Bornstein (9) of 30–45 nmol/L (10–15 ng/mL), which was based on older immunoassays.

On the other hand, lowering the cutoff level for 17OHP will lead to an increased number of false-positive results, and a more widespread use of genetic testing. In our validation cohort only 2/63 (3.2%) had elevated stimulated 17OHP without having NCCAH, and we consider this not a clinically significant problem.

Based on our data, albeit with a limited number of patients with NCCAH, accumulation of 21DF is more frequent in homozygote patients with NCCAH compared with heterozygote carriers. This suggests that there is some 17OHP accumulation before the enzyme 11β-hydroxylase gets active and converts 17OHP to 21DF. One of 3 homozygote patients with NCCAH showed elevated basal 21DF and normal basal 17OHP. This implies an additional diagnostic performance using 21DF as a supplement to 17OHP, but the numbers are too small to perform any accuracy calculations for 21DF.

From a clinical viewpoint, it is important to find the low graded NCCAH and the heterozygote carriers. Patients who are admitted with suspicion of NCCAH typically present with infertility and/or hirsutism. If the 21OHD activity is reduced and androgen overproduction is seen, glucocorticoid treatment could be a treatment option for a period or permanently. Glucocorticoid treatment may improve fertility, and a formal diagnosis will make it easier to get dermatologic help (electrolysis) for the hirsutism. In addition, heterozygote carriers are frequent in the community (the prevalence varying from 1/25-1/10 adults) (19), and identifying them could be of importance because of the potential risk of offspring with more severe NCCAH if a deletion, macro/micro conversions or variants of a pseudogene for CYP21A2 on chromosome 6 is inherited from both parents.

The approach presented here also can be used to diagnose patients with rarer enzyme defects, as the LC-MS/MS methods are traceable to international reference materials, enabling comparison of results across methods, time, and locations. In a patient with 11β-hydroxylase deficiency, corticosterone and cortisol may be lowered, as they are located downstream to the enzyme defect. Also, DOC and 11DF may be increased, because of accumulation. Cortisol stimulated >485 nmol/L (176 ng/mL, 60 minutes), corticosterone stimulated >41 nmol/L (14 ng/mL, 60 minutes), DOC <1.7 nmol/L (0.56 ng/mL, 60 minutes), and 11DF <5.1 nmol/L (1.8 ng/mL, 60 minutes) may indicate normal 11β-hydroxylase activity. In a patient with 3β-hydroxysteroid dehydrogenase deficiency, 17OHP, and A4 may be downregulated. The 17OHPreg and DHEA may be upregulated. The 17OHP stimulated >1.2 nmol/L (0.40 ng/mL, 60 minutes), A4 stimulated (60 minutes) >11 nmol/L (3.2 ng/mL, women) or 7.3 nmol/L (2.1 ng/mL, men), 17OHPreg <41 nmol/L (14 ng/mL, 60 minutes), and DHEA <115 nmol/L (33 ng/mL, 60 minutes) may indicate normal 3β-hydroxysteroid dehydrogenase activity.

Our data strongly show that even when we have the ability to perform a full steroid profiling of the patients, there is a need for cosyntropin stimulation with 17OHP measurements for the diagnostics of NCCAH. The 17OHP remains the best marker for the diagnosis, but stimulated 21DF could be an

important supplement to increase the diagnostic accuracy. To identify NCCAH and also heterozygote carriers, it is necessary to lower the diagnostic cutoff level from today's 30–45 nmol/L (10–15 ng/mL) for stimulated 17OHP, as we show here, and have shown previously in a smaller cohort of patients (14).

The 17 patients in our study who used oral estrogens showed overall lower 17OHP than healthy controls. This suggests that the use of oral estrogens could interfere with 17OHP as a screening test for NCCAH. Some of the healthy controls not using oral estrogens may have been tested in the late follicular or early luteal phase. This may be an explanation for 17OHP values in this group being higher compared to women on estrogen supplements. Hence, the patient should repeat the test a minimum of 4 weeks after estrogens have been withdrawn, preferably in the follicular phase in regularly menstruating women. In addition, cortisol, and cortisone and corticosterone were elevated in healthy controls on estrogens, as suspected, as these steroids are binding to cortisol binding globulin, which is increased when oral estrogens are used (20).

One major weakness of our study is the low number of individuals with diagnosed NCCAH in the validation cohort. Furthermore, the lack of patients with rare enzyme defect makes it impossible to validate the cutoff levels suggested for diagnosing these conditions.

CONCLUSION

In conclusion, the cosyntropin stimulation test still is needed in the diagnostic workup of patients with NCCAH. The 17OHP 60 minutes after stimulation with cosyntropin is the best biomarker, and the cutoff (8.5 nmol/L/2.8 ng/mL) is substantially lower than recommended in current European guidelines. Basal and stimulated 21DF could be a valuable supplement in diagnosing NCCAH and possibly also other rare enzyme defects in the adrenals. As we have identified a high degree of heterozygote and homozygote NCCAH cases among female patients with PCOS (12.5%), we propose screening for the condition to be more frequent in the diagnostic workup of patients with infertility and oligomenorrhea.

Acknowledgments: The authors thank Mrs. Nina Jensen, Mona Eliassen, Gro M. Olderøy, Elisabeth T. Halvorsen, Kari Julien, and Liv Hanne Bakke for handling samples and registration forms.

DIALOG: You can discuss this article with its authors and other readers at https://www.fertstertdialog.com/posts/34731

REFERENCES

1. Costa-Barbosa FA, Carvalho VM, Nakamura OH, Bachega TA, Vieira JG, Kater CE. Zona fasciculata 21-hydroxysteroids and precursor-to-product ratios in 21-hydroxylase deficiency: further characterization of classic and non-classic patients and heterozygote carriers. J Endocrinol Invest 2011;34:587–92.
2. Speiser PW, White PC. Congenital adrenal hyperplasia. N Engl J Med 2003;349:776–88.
3. Forest MG. Recent advances in the diagnosis and management of congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Hum Reprod Update 2004;10:469–85.
4. White PC, Speiser PW. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. Endocr Rev 2000;21:245–91.
5. Azziz R, Dewailly D, Owerbach D. Clinical review 56: nonclassic adrenal hyperplasia: current concepts. J Clin Endocrinol Metab 1994;78:810–5.
6. Tonetto-Fernandes V, Lemos-Marini SH, Kuperman H, Ribeiro-Neto LM, Verreschi IT, Kater CE. Serum 21-deoxycortisol, 17-hydroxyprogesterone, and 11-deoxycortisol in classic congenital adrenal hyperplasia: clinical and hormonal correlations and identification of patients with 11beta-hydroxylase deficiency among a large group with alleged 21-hydroxylase deficiency. J Clin Endocrinol Metab 2006;91:2179–84.
7. Fiet J, Gueux B, Gourmelen M, Kuttenn F, Vexiau P, Couillin P, et al. Comparison of basal and adrenocorticotropin-stimulated plasma 21-deoxycortisol and 17-hydroxyprogesterone values as biological markers of late-onset adrenal hyperplasia. J Clin Endocrinol Metab 1988;66:659–67.
8. Ueland GA, Dahl SR. Supplemental material: adrenal steroid profiling as a diagnostic tool to differentiate PCOS from non-classical congenital adrenal hyperplasia. Pinpointing easy screening possibilities and normal cut-off levels using LC-MS/MS. Zenodo. Available at: https://zenodo.org/record/6503942#.YpRmkqhBxaQ. Accessed May 6, 2022.
9. Merke DP, Bornstein SR. Congenital adrenal hyperplasia. Lancet 2005;365:2125–36.
10. Kushnir MM, Rockwood AL, Bergquist J. Liquid chromatography-tandem mass spectrometry applications in endocrinology. Mass Spectrom Rev 2010;29:480–502.
11. Stanczyk FZ, Clarke NJ. Advantages and challenges of mass spectrometry assays for steroid hormones. J Steroid Biochem Mol Biol 2010;121:491–5.
12. Rosner W, Auchus RJ, Azziz R, Sluss PM, Raff H. Position statement: utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. J Clin Endocrinol Metab 2007;92:405–13.
13. Rosner W, Vesper H. Preface. CDC workshop report improving steroid hormone measurements in patient care and research translation. Steroids 2008;73:1285.
14. Ueland GA, Methlie P, Oksnes M, Thordarson HB, Sagen J, Kellmann R, et al. The short cosyntropin test revisited: new normal reference range using LC-MS/MS. J Clin Endocrinol Metab 2018;103:1696–703.
15. Dahl SR, Nermoen I, Bronstad I, Husebye ES, Lovas K, Thorsby PM. Assay of steroids by liquid chromatography-tandem mass spectrometry in monitoring 21-hydroxylase deficiency. Endocr Connect 2018;7:1542–50.
16. Reed AH, Henry RJ, Mason WB. Influence of statistical method used on the resulting estimate of normal range. Clin Chem 1971;17:275–84.
17. Harris EK, Boyd JC. On dividing reference data into subgroups to produce separate reference ranges. Clin Chem 1990;36:265–70.
18. New MI, Ghizzoni L, Meyer-Bahlburg H, Khattab A, Reichman D, Rosenwaks Z. Fertility in patients with nonclassical congenital adrenal hyperplasia. Fertil Steril 2019;111:13–20.
19. Livadas S, Bothou C. Management of the female with non-classical congenital adrenal hyperplasia (NCCAH): a patient-oriented approach. Front Endocrinol (Lausanne) 2019;10:366.
20. Panton KK, Mikkelsen G, Irgens WO, Hovde AK, Killingmo MW, Oien MA, et al. New reference intervals for cortisol, cortisol binding globulin and free cortisol index in women using ethinyl estradiol. Scand J Clin Lab Invest 2019;79:314–9.

Perfil de esteroides adrenales como herramienta diagnóstica para diferenciar síndrome de ovario poliquístico de hiperplasia adrenal congénita no clásica: señalando posibilidades de fácil cribado y niveles de corte normales utilizando cromatografía líquida con espectrometría de masas en tandem.

Objetivo: Definir niveles de corte basados en cromatografía líquida con espectrometría de masas en tándem (LC-MS/MS) y paneles de hormonas esteroideas, para mejorar el diagnóstico de hiperplasia adrenal congénita no clásica (NCCAH) y otros defectos enzimáticos parciales en las adrenales.

Diseño: Análisis prospectivo de cohortes.

Lugar: Centro endocrinológico terciario basado en hospital universitario.

Paciente(s): Ciento veintiún adultos saludables y 65 pacientes evaluadas por posible NCCAH (cohorte de validación).

Intervención(es): Los puntos de corte determinados por LC-MS/MS para 11 esteroides (basal y estimulado por cosintropina) fueron definidos por los percentilos 2.5% y 97.5% en sujetos sanos. La cohorte de validación fue utilizada para comparación.

Medida(s) de resultado(s) principal(es): Porcentaje de pacientes diagnosticadas con NCCAH entre las pacientes con sintomatología tipo sindrome de ovario poliquístico (PCOS). Evaluación de los niveles de corte definidos basados en LC-MS/MS para hormonas esteroideas entre este grupo de pacientes.

Resultado(s): De las 65 pacientes tipo PCOS evaluadas por posible NCCAH, 8 (12.5%) fueron halladas y genéticamente verificadas, y 2 tuvieron hiperplasia adrenal congénita clásica. La 17-hidroxiprogesterona (17OHP) estimulada por cosintropina mostró la mejor eficacia diagnóstica para NCCAH con un área bajo la curva de 0.95 (0.89-1.0) con una sensibilidad de 86% y una especificidad de 88%. En pacientes homocigotas, los niveles de 21-desoxicortisol y 17OHP estaban elevados, en pacientes heterocigotas sólo 17OHP (basal y estimulado) estaba elevado. Cuatro pacientes sanas en la cohorte de validación tuvieron 17OHP por encima del punto de corte basal.

Conclusión(es): El sindrome NCCAH es frecuente en pacientes con sospecha de PCOS, y debería ser considerado como un cribado de rutina cuando se evalúa infertilidad. Nosotros sugerimos el empleo de perfiles de esteroides séricos, incluyendo 21-desoxicortisol, junto con la prueba de estimulación con cosintropina de 17OHP. Nuestros datos respaldan un punto de corte de 8.5 nmol/L (2.8 ng/mL) 60 minutos después de la estimulación con cosintropina, cuando se mide con LC-MS/MS, significativamente más bajo que las guías Europeas actuales.