Classic and 11-oxygenated androgens in serum and saliva across adulthood

Schiffer, Lina; Kempegowda, Punith; Sitch, Alice J; Adaway, Joanne E; Shaheen, Fozia; Ebbehoj, Andreas; Singh, Sumitabh; Mctaggart, Malcom P; O'reilly, Michael W; Prete, Alessandro; Hawley, James M; Keevil, Brian G; Bancos, Irina; Taylor, Angela E; Arlt, Wiebke

DOI:
10.1093/ejendo/lvac017

European Journal of Endocrinology, 2023, **188**, 1–15
https://doi.org/10.1093/ejendo/lvac017
Advance access publication 10 January 2023
Original Research

Classic and 11-oxygenated androgens in serum and saliva across adulthood: a cross-sectional study analyzing the impact of age, body mass index, and diurnal and menstrual cycle variation

Lina Schiffer,[1,†] Punith Kempegowda,[1,2,†] Alice J. Sitch,[3,4] Joanne E. Adaway,[5] Fozia Shaheen,[1] Andreas Ebbehoj,[6,7] Sumitabh Singh,[6] Malcom P. McTaggart,[5] Michael W. O'Reilly,[1,8] Alessandro Prete,[1,2] James M. Hawley,[1,5] Brian G. Keevil,[5] Irina Bancos,[6] Angela E. Taylor,[1] and Wiebke Arlt[1,2,4,*]

[1]Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, United Kingdom
[2]Centre for Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, United Kingdom
[3]Institute of Applied Health Research, University of Birmingham, Birmingham, United Kingdom
[4]National Institute for Health Research (NIHR) Birmingham Biomedical Research Centre, University of Birmingham and University Hospitals Birmingham NHS Foundation Trust, Birmingham, United Kingdom
[5]Department of Clinical Biochemistry, Wythenshawe Hospital, Manchester, United Kingdom
[6]Division of Endocrinology, Metabolism, Diabetes and Nutrition, Department of Internal Medicine, Mayo Clinic, Rochester, MN, United States
[7]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
[8]Endocrinology Research Group, Department of Medicine, Royal College of Surgeons in Ireland (RCSI) University of Medicine and Health Sciences, Dublin, Ireland

***Corresponding author:** Institute of Metabolism and Systems Research (IMSR), College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, UK. Email: w.arlt@bham.ac.uk

Abstract

Objective: 11-oxygenated androgens significantly contribute to the circulating androgen pool. Understanding the physiological variation of 11-oxygenated androgens and their determinants is essential for clinical interpretation, for example, in androgen excess conditions. We quantified classic and 11-oxygenated androgens in serum and saliva across the adult age and body mass index (BMI) range, also analyzing diurnal and menstrual cycle-dependent variation.

Design: Cross-sectional. Morning serum samples were collected from 290 healthy volunteers (125 men, 22-95 years; 165 women, 21-91 years). Morning saliva samples were collected by a sub-group (51 women and 32 men). Diurnal saliva profiles were collected by 13 men. Twelve women collected diurnal saliva profiles and morning saliva samples on 7 consecutive days during both follicular and luteal menstrual cycle phases.

Methods: Serum and salivary steroids were quantified by liquid chromatography–tandem mass spectrometry profiling assays.

Results: Serum classic androgens decreased with age-adjusted BMI, for example, %change kg/m^2 for 5α-dihydrotestosterone: men −5.54% (95% confidence interval (CI) −8.10 to −2.98) and women −1.62% (95%CI −3.16 to −0.08). By contrast, 11-oxygenated androgens increased with BMI, for example, %change kg/m^2 for 11-ketotestosterone: men 3.05% (95%CI 0.08-6.03) and women 1.68% (95%CI −0.44 to 3.79). Conversely, classic androgens decreased with age in both men and women, while 11-oxygenated androgens did not. Salivary androgens showed a diurnal pattern in men and in the follicular phase in women; in the luteal phase, only 11-oxygenated androgens showed diurnal variation.

Conclusions: Classic androgens decrease while active 11-oxygenated androgens increase with increasing BMI, pointing toward the importance of adipose tissue mass for the activation of 11-oxygenated androgens. Classic but not 11-oxygenated androgens decline with age.

Keywords: 11-oxygenated androgens, serum steroids, salivary steroids, liquid chromatography–tandem mass spectrometry, body mass index

† Joint first authors.

Received: March 27, 2022. **Revised:** September 28, 2022. **Editorial Decision:** December 8, 2022. **Accepted:** December 8, 2022

Significance

We analyzed classic and 11-oxygenated androgen concentrations from a large healthy volunteer cohort with broad ranges of age and body mass index who donated serum ($n = 290$) and saliva ($n = 83$) samples that underwent multi-steroid profiling by tandem mass spectrometry assays. In women, 11-oxygenated androgens were present in amounts at least equal amounts to those of classic androgens, while in men gonadal testosterone production ensured the predominance of classic androgens. 11-oxygenated androgen levels remained unchanged with age, while classic androgens decreased. Classic androgens testosterone and 5α-dihydrotestosterone decreased with body mass index whereas androgen receptor agonists 11-ketotestosterone and 11-hydroxytestosterone increased. 11-oxygenated androgens are major contributors to the circulating androgen pool, particularly in women, and their activation appears closely linked to adipose tissue mass.

Introduction

Circulating androgens and their precursors are produced by the gonads and the adrenal glands in both men and women. However, androgen biosynthesis in the gonads and adrenal glands is differentially regulated and generates different types of androgen precursors.

Gonadal androgen biosynthesis produces the classic androgen precursor androstenedione (A4) and the active classic androgens, testosterone (T), and 5α-dihydrotestosterone (DHT). Gonadal steroidogenesis begins with puberty and declines gradually with age in men and more rapidly in women during the peri-menopause, and is primarily regulated by pituitary gonadotrophins, that is, via the hypothalamic–pituitary–gonadal axis.[1]

Adrenal biosynthesis produces the classic androgen precursor dehydroepiandrosterone (DHEA), which serves as a substrate for the generation of active androgens in peripheral tissues.[2] DHEA can be inactivated to its sulfate ester DHEAS or activated in the classic androgen pathway in the adrenals contributing to circulating concentrations of A4 and, to a lesser degree, T. Adrenal DHEA biosynthesis declines with age in adults[3] and is stimulated by adrenocorticotropic hormone and hence follows the diurnal pattern of hypothalamic–pituitary–adrenal axis activation.[4,5]

The adrenal gland also produces significant amounts of the 11-oxygenated androgen precursor 11β-hydroxyandrostenedione (11OHA4),[6] which is generated through hydroxylation of A4 by steroid 11β-hydroxylase CYP11B1, the same enzyme that catalyzes the final step of cortisol biosynthesis.[7] While the ability of the adrenal to produce 11OHA4 is known for decades, 11OHA4 was previously considered an inert metabolite. However, in recent years it has been demonstrated that 11OHA4 can be activated in peripheral tissues to 11-ketoandrostenedione (11KA4) and 11-ketotestosterone (11KT).[7,8] Importantly, 11KT binds and activates the androgen receptor with similar potency and efficacy as T and the 11KT metabolite 11β-hydroxytestosterone (11OHT) is a partial androgen receptor agonist.[6,9,10] In peripheral tissues such as adipose, 11KT is generated from 11KA4 by aldo–keto reductase 1C3, AKR1C3, which can also convert A4 to T, but has a significantly higher catalytic activity for the activation of 11-oxygenated androgens.[11,12] The expression of AKR1C3 in adipose tissue is upregulated by insulin[13] and reduced by weight loss in women.[11]

11KT is the major circulating active androgen in women[14,15] and has been highlighted as a potential disease activity biomarker in women with polycystic ovary syndrome[14] and congenital adrenal hyperplasia.[16–18] To inform the understanding of the physiological variations of 11-oxygenated androgens in healthy women and men, we comprehensively studied the physiological variation of 11-oxygenated and classic androgens in serum and saliva, including diurnal and menstrual variation, and examined the influence of age and body mass index (BMI) in a large healthy volunteer cohort with a broad age range.

Participants and methods

Participants and sample collection

The collection of serum and saliva samples from healthy volunteers identified through advertisement received ethical approval by the Science, Technology, Engineering and Mathematics Ethical Review Committees of the University of Birmingham, UK (ERN_17-0494, ERN_17-0494B) and the Mayo Clinic, Rochester, MN, USA (IRB ID 18-009787). Inclusion criteria were age 18 years or above and provision of written informed consent prior to study participation. Exclusion criteria were any acute or chronic disease affecting steroid biosynthesis or metabolism, the intake of any medication known to interfere with steroid biosynthesis or metabolism except for use of hormonal contraceptives (combined oral contraceptives, contraceptive depot injection, or implant).

A standardized questionnaire was used to record demographic data including age and BMI; for women, the use of hormonal contraceptives and menopausal status were recorded. If no information on menopausal status was provided, women aged 50 years or older were considered postmenopausal. Premenopausal women not using hormonal contraception were asked on which day of their menstrual cycle the sample was collected, defining the day of the start of their last menstrual bleed as day 1.

We collected morning serum samples from 290 healthy volunteers (165 women, 57%; 125 men, 43%), with similar age and BMI distribution for men (median age 57 (range 22-95) years; median BMI 26 (range 19.0-43.3) kg m^{-2}) and women (median age 48 (range 21-91) years; median BMI 24.6 (range 17.3-43.5) kg m^{-2}). We included 76 (46%) premenopausal and 89 (54%) postmenopausal women. Fifteen of the premenopausal women (20%) either received hormonal oral contraceptives ($n = 7$; all combined estrogen/progestin) or contraceptive depot injections/implants ($n = 8$; all progestin-only); none of the postmenopausal women were on hormone replacement therapy (HRT). Menstrual cycle–dependent serum steroid concentrations were studied in 32 women, who reported the day of their menstrual cycle at the time of blood collection.

Morning saliva was collected by 83 healthy volunteers (51 women, 37 pre- and 14 post-menopausal; 32 men). Twenty-five volunteers (12 premenopausal women, age 24-39 years; 13 men, age 20-35 years) additionally collected

a 7-timepoint saliva profile (07:00 hours, 09:00 hours, 11:00 hours, 13:00 hours, 15:00 hours, 19:00 hours, 23:00 hours). Twelve premenopausal women (eight not on hormonal contraceptives) collected diurnal saliva profiles during both the follicular and luteal phases of the menstrual cycle. In addition, they collected morning saliva samples for 7 consecutive days both in the follicular and the luteal phases (days 1-7 and days 18-24, respectively).

All blood samples were collected in the morning between 8:30 and 10:25 and serum was stored at −80°C. Saliva samples were collected by passive drool; morning samples were collected between waking up and 10:00 hour. To avoid contamination by trace blood or other substances, participants were asked to avoid brushing their teeth for 2 hours, to avoid eating for 1 hour and to rinse their mouth with water 10-15 minutes prior to the saliva sample collection. Saliva samples were frozen at −20°C, then defrosted, and centrifuged at 2500 *g* for 5-10 minutes; the clear supernatant was aliquoted and stored at −80°C until analysis.

Serum steroid analysis by liquid chromatography–tandem mass spectrometry

Unconjugated steroids were quantified using a previously published and validated multi-steroid profiling liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay.[19] In brief, 200 µL of serum were mixed with stable isotope-labeled internal standards (Progesterone-d9, 17α-hydroxyprogesterone (17OHP)-d8, cortisol-d4, cortisone-d7, DHEA-d6, A4-d7, T-d3, DHT-d3, 11K4-d10, 11KT-d3, 11OHA4-d7, 5α-dihydroprogesterone (5αDHP)-d4, allopregnanolone (alloP)-d4) and following protein precipitation with 50 µL acetonitrile samples were extracted by liquid–liquid extraction with 1 mL methyl *tert*-butyl ether. The dried organic phase was reconstituted in the mobile phase and steroids were chromatographically separated using a Phenomenex Luna Omega C18 column (1.6 µm, 100Å, 2.1 mm × 50 mm) and a water–methanol gradient. Ammonium fluoride was introduced by post-column infusion and steroid quantification was performed on a Waters Xevo TQ-XS mass spectrometer using electrospray ionization in positive ion mode.

In brief, DHEAS was extracted and quantified using a separate validated assay.[20,21] Internal standard (DHEAS-d2) was added to 20 µL of serum followed by 20 µL of $ZnSO_4$ then100 µL of acetonitrile. The samples were then centrifuged and 100 µL of the solution was transferred to a new vial, dried, and reconstituted in 200 µL of methanol/water prior to LC–MS/MS analysis.[21] DHEAS was quantified against a calibration series ranging from 250 to 8000 ng mL^{-1}.

Saliva steroid analysis by LC–MS/MS

As previously described,[22,23] 300 µL of saliva were mixed with isotopically labelled internal standards and extracted by supported liquid extraction using 1 mL of dichloromethane. Progesterone, 17α-hydroxyprogesterone (17OHP), A4, T, 11OHA4, and 11KT were quantified using previously published assays[22,23] on a Waters Xevo TQ-S mass spectrometer. For a subset of morning saliva samples, 17OHP, A4, T, 11OHA4, and 11KT were measured by a modified version of the published assay using a Waters Xevo TQ-XS mass spectrometer. For this assay, chromatography was performed using a Waters Acquity HSS T3 (1.8 µm, 100 Å, 2.1 mm × 50 mm) column using a gradient of 2 mmol L^{-1} ammonium acetate and 0.1% (v/v) formic acid in water and acetonitrile. The lower limit of quantitation was 5 pmol L^{-1} for T, 10 pmol L^{-1} for A4, 12.5 pmol L^{-1} for 17OHP, 6 pmol L^{-1} for 11KT, and 45 pmol L^{-1} for 11OHA4. The assay was linear up to 50 000 pmol L^{-1} for T, 100 000 pmol L^{-1} for A4, 150 000 pmol L^{-1} for 17OHP, 100 000 pmol L^{-1} for 11KT, and 200 000 pmol L^{-1} for 11OHA4. Recovery was shown to be 92.5%-109.8% for all analytes, inter-assay precision was <8.6% (<13.7% at lower limit of quantitation, LLOQ) and inter-assay bias from nominal concentrations was <8.8% (<15.6% at LLOQ).

The analytical performance of the two assays measuring salivary 17OHP, A4, T, 11OHA4, and 11KT was compared using saliva samples from multiple donors ($n = 46$-59). The mean relative difference between the assays was within −5% and 5.8% and the concentrations determined with the two assays strongly correlated ($r > 0.93$, $P < .0001$). The data generated by the two assays was hence combined for analysis.

Statistical analysis

Statistical analysis was performed using Stata v.16 and SPSS version 27 (IBM Corp.) with graphs generated using Prism 9 for macOS Version 9.2.0 (GraphPad Software, LLC). Data were summarized using medians and interquartile ranges (IQRs) and presented separately for men and women by age group (<50 years and ≥50 years); for pre- and post-menopausal women; and for women on hormonal contraceptives and women not on hormonal contraceptives. Statistical comparisons were made used the Wilcoxon signed rank test for paired data and Mann–Whitney *U*-test for unpaired data as appropriate.

For our formal statistical analysis, a multivariable linear model for each steroid was fitted to estimate the association with both age and BMI, with further adjustment for recruitment center. Models were fitted for male and female participants separately. Each steroid outcome was log-transformed prior to regression analysis, due to non-normality of data, and results were expressed as approximate percentage changes per unit change in the characteristic,[24] with corresponding 95% confidence intervals (CIs).

Steroid concentrations below the LLOQ were replaced by 0.5×LLOQ for all statistical comparisons; however, raw data without adjustment are used for illustrative purposes in the figures. We have not made any formal corrections for multiple testing. However, due to the number of tests performed in this exploratory analysis, if readers do wish to consider statistical significance, we would advise a lower level of significance <0.05 to be used.

Results

Serum and saliva androgen in men and women

Serum concentrations of the classic androgen precursors DHEA and A4 were similar in men and women while, expectedly, the circulating concentrations of T and DHT were higher in men (Figure 1A). Serum concentrations of both 11-oxygenated androgen precursors (11OHA4 and 11KA4) and active 11-oxygenated androgens (11KT, 11OHT) were similar in men and women (Figure 1B). Serum concentrations of 11OHA4 were highest amongst the 11-oxygenated androgens, higher than serum A4 and similar to serum DHEA. Sex- and age-specific serum concentration ranges for classic

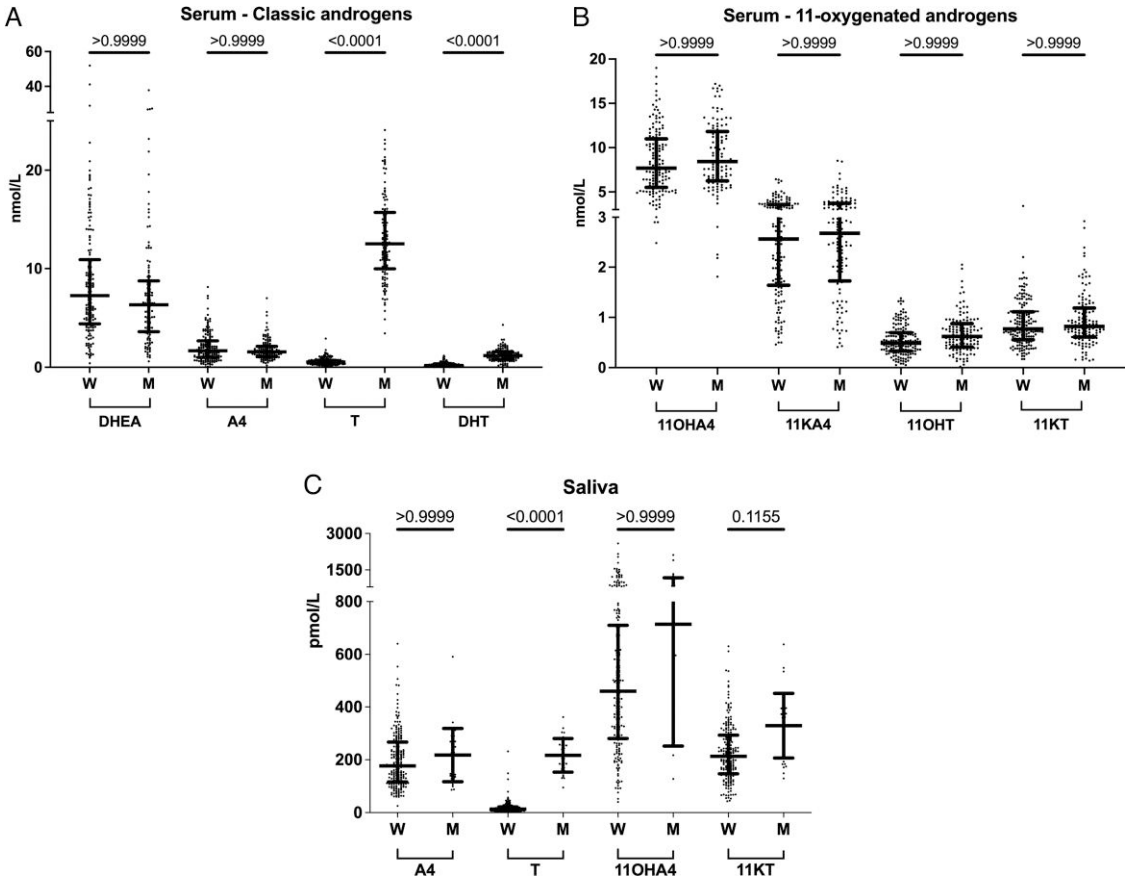

Figure 1. Classic (A) and 11-oxygenated (B) androgen concentrations in serum from 125 men and 165 women and saliva (C) from 32 men and 51 women. Individual data points, median, and interquartile range are indicated.

and 11-oxygenated androgens are summarized in Table 1; results for other steroids measured are provided in Table S1.

Salivary T, but not A4, concentrations were higher in men than in women. Salivary 11OHA4 and 11KT concentrations showed similar concentrations in men and women. Salivary 11KT was higher than T in both men and women (Figure 1C). Salivary and serum concentrations of A4, T, 11OHA4, and 11KT from the same individuals correlated closely (Figure S1).

Differential effects of age and BMI on classic and 11-oxygenated serum androgens

Exploratory analyses showed, that in the cohort providing serum samples age and BMI were positively correlated in both men ($r = 0.3482$, $P < .0001$) and women ($r = 0.4928$, $P < .0001$; Figure S2A and B).

In the classic androgen pathway, both androgen precursors (DHEAS, DHEA, A4) and active androgens (T, DHT) declined as age increased in both women (Figure 2) and men (Figure S3). The linear regression model adjusting for BMI demonstrated a clear impact of age with decreasing concentrations of all classic androgens except for DHT in men (Figure 3A and B, Table S2). In keeping with this, postmenopausal women had lower serum DHEAS, DHEA, and A4 compared to premenopausal women (all $P < .001$; Table S3).

Descriptive analyses indicated no clear association between 11-oxygenated androgen concentrations and age except for 11OHT (Figure 2). However, after adjustment for BMI (using

Table 1. Sex- and age-specific serum steroid concentrations (median and 5th-95th centile range) of classic and 11-oxygenated androgens.

	Men <50 years ($n = 48$)	Men ≥50 years ($n = 76$)	Women <50 years ($n = 84$)	Women ≥50 years ($n = 81$)
Age (years) median (min-max)	34 (22-47)	68 (50-95)	30 (21-49)	64 (51–91)
BMI (kg/m^2) median (min-max)	24 (19.0-31.8)	26.9 (20.6-43.3)	22.4 (17.3-37.3)	25.7 (18.4–43.5)
DHEAS (μmol L^{-1})	6.0 (2.7-12.0)	2.3 (0.82-6.6)	4.6 (1.6-8.2)	1.5 (0.41–4.1)
DHEA (nmol L^{-1})	8.1 (2.5-27.0)	5.0 (1.5-12.0)	9.6 (4.2-20.0)	5.0 (1.3-16.0)
A4	1.5 (<0.70-5.0)	1.7 (<0.70-3.2)	2.3 (0.71-5.3)	1.1 (<0.70-2.9)
T	13.0 (8.3-19.0)	12.0 (6.5-22.0)	0.61 (<0.35-1.2)	0.45 (<0.35-1.2)
DHT	1.4 (0.60-2.3)	1.1 (<0.34-2.2)	<0.34 (<0.34-0.80)	<0.34 (<0.34-0.36)
11OHA4	7.6 (2.9-14.0)	8.5 (4.6-16.0)	7.5 (3.4-14.0)	8.4 (3.9-16.0)
11KA4	3.5 (1.42-6.2)	2.1 (0.60-5.2)	3.2 (1.6-5.4)	1.8 (0.64-4.2)
11OHT	0.47 (<0.33-1.1)	0.72 (<0.33-1.5)	0.39 (<0.33-0.78)	0.63 (<0.33-1.2)
11KT	0.86 (<0.33-2.2)	0.81 (0.36-1.8)	0.70 (<0.33-1.6)	0.94 (0.37-1.7)

Values below the lower limit of quantification (LLOQ) are shown as less than (<) the steroid-specific LLOQ.

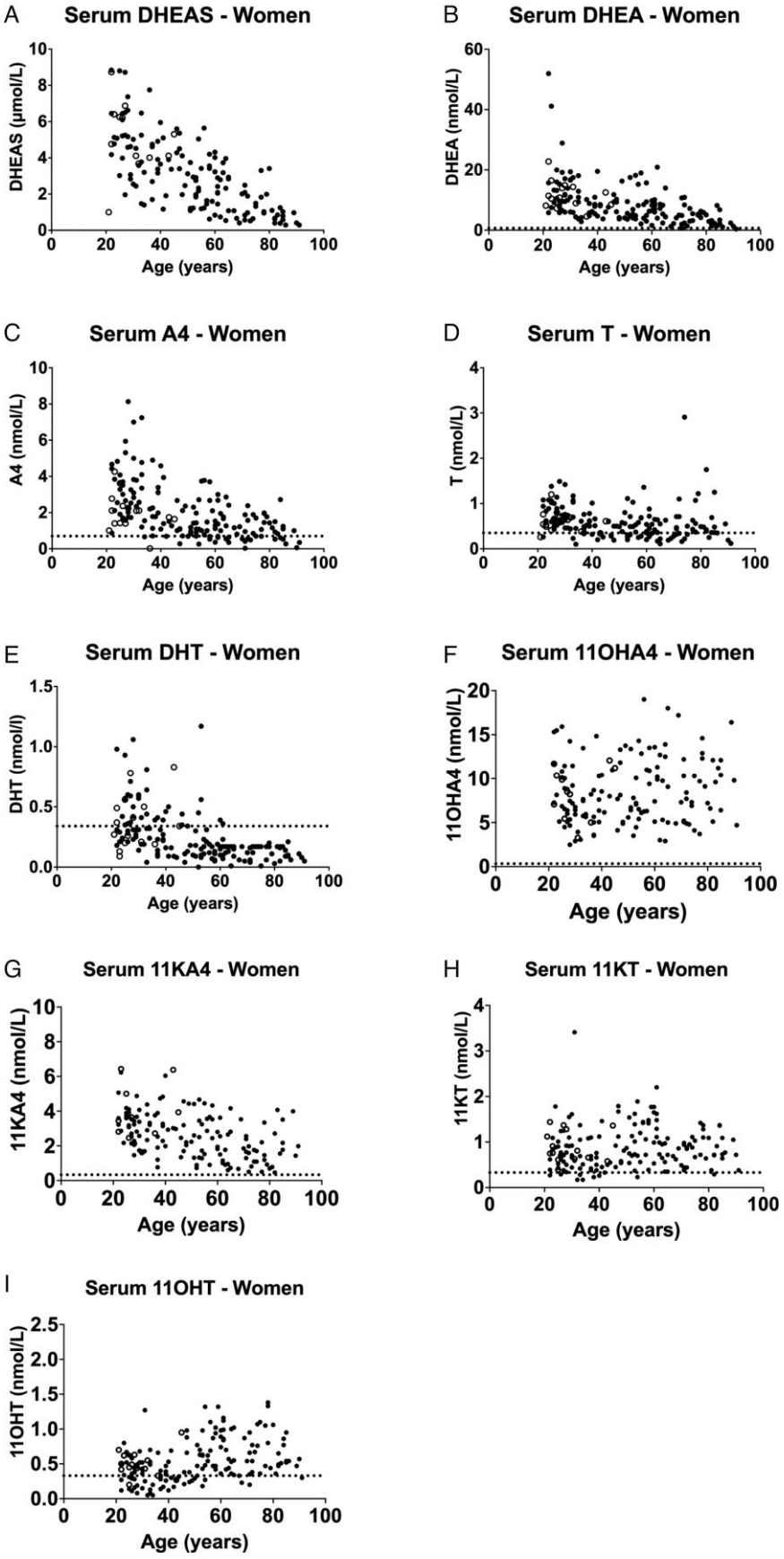

Figure 2. Serum concentrations of classic androgens across the adult age range in women ($n = 165$). Women on hormonal contraceptives are shown as open circles. The dotted line indicates the lower limit of quantification.

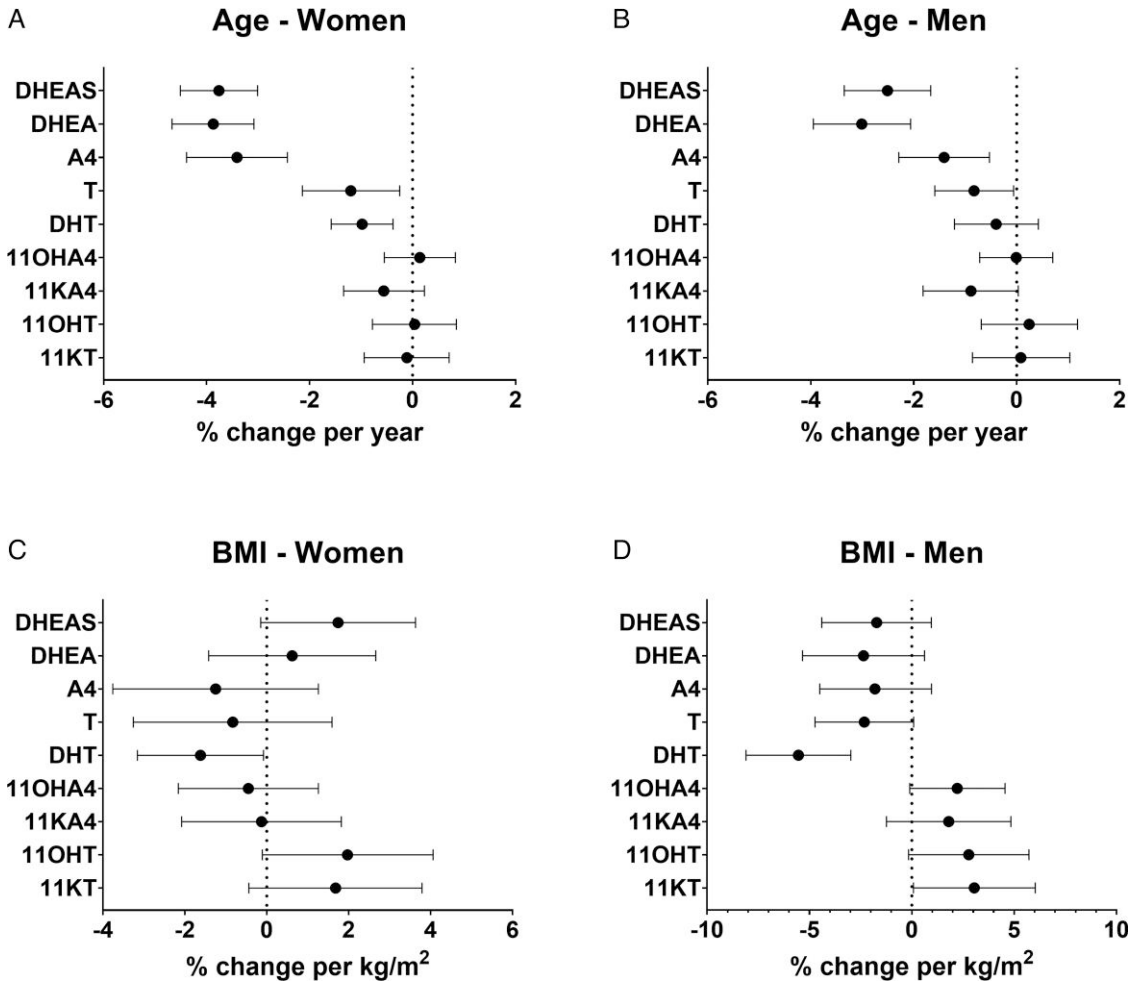

Figure 3. Impact of increasing age (%change per year) and body mass index (%change kg^{-1} m^{-2}) on classic and 11-oxygenated androgens in women ($n = 165$) and men ($n = 125$). Results obtained by linear regression analysis using log-transformed outcomes, with results expressed as percentage changes per unit change.

the linear regression model), serum 11OHT was similar across ages (women: %change kg/m^2 0.04 (95% CI −0.78 to 0.85); men: 0.24 (−0.69 to 1.18)), while 11KA4 decreased slightly in older subjects (women: −0.56 (95% CI −1.34 to 0.23); men: −0.89 (−1.82 to 0.03)) (Figure 3A and B, Table S2).

Descriptive analyses explored the relationship between circulating active androgen concentrations and BMI in women (Figure 4) and men (Figure S4). The linear regression analysis, adjusting for the effect of age, showed a clear negative association of BMI with DHT in men (%change kg/m^2 −5.54 (95% CI −8.10 to −2.98)), as well as a trend for increases in all four 11-oxygenated androgens in men with increasing BMI. In women, DHT was also negatively associated with BMI (−1.62 (−3.16 to −0.08)), but of the 11-oxygenated androgens only 11KT (1.68 (−0.44 to 3.79)) and 11OHT (1.97 (−0.11 to 4.04)) trended to increase with increases in BMI (Figure 3C and D, Table S2). Of note, DHEAS also tended to increase with BMI in women (% change kg/m^2 1.74 (95% CI −0.15 to 3.63)), but to decrease in men (−1.72 (−4.40 to 0.95)) (Figure 3C and D).

Menstrual cycle variations in serum and saliva steroids

Serum concentrations of progesterone ($P = .0034$) and its metabolites 5αDHP and alloP (both $P < .001$) were significantly increased during the luteal phase (Figure S5). Morning salivary progesterone and 17OHP concentrations were higher during the luteal phase, reflective of ovulation and corpus luteum formation (Figure 5A and B, Figure S6).

Salivary A4, T, 11OHA4, and 11KT (Figure 5C–F, Figure S6) and serum concentrations of classic and 11-oxygenated androgens (Figure 6, Figure S7) did not differ significantly between follicular and luteal phases of the menstrual cycle except for serum DHEA, which was higher in the luteal phase ($P = .0128$), and T which showed a trend toward higher concentrations in the luteal phase ($P = .0749$).

Diurnal variation of salivary steroids in premenopausal women and men

Salivary progesterone concentrations were below the limit of quantification at all time points during the follicular phase in all but one woman. Progesterone increased during the luteal phase without demonstrating a diurnal pattern (Figure 7A).

Salivary concentrations of 17OHP, A4, and T as well as 11OHA4 and 11KT showed a clear diurnal pattern during the follicular phase in women (Figure 7) and also in men (Figure S7), with the highest concentrations observed in the morning followed by a gradual decline with a nadir in the late evening. 11OHA4, which is produced by CYP11B1 in the adrenal gland, and its downstream metabolite 11KT, showed stronger diurnal variation than A4 and T (Figure 7,

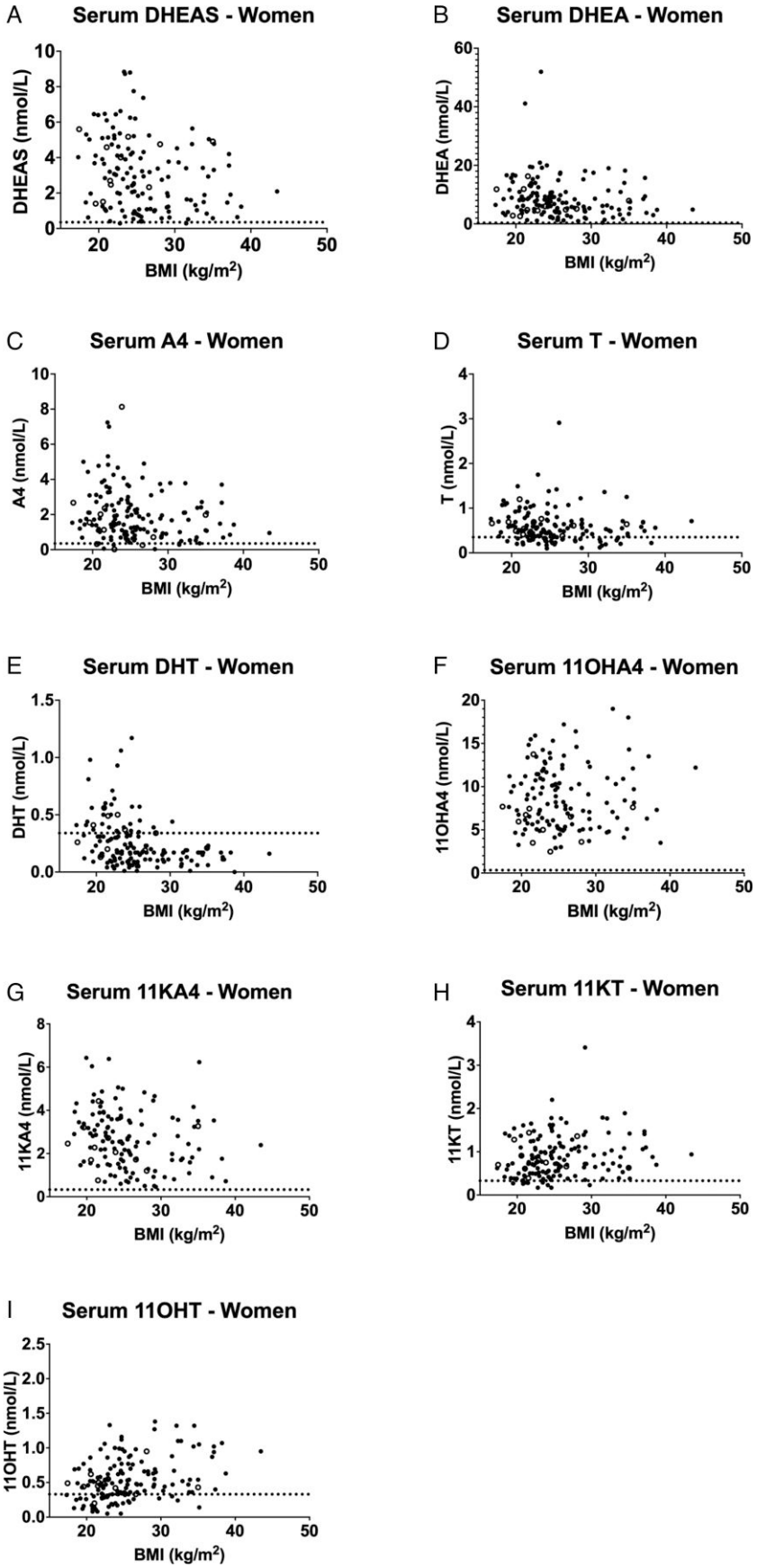

Figure 4. Serum concentrations of active classic and 11-oxygenated androgens in relation to body mass index (BMI) in women ($n = 165$). Women on hormonal contraceptives are shown as open circles. The dotted line indicates the lower limit of quantification.

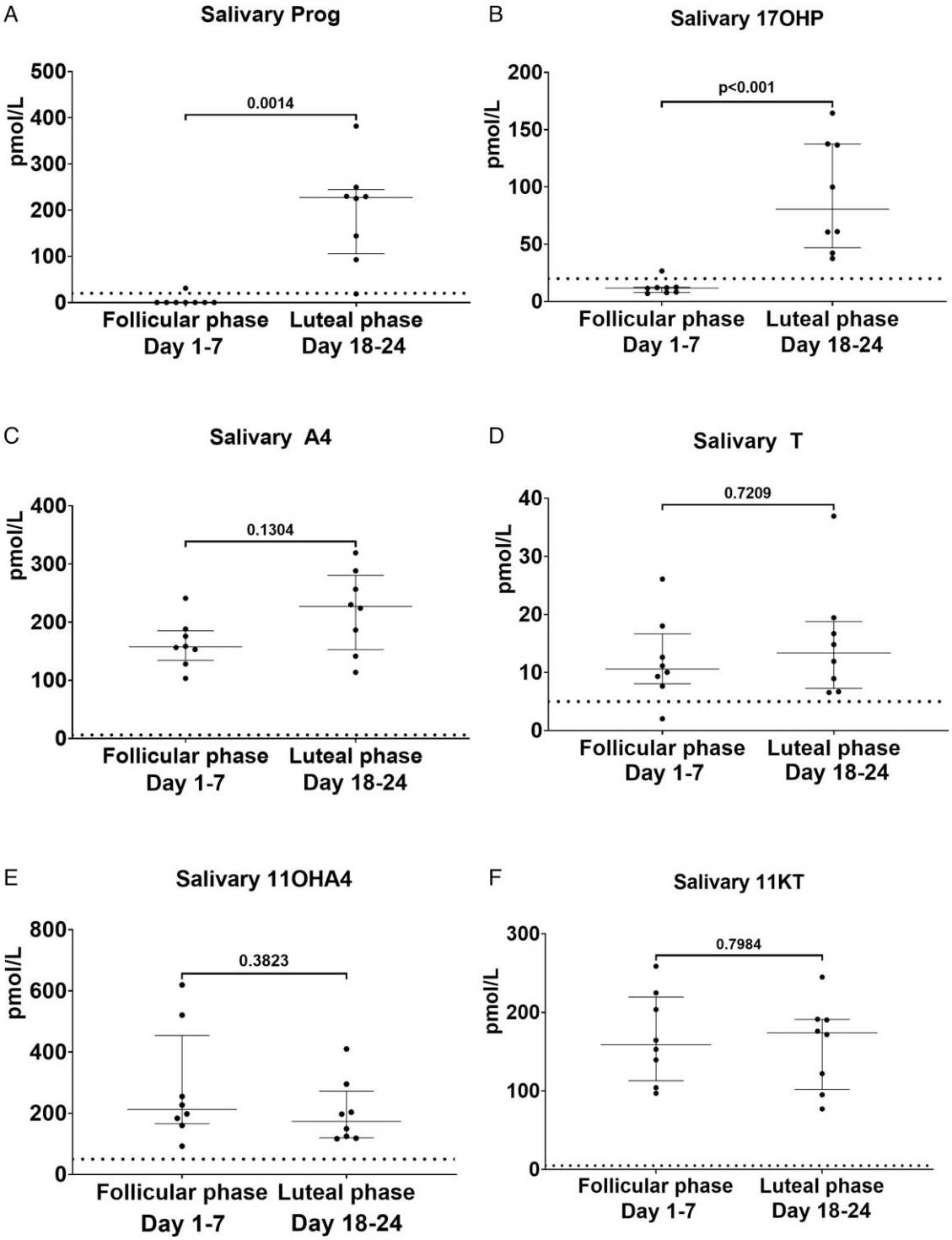

Figure 5. Salivary steroids during follicular and luteal phases of the menstrual cycle. Eight women collected daily morning saliva samples on days 1-7 of the menstrual cycle (follicular phase) and days 18-24 (luteal phase) and the median values of these collections are shown for every individual. The dotted line indicates the lower limit of quantification. Median and interquartile ranges are indicated.

Figure S8). A clear diurnal pattern was also maintained by serum 11-oxygenated androgens throughout the menstrual cycle while the diurnal pattern for A4 and T was less distinct in the luteal phase (Figure 7).

Impact of hormonal contraceptives on serum and salivary steroids

Premenopausal women with and without hormonal contraceptive use had similar age and BMI distributions (Table 2). Expectedly, women on hormonal contraceptives had significantly lower serum concentrations of progesterone and its metabolites 5αDHP, alloP, and 17OHP (Table 2).

Serum A4 concentrations were significantly lower in women taking hormonal contraceptives ($P < .001$). However, neither the serum concentration of T nor those of the adrenal-derived androgens and their precursors (DHEA, 11OHA4, 11KA4, and 11KT) were different between women taking and not taking contraceptives.

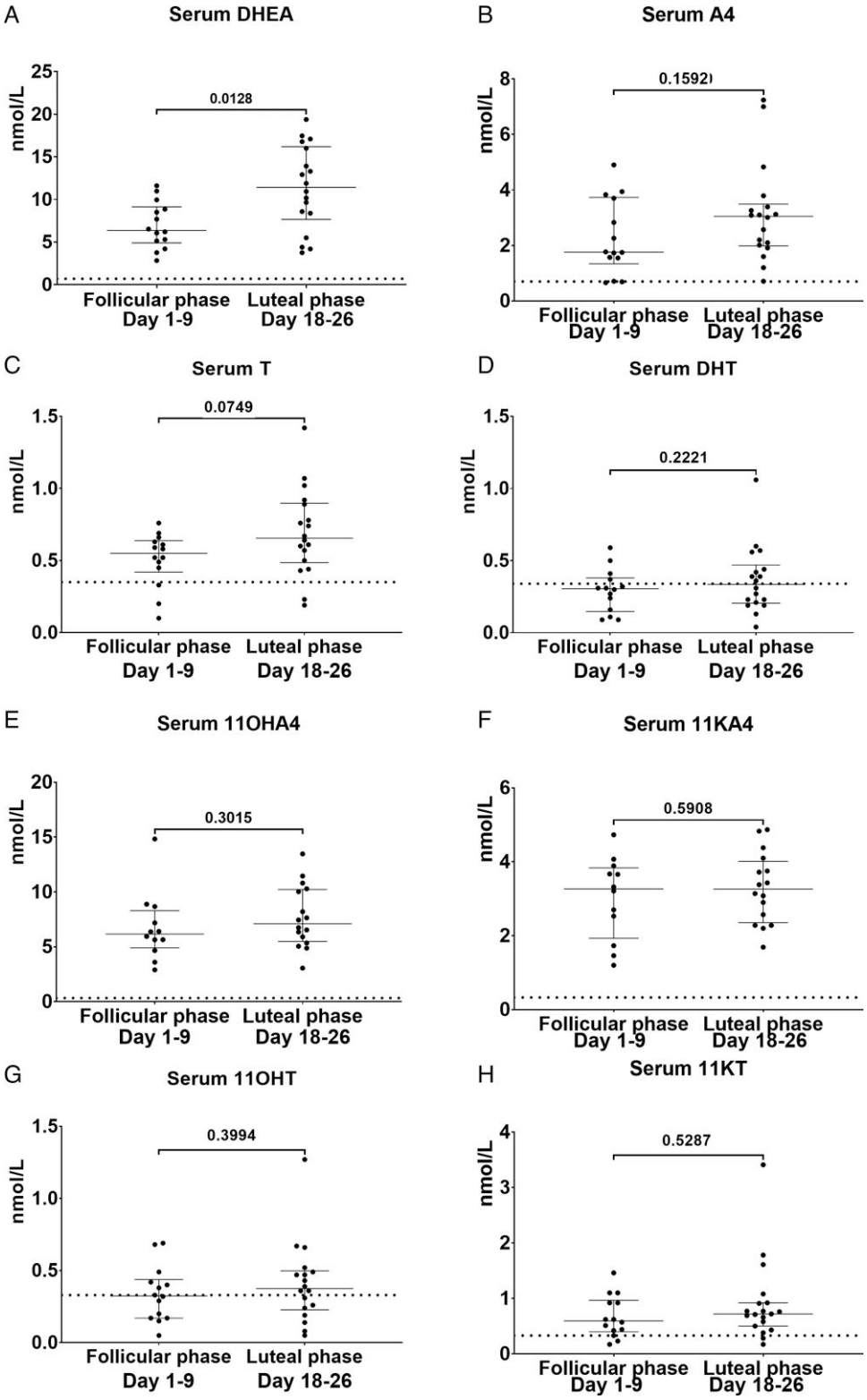

Figure 6. Serum androgens during follicular and luteal phases of the menstrual cycle. Women indicated on which day of their menstrual cycle their serum sample was collected. Samples collected on or between days 1 and 9 were categorized as the follicular phase ($n = 12$-14), samples collected on or between days 18 and 26 were categorized as the luteal phase ($n = 16$-18). The dotted line indicates the lower limit of quantification. The median and interquartile range are indicated.

Mirroring the observations in serum, salivary 17OHP and A4 concentrations were significantly decreased in women taking hormonal contraceptives (all $P < .001$; Table 2). Interestingly, while we did not observe differences in serum T and 11KT, salivary T and 11KT concentrations were significantly lower in women on hormonal contraceptives (all $P < .001$; Table 2).

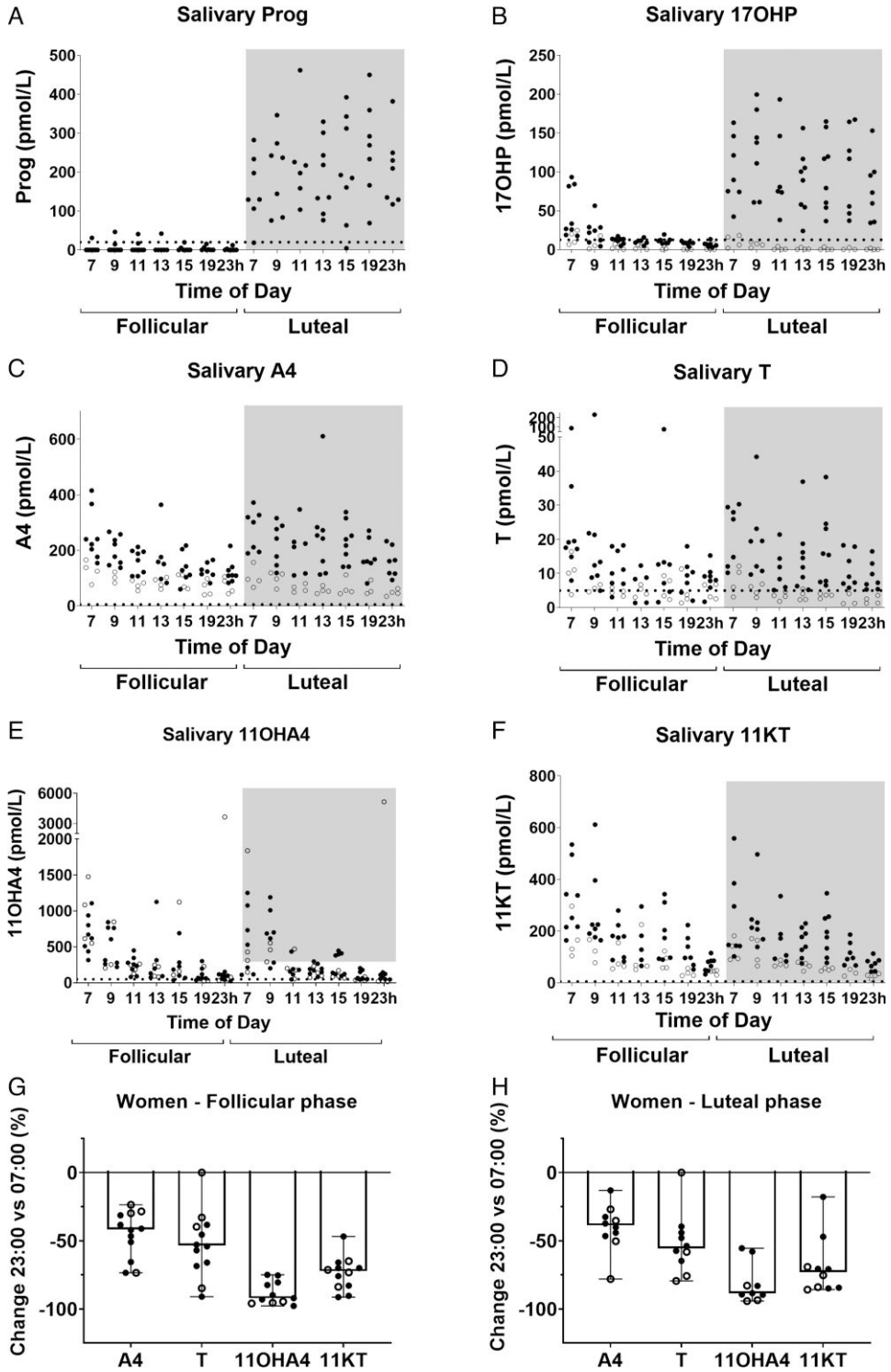

Figure 7. Diurnal variation of salivary steroids in premenopausal women during follicular and luteal phases of the menstrual cycle. Eight women not taking any hormonal contraceptives (solid circles) and 4 women on contraceptives (open circles) collected seven saliva samples at defined times over 24 hours on a day during the follicular phase (days 1-7) and on a day during the luteal phase (days 18-24, grey shaded area). Women were allowed to choose on which day during the respective phase they completed the sample collection series. The dotted line indicates the lower limit of quantification. Percentage change in salivary steroid concentrations over the day were calculated for follicular (G) and luteal phases (H). Bars represent the median and whiskers represent the range.

Discussion

The active 11-oxygenated androgen 11KT and its 11-oxygenated precursors have been established as useful biochemical markers of adrenal androgen excess over recent years,[14,16,18,25] in addition to the classic androgens, testosterone, and 5α-DHT. Aiming to improve the utility of 11-oxygenated androgen measurements in the clinical laboratory, we set out to characterize the physiological variation of

Table 2. Effect of hormonal contraceptives (HCs) on serum and salivary steroid concentrations.

	Women not on HC Median (IQR)	Women on HC Median (IQR)	*P*
Serum steroids			
n	61	15	
Age (years)	30.0 (26.0-37.0)	26.0 (23.0-32.0)	.060
BMI (kg/m^2)	22.1 (20.8-24.6)	21.6 (20.1-26.6)	.993
Pregnenolone	3.0 (1.3-5.3)	2.6 (1.9-3.0)	.014
Progesterone	2.8 (<0.3-34.5)	<0.3 (<0.3, < 0.3)	<.001
5αDHP	<0.6 (<0.6-1.7)	<0.6 (<0.6 to <0.6)	.006
alloP	<0.6 (<0.6-2.3)	<0.6 (<0.6 to <0.6)	.002
17OHPreg	4.7 (<3.0-6.0)	5.8 (4.0-7.6)	.480
17OHP	2.9 (0.9-5.4)	0.7 (<0.6-1.7)	.062
Cortisol	252 (205-380)	314 (252-491)	.050
Cortisone	47.1 (41.0-54.4)	52.8 (46.5-59.5)	.030
DHEA	9.6 (6.0-14.6)	10.8 (8.5-14.4)	.422
DHEAS	4.6 (3.2-5.6)	5.0 (4.0-6.4)	.327
A4	2.7 (1.8-3.9)	2.1 (1.4—2.4)	<.001
T	0.6 (0.5-0.9)	0.6 (0.4-0.7)	.620
DHT	<0.3 (<0.3-0.5)	<0.3 (<0.3-0.5)	.620
11OHA4	7.2 (5.3-10.0)	8.5 (5.4-11.2)	.357
11KA4	3.2 (2.3-3.8)	3.4 (2.5-5.0)	.414
11OHT	0.4 (<0.3-0.5)	0.5 (0.4-0.6)	1.000
11KT	0.7 (0.4-1.0)	0.8 (0.6-1.3)	.620
Salivary steroids			
n	32	9	
Age (years)	29.0 (28.0-35.0)	25.0 (24.0-27.0)	<.001
BMI (kg/m^2)	22.0 (20.3-24.9)	21.0 (21.0-24.1)	.967
17OHP	56.1 (24.9-110)	6.3 (6.3-18.2)	<.001
A4	220 (154-301)	103 (83-140)	<.001
T	16.8 (10.3-25.9)	10.5 (8.0-14.6)	<.001
11OHA4	476 (311-744)	417 (226-615)	.120
11KT	231 (177-315)	139 (95.9-201)	<.001

All concentrations are given in nmol L^{-1} for all serum steroids, except for DHEAS, which is in µmol L^{-1}; salivary steroid concentrations are in pmol L^{-1}. Values below the lower limit of quantification (LLOQ) are shown as less than (<) the steroid-specific LLOQ.

11-oxygenated androgen concentrations in healthy individuals by multi-steroid profiling in serum and saliva. The effect of age,[15,26] BMI,[15,26] menopause,[27] and variations due to menstrual cycle[26] and diurnal rhythm[28,29] have previously been studied for 11-oxygenated androgen concentrations in serum in several cohorts, however, most of those were of limited size (Table 3). In this study, we comprehensively analyzed the impact of all these variables on classic and 11-oxygenated androgens and their precursors in the second largest cohort to date, analyzing serum steroids in 290 healthy volunteers and salivary steroids in a sub-group of 83 volunteers recruited from a community setting in the UK.

A key finding of our study is the differential and sex-specific impact of BMI on circulating 11-oxygenated androgen concentrations. In men, the levels of all four 11-oxygenated androgens increased with increasing BMI. In women, however, only the AR agonists 11KT and 11OHT are increasing with BMI, while there was no change in the concentrations of their precursors 11OHA4 and 11KA4. This points to adipose tissue as an important determinant and source of active 11-oxygenated androgens, in line with previous findings on increased 11-oxygenated androgens in women with polycystic ovary syndrome with androgen excess, insulin resistance, and obesity.[14] The most important enzyme for the activation of 11-oxygenated androgens is AKR1C3, which converts 11KA4 to 11KT. We have previously shown that AKR1C3 expression in subcutaneous, but not omental, adipose tissue from women correlates significantly with BMI, is reduced after weight loss[11] and upregulated by insulin.[13] It should be noted that the catalytic activity of AKR1C3 is significantly higher for the conversion of 11KA4 to 11KT compared to the conversion of A4 to T.[11,12] Therefore, we suggest that increased fat mass and increased insulin resistance will upregulate AKR1C3 activity, which consequently is likely to increase the generation of the active 11-oxygenated androgens 11KT and 11OHT; the latter is formed as a downstream product from 11KT by the 11-oxoreductase activity of HSD11B1, an enzyme abundantly expressed in adipose tissue.

While studies on AKR1C3 in male adipose tissue are missing, it is plausible that increased AKR1C3 activity in adipose tissue can explain BMI-related increases in circulating 11KT concentrations in both sexes. However, in men, the metabolic impact of the decrease in T and DHT with increasing BMI will be much more significant than any increase in 11-oxygenated androgens. While we found 11-oxygenated androgen concentrations to be similar between men and women, gonadal T production by far exceeded the circulating 11-oxygenated androgen pool in men, irrespective of changes due to increasing age and BMI.

In contrast to our study, where we found a significant positive correlation of age and BMI, in the cohort studied by Davio *et al.*[15] no association between age and BMI was observed. A likely explanation for this might be that in the US American cohort the younger individuals already had high rates of obesity, though this cannot be verified, as no age-specific breakdown of BMI ranges was provided by Davio *et al.*[15] However, they reported a positive association of 11KT with BMI in men and additionally a positive association of 11OHT and a negative association of 11KA4 with BMI in women.[15] Collectively, their observations and our current findings indicate that the peripheral activation of 11-oxygenated androgens appears to be of importance for their biological action.

Of note, the classic androgen precursor DHEAS tended to increase with BMI in women, but not in men, which is interesting considering that in a recent study increases in DHEAS were the most prevalent change observed in 270 premenopausal women with PCOS.[30] In line with previous observations[15,26,27,31,32] we found that serum concentrations of the classic androgen precursors DHEA and A4 and the active androgens T and DHT decline with age. Despite the age-related decrease in DHEA and A4, which are the immediate precursors of 11-oxygenated androgen biosynthesis in the adrenal gland, we did not find evidence for changes in the serum concentrations of 11-oxygenated androgens. The generation of C19 steroids, that is, androgens, from their C21 precursors takes place predominantly in the zona reticularis of the adrenal gland, which co-expresses the enzymes CYP17A1 and CYB5A,[33] which are crucial for biosynthesis of DHEA, the immediate precursor of A4. The zona reticularis also expresses AKR1C3, which converts A4 to T.[34] Due to the age-related reduction of the zona reticularis[35,36] the adrenal production of A4 and T is expected to decrease with age. However, the enzyme CYP11B1, which is required for the formation of 11OHA4 from A4, is expressed in the zona fasciculata, which has not been shown to change with age.[35] The decreased levels of adrenal A4 in older individuals appear to still serve as a sufficient substrate pool to feed the generation of 11-oxygenated androgens by CYP11B1. Consistent with this hypothesis, a recent preliminary report indicated that the CYP11B1 K_m value

Table 3. Previously reported serum concentrations (nmol L^{-1}) of classic and 11-oxygenated androgens as measured in healthy subjects by tandem mass spectrometry.

	Subject	DHEA	A4	T	DHT	11OHA4	11KA4	11KT	11OHT
Previously reported data in healthy women									
Caron et al. (2021)[44]	10 women, follicular phase age 20-40 years mean [SEM]	11.2 [0.8]	2.8 [0.1]	0.6 [0.0]	0.2 [0.0]	5.3 [0.4]	0.8 [0.1]	0.8 [0.1]	0.3 [0.0]
	10 women, luteal phase age 20-40 years mean [SEM]	9.4 [0.5]	3.8 [0.1]	0.6 [0.0]	0.2 [0.0]	4.6 [0.4]	0.8 [0.1]	0.9 [0.1]	0.3 [0.0]
	10 postmenopausal women, age 51-70 years mean [SEM]	14.5 [0.5]	3.0 [0.2]	1.1 [0.1]	0.2 [0.0]	11.6 [0.6]	1.2 [0.0]	1.8 [0.1]	0.8 [0.0]
Davio et al. (2020)[14]	271 women age 55.0 ± 20.2 years mean (SD)	n.m.	1.9 (1.2)	1.0 (0.5)	n.m.	4.4 (2.5)	0.5 (0.2)	0.9 (0.4)	0.5 (0.4)
Nanba et al. (2019)[26]	100 premenopausal women, age 27-39 years median [IQR]	8.1 [5.4-14.0]	2.1 [1.6-3.0]	1.0 [0.8-1.4]	n.m.	0.1 [0.0-0.1]	1.2 [0.9-1.8]	0.9 [0.6-1.3]	0.5 [0.3-0.8]
	100 postmenopausal women, age 62-78 years median [IQR]	2.7 [1.8-4.7]	1.0 [0.8-1.4]	0.7 [0.5-1.1]	n.m.	6.5 [4.7-9.6]	1.1 [0.8-1.5]	0.9 [0.7-1.2]	0.7 [0.4-0.9]
Skiba et al. (2019)[25]	163 women, follicular phase, age 18-19 years median (min-max)	5.3 (1.0-21.3)	1.8 (0.6-5.4)	0.3 (0.0-0.9)	n.m.	n.m.	8.8 (0.1-28.8)	1.3 (0.3-7.6)	n.m.
	184 women, mid-cycle Age 18-19 years $n = 184$; median (min-max)	4.9 (0.4-23.5)	2.1 (0.6-7.9)	0.4 (0.1-1.0)	n.m.	n.m.	7.7 (1.1-31.7)	1.3 (0.0-5.8)	n.m.
	241 women, luteal phase age 18-19 years median (min-max)	4.9 (0.1-23.5)	2.0 (0.5-7.9)	0.3 (0.0-1.0)	n.m.	n.m.	8.0 (0.1-31.7)	1.3 (0.0-7.6)	n.m.
O'Reilly et al. (2017)[13]	49 women; median age 28 (IQR 23-32) years median [IQR]	7.1 [4.2-11.8]	n.m.	0.3 [0.2-0.5]	n.m.	6.8 [4.9-12.5]	2.7 [2.0-3.9]	1.5 [1.2-1.8]	0.2 [0.1-0.3]
Turcu et al. (2016)[15]	19 men and 19 women, reported together, no details on age median [IQR]	6.1 [4.1-11.0]	n.m.	0.9 [0.4-10.7]	n.m.	3.9 [2.3-5.1]	1.0 [0.7-1.4]	1.7 [1.0-2.6]	0.5 [0.3-0.7]
Previously reported data in healthy men									
Caron et al. (2021)[44]	9 men age 21-62 years mean [SEM]	8.2 [0.3]	2.4 [0.1]	18.7 [0.6]	1.3 [0.0]	4.4 [0.2]	0.5 [0.0]	1.1 [0.3]	0.6 [0.0]
	10 men age 60-72 years $n = 10$; mean [SEM]	4.9 [0.2]	2.4 [0.1]	19.6 [0.8]	1.3 [0.1]	6.8 [0.3]	0.8 [0.0]	1.5 [0.1]	0.5 [0.0]
Schröder et al. 2021	12 men; mean age 36 (min-max 23-68) years median [IQR]	n.m.	n.m.	14.2 [10.3-18.1]	n.m.	3.7 [3.0-5.3]	0.5 [0.4-0.6]	0.7 [0.6-0.9]	0.4 [0.3-0.6]
Davio et al. (2020)[14]	319 men age 57.1 ± 18.7 years mean (SD)	n.m.	1.6 (0.7)	16.0 (6.8)	n.m.	5.1 (2.5)	0.6 (0.3)	0.9 (0.5)	0.6 (0.3)
Turcu et al. (2021)[27]	10 men; median age 24 (range 19-29) years median [IQR] circadian peak and nadir	n.m.	2.7 [2.7-2.8]	13.5 [13.0-14.0]	n.m.	8.6 [8.2-9.0]	2.1 [2.0-2.1]	1.4 [1.3-1.4]	0.6 [0.6-0.6]
		n.m.	1.1 [1.0-1.2]	9.4 [8.7-10.1]	n.m.	0.7 [0.4-1.1]	0.4 [0.3-0.5]	0.3 [0.3-0.3]	0.0 [0.0-0.1]
	10 men; median age 63 (range 61-75) years median [IQR] circadian peak and nadir	n.m.	2.1 [2.0-2.1]	12.7 [12.2-13.3]	n.m.	7.3 [6.9-7.7]	1.5 [1.4-1.6]	1.0 [1.0-1.0]	0.5 [0.5-0.5]
		n.m.	1.0 [0.9-1.0]	9.6 [8.9-10.2]	n.m.	1.7 [1.4-2.1]	0.6 [0.5-0.7]	0.4 [0.4-0.4]	0.2 [0.2-0.2]

n.m., not measured.

for A4 is significantly lower than that for its other substrates 11-deoxycortisol and 11-deoxycorticosterone.[37]

Davio *et al.*[15] recently studied a large cohort comprising healthy men ($n = 319$) and women ($n = 271$). They reported that the 11-hydroxy androgens (11OHA4 and 11OHT) increased with age in women, while the 11-keto androgens (11KA4 and 11KT) decreased with age in men. In our study of 125 men and 165 women, we observed an apparent increase in 11OHT with age that vanished after adjustment for BMI while 11KA4 decreased with age after adjusting for BMI.

The absolute concentration we report for serum 11OHA4 and 11KA4 are higher compared to values reported by other laboratories[4,15] (Table 3), which can be explained by differences in the internal standards used and indicates that absolute concentrations determined by different assays cannot be directly compared unless a cross-validation of the assays has been performed.

Using saliva day profiles, we have shown in this study that 11-oxygenated androgen concentrations follow a clear diurnal pattern in both sexes, with peak concentrations in the morning and lowest concentrations in the late evening, reflecting their adrenal origin and regulation of their biosynthesis through the hypothalamic–pituitary–adrenal axis. This pattern was more pronounced for the direct adrenal precursor 11OHA4 compared to 11KT, which is predominantly generated from 11OHA4. As participants did not collect saliva samples during the night, we cannot draw any conclusions on the exact timing of the early morning peak in androgen concentrations. Previous work had already established the diurnal pattern of 11-oxygenated androgens for men in serum[28] and for men and women in saliva.[29] We now show that in women this pattern is maintained across the menstrual cycle, while it is reduced in the luteal phase for the classic androgens A4 and T. Additionally, we establish that the morning concentrations of 11-oxygenated androgens in serum and saliva do not vary between follicular and luteal phases. While it is usually recommended to collect samples for the analysis of androgen excess in the follicular phase, this does not appear to be relevant for the measurement of 11-oxygenated androgens.

Of note, across the entire cohort, the concentrations of 11KT were comparatively higher in saliva than in serum in relation to the other steroid analytes. This can be explained by high HSD11B2 expression in the salivary gland[38,39] leading to the local conversion of 11β-hydroxysteroids to their 11-keto counterparts, as has been shown for glucocorticoids[40] explaining the relevance of salivary cortisone as a marker for circulating cortisol.

Interestingly, we observed that the increases in progesterone concentrations during the luteal phase of the menstrual cycle result in simultaneous increases in its metabolites 5αDHP and alloP, which are also intermediates of the alternative DHT biosynthesis pathway.[41] The alternative DHT biosynthesis pathway has been established as a source of active androgens in congenital adrenal hyperplasia and during fetal development.[42,43] This raises the question as to whether the alternative pathway of DHT biosynthesis might contribute to the generation of active androgens in conditions of elevated circulating progesterone such as the luteal phase of the menstrual cycle or pregnancy.

Previous studies on the effect of hormonal contraceptives on total circulating T concentrations in women have led to contradictory results, with some reporting reduced serum T concentrations,[44] while others did not find any differences compared to women not taking hormonal contraceptives.[32,45,46] In this study we observed significantly reduced concentrations of T and 11KT in the saliva of women on hormonal contraceptives compared to women not taking hormonal contraceptives, but not in the serum. There are at least two possible explanations for this. First, contraceptives could differentially impact on the peripheral generation of T and 11KT from their circulating precursors. Second, estrogen-mediated increases in hepatic SHBG production could reduce the fraction of free T and 11KT in circulation. Consequently, fewer molecules of T and 11KT may diffuse across the epithelial cells in the salivary gland, thereby leading to reduced salivary concentrations, while the total circulating levels (protein bound plus free) as measured by our assay remain unaltered.

The strengths of our study are (1) the inclusion of large numbers of participants whose health status was verified, allowing for analysis of subgroups including a sex-specific analysis; (2) steroid measurements by validated tandem mass spectrometry assays, (3) concurrent measurement of androgens from the classic, 11-oxygenated and alternative DHT pathways, and (4) steroid measurements in both serum and saliva including assessment of diurnal and menstrual cycle variations.

Our study has several limitations. First, our study was cross-sectional by design, thus, future longitudinal studies with clinically well-characterized cohorts are needed to assess the physiological role of sustained 11-oxygenated androgen concentrations with ageing. We used a longitudinal design to study alterations in salivary androgens across the menstrual cycle, but the relatively low number of participants represents a limitation. Second, though we measured salivary steroids that are reflective of free, unbound serum steroid concentrations, we measured the total concentrations of the analytes in serum and, therefore, cannot conclude on the variation of their free or bioavailable serum concentrations. This is of particular importance in the context of the menstrual cycle and hormonal contraceptives, as estrogens increase hepatic SHBG production. Of note, the affinity of the 11-oxygenated androgens for SHBG has not been determined to date. Third, the sensitivity of our LC–MS/MS assay did not allow for accurate quantification of DHT and 11OHT in all serum samples from women due to the very low female serum concentrations.

In conclusion, 11-oxygenated androgens form a stable circulating pool of androgens during adulthood, while classic androgens decline with age and are subject to menstrual cycle-dependent variation. BMI is a key factor in the regulation of 11-oxygenated androgen activation in peripheral tissues, namely adipose tissue, warranting further investigation.

Declarations

All research reported in this paper complies with the Declaration of Helsinki. W.A. is Editor-in-Chief of EJE, I.B. is an Associate Editor, and A.S. is an Advisory Editor. They were not involved in the review or editorial process for this paper, on which they are listed as authors.

Supplementary material

Supplementary material is available at *European Journal of Endoicrinology* online.

Funding

This work was supported by the Wellcome Trust (Investigator Award WT209492/Z/17/Z to W.A.), the Diabetes UK (Sir George Alberti Research Training Fellowship 18/0005782 to A.P.), the National Institute of Diabetes and Digestive and Kidney Diseases (K23DK121888), and the National Institute of Aging (AG71934) of the U.S. National Institutes of Health (to I.B.), and the Health Research Board (Emerging Clinician Scientist Award ECSA-2020-001, to M.W.O'R.). W.A. and A.J.S. receive support from the NIHR Birmingham Biomedical Research Centre at the University Hospitals Birmingham NHS Foundation Trust and the University of Birmingham (grant reference number BRC-1215-20009).

Conflicts of interest: The authors declare no conflict of interest.

References

1. Andersen CY, Ezcurra D. Human steroidogenesis: implications for controlled ovarian stimulation with exogenous gonadotropins. *Reprod Biol Endocrinol*. 2014;12(1):128. https://doi.org/10.1186/1477-7827-12-128
2. Schiffer L, Barnard L, Baranowski ES, *et al*. Human steroid biosynthesis, metabolism and excretion are differentially reflected by serum and urine steroid metabolomes: A comprehensive review. *J Steroid Biochem Mol Biol*. 2019;194:105439. https://doi.org/10.1016/j.jsbmb.2019.105439
3. Arlt W. Dehydroepiandrosterone and ageing. *Best Pract Res Clin Endocrinol Metab*. 2004;18(3):363-380. https://doi.org/10.1016/j.beem.2004.02.006
4. Nieschlag E, Loriaux DL, Ruder HJ, Zucker IR, Kirschner MA, Lipsett MB. The secretion of dehydroepiandrosterone and dehydroepiandrosterone sulphate in man. *J Endocrinol*. 1973;57(1):123-134. https://doi.org/10.1677/joe.0.0570123
5. Rosenfeld RS, Hellman L, Roffwarg H, Weitzman ED, Fukushima DK, Gallagher TF. Dehydroisoandrosterone is secreted episodically and synchronously with cortisol by normal man. *J Clin Endocrinol Metab*. 1971;33(1):87-92. https://doi.org/10.1210/jcem-33-1-87
6. Rege J, Nakamura Y, Satoh F, *et al*. Liquid chromatography-tandem mass spectrometry analysis of human adrenal vein 19-carbon steroids before and after ACTH stimulation. *J Clin Endocrinol Metab*. 2013;98(3):1182-1188. https://doi.org/10.1210/jc.2012-2912
7. Swart AC, Schloms L, Storbeck KH, *et al*. 11β-hydroxyandrostenedione, The product of androstenedione metabolism in the adrenal, is metabolized in LNCaP cells by 5α-reductase yielding 11β-hydroxy-5α-androstanedione. *J Steroid Biochem Mol Biol*. 2013;138:132-142. https://doi.org/10.1016/j.jsbmb.2013.04.010
8. Storbeck KH, Bloem LM, Africander D, Schloms L, Swart P, Swart AC. 11β-Hydroxydihydrotestosterone And 11-ketodihydrotestosterone, novel C19 steroids with androgenic activity: a putative role in castration resistant prostate cancer? *Mol Cell Endocrinol*. 2013;377(1-2):135-146. https://doi.org/10.1016/j.mce.2013.07.006
9. 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. https://doi.org/10.1371/journal.pone.0159867
10. Rege J, Turcu AF, Kasa-Vubu JZ, *et al*. 11-Ketotestosterone Is the dominant circulating bioactive androgen during normal and premature adrenarche. *J Clin Endocrinol Metab*. 2018;103(12):4589-4598. https://doi.org/10.1210/jc.2018-00736
11. Quinkler M, Sinha B, Tomlinson JW, Bujalska IJ, Stewart PM, Arlt W. Androgen generation in adipose tissue in women with simple obesity–a site-specific role for 17beta-hydroxysteroid dehydrogenase type 5. *J Endocrinol*. 2004;183(2):331-342. https://doi.org/10.1677/joe.1.05762
12. Barnard M, Quanson JL, Mostaghel E, Pretorius E, Snoep JL, Storbeck KH. 11-Oxygenated Androgen precursors are the preferred substrates for aldo-keto reductase 1C3 (AKR1C3): implications for castration resistant prostate cancer. *J Steroid Biochem Mol Biol*. 2018;183:192-201. https://doi.org/10.1016/j.jsbmb.2018.06.013
13. O'Reilly MW, Kempegowda P, Walsh M, *et al*. AKR1C3-mediated Adipose androgen generation drives lipotoxicity in women with polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2017;102(9):3327-3339. https://doi.org/10.1210/jc.2017-00947
14. O'Reilly MW, Kempegowda P, Jenkinson C, *et al*. 11-Oxygenated C19 steroids are the predominant androgens in polycystic ovary syndrome. *J Clin Endocrinol Metab*. 2017;102(3):840-848. https://doi.org/10.1210/jc.2016-3285
15. Davio A, Woolcock H, Nanba AT, *et al*. Sex differences in 11-oxygenated androgen patterns across adulthood. *J Clin Endocrinol Metab*. 2020;105(8):e2921-9. https://doi.org/10.1210/clinem/dgaa343
16. Turcu AF, Nanba AT, Chomic R, *et al*. Adrenal-derived 11-oxygenated 19-carbon steroids are the dominant androgens in classic 21-hydroxylase deficiency. *Eur J Endocrinol*. 2016;174(5):601-609. https://doi.org/10.1530/EJE-15-1181
17. Jones CM, Mallappa A, Reisch N, *et al*. Modified-Release and conventional glucocorticoids and diurnal androgen excretion in congenital adrenal hyperplasia. *J Clin Endocrinol Metab*. 2017;102(6):1797-1806. https://doi.org/10.1210/jc.2016-2855
18. Bacila I, Adaway J, Hawley J, *et al*. Measurement of salivary adrenal-specific androgens as biomarkers of therapy control in 21-hydroxylase deficiency. *J Clin Endocrinol Metab*. 2019;104(12):6417-6429. https://doi.org/10.1210/jc.2019-00031
19. Schiffer L, Shaheen F, Gilligan LC, *et al*. Multi-steroid profiling by UHPLC-MS/MS with post-column infusion of ammonium fluoride. *J Chromatogr B*. 2022;1209:123413. https://doi.org/10.1016/j.jchromb.2022.123413
20. Foster MA, Taylor AE, Hill NE, *et al*. Mapping the steroid response to major trauma from injury to recovery: a prospective cohort study. *J Clin Endocrinol Metab*. 2020;105(3):925-937. https://doi.org/10.1210/clinem/dgz302
21. Chadwick CA, Owen LJ, Keevil BG. Development of a method for the measurement of dehydroepiandrosterone sulphate by liquid chromatography-tandem mass spectrometry. *Ann Clin Biochem*. 2005; 42(Pt 6):468-474. https://doi.org/10.1258/000456305774538175
22. Schiffer L, Adaway JE, Arlt W, Keevil BG. A liquid chromatography-tandem mass spectrometry assay for the profiling of classical and 11-oxygenated androgens in saliva. *Ann Clin Biochem*. 2019;56(5):564-573. https://doi.org/10.1177/0004563219847498
23. Schiffer L, Adaway JE, Baranowski ES, Arlt W, Keevil BG. A novel high-throughput assay for the measurement of salivary progesterone by liquid chromatography tandem mass spectrometry. *Ann Clin Biochem*. 2019;56(1):64-71. https://doi.org/10.1177/0004563218780904
24. Cole TJ. Sympercents: symmetric percentage differences on the 100 logI scale simplify the presentation of log transformed data. *Stat Med*. 2000;19(22):3109-3125. https://doi.org/10.1002/1097-0258(20001130)19:22<3109::AID-SIM558>3.0.CO;2-F
25. 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. *J Steroid Biochem Mol Biol*. 2018;178:221-228. https://doi.org/10.1016/j.jsbmb.2017.12.016
26. Skiba MA, Bell RJ, Islam RM, Handelsman DJ, Desai R, Davis SR. Androgens during the reproductive years: what is normal for women? *J Clin Endocrinol Metab*. 2019;104(11):5382-5392. https://doi.org/10.1210/jc.2019-01357

27. Nanba AT, Rege J, Ren J, Auchus RJ, Rainey WE, Turcu AF. 11-Oxygenated C19 steroids do not decline with age in women. *J Clin Endocrinol Metab*. 2019;104(7):2615-2622. https://doi.org/10.1210/jc.2018-02527
28. Turcu AF, Zhao L, Chen X, *et al.* Circadian rhythms of 11-oxygenated C19 steroids and Δ5-steroid sulfates in healthy men. *Eur J Endocrinol*. 2021;185(4):K1-K6. https://doi.org/10.1530/EJE-21-0348
29. Nowotny HF, Auer MK, Lottspeich C, *et al.* Salivary profiles of 11-oxygenated androgens follow a diurnal rhythm in patients with congenital adrenal hyperplasia. *J Clin Endocrinol Metab*. 2021;106-(11):e4509-e4519. https://doi.org/10.1210/clinem/dgab446
30. Elhassan YS, Idkowiak J, Smith K, *et al.* Causes, patterns, and severity of androgen excess in 1205 consecutively recruited women. *J Clin Endocrinol Metab*. 2018;103(3):1214-1223. https://doi.org/10.1210/jc.2017-02426
31. Haring R, Hannemann A, John U, *et al.* Age-specific reference ranges for serum testosterone and androstenedione concentrations in women measured by liquid chromatography-tandem mass spectrometry. *J Clin Endocrinol Metab*. 2012;97(2):408-415. https://doi.org/10.1210/jc.2011-2134
32. Eisenhofer G, Peitzsch M, Kaden D, *et al.* Reference intervals for plasma concentrations of adrenal steroids measured by LC-MS/MS: impact of gender, age, oral contraceptives, body mass index and blood pressure status. *Clin Chim Acta*. 2017;470:115-124. https://doi.org/10.1016/j.cca.2017.05.002
33. Dharia S, Slane A, Jian M, Conner M, Conley AJ, Parker CR, Jr. Colocalization of P450c17 and cytochrome b5 in androgen-synthesizing tissues of the human. *Biol Reprod*. 2004; 71(1): 83-88. https://doi.org/10.1095/biolreprod.103.026732
34. Nakamura Y, Hornsby PJ, Casson P, *et al.* Type 5 17beta-hydroxysteroid dehydrogenase (AKR1C3) contributes to testosterone production in the adrenal reticularis. *J Clin Endocrinol Metab*. 2009;94(6):2192-2198. https://doi.org/10.1210/jc.2008-2374
35. Tezuka Y, Atsumi N, Blinder AR, *et al.* The age-dependent changes of the human adrenal cortical zones are not congruent. *J Clin Endocrinol Metab*. 2021;106(5):1389-1397. https://doi.org/10.1210/clinem/dgab007
36. Nakamura Y, Fujishima F, Hui XG, *et al.* 3βHSD And CYB5A double positive adrenocortical cells during adrenal development/aging. *Endocr Res*. 2015;40(1):8-13. https://doi.org/10.3109/07435800.2014.895377
37. Barbier ID, Gent R, Du Toit T, Swart AC. https://doi.org/10.1210/jendso/bvab048.132 2021.
38. Hirasawa G, Sasano H, Takahashi K, *et al.* Colocalization of 11 beta-hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human epithelia. *J Clin Endocrinol Metab*. 1997;82 (11):3859-3863. https://doi.org/10.1210/jcem.82.11.4337
39. Smith RE, Maguire JA, Stein-Oakley AN, *et al.* Localization of 11 beta-hydroxysteroid dehydrogenase type II in human epithelial tissues. *J Clin Endocrinol Metab*. 1996;81(9):3244-3248. https://doi.org/10.1210/jcem.81.9.8784076
40. Debono M, Harrison RF, Whitaker MJ, *et al.* Salivary cortisone reflects cortisol exposure under physiological conditions and after hydrocortisone. *J Clin Endocrinol Metab*. 2016;101(4): 1469-1477. https://doi.org/10.1210/jc.2015-3694
41. Auchus RJ. The backdoor pathway to dihydrotestosterone. *Trends Endocrinol Metab*. 2004;15(9):432-438. https://doi.org/10.1016/j.tem.2004.09.004
42. Reisch N, Taylor AE, Nogueira EF, *et al.* Alternative pathway androgen biosynthesis and human fetal female virilization. *Proc Natl Acad Sci U S A*. 2019;116(44):22294-9. https://doi.org/10.1073/pnas.1906623116
43. Arlt W, Walker EA, Draper N, *et al.* Congenital adrenal hyperplasia caused by mutant P450 oxidoreductase and human androgen synthesis: analytical study. *Lancet*. 2004;363(9427):2128-2135. https://doi.org/10.1016/S0140-6736(04)16503-3
44. Søeborg T, Frederiksen H, Mouritsen A, *et al.* Sex, age, pubertal development and use of oral contraceptives in relation to serum concentrations of DHEA, DHEAS, 17α-hydroxyprogesterone, Δ4-androstenedione, testosterone and their ratios in children, adolescents and young adults. *Clin Chim Acta*. 2014;437:6-13. https://doi.org/10.1016/j.cca.2014.06.018
45. Bae YJ, Zeidler R, Baber R, *et al.* Reference intervals of nine steroid hormones over the life-span analyzed by LC-MS/MS: effect of age, gender, puberty, and oral contraceptives. *J Steroid Biochem Mol Biol*. 2019;193:105409. https://doi.org/10.1016/j.jsbmb.2019.105409
46. Bermon S, Garnier PY, Hirschberg AL, *et al.* Serum androgen levels in elite female athletes. *J Clin Endocrinol Metab*. 2014;99(11): 4328-4335. https://doi.org/10.1210/jc.2014-1391