Screening for Nonclassic Congenital Adrenal Hyperplasia in the Era of Liquid Chromatography-Tandem Mass Spectrometry

Alexander D Chesover,1 Heather Millar,2 Lusia Sepiashvili,3 Khosrow Adeli,3 Mark R Palmert,1,4 and Jill Hamilton1

1Division of Endocrinology, Department of Paediatrics, The Hospital for Sick Children, University of Toronto, Toronto M5G 1H4, Canada; 2Section of Gynaecology, Division of Endocrinology, Department of Obstetrics and Gynaecology, The Hospital for Sick Children, University of Toronto M5G 1H4, Toronto, Canada; 3Department of Paediatric Laboratory Medicine, The Hospital for Sick Children, Toronto M5G 1H4, Canada; 4Department of Physiology, University of Toronto, Toronto M5S 1A8, Canada

ORCiD numbers: 0000-0002-6280-5053 (A. D. Chesover); 0000-0002-1958-2800 (J. Hamilton).

Context: Screening for and diagnosing non classic congenital adrenal hyperplasia (NCCAH) uses serum 17-hydroxyprogesterone (17OHP) thresholds established from immunoassay data; however, a new liquid-chromatography tandem mass spectrometry (LC-MS/MS) method results in lower 17OHP values. The evolution of immunoassays is also challenging our diagnostic cut-off for glucocorticoid insufficiency and few data re-evaluate the utility of testing for glucocorticoid insufficiency in NCCAH.

Objective: (1) Evaluate the 17OHP threshold that predicts NCCAH in children using LC-MS/MS, and (2) determine the prevalence of glucocorticoid insufficiency in NCCAH.

Methods: A retrospective chart review of pediatric patients who underwent ACTH stimulation tests with cortisol and 17OHP measurements from 2011 to 2018 for assessment of NCCAH. Other adrenal pathologies were excluded. A cortisol < 415 nmol/L defined glucocorticoid insufficiency. Published correlation data determined a 17OHP of 3.3 nmol/L by LC-MS/MS was equivalent to 6 nmol/L by immunoassay. Data analysis was by measures of diagnostic accuracy.

Results: Of 188 patients included, 23 (12%) had NCCAH (21/23 had genetic confirmation); the remaining 2 had peak 17OHP > 30 nmol/L. Baseline 17OHP ≥ 6 nmol/L most accurately screened for NCCAH—sensitivity and specificity 96%. Almost all genetically confirmed NCCAH (20/21) had peak 17OHP > 30 nmol/L; all subjects with other diagnoses peaked < 30 nmol/L. Glucocorticoid insufficiency was present in 55% with NCCAH.

Conclusions: Despite the increased specificity of LC-MS/MS, a baseline 17OHP ≥ 6 nmol/L most accurately screened for NCCAH; this supports current practice guidelines. This threshold identified all with glucocorticoid insufficiency, notably prevalent in our cohort and for whom glucocorticoid stress dosing should be considered.

© Endocrine Society 2019.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs licence (http://creativecommons.org/licenses/by-nc-nd/4.0/), which permits non-commercial reproduction and distribution of the work, in any medium, provided the original work is not altered or transformed in any way, and that the work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Freeform/Key Words: congenital adrenal hyperplasia, 17-hydroxyprogesterone, mass spectrometry, 21-hydroxylase, cortisol, pediatrics
Congenital adrenal hyperplasia (CAH) is caused by an enzymatic defect in steroid biosynthesis, most commonly by a mutation in CYP21A2 (the gene encoding 21-hydroxylase), and its inheritance is autosomal recessive. Nonclassic congenital adrenal hyperplasia (NCCAH) has a milder phenotype, where usually 20–50% of enzyme activity is retained; it presents in childhood with signs of androgen excess and a variable degree of glucocorticoid insufficiency.

Diagnosis in childhood has important clinical implications. Androgen excess, for which glucocorticoid treatment is recommended [1, 2], can cause premature closure of growth plates and short adult height. An individual with NCCAH has an increased chance of having offspring with NCCAH or classic CAH [3]; genetic counselling can support family planning. Children with NCCAH can have glucocorticoid insufficiency [1, 4] for which providing glucocorticoid stress dose advice is recommended to reduce the risk of adrenal crisis [2].

NCCAH can be clinically indistinguishable from, for example, benign premature pubarche or polycystic ovarian syndrome—conditions also seen in pediatrics. Individuals with symptoms or clinical features may undergo screening using a morning serum 17-hydroxyprogesterone (17OHP). Clinical practice guidelines recommend that a result less than 6 nmol/L excludes CAH and greater than 30 nmol/L is diagnostic; 6–30 nmol/L prompts a 250 mcg ACTH stimulation test where a 60-minute peak above 30 nmol/L is diagnostic [2]. These thresholds are for the screening and diagnosis of CAH from 21-hydroxylase deficiency and not other enzyme defects.

Liquid-chromatography tandem mass spectrometry (LC-MS/MS) has become the recommended method for 17OHP measurement for NCCAH screening [2]; however, immunoassay data informs the current 17OHP screening and diagnostic thresholds [5–8]. In comparison to immunoassays, LC-MS/MS offers increased analytical specificity, the ability for multiplex analysis (simultaneously measure multiple analytes), and the advantage of using minute specimen volumes. In 2011, our institution introduced LC-MS/MS to measure 17OHP and based on method comparison data, 6 nmol/L on immunoassay is equivalent to 3.3 nmol/L on LC-MS/MS [9].

There is uncertainty whether using a more specific 17OHP assay warrants lowering the threshold for NCCAH screening. We hypothesize the change to LC-MS/MS increases false-negative screening results, therefore prompting a reduction of the threshold 17OHP value to exclude NCCAH.

Glucocorticoid insufficiency is traditionally defined by a peak cortisol less than 500 nmol/L on a 250 mcg ACTH stimulation test [10]. However, this threshold is being challenged in light of more specific immunoassays [11, 12]. A systematic review suggests a threshold of 415 nmol/L is more appropriate [13].

A more accurate NCCAH screening approach would increase the rate of diagnosis and allow patient care to address target height, genetic counselling, and glucocorticoid insufficiency. Thus, we performed a retrospective analysis of ACTH stimulation tests since changing to LC-MS/MS measurement of 17OHP to determine the 17OHP threshold that best screens for NCCAH in children. A secondary aim is to establish the prevalence of glucocorticoid insufficiency in our cohort of patients and whether this can be predicted by the screening 17OHP.

1. Methods

A. Study Design

Chart review was performed for patients under age 18 years undergoing an ACTH stimulation test at The Hospital for Sick Children, Toronto, when both 17OHP and cortisol results were measured. The 17OHP measurement at time 0 minutes of the ACTH stimulation test (baseline 17OHP) was regarded as the screening 17OHP for NCCAH. Data were collected from 2011—from the time LS-MS/MS for 17OHP was introduced—until 2018. Clinical, biochemical, and genetic data were collected from the electronic medical record.
NCCAH was confirmed by molecular genetic analysis of CYP21A2 or, if unavailable, by a 60-minute peak 17OHP (after ACTH stimulation) above 30 nmol/L. NCCAH was distinguished from classic CAH by an onset of symptoms after infancy, absence of salt-wasting, and associated genetic mutation (where available). Other diagnoses were confirmed by documentation in the chart or, if unavailable, an alternative diagnosis was presumed by a peak 17OHP less than 30 nmol/L. Glucocorticoid insufficiency was defined as a peak cortisol less than 415 nmol/L at 60 min during a 250 mcg ACTH stimulation test.

B. Assays

17OHP quantification was achieved using an LC-MS/MS method validated for simultaneous measurement of eight steroid hormones on an AB Sciex 4000 QTRAP mass spectrometer with a total run time of 10 minutes. 17OHP assay performance characteristics were as follows: low limit of quantification of 0.05 nmol/L (CV of 6.1%), analytical measurement range of 0.05–160 nmol/L, and intra and interassay imprecision less than 10% [9]. Prior to September 2013, cortisol was measured on the Siemens Immulite 2500, as described previously [9]. From September 2013, cortisol was measured by a chemiluminescent immunoassay on the Abbot Architect i2000. Both methods were controlled according to the manufacturer’s instructions.

C. Statistical Analyses

All patients meeting the above inclusion criteria underwent chart review. Patients with other adrenal pathology were excluded from further analysis. Patients taking glucocorticoids at the time of testing were excluded from analysis to determine the prevalence of glucocorticoid insufficiency.

Descriptive statistics characterised the cohort using mean, standard deviation, median, and range, where appropriate. The accuracy of 17OHP to screen for and diagnose NCCAH was evaluated by: sensitivity, specificity, false-positive and false-negative rates, positive and negative predictive values, and receiver operating characteristic curve analysis. Youden’s index was used to generate the most accurate cut-off by optimising both sensitivity and specificity [14].

D. Ethics

This study received research ethics board approval from The Hospital for Sick Children, Toronto.

2. Results

In total, 188 patients met inclusion criteria: 23/188 (12%) had NCCAH and 165/188 (88%) had Other Diagnoses. Genotype confirmation was available for 21/23 with NCCAH (Table 1). Seven individuals were excluded for: adrenocortical carcinoma (n = 2), adrenal insufficiency (n = 2), previously diagnosed simple virilized CAH (n = 1), X-linked adrenoleukodystrophy (n = 1), and suspected autoimmune adrenal insufficiency (n = 1). A total of 181 (158 female; 87%) were included in final analyses (Fig. 1). The majority of those included with documented Other Diagnoses had hyperandrogenism (31/77)—from polycystic ovarian syndrome, premature pubarche, adrenarche, hirsutism, or Leydig cell tumor. There was no difference in sex between the NCCAH and the Other Diagnoses group ($P = 0.66$). Those with NCCAH were younger (10.4 ± 3.8 years) than those with Other Diagnoses (12.8 ± 4.2 years); $P = 0.009$.

A. Baseline 17OHP

The median baseline 17OHP in NCCAH was 16.0 nmol/L [2.5–110] and in Other Diagnoses was 1.5 nmol/L [0.1–11.9]; $P < 0.001$ (Fig. 2A). The baseline 17OHP was above 6 nmol/L in
Table 1. Genotype of Patients With Nonclassic Congenital Adrenal Hyperplasia

| Genotype                     | n = 23 |
|------------------------------|--------|
| V282L/V282L                  | 9      |
| V282L/I173N                  | 3      |
| V282L/deletion               | 2      |
| V282L/R484P                  | 1      |
| V282L/P454S                  | 1      |
| V282L/Q319*                  | 1      |
| V282L/V384G a                | 1      |
| I173N/A435V                  | 1      |
| P454S/gene conversion event b| 1      |
| P30L/splicing variant c      | 1      |
| Unknown                      | 2      |

All listed variants are known to cause congenital adrenal hyperplasia unless otherwise stated.

- c.1151T>G, variant of unknown significance, predicted to substitute a moderately conserved valine to glycine, and in silico analysis predicts to be both damaging and benign by two different methods.

- Introduces 4 mutations into promotor region: c.-126>C>T, c.-113>G>A, c.-110>T>C, and c.-103>A>G.

- c.293-13A>C > G

Figure 1. Diagram showing, from 188 patients included for chart review, how many patients were: excluded, included in final analyses, and diagnosed with NCCAH. aWhen molecular genetic diagnosis was not available and the peak 17OHP 60 minutes after ACTH stimulation was > 30 nmol/L. Two patients without molecular genetic diagnosis had peak 17OHP of 198 and 91.9 nmol/L. bWhen another diagnosis was recorded in the patient chart. cWhen another diagnosis was not recorded in the patient chart and the peak 17OHP 60 minutes after ACTH stimulation was < 30 nmol/L. Abbreviations: 17OHP, 17-hydroxyprogesterone; NCCAH, nonclassic congenital adrenal hyperplasia.

all NCCAH patients with the exception of one individual—this patient had genetically confirmed NCCAH and a peak 17OHP 73.8 nmol/L with a baseline 17OHP 2.5 nmol/L. A baseline 17OHP cut-off of ≥ 6 nmol/L has the same sensitivity and negative predictive value (NPV) but better specificity and positive predictive value (PPV) than using ≥ 3.3 nmol/L to screen for NCCAH (Table 2). The area under the receiver operating characteristic (ROC) curve (Fig. 3A) is 0.98 (95% CI 0.96–1.00, P < 0.001), which generates an optimal cut-off for diagnosing NCCAH as a baseline 17OHP level of 5.8 nmol/L.

B. Peak 17OHP

The median peak 17OHP in NCCAH was 161 nmol/L [15.3–358] and in Other Diagnoses 4.1 nmol/L [1.0–26.1]; P < 0.001 (Fig. 2B). Peak 17OHP was above 30 nmol/L in all NCCAH
patients except one individual who had genetically confirmed NCCAH with a peak 17OHP 15.3 nmol/L and a baseline 17OHP 8.7 nmol/L (no glucocorticoid treatment at the time of this testing).

The peak 17OHP was below 30 nmol/L in all Other Diagnoses. The peak 17OHP was between 20–30 nmol/L in 3/158 with Other Diagnoses; this is highlighted in light of the negative bias of the LC-MS/MS assay, which means that 20 nmol/L by LC-MS/MS equates to 30 nmol/L on our previous immunoassay [9]. One of these three was a CAH carrier, one had aldosterone synthase deficiency, and for one there was insufficient clinical data. A peak 17OHP cut-off of > 30 nmol/L has the same sensitivity and NPV but better specificity and PPV than using a cut-off of > 20 nmol/L (Table 3). The area under the ROC curve (Fig. 3B)

![Figure 2](image-url)
is 0.999 (95% CI 0.995–1.000, $P < 0.001$), which generates an optimal cut-off for diagnosing NCCAH as a peak 17OHP level of 14.8 nmol/L.

**C. Glucocorticoid Insufficiency**

When using the traditional peak cortisol of 500 nmol/L 60 minutes after ACTH stimulation as a diagnostic cortisol cut-off, 19/22 (86%) with NCCAH and 3/158 (2%) with Other Diagnoses had glucocorticoid insufficiency. Alternatively, using 415 nmol/L as a cortisol...
cut-off diagnosed 12/22 (55%) of the NCCAH group and none with Other Diagnoses as having glucocorticoid insufficiency.

All of those with NCCAH and glucocorticoid insufficiency, when using either diagnostic cortisol cut-off, had a baseline 17OHP ≥ 6 nmol/L.

When defining glucocorticoid insufficiency as cortisol less than 415 nmol/L, 7/12 (58%) were subsequently treated with glucocorticoids, 2/12 (17%) were not, and in 3/12 (25%) this was not documented in the medical record.

### 3. Discussion

We found that a baseline 17OHP of ≥ 6 nmol/L measured by LC-MS/MS remains the most accurate cut-off for NCCAH screening; and > 30 nmol/L at 60 minutes after a 250 mcg ACTH stimulation test remains the most accurate cut-off for NCCAH diagnosis. These data do not support our hypothesis of the need to reduce these thresholds in light of the negative bias demonstrated by LC-M/MS against immunoassay [9]. Despite these results supporting the ongoing use of the current guidelines for NCCAH screening and diagnosis [2], this is a valuable re-evaluation of our clinical cut-offs in the current era of implementing more specific assays.

We also demonstrated that a significant proportion of individuals with NCCAH have glucocorticoid insufficiency – both at the traditional cortisol cut-off of 500 nmol/L and lower cortisol cut-off of 415 nmol/l. This finding should prompt consideration of glucocorticoid stress dose education for these patients. All patients with glucocorticoid insufficiency, regardless of which diagnostic threshold was used, were identified using a 17OHP screening cut-off of ≥ 6 nmol/L.

The calculated sensitivity and specificity for baseline 17OHP in diagnosing NCCAH in this study is comparable to previous studies (which all used immunoassay), but accuracy will vary across different populations. For example, in 238 patients with precocious puberty, 10/238 had NCCAH and a baseline 17OHP cut-off of ≥ 6 nmol/L was 100% sensitive and 99% specific [6]. However, in two other studies 6/280 (2.1%) and 4/58 (7%) patients with genetically confirmed NCCAH had a baseline 17OHP less than 6 nmol/L [15, 16]; a finding that is comparable to our data showing 1/23 (4%) had a baseline 17OHP less than 6 nmol/L.

LC-MS/MS is a more specific assay than immunoassay both in adult and pediatric healthy subjects [9, 17–19]. A comparison in 55 adults with CAH concluded that LC-MS/MS is preferred to immunoassay for monitoring glucocorticoid therapy due to its increased specificity and decreasing the risk of glucocorticoid overtreatment [20]. An observational study of 39 women with hyperandrogenism and 29 controls showed that using LC-MS/MS to screen for CAH reduced false positive results and the number of unnecessary ACTH stimulation tests [21]. However, their cohort was small: only 2 patients were diagnosed with CAH; their study was only in adult women; and their screening 17OHP cut-off for CAH was ≥ 5.1 nmol/L, whereas current guidelines use ≥ 6 nmol/L [2]. The authors’ conclusion for improved diagnostic accuracy using LC-MS/MS was extrapolated from method comparison data alone, whereas our study used the method comparison data but also clinically re-evaluated the 17OHP cut-off for screening when measured by LC-MS/MS.

### Table 3. Accuracy of Peak 17OHP to Diagnose NCCAH

| 17OHP (nmol/L) | NCCAH (n = 23) | Other Diagnoses (n = 151) | Sensitivity | Specificity | Positive Predictive Value | Negative Predictive Value | False Negative | False Positive |
|---------------|----------------|--------------------------|-------------|-------------|--------------------------|---------------------------|---------------|---------------|
| > 20          | 22             | 3                        | 96%         | 98%         | 88%                      | 99%                       | 4%            | 2%            |
| > 30          | 22             | 0                        | 96%         | 100%        | 100%                     | 99%                       | 4%            | 0%            |

Abbreviations: 17OHP, 17-hydroxyprogesterone; NCCAH, nonclassic congenital adrenal hyperplasia.
The clinical efficacy of NCCAH screening requires a high NPV to confidently rule out the diagnosis; both cut-offs in our cohort, for baseline and peak 17OHP, resulted in a 99% NPV. On the other hand, a high PPV increases clinical confidence in correctly identifying a patient with NCCAH after a positive screening result; using the higher cut-offs improved the PPV for baseline 17OHP from 47% to 76% and for peak 17OHP from 88% to 100%. In addition, the ROC curve analyses are highly significant and demonstrate excellent diagnostic accuracy using the cut-offs recommended in current clinical practice guidelines even when using LC-MS/MS [2].

Our reported prevalence of glucocorticoid insufficiency in patients with NCCAH (55%) is comparable to another pediatric cohort [1] but higher than other reports [4, 22]. Individuals with NCCAH and glucocorticoid insufficiency are at a potentially increased risk of morbidity and mortality from adrenal crisis [7, 23]; however, crises have not been reported in other published cohorts [22, 24, 25]. Clinical practice guidelines recommend glucocorticoid stress dosing for patients with NCCAH and adrenal insufficiency, but this is a conditional recommendation based on very low quality evidence [2]. Re-evaluating the cortisol threshold for diagnosing primary adrenal insufficiency (from any cause) is outside the scope of this study; but this work should be undertaken to better identify individuals with NCCAH who may be at risk of adrenal insufficiency [11, 12, 26].

A strength of this study is the focus on addressing the screening and diagnostic cut-offs for NCCAH in pediatrics rather than a correlation between immunoassay and LC-MS/MS data in either healthy controls or established patients. The glucocorticoid insufficiency data contributes to a small and varied literature. Inherent challenges in defining primary glucocorticoid insufficiency include the diagnostic thresholds of newer immunoassays; the variable, sometimes subtle, symptoms of adrenal insufficiency; and the rarity of adrenal crisis [13, 23].

The study’s limitations include a small NCCAH population, which risks inflating the sensitivity and specificity of the 17OHP cut-off if patients with NCCAH and a baseline 17OHP less than 6 nmol/L exist but are not included. The baseline 17OHP samples were spread throughout the day (data not shown), whereas a screening 17OHP should be performed at 8:00 AM due to the diurnal variability of 17OHP—afternoon sampling could result in a false-negative screen. Nevertheless, the data performed comparably to previous studies in distinguishing NCCAH from other diagnoses by baseline or peak 17OHP, suggesting this variability in timing did not significantly confound the results. Those with Other Diagnoses were identified based on clinical records or their ACTH stimulation test results and not by molecular genetic analysis of CYP21A2. This is a common limitation of similar studies [5, 6, 8] and risks not identifying those with NCCAH who were presumed to have an alternative diagnosis, which could falsely elevate the accuracy of the screening test. Glucocorticoid insufficiency was diagnosed using an empirical cut-off for peak cortisol based on current clinical guidelines and best available evidence. This outcome would be better defined by clinical and biochemical evidence of an adrenal crisis; however, this is fortunately a rare event, particularly as those at risk are treated with glucocorticoids, so not a practical outcome measure for this study.

In summary, we found that the 17OHP screening and diagnostic thresholds should remain unchanged for NCCAH despite a more specific LC-MS/MS method compared with immunoassay. Glucocorticoid insufficiency in NCCAH was found to be comparable to previous reports, suggesting that a significant proportion of patients may require glucocorticoid stress dosing during illness. To provide optimal patient care, it is critical we re-evaluate screening and diagnostic cut-offs in the light of evolving assays—both locally when assays differ from reported diagnostic cut-offs and to inform best practice guidelines.

Additional Information

**Correspondence:** Jill Hamilton, The Hospital for Sick Children. 555 University Avenue, Toronto, Ontario, M5G 1X8, Canada. E-mail: jill.hamilton@sickkids.ca.

**Disclosure Summary:** No grants, fellowships, or other funding supported the writing of this paper. The authors have no conflicts of interest.
References

1. Stoupa A, González-Briceño L, Pinto G, et al. Inadequate cortisol response to the tetracosactide (Synacthen®) test in non-classic congenital adrenal hyperplasia: an exception to the rule? *Horm Res Paediatr.* 2015;83(4):262–267.

2. Speiser PW, Arlt W, Auchus RJ, et al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab.* 2018;103(11):4043–4088.

3. Moran C, Aziz R, Weintrub N, et al. Reproductive outcome of women with 21-hydroxylase-deficient nonclassic adrenal hyperplasia. *J Clin Endocrinol Metab.* 2006;91(9):3451–3456.

4. Karachaliou FH, Kafetzi M, Dracopoulou M, et al. Cortisol response to adrenocorticotropic testing in non-classical congenital adrenal hyperplasia (NCCAH). *J Pediatr Endocrinol Metab.* 2016;29(12):1365–1371.

5. Aziz R, Hincapie LA, Knochenhauer ES, Dewaily D, Fox L, Boots LR. Screening for 21-hydroxylase-deficient nonclassic adrenal hyperplasia among hyperandrogenic women: a prospective study. *Fertil Steril.* 1999;72(5):915–925.

6. Armengaud JB, Charkaluk ML, Trivin C, et al. Precocious pubarche: distinguishing late-onset congenital adrenal hyperplasia from premature adrenarche. *J Clin Endocrinol Metab.* 2009;94(8):2835–2840.

7. Bidet M, Bellanné-Chantelot C, Galand-Portier MB, et al. Clinical and molecular characterization of a cohort of 161 unrelated women with nonclassical congenital adrenal hyperplasia due to 21-hydroxylase deficiency and 330 family members. *J Clin Endocrinol Metab.* 2009;94(5):1570–1578.

8. Török D, Halász Z, Garami M, Homoki J, Fekete G, Sólyom J. Limited value of serum steroid measurements in identification of mild form of 21-hydroxylase deficiency. *Exp Clin Endocrinol Diabetes.* 2003;111(1):27–32.

9. Kyriakopoulou L, Yazdanpanah M, Colantonio DA, Chan MK, Daly CH, Adeli K. A sensitive and rapid mass spectrometric method for the simultaneous measurement of eight steroid hormones and CALIPER pediatric reference intervals. *Clin Biochem.* 2013;46(7-8):642–651.

10. Bornstein SR, Alloio B, Arlt W, et al. Diagnosis and treatment of primary adrenal insufficiency: an Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab.* 2016;101(2):364–389.

11. Kline GA, Buse J, Krause RD. Clinical implications for biochemical diagnostic thresholds of adrenal hyperplasia in healthy adults. *Clin Biochem.* 2019;68:129–138.

12. Kline GA, Holmes DT. De-evolution of diagnostic testing for adrenal insufficiency. *Ann Intern Med.* 2003;139(3):194–204.

13. Fluss R, Faraggi D, Reiser B. Estimation of the Youden Index and its associated cutoff point. *Biom J.* 2005;47(4):458–472.

14. Livadas S, Dracopoulou M, Dastamani A, et al. The spectrum of clinical, hormonal and molecular findings in 280 individuals with nonclassical congenital adrenal hyperplasia caused by mutations of the CYP21A2 gene. *Clin Endocrinol (Oxf).* 2015;82(4):543–549.

15. Bachega TA, Billeber AE, Marcondes JA, Madureira G, Arnhold IJ, Mendonca BB. Influence of different genotypes on 17-hydroxyprogesterone levels in patients with nonclassical congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Clin Endocrinol (Oxf).* 2000;52(5):601–607.

16. van der Veen A, van Faassen M, de Jong WHA, van Beek AP, Dijck-Brouwer DAJ, Kema IP. Development and validation of a LC-MS/MS method for the establishment of reference intervals and biological variation for five plasma steroid hormones. *Clin Biochem.* 2013;46(7-8):642–651.

17. Fanelli F, Belluomo I, Di Lallo VF, et al. Serum steroid profiling by isotopic dilution-liquid chromatography-mass spectrometry: comparison with current immunoassays and reference intervals in healthy adults. *Steroids.* 2011;76(3):244–253.

18. Dahl SR, Nermoen I, Brønstad I, Husebye ES, Lavås K, Thorsby PM. Assay of steroids by liquid chromatography-tandem mass spectrometry in monitoring 21-hydroxylase deficiency. *Endocr Connect.* 2018;7(12):1542–1550.

19. Ambroziuk U, Kępczyńska-Nyk A, Kurylowicz A, et al. LC-MS/MS improves screening towards 21-hydroxylase deficiency. *Gynecol Endocrinol.* 2015;31(4):296–300.

20. Pinto G, Tardy V, Trivin C, et al. Follow-up of 68 children with congenital adrenal hyperplasia due to 21-hydroxylase deficiency: relevance of genotype for management. *J Clin Endocrinol Metab.* 2003;88(6):2624–2633.
23. Falhammar H, Frisén L, Norrby C, et al. Increased mortality in patients with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J Clin Endocrinol Metab.* 2014;99(12):E2715–E2721.

24. Nandagopal R, Sinaii N, Avila NA, et al. Phenotypic profiling of parents with cryptic nonclassic congenital adrenal hyperplasia: findings in 145 unrelated families. *Eur J Endocrinol.* 2011;164(6):977–984.

25. Ghizzoni L, Cappa M, Vottero A, et al. Relationship of CYP21A2 genotype and serum 17-hydroxyprogesterone and cortisol levels in a large cohort of Italian children with premature pubarche. *Eur J Endocrinol.* 2011;165(2):307–314.

26. Ueland GÅ, Methlie P, Øksnes M, et al. The short cosyntropin test revisited: new normal reference range using LC-MS/MS. *J Clin Endocrinol Metab.* 2018;103(4):1696–1703.