RESEARCH

Assay of steroids by liquid chromatography–tandem mass spectrometry in monitoring 21-hydroxylase deficiency

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Abstract

Immunoassays of steroid hormones are still used in the diagnosis and monitoring of patients with congenital adrenal hyperplasia. However, cross-reactivity between steroids can give rise to falsely elevated steroid levels. Here, we compare the use of immunoassays and liquid chromatography–tandem mass spectrometry (LC–MS/MS) in the monitoring of patients with classic 21-hydroxylase deficiency (21OHD). Steroid profiles in different mutation groups (genotypes) were also compared. Fifty-five patients with classic 21OHD (38 women) were studied. Blood samples were collected in the morning after an overnight medication fast. LC–MS/MS and immunoassays were employed to assay 17-hydroxyprogesterone (17OHP), testosterone and androstenedione. In addition, 21-deoxycortisol (21DF), 11-deoxycortisol (11DF), corticosterone, deoxycorticosterone, cortisone and cortisol were analyzed by LC–MS/MS. Testosterone, androstenedione and 17OHP levels were consistently lower (by about 30–50%) when measured by LC–MS/MS compared with immunoassays, with exception of testosterone in men. There was a significant correlation between 21DF and 17OHP ($r=0.87$, $P<0.001$), but three patients had undetectable 21DF. Subjects with no enzyme activity had significantly lower mean 11DF concentrations than subjects with residual activity. The use of LC–MS/MS gives a more specific view of adrenal steroid levels in 21OHD compared with immunoassays, which seem to considerably overestimate the levels of 17OHP and androstenedione. Falsely elevated levels of 17OHP and androstenedione could lead to overtreatment with glucocorticoids.

Key Words

- 21-hydroxylase deficiency
- congenital adrenal hyperplasia
- LC–MS/MS
- monitoring
- adrenal glucocorticoid quantitation
- adrenal androgens quantitation

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Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders with impaired biosynthesis of adrenal glucocorticosteroids and defects in cortisol biosynthesis. More than 95% of cases are caused by mutations in *CYP21A2* encoding 21-hydroxylase, a key enzyme in the aldosterone and cortisol pathway (1, 2). The classic form of 21-hydroxylase deficiency (21OHD) includes the salt-wasting (SW) form with complete lack

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of cortisol and aldosterone production, and the simple virilizing (SV) form where sufficient aldosterone is produced to avoid salt wasting. Both are accompanied by adrenocorticotropic hormone (ACTH)-driven increase in adrenal androgens with a virilizing effect on girls. Untreated, premature puberty with accelerated bone age will ensue. The prevalence of 21OHD shows large ethnic and geographic differences; the worldwide incidence is approximately 1/15,000 based on newborn screening (3).

The aim of the medical treatment is to provide sufficient glucocorticoid and mineralocorticoid replacement to suppress ACTH and adrenal androgen production without inducing glucocorticoid side effects (4). Monitoring of replacement therapy is difficult and lacks standardization in adults. Clinical assessment is not sensitive enough to identify subtle over- and under-treatment. Assay of the intermediates 17-hydroxyprogesterone (17OHP) and androstenedione is often used, but results are often difficult to interpret as cross-reactivity between steroids makes results unreliable (5). It is recommended to aim for a 17OHP concentration just above the upper reference level to avoid overtreatment with glucocorticoids (6) and in the recently published updated Endocrine Society Clinical Practice Guidelines (CAH due to steroid 21-hydroxylase deficiency) consistently timed hormone measurements relative to medication schedule and time of day is recommended (1). Drug fasting morning values of androstenedione and testosterone should be in the normal range for the patient's age and sex. However, current immunoassays for testosterone are prone to interferences in the female reference range (low levels of testosterone) and can be inaccurate (7, 8, 9).

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has emerged as the method of choice for determination of steroid hormones. Advantages include its superior specificity compared to immunoassays, the possibility for multiplexing and low sample volume (10). This enables simultaneous analyses of other steroids that might aid diagnosis and monitoring. One such candidate is 21-deoxycortisol (21DF), which is elevated in patients with 21OHD (11) and a better marker than 17OHP for diagnosing infants (12, 13). The formation of cortisol via 21DF is an alternative pathway which is activated in patients with 21OHD (Fig. 1), but is lacking in healthy individuals (11).

Here, we compare immunoassays with LC–MS/MS in 21OHD patients in order to establish more precise biochemical monitoring in CAH, and, for the first time, LC–MS/MS steroid hormone concentrations among different genotypes and phenotypes. In addition, we studied hormone panels and looked for individual variations and examined if other steroid precursors such as 21DF could provide better information for monitoring patients with 21OHD.

Subjects and methods

Study population

Serum was obtained from 55 adult patients with 21OHD (17 men, 38 women; mean age 40.3 years (range 19–72) recruited from a previous study (14). Written informed consent was obtained. The patients were categorized as SW or SV phenotype, as defined by medical history and laboratory assessment (14). The diagnosis was verified by genotyping of the *CYP21A2*. Blood samples were collected in the morning before breakfast and medication. The Regional Ethics Committee of Western Norway and the Data Inspectorate of Norway approved the study. The study was performed according to the Helsinki Declaration.

Steroid hormone quantitation by immunoassay

Androstenedione and testosterone were analyzed with the Immulite 2000 competitive chemiluminescent immunoassay (Siemens). The androstenedione assay had a upper limit of detection of 35 nmol/L. Samples above this limit were not diluted. 17OHP was measured by a competitive radioimmunoassay (RIA) method (Siemens) with an upper limit of detection of 47 nmol/L. Samples above this limit were diluted to provide an accurate concentration (15).

Steroid hormone quantitation by LC–MS/MS

Serum concentrations of testosterone, androstenedione 17OHP, 21DF, 11-deoxycortisol (11DF), deoxycortisosterone (DOC), corticosterone, cortisone and cortisol were determined by an inhouse LC–MS/MS method developed at the Hormone Laboratory in Oslo. In brief, steroid hormones were extracted from serum with ethyl acetate using supported liquid extraction. Separation of steroid hormones was done by reversed-phase liquid chromatography using a high-strength silica-pentafluorophenyl (HSS-PFP) column. Detection was performed by positive electrospray ionization tandem mass spectrometry (triple quadrupole) as outlined in the Supplementary Subjects and methods (see section on Supplementary data given at the end of this paper).

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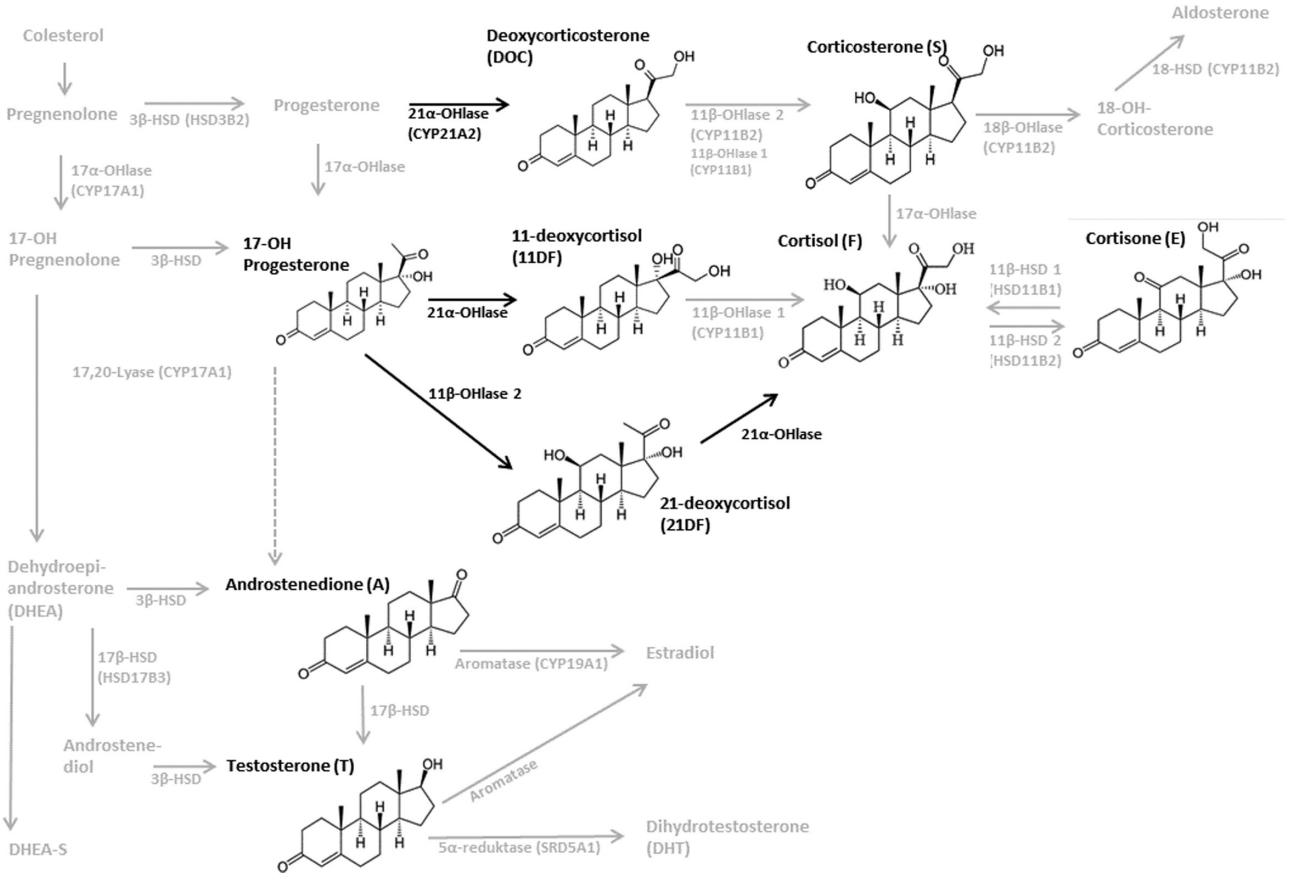

Figure 1
Steroid hormone synthesis. Analyzed steroids are marked with bold. The dashed line indicates formation of androstenedione from 17-hydroxyprogesterone.

LC–MS/MS reference values for adults (age ≥18) are given in Table 1. The reference values were obtained from literature and their validity was confirmed with 20 routine samples (assumed healthy women, men and children) (9, 15, 16, 17, 18). The reference value for 21DF was established inhouse by carefully evaluating individuals (n=42) which had a detectable 21DF concentration (>0.25 nmol/L) obtained from routine samples. Our reference value of >0.7 nmol/L is in agreement with a published reference value for 21DF (19).

Genetic analysis and enzyme activity

DNA was isolated from peripheral blood lymphocytes. Mutations in the *CYP21A2* gene were identified by direct DNA sequencing and deletions were determined by real-time PCR (20). *CYP21A2* mutations were divided into four groups according to their enzyme activity (*in vitro*): group Null (no enzyme activity), group A (<2%), group B (2–10%) and group C (10–75%) (21, 22).

Statistical analysis

The results are given as mean (±S.D.). For correlation and method comparison Passing–Bablok regression was performed with Y=LC–MS/MS and X=immunoassay (MedCalc Software 16.1). A difference in concentration between two methods <10% is regarded as acceptable (Y=1±0.1 X). Pearson correlation coefficients (r) were assessed for correlations between steroid hormones, and steroid hormones in different mutation groups were compared by one-way ANOVA if normally distributed and Kruskal–Wallis if not normally distributed. Statistical significance was defined as P<0.05.

Results

Comparison of LC–MS/MS and immunoassay

The concentrations of testosterone, androstenedione and 17OHP were generally lower when assayed with

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Table 1 LC–MS/MS reference values for steroid hormones, current immunoassays values in parenthesis.

Analyte	Reference values, adults ≥18 years (nmol/L)	References
Cortisol	120–600	Hormone Laboratory Haukeland University Hospital Bergen (18)
Cortisone	13–92	Kulle *et al.* (9)
Corticosterone	1.3–36	Fanelli *et al.* (15)
11-Deoxycortisol	≤3.2	Fanelli *et al.* (15)
21-Deoxycortisol	≤0.7	Own data
17-Hydroxyprogesterone		Mayo Medical Laboratories (17)
Females, follicular phase	≤2.4 (1.2–9.9)	
Females, luteal phase	≤8.6 (1.2–9.9)	
Males	≤6.7 (1.8–7.8)	
Deoxycortisol	≤0.41	Costa-Barbosa *et al.* (11)
Testosterone	≤1.9 (0.3–1.9)	Kushnir *et al.* (16)
Females, postmenopausal	≤1.1 (0.2–1.1)	
Males, age 18–40	7.2–24 (8.0–35)	
Males, age ≥41	4.6–24	
Androstenedione		Mayo Medical Laboratories (17)
Females	1.4–5.2 (<11.5)	
Males	1.0–7.0 (2.1–10.8)	

LC–MS/MS compared with immunoassay. Testosterone concentrations were 30% lower in females (Fig. 2A), but only a small difference in concentration was observed in males (Fig. 2B). Androstenedione concentrations were 30% lower (males and females combined) (Fig. 2E). Concentrations above 35 nmol/L ($n=12$) obtained by immunoassay were excluded (above measuring range for immunoassay). Method comparison for 17OHP (males and females combined) revealed overall 23% lower values when samples >100 nmol/L (immunoassay) were excluded (Fig. 2D). When all samples were included, the difference was even greater (57%) (Fig. 2C).

21-Deoxycortisol (21DF)

The correlation between 17OHP and 21DF was good when all samples were included ($r=0.87$, $P\leq0.001$) (Fig. 3A). When samples with a 17OHP concentration >100 nmol/L were excluded, less correlation between 17OHP and 21DF was observed ($r=0.60$, $P\leq0.001$) (Fig. 3B). Three of the patients did not have measurable concentrations of 21DF. In these patients, the mutations were in group Null and A including del/I2 splice, I2 splice/I2 splice and del/del.

Genotype, phenotype and steroid hormone profile

Thirty-one individuals were classified in category Null and A, leading to the SW phenotype in 29 patients. Twenty-four individuals were classified in category B and C, of whom 22 had the SV phenotype. Thus, two individuals had a SV phenotype even though genetic analysis indicated no enzyme activity and two individuals had a SW phenotype despite a genetic analysis indicating 2–10% enzyme activity (group B).

Subjects in group Null had lower mean 11DF concentrations compared with subjects in group B; mean (±S.D.) 0.153 (0.20) vs 0.513±(0.63) nmol/L ($P=0.052$). Concentrations of other steroid hormones did not differ significantly between the genotypes; however, large individual differences were observed (Fig. 4).

Patient cases

Four individuals had a phenotype which did not correspond to genetic analysis, and their individual hormone concentrations are given in Table 2. The steroids assayed by LC–MS/MS distal to the block fitted well with the phenotype.

One woman had a high testosterone concentration of 12.7 nmol/L assayed with immunoassay but 3.8 nmol/L with LC–MS/MS.

Discussion

It is well known that immunoassays and LC–MS/MS methods measure different concentrations of steroid hormones, primarily because immunoassays are more prone to cross-reactivity, especially in the low concentration range (5, 7). In our cohort of 21OHD patients, acceptable correlations ($r>0.65$) were achieved for 17OHP, androstenedione and testosterone (female and male range). As expected, the concentrations obtained

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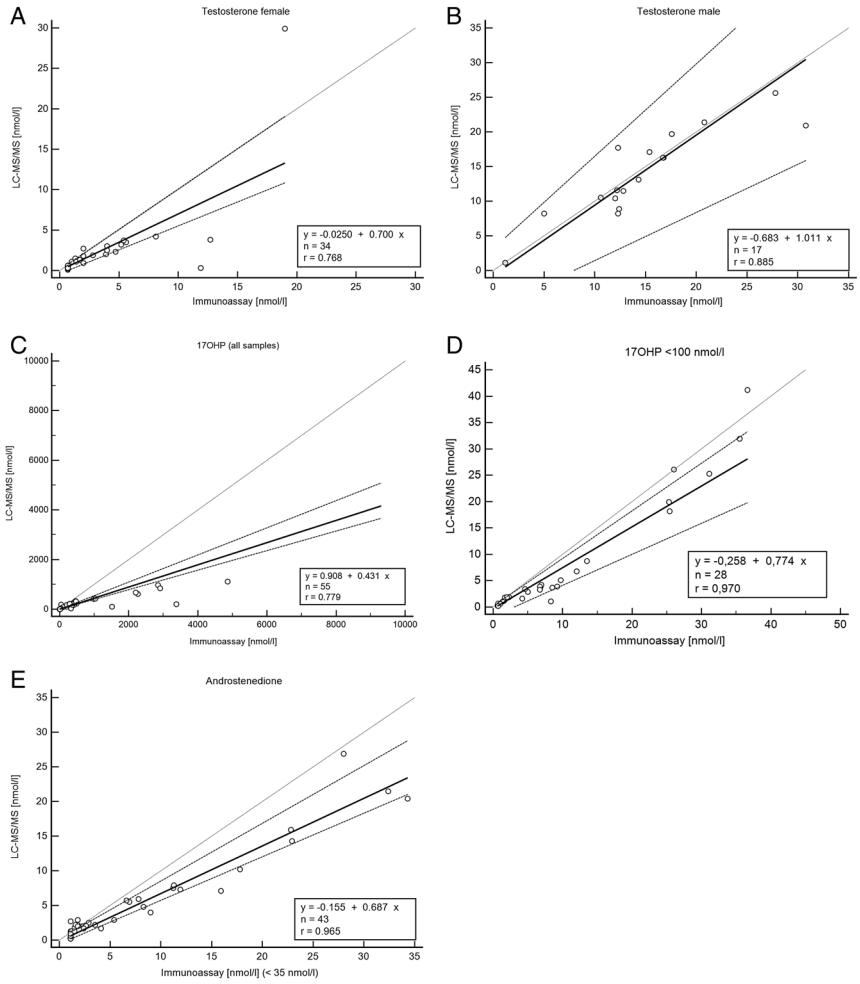

Figure 2
Comparison of LC–MS/MS (Y) and immunoassay (X) methods. Statistical analyses were carried out using the Passing–Bablok regression. The dashed lines indicate the 95% confidence interval for the regression line. (A) testosterone females; (B) testosterone males; (C) 17OHP all samples included; (D) 17OHP samples < 100nmol/l included; (E) androstenedione.

with LC–MS/MS were lower compared to concentrations obtained with immunoassay. A difference in concentration of 10% between two methods obtained from Passing–Bablok regression is generally regarded as acceptable, that is, methods giving comparable concentrations (internal laboratory procedure). Large differences imply that the methods measure different concentrations and thus different reference values must be employed.

Immunoassays overestimate especially 17OHP and androstenedione concentrations in patients with 21OHD because of a general lack of specificity and a high degree of interference in these methods. This may in turn lead to overtreatment with glucocorticoids. In our study, testosterone was highly overestimated in one woman, where LC–MS/MS measured a testosterone concentration of 3.8 nmol/L and immunoassay 12.7 nmol/L. A testosterone level of 12.7 nmol/L would normally result in intensification of the treatment and more likely overtreatment with glucocorticoids. We also performed a chromosome analysis due to this high value which showed a normal 46 XX karyotype.

Thus, LC–MS/MS methods are superior to immunoassays in this regard.

Many of the study patients had high levels of 17OHP in the morning which could indicate that they were not treated appropriately. However, higher doses of glucocorticoids may give more side effects such as obesity and osteoporosis. Biochemical monitoring of therapy in CAH is controversial and difficult as the serum hormones fluctuate with time of day and intake of glucocorticoid tablets. Hence, target concentrations are not clearly defined. A random test of serum hormones has limited value, but the therapeutic goal has until now been assessed by measurement of 17OHP in the mornings before intake of glucocorticoid tablets (6). A way to overcome this could be diurnal 17OHP curve analyzed from dried blood spots done at the patient's home (23). It is, however, only available in certain countries.

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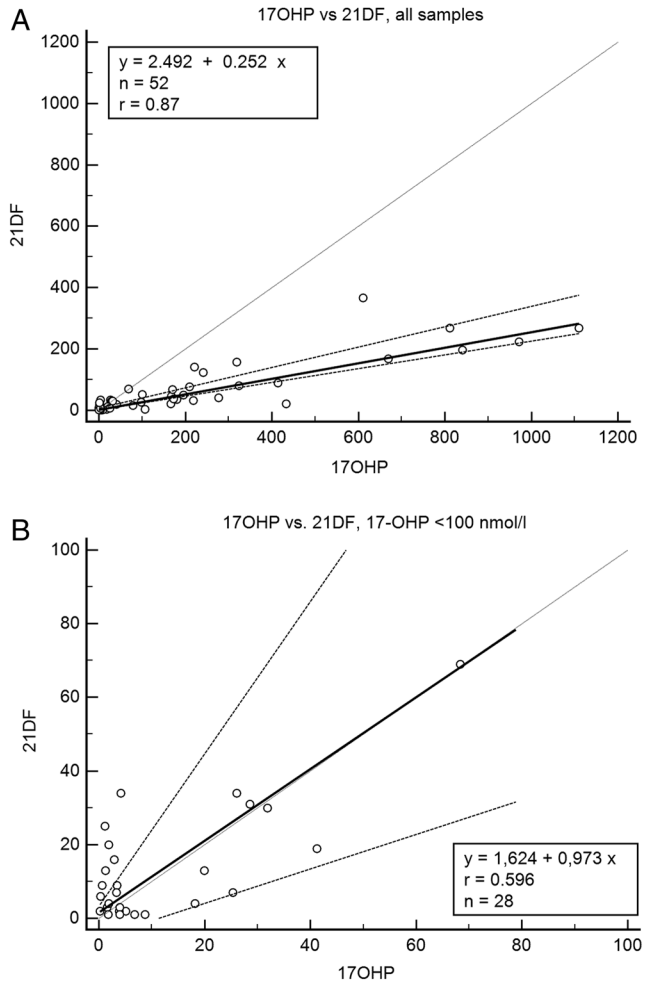

Figure 3
Passing–Bablok regression of 21-deoxycortisol (21DF) (Y) and 17-hydroxyprogesterone (17OHP) (X) with LC–MS/MS. The dashed lines indicate the 95% confidence interval for the regression line. (A) all samples included; (B) samples with 17OHP <100nmol/l included.

When methods for determinations for steroid hormones are changed from immunoassay to LC–MS/MS, it is thus important that not only reference values are changed. Cut-off values for diagnosis and target levels for treatment must also be defined.

Previous studies have found that the measurement of 21DF may be helpful in the diagnosis of CAH (13). We did not find evidence for that 21DF is better than 17OH in monitoring patients with CAH. In the screening for newborn errors of metabolism, 21DF may be a better discriminator for CAH compared to 17OHP. Especially in premature infants, 17OHP is often elevated in the first few days (12). The correlation between 17OHP and 21DF concentrations was good in our treated patients with CAH. A positive 21DF concentration (>0.7 nmol/L) strongly indicates that the 21-hydroxylase activity is impaired. However, it is not clear whether overtreatment with glucocorticoids cause a decline in 21DF. If 21DF could be a better marker compared to 17OHP needs further evaluation. Compared to 17OHP, 21DF may be better for diagnosing adults with non-classic CAH due to 21OHD (24, 25). Whether 21DF is better than 17OHP to differentiate other disorders with elevated 17OHP, such as PCOS needs to be evaluated (26, 27).

There was no apparent association between patients who had negative 21DF and their medication (fludrocortisone dose and hydrocortisone equivalents). All three subjects with negative 21DF were females with a SW phenotype treated with prednisolone. Genetic analysis showed mutations consistent with group Null in one subject, while the other two were in group A. In these patients, the mutations were del/I2 splice, I2 splice/I2 splice and del/del, but other patients had these mutations and measurable 21DF concentrations. 21DF is most likely not superior to 17OHP in monitoring patients treated for 21OHD.

Patients who have a SW genotype, but present a SV phenotype have higher concentrations of the hormones after the 21OH enzymatic block (11DF, DOC, corticosterone, cortisol, cortisone) compared to patients who have a SV genotype but SW phenotype. LC–MS/MS analyses of these hormones fit well with the phenotype and provide valuable information. The patient examples in Table 2 show that the analysis of only a few steroid hormones may be misleading in the follow-up of CAH patients.

Another advantage of LC–MS/MS is that several hormones and glucocorticoid medication can be measured in one sample/run and an adrenal steroid profile can be established. By multiplexing, valuable information is gained. Furthermore, the updated Clinical Practice Guidelines (CAH due to steroid 21-hydroxylase deficiency) from November 2018 state that LC–MS/MS is the gold standard for blood and saliva measurements of steroids (1).

In our study of treated CAH patients, there seems to be a discrepancy between mutations detected and adrenal steroid hormone profile. This discrepancy is reduced when using LC–MS/MS which provides the ability to simultaneously analyze steroid hormones after the 21OH enzymatic block. Also, there is a lack of similarity in steroid hormone profile and clinical phenotype (SW vs SV). In compound heterozygous cases, the mildest mutation defines the genotype group. The correlation

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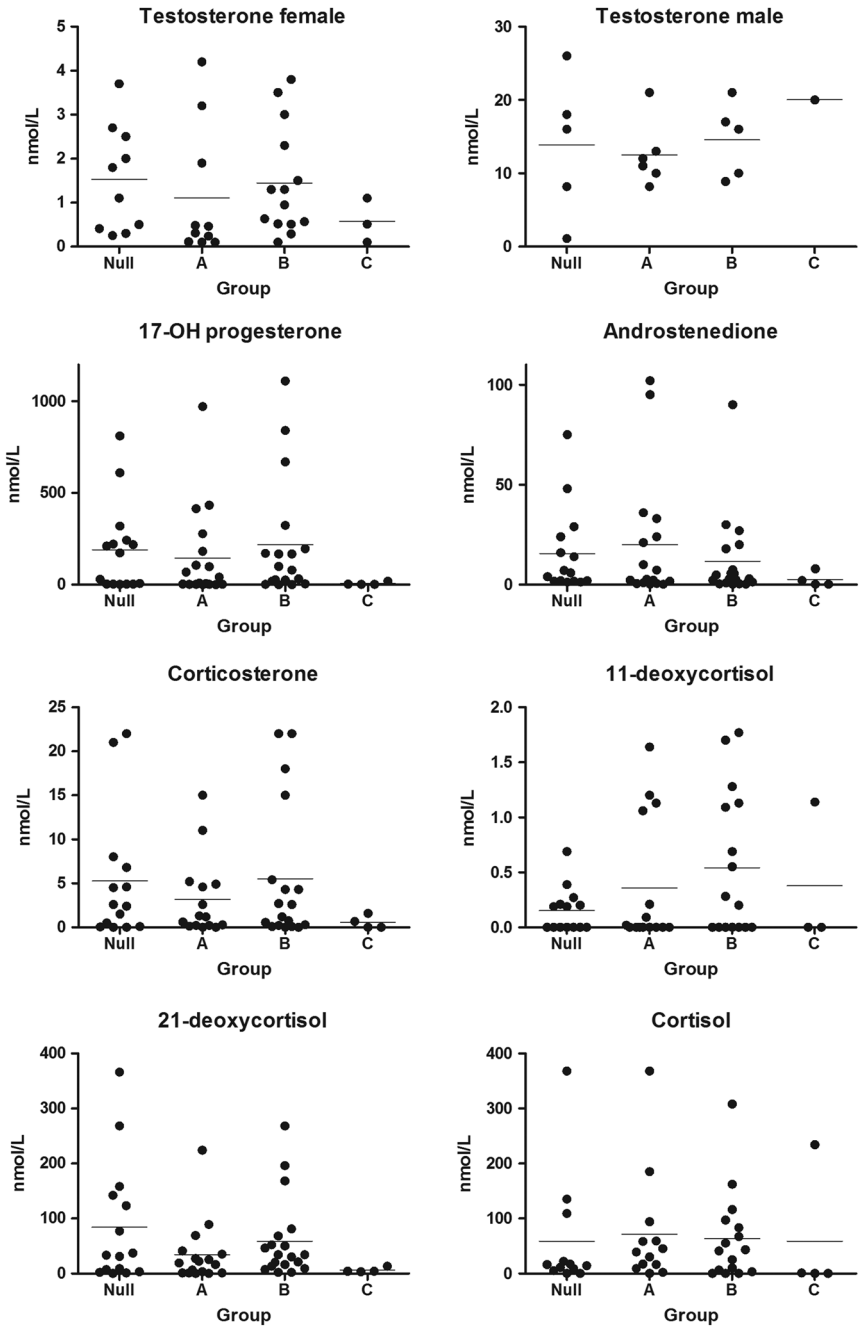

Figure 4
LC–MS/MS hormone profile (nmol/L) in different mutation groups group; Null (complete enzyme impairment), group A (almost complete enzyme impairment (<2%), group B (severe enzyme impairment, 2–10%) and group C (partial impairment, 10–75%). Mean values are marked.

between genotype and clinical phenotype in 21OHD is usually high, however, not 100% (22), which may be due to artifacts when using PCR-based genotyping (28, 29, 30). A source of phenotype–genotype variability is the leakiness of splice mutations (31). Thus, treatment should be given according to the clinical picture.

In conclusion, LC–MS/MS methods are superior to immunoassays in monitoring patients with 21OHD on corticosteroid replacement therapy as they are more specific and can be multiplexed. Immunoassays seem to overestimate high concentrations of 17OHP and androstenedione considerably. With the possibility of obtaining steroid hormone profiles, LC–MS/MS methods provide a more complete picture of the whole hormone cascade compared to immunoassay methods. Some of the examples show that the analysis of only a few steroid hormones may be misleading in the follow-up of CAH patients. 21DF is most likely not superior to 17OHP in monitoring patients treated for 21OHD, but further investigation is needed.

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Table 2 Steroid hormone profile with LC–MS/MS; (nmol/L) in two patients with SW genotype but SV phenotype and two patients with SV genotype but SW phenotype.

	Genotype	Phenotype	Age	17OHP	21DF	11DF	DOC	B	F	E	T	A	Medication
F1	SW	SV	34	6.8 (H) (12)	1 (H)	0.69	0.11	1.5	135	38	0.5 (0.7)	4.0 (9.0)	Prednisolone 2.5 mg morning
F2	SW	SV	42	414 (H) (980)	89 (H)	1.6	0.25	11	185	42	4.2 (H) (8.1)	24 (H) (35)	Prednisolone 5 mg morning
M1	SV	SW	51	3.3 (7)	7 (H)	0	0	0 (L)	0 (L)	0 (L)	16 (16.7)	1.7 (2.4)	Dexamethasone 0.75 mg morning
M2	SV	SW	50	5.1 (10)	2 (H)	0	0.09	0 (L)	3 (L)	1.6 (L)	17 (15.4)	0.93 (1.1)	Hydrocortisone 20 mg + fludrocortisone 0.05 mg morning

Levels with immunoassays in parenthesis; reference values in Table 1. (H) Above reference, (L) below reference.
11DF, 11-deoxycortisol; 17OHP, 17-hydroxyprogesterone; 21DF, 21-deoxycortisol; A, androstenedione; B, corticosterone; DOC, deoxycorticosterone; E, cortisone; F, cortisol; F, female; M, male; SV, simple virilizing; SW, salt wasting; T, testosterone.

Supplementary data
This is linked to the online version of the paper at https://doi.org/EC-18-0453.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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