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3 **A liquid chromatography-tandem mass spectrometry assay for the**
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5 **profiling of classical and 11-oxygenated androgens in saliva**
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51
52 and LS have conducted the validation of the assay and the measurement of saliva samples.

53
54 All authors have contributed to the writing and editing of the manuscript. All authors have
55
56 accepted responsibility for the entire content of this submitted manuscript and approved
57
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Abstract

Background: Classical and 11-oxygenated androgens both contribute to the androgen pool.

Regular monitoring of the androgen status is required in disorders of steroidogenesis and multiplexing of androgens improves the diagnostic ability of an assay. Due to the cheap non-invasive collection, saliva is advantageous when multiple samples are required. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers sensitive, simultaneous quantification of steroids with short run times. Here, we have developed an LC-MS/MS assay for the simultaneous measurement of 17-hydroxyprogesterone, androstenedione, testosterone, 11 β -hydroxyandrostenedione and 11-ketotestosterone in saliva.

Methods: Samples (300 μ L unstimulated whole saliva) were prepared by supported liquid extraction with dichloromethane and were reconstituted in 40% methanol. After on-line solid phase extraction with C18 cartridges, liquid chromatography was performed on a C8 column using a water/methanol gradient containing 0.1% formic acid and 2 mmol/L ammonium acetate. A Waters TQ-S mass spectrometer was used for quantification.

Results: Total run time was 6.4 minutes. For all analytes recovery was between 89% and 109%, ion suppression between 86% and 105%. Intra- and inter-assay comparisons showed a coefficient of variation <10% and the bias between measured and nominal concentration varied between -8% and 10%. Interference with a large set of natural and synthetic steroids was excluded. The assay was applied for the measurement of the androgen profile in healthy men (n=17) and women (n=10) which confirmed the sensitivity of the assay to be appropriate.

Conclusion: We present a novel LC-MS/MS assay for the comprehensive profiling of classical and 11-oxygenated androgens with potential for routine clinical application.

Introduction

The determination of androgen status in female and male patients with disorders of steroidogenesis, in particular in congenital adrenal hyperplasia (CAH), is essential to assess the quality of their treatment and to stratify their androgen-related cardio-metabolic risk. However, adrenal and gonadal androgen biosynthesis are complex and there is extensive peripheral activation of androgen precursors (1). Multiplexing of analytes is therefore required to improve the diagnostic ability of an androgen assay (2-4). The simultaneous measurement of the classical androgen testosterone and its precursor androstenedione in serum or saliva has previously been established as a sensitive marker for metabolic risk in polycystic ovary syndrome (PCOS), a syndrome of androgen excess in women (2, 5-7). Additionally, recent research on androgen biosynthesis pathways has identified the class of 11-oxygenated androgens as a significant driver of androgenic activity in addition to the classical androgens like testosterone (8). The 11-oxygenated androgen pathway is initiated by adrenal conversion of androstenedione to 11 β -hydroxyandrostenedione (11OHA4), a major adrenal androgen precursor (9). 11OHA4 can be further converted to 11-ketotestosterone (11KT), which activates the androgen receptor with the same potency and efficacy as testosterone (10, 11). In both common (PCOS) and rare (CAH) androgen excess conditions, 11-oxygenated androgens make the major contribution to the circulating androgen pool (12-15). It is therefore essential to include these analytes in the routine assessment of androgen status. Treatment monitoring in CAH requires regular and frequent sample collections and analysis. The collection of saliva for steroid analysis represents an excellent option for this, as it can be conducted by the patients on their own and at home, without the need for clinic attendance or support by clinical personnel. Additionally, a significant proportion of androgens in circulation is bound to sex-hormone binding globulin and therefore considered

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3 bio-inactive according to the free hormone hypothesis (16). Levels in saliva, however,
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5 represent the free, unbound fraction of the hormone and more likely reflect bioactive
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7 concentrations (17). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is
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9 currently the tool of choice for steroid analysis due to its outstanding selectivity and short run
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11 times (18, 19). Additionally, LC-MS/MS has the sensitivity to accurately quantify low analyte
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13 levels, as are expected in saliva, and allows for multiplexing of analytes of interest.
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17 We, therefore, developed and validated an LC-MS/MS assay for the simultaneous
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19 measurement of the classical androgens testosterone and androstenedione and the 11-
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21 oxygenated androgens 11OHA4 and 11KT. Additionally, the assay includes 17-
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23 hydroxyprogesterone (17OHP), which is the accepted reference standard for the diagnosis
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25 and monitoring of treatment efficiency in CAH (20, 21). The structures of the analytes are
26
27 shown in **Figure 1**. This specific combination of analytes in the presented assay can
28
29 comprehensively support the assessment of androgen status in both female and male
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31 patients with disorders of androgen excess and deficiency.
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40 **Material, subjects and methods**

41 *Collection of saliva samples*

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43 Unstimulated whole saliva was collected by the passive drool technique as previously
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45 described (22). The passive drool technique was chosen because commercially available
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47 collection devices, like Salivettes®, can affect analyte recovery and results of the
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49 measurement due to unspecific adsorption of non-polar steroids such as testosterone (23,
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51 24). To avoid blood or other contaminations of the sample, participants were asked to avoid
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53 brushing teeth two hours before the sample collection, avoid eating one hour before the
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55 sample collection and to rinse their mouth with water 10-15 min prior to sample collection.
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3 Immediately after collection samples were frozen at -20 °C. Samples were thawed,
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5 centrifuged at 2,500g for 5-10 min and the clear supernatant was aliquoted and stored at -80
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7 °C for analysis.
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10 The use of human saliva samples has been ethically approved by the Science, Technology,
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12 Engineering and Mathematics Ethical Review Committee of the University of Birmingham
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14 (ERN_17-0494). All participants gave written informed consent prior to the sample collection.
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16 Samples were anonymised and no identifying information was available at the laboratory.
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18 Healthy volunteers provided morning samples collected between awakening and 10 a.m.
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20 Male participants (n=17) were between 20 and 35 years of age. Female participants (n=10,
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22 age range 26-39) had a regular menstrual cycle, did not take oral contraceptives and collected
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24 the sample on day 21 of the menstrual cycle. The start of the menstrual bleed was defined as
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26 day 1.
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35 *Preparation of stock solutions, standards and QCs*

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37 17OHP, androstenedione and testosterone were purchased as solutions in methanol
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39 (Cerilliant, Sigma Aldrich, Poole, UK). 11OHA4 and 11KT were purchased as powder (Sigma
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41 Aldrich, Poole, UK). Stock solutions were prepared by dissolving or diluting the respective
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43 steroid in ultra-pure methanol (Fluka, Poole, UK) and were stored at -20 °C. Two separately
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45 prepared stock solutions were used for the preparation of standards and quality controls
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47 (QCs) by dilution in a surrogate saliva matrix consisting of phosphate-buffered saline (pH 7.4)
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49 supplemented with 0.1% (w/v) bovine serum albumin (PBS/BSA). The standards and QCs
50
51 contained a mixture of all analytes at the desired concentrations. Nine standards including a
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53 zero sample without analytes were used. Concentrations are summarised in **Table 1**. The
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55 internal standard mixture in methanol contained [¹³C₃]- (2,3,4)-17-alpha-
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3 hydroxyprogesterone ($[^{13}\text{C}_3]$ -17OHP; Cerilliant, Round Rock, USA), D_7 -(2,2,4,6,6,16,16)-4-
4 androstene-3,17-dione (D_7 - androstenedione; CDN isotopes, Pointe-Claire, Canada), $[^{13}\text{C}_3]$ -
5 (2,3,4)-testosterone (Isosciences, King of Prussia, USA), D_7 -2,2,4,6,6,16,16-4-androsten-11 β -
6 ol-3,17-dione (D_7 -11OHA4; CDN Isotopes, Pointe-Claire, Canada), D_3 -16,16,17-11-
7 ketotestosterone (D_3 -11KT; Isosciences, Philadelphia, USA).
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18 *Sample preparation by Supported Liquid Extraction*

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20 Samples were extracted by Supported Liquid Extraction (SLE) in a 96-well format and a set of
21 standards and QCs was added to each plate. 300 μL of saliva sample, standard or QC were
22 pipetted into the wells of a 2-mL square well 96-well plate (Porvair, Leatherhead, UK). 20 μL
23 of the internal standard (IS) mixture (**Table 1**) in methanol was added using an Eppendorf
24 multipipette. The plate was mixed for 1 minute. Samples were transferred onto the wells of a
25 SLE plate (SLE+, Biotage, Uppsala, Sweden) and a vacuum was applied to draw the entire
26 sample into the matrix of the plate. The plate was incubated at room temperature for 20
27 minutes. For elution a fresh 96-well plate was placed under the SLE plate and 1 mL
28 dichloromethane (Chromasolv Plus, Sigma Aldrich, Poole, UK) was added onto each well of
29 the SLE plate. The solvent was allowed to pass through into the collection plate until no
30 residual solvent was visible on the wells. Subsequently positive pressure was applied for 30
31 seconds to transfer the remaining solvent into the collection plate. The dichloromethane was
32 evaporated using a Vacmaster 96 (Biotage, Uppsala, Sweden) and samples were reconstituted
33 in 100 μL 40% (v/v) ultra-pure methanol in ultra-pure Milli-Q water (Merck-Millipore,
34 Hertfordshire, UK).
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Online solid phase extraction and Liquid chromatography

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3 Online solid phase extraction and chromatography were carried out using a Waters Online
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5 SPE Manager coupled to an Acquity ultra high pressure liquid chromatography system
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7 (Waters, Manchester, UK). A Waters XBridge C18 cartridge was conditioned with 0.5 mL
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9 methanol and equilibrated with 0.5 mL water. 50 µL sample were injected onto the cartridge
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11 using 0.5 mL water. The cartridge was washed with 0.25 mL 30% (v/v) methanol in water and
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13 the analytes were subsequently eluted onto the analytical Kinetex® C8 column (100 x 3 mm,
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15 2.6 µm; Phenomenex, Torrance, USA) using 55% of mobile phase A (2 mmol/L ammonium
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17 acetate in 0.1% (v/v) formic acid in distilled Milli-Q water) and 45% of mobile phase B (2
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19 mmol/L ammonium acetate in 0.1% formic acid in ultra-pure methanol). The flow rate was
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21 0.45 mL/min and the column temperature was 45°C. For chromatographic separation a linear
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23 gradient from 45 to 85% mobile phase B over 4 minutes was applied; followed by one minute
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25 at 100% B and one minute of re-equilibration at starting conditions. The eluate was directly
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27 injected into the mass spectrometer.
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37 *Mass spectrometry*

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39 Quantification was performed on a Waters XEVO TQ-S mass spectrometer operated in
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41 positive ion mode. The source offset was maintained at 30 V with a source temperature of
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43 150 °C. The capillary voltage was 0.75 kV and the desolvation temperature and gas flow were
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45 550°C and 1200 L/h. Multiple reaction monitoring mode was used with a dwell time of 0.016
46
47 s. All mass transitions are summarised in **Table 1** together with respective cone voltages and
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49 collision energies. MassLynx NT 4.1 software was used for system control and the MassLynx
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51 TargetLynx programme for data processing. Peak height ratios of analyte and IS, 1/x weighting
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53 and linear least square regression were used to produce the standard curves.
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Validation

The assay was validated according to accepted guidelines (25, 26).

Matrix effect. Several saliva samples (n=5 for 17OHP, androstenedione and testosterone; n=6 for 11OHA4 and 11KT) or distilled water were extracted by SLE as described above. Dried extracts were reconstituted in 100 μ L of 40% methanol and spiked with 10 μ L of a steroids stock in methanol resulting in the final concentrations indicated in **Table 2**. As a background control to determine the signal from the non-spiked sample, a corresponding volume of water was added to the reconstituted extract of saliva samples or water. The matrix effect was calculated from the peak height determined in extracts of saliva compared to peak height determined in the extract of water after deducting the respective background signal: $100 \cdot \frac{(\text{height}_{\text{H}_2\text{O}} - \text{height}_{\text{saliva}})}{\text{height}_{\text{H}_2\text{O}}}$. Matrix effects between 80% and 120% were considered acceptable.

Recovery. Recovery includes extraction efficiency and matrix effects. Different saliva samples (n= 5 for 17OHP, androstenedione and testosterone; n=6 for 11OHA4 and 11KT) or 50% (v/v) methanol were spiked with different concentrations of the analytes. As a background control for analyte levels before spiking, an equivalent volume of water was added to the samples. The samples were extracted as described above. Recovery was calculated from the concentrations measured in saliva compared to the concentrations measured in 50% (v/v) methanol deducting the respective background control. A recovery between 80% and 120% was considered acceptable.

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3 *Specificity.* Solutions of common endogenous and synthetic steroids were prepared at
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Specificity. Solutions of common endogenous and synthetic steroids were prepared at
supraphysiological concentrations in 50% (v/v) methanol in distilled water and analysed, after
direct injection, for interference in all channels (**Table 1**) at the relevant retention times. The
following steroids were tested: 21-deoxycortisol, aldosterone, corticosterone, cortisol,
cortisone, DHEA, dexamethasone, pregnenolone, DHEAS, budesonide, fludrocortisone,
fludrocortisone acetate, fluocinolone, triamcinolone, triamcinolone acetonide, prednisolone,
prednisone, 21-hydroxyprogesterone, epitestosterone, beclomethasone, 11-deoxycortisol,
dihydrotestosterone, cyproterone acetate, levonorgestrel, desogestrel, norethisterone and
ethinylestradiol.

Dilution linearity. Different saliva samples (n=6 for 17OHP, androstenedione and
testosterone; n=4 for 11OHA4 and 11KT) were spiked with the top concentration of the
standard range for the respective analyte (see **Table 1**) and serially diluted (factor 2) with
PBS/BSA. Measured concentrations were plotted against calculated theoretical
concentrations and curves were judged linear if the correlation coefficient of linear regression
(R^2) was >0.9900 . The bias between calculated theoretical and measured concentrations was
calculated and a bias $\leq 10\%$ was considered acceptable.

Precision and bias. Saliva samples with three different concentrations and PBS/BSA-based
samples spiked with different concentrations of the analytes were analysed in ten different
runs to assess inter-assay imprecision as well as ten times within the same run for intra-assay
imprecision. The saliva samples were pooled female saliva, pooled male saliva and pooled
mixed male and female saliva to allow to cover the range of concentrations and get the
volume required for replicate analysis. The percentage coefficient of variation (CV) calculated

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3 from the ratio of standard deviation and mean. The bias (spiked PBS/BSA-based samples only)
4 was calculated as percentage deviation between the mean observed and nominal
5 concentrations. CV and bias were considered acceptable if $\leq 10\%$.
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13 *Lower limit of quantification (LLOQ).* The lower limit of quantification was defined as the
14 lowest concentration for which 10 replicates of PBS/BSA based samples gave a bias and CV of
15 less than 20%.
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23 *Post-extraction stability.* Saliva samples (n=17 for 17OHP, n=18 for androstenedione, n=16 for
24 T, n=10 for 11OHA4 and 11KT) were extracted in duplicate and reconstituted for analysis. The
25 first plate was measured immediately after extraction, while the second plate was stored in
26 a refrigerator for and overnight period before being analysed (10 °C for 22h for 17OHP,
27 androstenedione and testosterone or at 4 °C for 18h for 11OHA4 and 11KT). Percentage bias
28 between the results of the two measurements was calculated.
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40 *Carry-over.* A PBS/BSA-based sample containing 86,667 pmol/L 17OHP, 100,000 pmol/L
41 androstenedione, 99300 pmol/L testosterone, 250,000 pmol/L 11OHA4 and 100,000 pmol/L
42 11KT was analysed followed by sample containing only PBS/BSA. Percentage carry-over of the
43 analytes into the following sample was calculated from the peak height determined for the
44 two samples.
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54 **Results**

55 *Validation of the analytical performance*

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3 All analytes eluted as discrete, identifiable peaks with co-elution of their respective
4 deuterated or ^{13}C -labelled IS. **Figure 2** shows the chromatograms of a saliva sample. T and
5 17OHP co-eluted, but due to the difference in m/z of the parent ions (m/z of 17OHP 331.2
6 and $^{13}\text{C}_3$ -17OHP 334.2 and m/z testosterone 289.1 and $^{13}\text{C}_3$ -testosterone 292.15), no
7 interference was observed. The total run time of the assay was 6.4 minutes injection-to-
8 injection. For each batch, individual standard curves for each analyte were generated by
9 plotting the ratio of analyte to IS peak height against the nominal concentrations. Standard
10 curves were linear ($R^2 > 0.99$) over the entire range given in **Table 1** and correlated with the
11 respective nominal concentrations with reproducibility between batches. The evaluation of
12 matrix effects ($n \geq 5$ different saliva samples) showed percentage signals with mean values
13 between 88 and 105% for three different concentrations per analyte and indicated no
14 significant ion suppression or enhancement by the saliva matrix (**Table 2**). For recovery ($n \geq 5$
15 different saliva samples), combining extraction efficiency and matrix effects, mean values
16 were between 89 and 109.4% for three different concentrations of all analytes (**Table 2**).
17 Interference was excluded for a large set of natural and synthetic steroids (see Material and
18 Methods section *Specificity* for a complete list) for all analyte and IS channels by directly
19 injecting supraphysiological concentrations. Serial dilutions of saliva after the addition of the
20 analytes at concentrations of the respective highest standard (**Table 1**) showed linearity with
21 R^2 values > 0.99 , when measured concentrations were plotted against the theoretical
22 concentrations. The bias between the measured and theoretical concentrations was $1.0 \pm$
23 5.9% for 17OHP, $-9.1 \pm 10.5\%$ for androstenedione, $-2.2 \pm 6.9\%$ for testosterone, $8.8 \pm 6.3\%$
24 for 11OHA4 and $7.3 \pm 4.6\%$ for 11KT.

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The evaluation of the precision of the assay within and between batches is summarised in
Table 3. For spiked PBS/BSA-based samples, the intra-assay CV was $< 8.1\%$ and the intra-assay

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3 bias ranged between -6.6 and 4.2% for all analytes. The respective inter-assay CV was <10.0%
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5 and the inter-assay bias between -3.1 and 10.2%. For selected saliva samples with three
6
7 different concentrations of each analyte, the intra-assay CV was <6.8% and the inter-assay CV
8
9 was <9.3%. The lower limits of quantification were 12.5 pmol/L for 17OHP (1.875 fmol on
10
11 column), 6.25 pmol/L for androstenedione (0.9375 fmol on column), 5 pmol/L for
12
13 testosterone (0.75 fmol on column), 50 pmol/L for 11OHA4 (7.5 fmol on column), 5 pmol/L
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15 for 11KT (0.75 fmol on column). Post-extraction stability was tested for overnight storage.
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17 The bias between the direct and post-storage analysis was between -8.4 and 8.8% for 17OHP,
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19 -14.9 and 4.9% for androstenedione, -30.6 and 19.9% for testosterone, -8.8 and 4.8% for
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21 11OHA4 and -8.3 and -0.5% for 11KT. Carry-over was 0.15% for 17OHP, 0.62% for
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23 androstenedione, 0.65% for T, 0.075% for 11OHA4 and 0.036% for 11KT.
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32 *Salivary androgens in healthy volunteers*

34 We applied the assay to saliva samples collected by healthy male (n=17) and female
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36 volunteers (n=10) between awakening and 10 a.m. Female participants had a regular
37
38 menstrual cycle and provided samples collected on day 21 of the cycle. Results are
39
40 summarised in **Figure 3**. Concentrations were above the LLOQ for all samples and analytes.
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42 For male participants median concentrations (range) were 56 (18-133) pmol/L for 17OHP, 147
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44 (86-310) pmol/L for androstenedione, 187 (130-320) pmol/L for testosterone, 450 (127-2114)
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46 pmol/L for 11OHA4, 276 (129-548) pmol/L for 11KT. For female participants median (range)
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48 values were 117 (27-201) pmol/L for 17OHP, 242 (107-367) pmol/L for androstenedione, 18
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50 (7-149) pmol/L for testosterone, 448 (201-2039) pmol/L for 11OHA4, 233 (139-535) pmol/L
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52 for 11KT. As expected, women had higher 17OHP levels than men (p=0.0009), but lower
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54 testosterone levels (p<0.0001). No differences between men and women were observed for
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3 analytes that are primarily of adrenal origin: androstenedione, 11OHA4 and 11KT. 11OHA4
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5 showed the widest inter-individual variation followed by 11KT.
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10 **Discussion**

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12 We present a novel LC-MS/MS assay for the simultaneous measurement of highly clinically
13 relevant steroids derived from the classic androgen pathway, androstenedione and
14 testosterone, and the 11-oxygenated androgen pathway, 11OHA4 and 11KT, as well as the
15 precursor steroid 17OHP in saliva. This specific combination of analytes allows for the
16 comprehensive single assay assessment of androgen status including monitoring of
17 biochemical control in CAH, which is required over the entire course of life for treatment
18 titration and risk stratification. The use of saliva facilitates the collection of multiple samples
19 over long periods of time due the simple, self-administered collection technique. While
20 previously published assays for salivary androgens were limited to the measurement of
21 combinations of testosterone, androstenedione and 17OHP (7, 27-29), our new assay enables
22 a complete assessment of the androgen profile, by including 11-oxygenated androgens, which
23 have recently been shown to significantly contribute to the circulating androgen burden in
24 PCOS and CAH (12, 13). The LLOQs for 17OHP, androstenedione and testosterone of our assay
25 are comparable (27, 28) or superior (29) to previously published assays. The short run time of
26 the assay (6.4 minutes) compares favourably to other assays (9.5 to 10 min) (27-29) and
27 allows for the implementation of the assay into routine practise of clinical laboratories. We
28 performed a strict validation of the developed assay that guarantees its accurate, precise and
29 reproducible performance according to accepted criteria (25, 26). The assay was
30 demonstrated to be highly selective excluding interference with a large set of natural and
31 synthetic steroids. There are no significant effects of the saliva matrix and the assay has
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3 excellent recovery, intra- and inter-assay precision and linearity. The high bias between the
4 results for testosterone before and after storage of the extracted samples indicate limited
5 post-extraction stability of testosterone. If the accurate quantification of testosterone is
6 required, the samples thus need to be analysed without post-extraction storage. However, all
7 other analytes are stable for an overnight period.
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10 We have successfully applied the assay for preliminary measurements of the salivary
11 androgen profile in healthy male and female volunteers. This is the first time 11-oxygenated
12 androgens have been measured in saliva. Measured concentrations were above the LLOQ for
13 all analytes demonstrating appropriate sensitivity of the assay to quantify the low picomolar
14 concentrations in saliva. For androstenedione and testosterone, the presented levels
15 compare well to data previously published by our laboratory (5, 7) and others (27, 28). Male
16 17OHP levels are also in the same range as previously published, but female 17OHP were
17 higher in our samples compared to the data published Mezzullo *et al.*, which can be explained
18 by age differences of the participants between the two studies and the collection of our
19 samples exclusively on day 21 of the menstrual cycle (28). We observed a comparably high
20 degree of inter-individual variability for salivary 11OHA4. 11OHA4 is the second most
21 abundant steroid produced by the adrenal gland after DHEAS and a sensitive marker for
22 adrenal steroid output (9, 14), which is maximal in the early morning with a subsequent
23 decline. The samples in this study have been collected between awakening and 10 a.m.
24 Differences in the timing of sample collection within this window are likely to be magnified
25 due to the higher morning production rate of 11OHA4 compared to other steroids generated
26 by down-stream metabolism in peripheral tissue or, at least in part, primary gonadal
27 production; this is a likely explanation for the observed higher variation in salivary 11OHA4.
28 It should be noted that, due to the limited number of participants and the limitation of female
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3 samples to a specific day of the menstrual cycle, the presented data does not yet allow the
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5 derivation of reference ranges.
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8 In conclusion, we have developed and validated a multi-steroid salivary LC-MS/MS assay for
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10 comprehensive assessment of both classic and 11-oxygenated androgen pathways with high
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12 potential for routine clinical use for assessment of androgen excess conditions and treatment
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14 monitoring in CAH.
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3 **Figure legends**
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7 **Figure 1:** Structures of 17-hydroxyprogesterone (17OHP), androstenedione, testosterone,
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9 11 β -hydroxyandrostenedione (11OHA4) and 11-ketotestosterone (11KT).
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14 **Figure 2: Chromatograms of a saliva sample for all target analytes and their internal**
15 **standards.** Quantifier transitions (*m/z*) are indicated. 17OHP, 17-hydroxyprogesterone; A4,
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17 androstenedione; T, testosterone; 11OHA4, 11 β -hydroxyandrostenedione; 11KT, 11-
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19 ketotestosterone.
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26 **Figure 3: Morning salivary androgen levels in healthy men (A, n=17) and women (B, n=10).**

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28 Samples were collected between awakening and 10 a.m. Participating women had a regular
29
30 menstrual cycle and collected the sample on day 21 of the cycle. Individual data points,
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32 median and interquartile range are depicted. 17OHP, 17-hydroxyprogesterone; A4,
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34 androstenedione; T, testosterone; 11OHA4, 11 β -hydroxyandrostenedione; 11KT, 11-
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References

1. Schiffer L, Arlt W, Storbeck KH. Intracrine androgen biosynthesis, metabolism and action revisited. *Molecular and cellular endocrinology*. 2018;465:4-26.
2. O'Reilly MW, Taylor AE, Crabtree NJ, Hughes BA, Capper F, Crowley RK, et al. Hyperandrogenemia predicts metabolic phenotype in polycystic ovary syndrome: the utility of serum androstenedione. *The Journal of clinical endocrinology and metabolism*. 2014;99(3):1027-36.
3. Munzker J, Hofer D, Trummer C, Ulbing M, Harger A, Pieber T, et al. Testosterone to dihydrotestosterone ratio as a new biomarker for an adverse metabolic phenotype in the polycystic ovary syndrome. *The Journal of clinical endocrinology and metabolism*. 2015;100(2):653-60.
4. Elhassan YS, Idkowiak J, Smith K, Asia M, Gleeson H, Webster R, et al. Causes, Patterns, and Severity of Androgen Excess in 1205 Consecutively Recruited Women. *The Journal of clinical endocrinology and metabolism*. 2018;103(3):1214-23.
5. Munzker J, Lindheim L, Adaway J, Trummer C, Lerchbaum E, Pieber TR, et al. High salivary testosterone-to-androstenedione ratio and adverse metabolic phenotypes in women with polycystic ovary syndrome. *Clinical endocrinology*. 2017;86(4):567-75.
6. Sathyapalan T, Al-Qaissi A, Kilpatrick ES, Dargham SR, Keevil B, Atkin SL. Salivary and serum androgens with anti-Mullerian hormone measurement for the diagnosis of polycystic ovary syndrome. *Scientific reports*. 2018;8(1):3795.
7. Sathyapalan T, Al-Qaissi A, Kilpatrick ES, Dargham SR, Adaway J, Keevil B, et al. Salivary testosterone measurement in women with and without polycystic ovary syndrome. *Scientific reports*. 2017;7(1):3589.
8. Pretorius E, Arlt W, Storbeck KH. A new dawn for androgens: Novel lessons from 11-oxygenated C19 steroids. *Molecular and cellular endocrinology*. 2017;441:76-85.
9. Rege J, Nakamura Y, Satoh F, Morimoto R, Kennedy MR, Layman LC, et al. Liquid chromatography-tandem mass spectrometry analysis of human adrenal vein 19-carbon steroids before and after ACTH stimulation. *The Journal of clinical endocrinology and metabolism*. 2013;98(3):1182-8.
10. Storbeck KH, Bloem LM, Africander D, Schloms L, Swart P, Swart AC. 11beta-Hydroxydihydrotestosterone and 11-ketodihydrotestosterone, novel C19 steroids with androgenic activity: a putative role in castration resistant prostate cancer? *Molecular and cellular endocrinology*. 2013;377(1-2):135-46.
11. Pretorius E, Africander DJ, Vlok M, Perkins MS, Quanson J, Storbeck KH. 11-Ketotestosterone and 11-Ketodihydrotestosterone in Castration Resistant Prostate Cancer: Potent Androgens Which Can No Longer Be Ignored. *PloS one*. 2016;11(7):e0159867.
12. O'Reilly MW, Kempegowda P, Jenkinson C, Taylor AE, Quanson JL, Storbeck KH, et al. 11-Oxygenated C19 Steroids Are the Predominant Androgens in Polycystic Ovary Syndrome. *The Journal of clinical endocrinology and metabolism*. 2017;102(3):840-8.
13. Turcu AF, Nanba AT, Chomic R, Upadhyay SK, Giordano TJ, Shields JJ, et al. Adrenal-derived 11-oxygenated 19-carbon steroids are the dominant androgens in classic 21-hydroxylase deficiency. *Eur J Endocrinol*. 2016;174(5):601-9.
14. Turcu AF, Mallappa A, Elman MS, Avila NA, Marko J, Rao H, et al. 11-Oxygenated Androgens Are Biomarkers of Adrenal Volume and Testicular Adrenal Rest Tumors in 21-Hydroxylase Deficiency. *The Journal of clinical endocrinology and metabolism*. 2017;102(8):2701-10.

15. Kamrath C, Wettstaedt L, Boettcher C, Hartmann MF, Wudy SA. Androgen excess is due to elevated 11-oxygenated androgens in treated children with congenital adrenal hyperplasia. *The Journal of steroid biochemistry and molecular biology*. 2018;178:221-8.
16. Mendel CM. The free hormone hypothesis: a physiologically based mathematical model. *Endocrine reviews*. 1989;10(3):232-74.
17. Vining RF, McGinley RA, Symons RG. Hormones in saliva: mode of entry and consequent implications for clinical interpretation. *Clin Chem*. 1983;29(10):1752-6.
18. Keevil BG. Novel liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for measuring steroids. *Best Pract Res Clin Endocrinol Metab*. 2013;27(5):663-74.
19. Adaway JE, Keevil BG, Owen LJ. Liquid chromatography tandem mass spectrometry in the clinical laboratory. *Ann Clin Biochem*. 2015;52(Pt 1):18-38.
20. Shibayama Y, Higashi T, Shimada K, Kashimada K, Onishi T, Ono M, et al. Liquid chromatography-tandem mass spectrometric method for determination of salivary 17alpha-hydroxyprogesterone: a noninvasive tool for evaluating efficacy of hormone replacement therapy in congenital adrenal hyperplasia. *Journal of chromatography B, Analytical technologies in the biomedical and life sciences*. 2008;867(1):49-56.
21. Honour JW. 17-Hydroxyprogesterone in children, adolescents and adults. *Ann Clin Biochem*. 2014;51(Pt 4):424-40.
22. Granger DA, Kivlighan KT, Fortunato C, Harmon AG, Hibel LC, Schwartz EB, et al. Integration of salivary biomarkers into developmental and behaviorally-oriented research: problems and solutions for collecting specimens. *Physiol Behav*. 2007;92(4):583-90.
23. Buttler RM, Bagci E, Brand HS, Heijer MD, Blankenstein MA, Heijboer AC. Testosterone, androstenedione, cortisol and cortisone levels in human unstimulated, stimulated and parotid saliva. *Steroids*. 2018;138:26-34.
24. Keevil BG, MacDonald P, Macdowall W, Lee DM, Wu FC. Salivary testosterone measurement by liquid chromatography tandem mass spectrometry in adult males and females. *Ann Clin Biochem*. 2014;51(Pt 3):368-78.
25. Honour JW. Development and validation of a quantitative assay based on tandem mass spectrometry. *Ann Clin Biochem*. 2011;48(Pt 2):97-111.
26. US Department of Health and Human Services FaDA. Guidance for Industry. Bioanalytical Method Validation. Rockville, MD: Center for Drug Evaluation and Research (CDER) and Center for Veterinary Medicine (CVM). 2001.
27. Turpeinen U, Hamalainen E, Haanpaa M, Dunkel L. Determination of salivary testosterone and androstendione by liquid chromatography-tandem mass spectrometry. *Clin Chim Acta*. 2012;413(5-6):594-9.
28. Mezzullo M, Fazzini A, Gambineri A, Di Dalmazi G, Mazza R, Pelusi C, et al. Parallel diurnal fluctuation of testosterone, androstenedione, dehydroepiandrosterone and 17OHprogesterone as assessed in serum and saliva: validation of a novel liquid chromatography-tandem mass spectrometry method for salivary steroid profiling. *Clin Chem Lab Med*. 2017;55(9):1315-23.
29. Liu J, Qiu X, Wang D, Li Y, Zong Y, Liu Y, et al. Quantification of 10 steroid hormones in human saliva from Chinese adult volunteers. *The Journal of international medical research*. 2018;46(4):1414-27.

Table 1: Standard and QC concentrations, mass transitions m/z , cone voltages and collision energies for target analytes and internal standards.

	Standards (pmol/L)	Quality controls (pmol/L)	Quantifier transition Qualifier transition	Cone voltage (V)	Collision energy (eV)
Target analyte (MW in g/mol)					
17OHP (330)	0; 8.727; 87.27; 174.54; 436.35; 872.7; 1,745.4; 4,363.5; 8,727	43.6; 174.25; 697; 6970	331.2 > 97.1 331.2 > 109.1	30 30	22 25
A4 (286)	0; 5.043; 50.43; 100.86; 252.15; 504.3; 1,008.6; 2,521.5; 5,034	25; 100.5; 402.1; 4021	287.15 > 97.1 287.15 > 109.1	20 20	22 22
T (288)	0; 5; 50; 100; 250; 500; 1,000; 2,500; 5,000	25;100; 400; 4000	289.1 > 97.0 289.1 >109.0	32 32	17 22
11OHA4 (302)	0; 25; 250; 500; 1,250; 2,500; 5,000; 12,500; 25,000	50; 200; 800; 8000	302.9 > 145.0 302.9 > 97.0	30 30	20 19
11KT (302)	0; 10; 100; 200; 500; 1,000; 2,000; 5,000; 10,000	50; 200; 800; 8000	302.9 > 259.1 302.9 > 121.1	30 30	20 22
Internal standard (MW in g/mol)					
¹³C₃-17OHP (333)	-	-	334.2 > 100.1	30	22
D₇-A4 (293)	-	-	294.1 > 100.0	20	20
¹³C₃-T (291)	-	-	292.15 > 100.1	32	17
D₇-11OHA4 (309)	-	-	309.9 > 147.0	30	20
D₃-11KT (305)	-	-	305.9 > 262.1	30	22

Table 2: Recovery and matrix effects. Values indicate the percentage concentration (recovery) or percentage height (matrix effect) compared to the respective control samples as described in Material and Methods (n=5 for 17OHP, A4 and T; n=6 for 11OHA4 and 11KT).

Analyte	Concentration (pmol/L)	Recovery (%)	Matrix effect (%)
17OHP	40	104.4 ± 7.1	105 ± 13.7
	80	105.3 ± 7.3	92.6 ± 13.3
	400	98.4 ± 11.8	93.0 ± 6.0
Androstenedione	40	105.5 ± 14.2	86.0 ± 14.3
	80	103.4 ± 11.8	90.1 ± 5.1
	400	102.1 ± 8.4	95.5 ± 7.7
Testosterone	40	108.6 ± 14.0	87.5 ± 17.4
	80	109.4 ± 9.5	92.2 ± 15.6
	400	102.1 ± 10.4	92.7 ± 4.5
11OHA4	500	89.0 ± 9.2	95.4 ± 7.7
	1000	97.1 ± 6.8	98.2 ± 12.1
	2000	91.8 ± 4.4	93.7 ± 6.9
11KT	250	96.4 ± 6.0	101.5 ± 6.1
	500	98.9 ± 3.8	88.0 ± 5.4
	1000	95.0 ± 3.2	89.5 ± 3.8

Table 3: Intra- and inter-assay precision and bias. Percentage CV was calculated for replicates (n=10) of saliva samples and spiked PBS/BSA-based samples. Percentage bias between observed and nominal concentrations was calculated for the spiked PBS/BSA-based samples.

		Intra-assay precision		Inter-assay precision	
Saliva samples					
Analyte	Mean concentration observed (pmol/L)	CV (%)		CV (%)	
17OHP	18	3.3		8.4	
	150	1.3		9.2	
	1061	1.6		5.4	
Androstenedione	133	2.2		2.6	
	308	1.5		3.7	
	695	3.9		3.0	
Testosterone	30	2.4		4.0	
	88	2.2		3.0	
	392	1.9		7.2	
11OHA4	187	5.3		9.3	
	926	6.8		8.6	
	2682	5.5		2.9	
11KT	128	2.3		3.2	
	397	2.2		3.3	
	1358	2.9		3.3	
Spiked PBS/BSA-based samples					
Analyte	Spiked concentration (pmol/L)	CV (%)	Bias (%)	CV (%)	Bias (%)
17OHP	25	8.0	-0.8	5.2	2
	50	3.3	-5.1	2.4	0.3
	100	3.5	-8.2	2.6	-0.4
Androstenedione	25	7.4	-4.1	2.6	-2
	50	5.9	-4.4	2.1	-1.6
	100	7.6	-6.6	5.2	-2.3

Testosterone	25	3.7	-4.0	1.8	-3.1
	50	3.9	-2.5	1.1	-3
	100	3.6	-3.8	3.8	-0.9
11OHA4	300	3.4	-5.2	5.2	8.6
	1500	4.4	-3.2	5.6	1
	3000	1.5	-3	3.3	2.3
	7500	3.7	-3.3	9.95	3.3
11KT	60	3.2	1.4	3.6	10.2
	300	2.4	2	3.8	5.9
	600	1.5	4.2	2.9	4.2
	1500	1.5	4	1.9	2.6

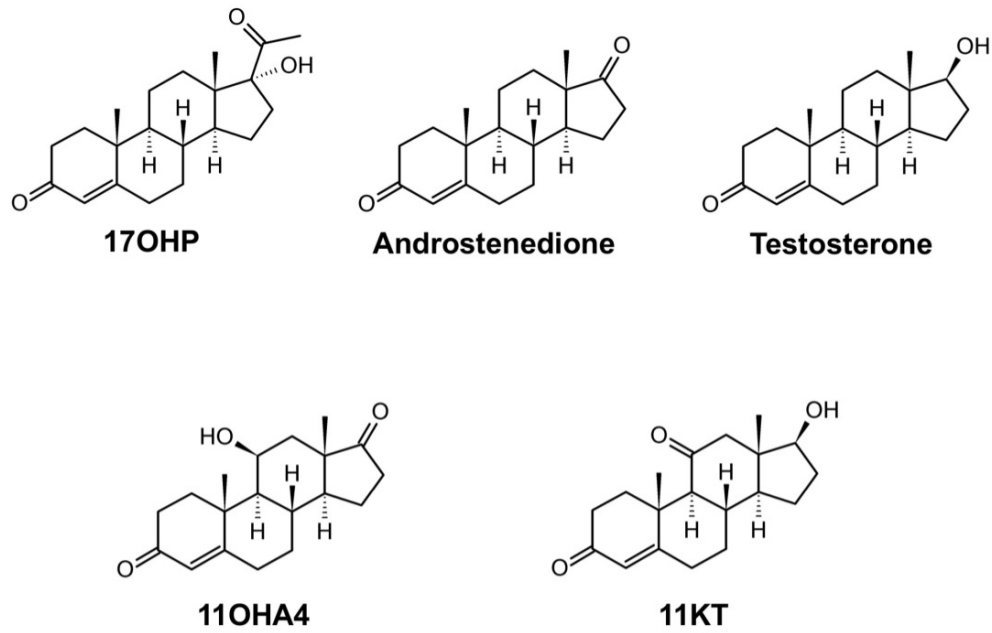
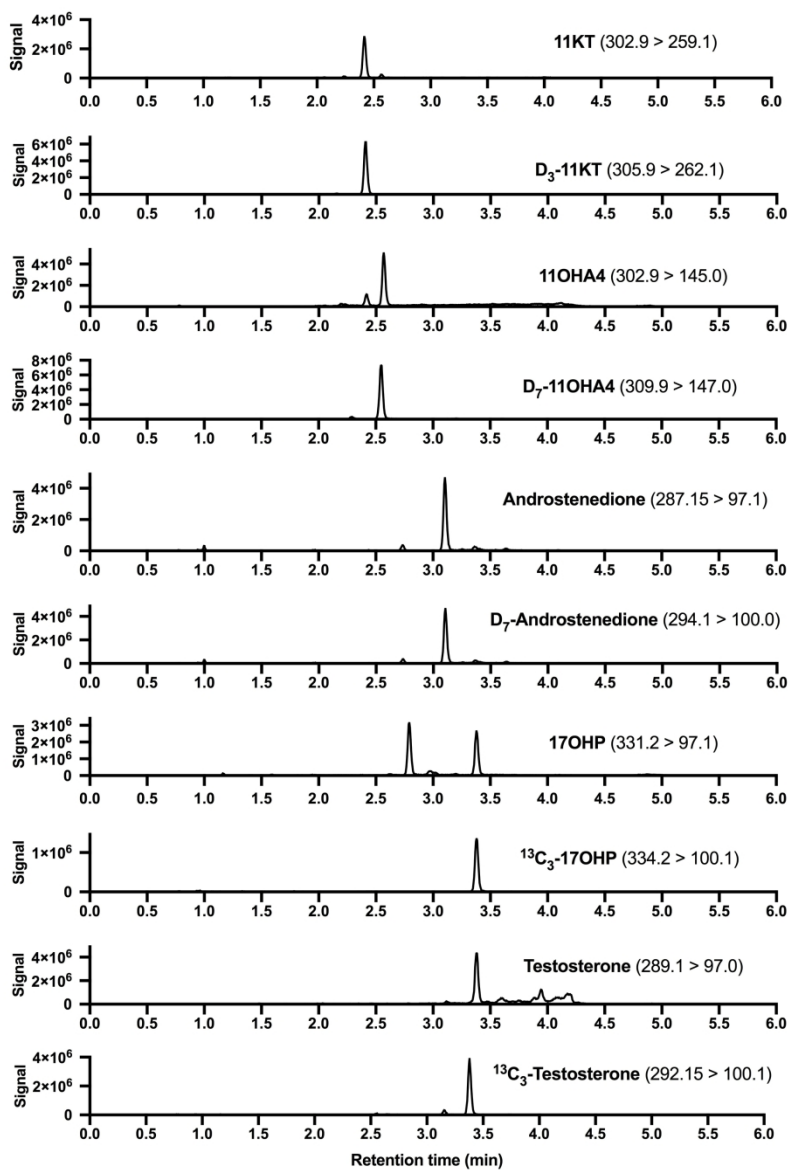


Figure 1: Structures of 17-hydroxyprogesterone (17OHP), androstenedione, testosterone, 11 β -hydroxyandrostenedione (11OHA4) and 11-ketotestosterone (11KT).

385x254mm (72 x 72 DPI)



45 Chromatograms of a saliva sample for all target analytes and their internal standards. Quantifier transitions
46 (m/z) are indicated. 17OHP, 17-hydroxyprogesterone; A4, androstenedione; T, testosterone; 11OHA4, 11 β -
47 hydroxyandrostenedione; 11KT, 11-ketotestosterone.

48 198x284mm (300 x 300 DPI)

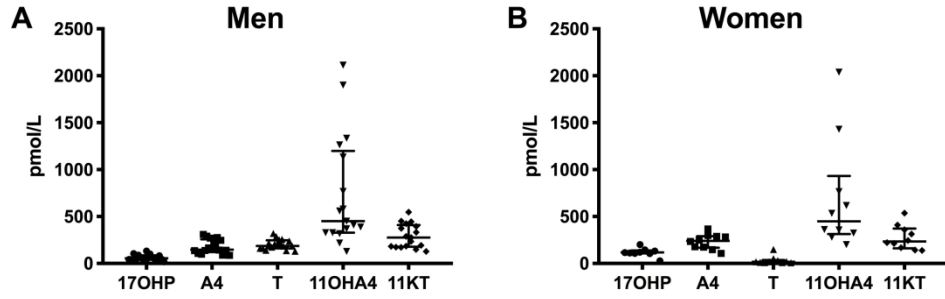


Figure 3: Morning salivary androgen levels in healthy men (A, n=17) and women (B, n=10). Samples were collected between awakening and 10 a.m. Participating women had a regular menstrual cycle and collected the sample on day 21 of the cycle. Individual data points, median and interquartile range are depicted. 17OHP, 17-hydroxyprogesterone; A4, androstenedione; T, testosterone; 11OHA4, 11 β -hydroxyandrostenedione; 11KT, 11-ketotestosterone.

239x75mm (300 x 300 DPI)