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Salivary Androgens in Adolescence and their Value as a Marker of Puberty – Results From The SCAMP Cohort

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Abstract (226/250)

Context: Salivary androgens represent non-invasive biomarkers of puberty that may have utility in clinical and population studies.

Objective: To understand normal age-related variation in salivary sex steroids and demonstrate their correlation to pubertal development in young adolescents.

Design, Setting, and participants: School-based cohort study of 1,495 adolescents at two time points for collecting saliva samples approximately two years apart.

Outcome measures: The saliva samples were analyzed for five androgens [Testosterone (T), androstenedione (A4), 17-hydroxyprogesterone (17-OHP), 11-ketotestosterone (11-KT) and 11β-hydroxyandrostenedione (11-OHA4)] using LC-MS/MS; in addition, salivary dehydroepiandrosterone (DHEA) and oestradiol (OE2) were analyzed by ELISA. Pubertal staging was self-reported using the Pubertal Development Scale (PDS).

Results: In 1,236 saliva samples from 903 boys aged between 11-16 years, salivary androgens except DHEA exhibited an increasing trend with an advancing age (ANOVA, $p<0.001$), with salivary T and A4 concentration showing the strongest correlation ($r=0.55$, $p<0.001$ and $r=0.48$, $p<0.001$, respectively). In a subgroup analysis of 155 and 63 saliva samples in boys and girls, respectively morning salivary T concentrations showed the highest correlation with composite PDS scores and voice-breaking category from PDS self-report in boys ($r=0.75$, $r=0.67$, respectively). In girls, salivary DHEA and OE2 had negligible correlations with age or composite PDS scores.

Conclusion: In boys aged 11-16 years, increase in salivary T and A4 is associated with self-reported pubertal progress and represent valid non-invasive biomarkers of puberty in boys.

Introduction

Pubertal progress is accompanied by a steady increase in a wide range of sex steroids that are produced by the adrenal glands and the gonads. Increase of serum testosterone (T) and free testosterone during puberty is well-known and there are several studies correlating T to Tanner stages of puberty (1, 2). Most circulating sex steroids are bound to proteins with only 0.5-3.0% available in plasma as an unbound steroid and it is this free form which exerts steroid activity at the level of the target tissue (3). Free sex steroids including androgens are small lipophilic compounds, and can also enter the salivary glands from the capillaries by passive diffusion and there is a good correlation between salivary and serum androgen concentrations from previous studies (4-6).

Salivary androgens, including T, androstenedione (A4), 17-hydroxyprogesterone (17-OHP), 11-ketotestosterone (11-KT), 11β-hydroxyandrostenedione (11-OHA4) have been described as markers of diagnosis or therapeutic control in male hypogonadism(7, 8) and 21-hydroxylase deficiency congenital adrenal hyperplasia (CAH) but in these situations have rarely been correlated to pubertal status(4, 9). In non-clinical health research, salivary androgens, and particularly T, have been generally measured by radioimmunoassay (RIA) (10-14). However, RIAs are not well suited for the measurement of salivary androgens as, in addition to the need for a high level of sensitivity, the presence of other steroid hormones as well as precursors and metabolites in the assay matrix leads to a greater chance of cross-reactivity. Liquid Chromatography Mass Spectrometry (LC-MS/MS) offers higher sensitivity and specificity with greater accuracy for steroid hormone measurement as well as a lower limit of quantification(15, 16) and has been used to describe age related changes in salivary testosterone in children, adolescents and adults (15-19). However, there is scarce information on the wider range of salivary sex steroids including these that are involved in the alternative pathways of steroid

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synthesis (20) and there is even less information on their relationship to puberty. Other considerations such as the effect of long-term storage of samples and the timing of sample collection have also not been studied sufficiently. The primary objective of the current study was to investigate age-related variations in a wide range of salivary steroids in healthy school children and adolescents and explore their relationship to pubertal development, with secondary objectives of investigating the effect of sample collection time and the stability of the long-term storage of the samples for measuring the sex steroids in saliva.

Methods

Study setting and population

The current study was part of a large school-based adolescent cohort study, SCAMP, conducted in 39 secondary schools across Greater London, UK (21). In addition to completing a series of questionnaires that included demographic details and concomitant medications, this sub-study of participants at 12 schools also took part in SCAMP 'Bio-Zone' sessions which involved providing saliva and urine samples and collection of anthropometric measurements. For the analysis of salivary steroids in the present study, a total 925 boys and 620 girls had at least one saliva sample analysed at one of two timepoints, T1 (between March 2015 and July 2016) and T2 (between February 2017 and July 2018). Figure 1 shows the cohort data relevant to this study. There were no reports of steroid hormonal therapy or the use of contraceptives. Saliva samples were collected from participants during school hours between March 2015 and July 2016 (T1); 706 boys with a median age of 12.3 yrs (range, 11.3, 13.2) and 473 girls with a median age 12.3 yrs (11.1, 13.2). Approximately two years later (T2) between February 2017 and July 2018, 563 boys with median age of 14.3 yrs (13.4, 15.8) and 422 girls with median age 14.3 yrs (13.4, 15.7) from 10 of the 12 schools had further saliva samples collected when participants were aged 13-15 years.

Self-reported pubertal development

Most SCAMP questionnaire data collection was conducted in schools, but for reasons of school time limitations and question sensitivity, some additional questions were asked via a home-based online questionnaire. All SCAMP participants were invited to complete an online questionnaire at home, which included 14 questions on puberty (seven for each sex) based on the validated Pubertal Developmental Scale (PDS) (22). Previous studies demonstrated that the association between professional-rated Tanner stage and PDS self-report were moderate to high and that PDS self-report can be used for pubertal assessment in research without the need for Tanner

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evaluation when the precise concordance is not required(23-26). Of the total SCAMP cohort, 608 boys and 530 girls (1138 in total) reported their pubertal development using the PDS. Participants had the option of repeating the questionnaire at a later date; if two questionnaires were completed within 12 months by the same participant, only the first completed was used for analysis (Figure 1). The questionnaire asked participants to self-report secondary sexual characteristic development from 1 (development not started) to 4 (development completed). Scale items included growth in height, pubic hair, axillary hair, and skin changes for both genders. Boys were asked additional items on deepening of voice and facial hair and girls were asked items on breast development and onset of menarche. PDS scores were then converted using an algorithm developed by Crockett (1988, unpublished) to one of five PDS-derived pubertal categories to summarize participants' pubertal development: pre-pubertal, early pubertal, mid-pubertal, late-pubertal and post-pubertal. To evaluate correlations between salivary sex steroid concentration and self-reported development, we selected questionnaires completed less than 90 days of a saliva collection, giving a validation sample of 155 and 63 questionnaire/sample pairs from boys and girls respectively.

Sample collection, stability analysis and assays

Saliva samples were collected during school hours directly into sterile polypropylene vials and the time of collection was recorded. To prevent contamination and dilution of the saliva sample participants were not permitted to eat or drink 30 mins before saliva sampling began. Saliva was collected by passive drool into 7ml polypropylene containers with polyethylene caps; participants were instructed to drool at least 2ml. No salivary stimulants were used to provoke salivary flow. Samples were then transported by Thermoporter before being aliquoted and stored long-term at -20°C until cryo-shipment to the laboratory for analysis. To investigate long-term stability of the analytes, saliva was also collected from 35 healthy adult volunteers (18 female), aliquotted, cryo-shipped to the laboratories and stored at -20°C until biochemical analysis. One aliquot from

each of the samples was analysed, and a further aliquot from each sample was analysed at 4 further timepoints up to a maximum of 608 days for steroids assayed by LC-MS/MS, and 664 days steroids assayed by ELISA immunoassay (Salimetric Assay, PA, USA).

Saliva samples for males were analysed for T, A4, 11KT, 17OHP, 11OHA4, and DHEA. Saliva samples for females were analysed for OE2 and DHEA only. Sample assay methods were the same for samples collected from both SCAMP participants and healthy adult volunteers, as follows. T, A4, 17-OHP, 11-OHA4 and 11-KT concentrations were determined by LC-MS/MS. The lower limit of quantification was 5 pmol/L for T; 10 pmol/L for A4; 12.5 pmol/L for 17-OHP; 45 pmol/L for 11-OHA4; 6 pmol/L for 11-KT. The inter- and intra-assay coefficient of variation (CV) at the lower limit of quantification was 13.6% (between-batch) and 7.5% (within-batch CV) for T; 12.8% and 3.3% for A4; 9.4% and 3.6% for 17-OHP; 6.3% and 3.4% for 11-OHA4; and 11.0% and 6.9% for 11-KT. OE2 and DHEA concentrations were determined by ELISA (Salimetric Assay, PA, USA). The lower limit of quantification was 0.365 pmol/L for OE2 and 0.0174 nmol/L for DHEA. The inter-assay CV was 8.2% and 12.5%, and the intra-assay CV was 7.1% and 5.6% for OE2 and DHEA respectively. To reduce bias when calculating references ranges or conducting statistical tests, concentrations measured below lower limit of quantification were imputed as the lower limit of quantification multiplied a factor of $\frac{1}{\sqrt{2}}$ (27).

Statistical Analysis

All salivary samples were included in the analysis, i.e., no samples were removed from the analysis due to being outliers. References ranges for all the measured steroids stratified by sex were calculated for 11-16 years by year increment. One-way ANOVA and post-hoc Games-Howell test were conducted to assess for difference in mean scores by age. Pearson correlation was used

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to investigate correlation of steroid concentration with age. Nonparametric quantile regression was used to fit percentile curves at 2.5%, 16%, 50%, 84% and 97.5%, conditional on age for each sex steroid separately(28). Correlations were evaluated between salivary sex steroid concentration and pubertal development from self-reported PDS scores and PDS-derived pubertal category ranks in participants that provided samples and completed self-report questionnaires. Spearman rank correlations were used to analyse the relationship between each PDS domain score, composite PDS (mean of all PDS domain scores), PDS-derived pubertal category ranks and salivary steroid concentrations. Salivary androgens exhibit diurnal variation and the times reported for specimen collection from previous studies are usually between 7-11:00 AM (4, 6, 13, 15, 16, 19). In the present study, to assess if diurnal changes affected the inferences, the same correlation analysis (between salivary steroid concentration and PDS) was conducted with subgroups of samples that were collected in early morning or later in the school day; cut-off of 11:00 AM was used based on previous studies (19). To further assess diurnal change, Wilcoxon-Mann-Whitney U Test was conducted in boys (stratified by age in 1-year intervals) to compare differences in concentrations for T and A4 with respect to time of collection, again for before and after 11:00 AM. The capacity of each biomarker to predict marked voice change (PDS score 3 and above) was evaluated using receiver operator characteristic (ROC) curve analysis, which provided sensitivity and specificity, area under the curve (AUC) measures and optimum cut-off values from bootstrapped maximised Youden-index. For stability analysis, linear mixed effects models with random intercepts for subjects were fitted to test for the significance of effect of storage time. For biomarkers that showed a significant effect of storage time, post-hoc estimations of degradation or accumulation kinetics were conducted. Prior to model fitting, the data were normalized to a percentage of the baseline (time = 0) concentration. For steroids that showed significant degradation, we assumed their kinetics followed a single first-order (SFO) exponential decline, i.e. that the rate of degradation was proportional to the concentration, a common kinetic profile of biomarkers in storage (29). For steroids that showed significant

increase in concentration, the data were fitted to linear models. Bayesian mixed effects models were fitted using MCMC estimation with Stan (30) using the brms package (31) to model between-subject heterogeneity of rate slopes. For this study, the posterior mean annual change in concentration is reported for each steroid deemed unstable. For degradation SFO models estimates of the population-average SFO rate constant (k), the half-life or time required for steroid to halve in concentration ($t_{1/2}$) and their respective 95% credible intervals for each unstable biomarker were also evaluated. All analyses were conducted using IBM SPSS Statistics (Version 20) predictive analytics software and R version 4.0.3, with packages *brms*, *cutpointr* and *np* (31-33).

Ethics

The North West Haydock Research Ethics Committee approved the SCAMP study protocol and subsequent amendments (ref 14/NW/0347). School headteachers consented to participation in SCAMP. Parents and adolescents were provided in advance with written information about the study and were given the opportunity to opt out of the research at any time. The study was conducted in accordance with the Declaration of Helsinki. Saliva samples from healthy adult volunteers that were used for the stability analysis were collected under the framework of the Imperial College Healthcare Tissue Bank which is approved by Wales REC3 to release human material for research (17/WA/0161). Consent has been obtained from each healthy adult participant of the stability analysis after full explanation of the purpose and nature of all procedures used. The samples for this project were issued from sub-collection reference number [MED_MT_19_036].

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Results

Age & sex related data

A total of 1,236 saliva samples were available from 903 boys aged between 11-16 with the median age of 12.3 yrs (11.3, 13.2) and 14.3 yrs (13.4, 15.8) at T1 and T2, respectively. 333 boys gave saliva samples at both T1 and T2. In these boys, all salivary androgens (T, A4, 17-OHP, 11-KT, 11-OHA4) except DHEA exhibited an increasing trend with advancing age (ANOVA, $p < 0.001$). Salivary T concentrations revealed the highest correlation with age ($r=0.55$, $p < 0.001$, $n=1,166$) followed by A4 ($r= 0.48$, $p < 0.001$, $n=1,167$), 11-KT ($r=0.30$, $p < 0.001$, $n=1,145$), 17-OHP ($r=0.27$, $p < 0.001$, $n=1,139$), 11-OHA4 ($r=0.15$, $p < 0.001$, $n=1,161$). Salivary DHEA concentration did not demonstrate a significant trend with advancing age in boys or girls (ANOVA, $p = 0.52$ and 0.29, respectively). 855 samples were available from 592 girls between 11-16 with the median age of 12.3 yrs (11.1, 13.2) and 14.3 yrs (13.4, 15.8) at T1 and T2, respectively. 262 participants gave saliva samples at both T1 and T2. For girls, salivary OE2 and DHEA concentrations showed a negligible correlation with age ($r=0.12$, $p=0.001$ and $r=0.05$, $p=0.167$, respectively). Table 1 and Figure 2 show percentiles for salivary T and A4 concentrations in boys aged between 11 to 16 years. Supplementary Table 1-3 show percentiles for all other salivary androgens in boys and Supplementary Table 4 show percentiles for salivary DHEA and OE2 in girls aged 11 to 16 years.

Specimen collection time and salivary hormone concentrations

The median times for saliva samples collection in boys and girls were 11:30 and 11:25 AM, respectively. Thirty-eight percent (462 out of 1224) and 42% (344 out of 825) participants provided saliva samples before 11:00 AM in boys and girls, respectively. Analysing within each 1-year age band, boys who had provided saliva samples before 11:00 AM had higher salivary T concentration (Wilcoxon-Mann-Whitney test: $p<0.001$ for age band 12-13 years and 14-15 years; $p=0.015$ for age band 13-14 years) compared to those who provided samples after 11:00 AM

(Supplementary Table 5 and Figure 3). On the other hand, also in boys, mean salivary 11-KT concentration in the group that provided saliva samples before 11:00 AM were consistently lower within every age group compared to those who supplied samples after 11:00 AM. Such consistent trends for every age group in saliva collected before and after 11:00 AM were not observed for A4, 17-OHP, DHEA and 11-OHA4 concentrations (Supplementary Table 5). In girls (Supplementary Table 6), there was no overall meaningful difference in mean salivary DHEA or OE2 when comparing saliva samples collected before and after 11:00 AM. Overall, salivary T concentrations revealed the strongest linear correlation with salivary A4 ($r= 0.76$; $p < 0.01$) while the other androgens showed a lesser degree of correlation between salivary T and 17-OHP ($r= 0.58$; $p<0.01$), 11-KT ($r=0.39$; $p<0.01$) or 11-OHA4 ($r=0.27$; $p<0.01$). Salivary 11-OHA4 concentrations showed a correlation with its precursor salivary A4 ($r=0.38$; $p<0.01$) and its downstream metabolite, 11-KT ($r=0.45$; $p<0.01$).

Correlation between salivary steroids and PDS scores

Of all the salivary androgens collected from boys, T had the highest correlation with PDS composite score ($r=0.64$, $p<0.01$) (Table 2). In descending order, the correlations between the remaining salivary steroid concentration and PDS composite score were: A4, 17-OHP, 11-KT, 11-OH-A4 and DHEA (Table 2). Salivary T and A4 were also moderately correlated with advancing PDS-derived pubertal category rank ($r=0.63$, $p<0.01$ and $r=0.50$, $p<0.01$ respectively). For correlations between salivary androgens and each component in the PDS self-report: voice-change was the individual component of the PDS with the highest correlation with T and A4 ($r=0.57$, $p<0.01$ and $r=0.46$, $p<0.01$ respectively) (Table 2). Subgroup analyses for time of collection revealed that correlations between T and A4 with the PDS composite score, with PDS-derived pubertal categories and with almost all individual PDS components were higher for samples collected before 11:00AM (Table 2). Conversely, correlations were lower when analysis was restricted to samples collected after 11:00AM. Salivary T and A4 concentrations also

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gradually increased with an increasing composite PDS score (Table 3). ROC curve analysis showed that specimens collected before 11:00 AM had a greater discriminative performance to predict marked voice-change (Figure 4). Salivary T and A4 collected before 11:00AM had an AUC of 0.93 and 0.83, respectively; when collected after 11:00AM, T and A4 had an AUC of 0.77 and 0.72; when samples that were collected at all times were analysed together, the AUC was 0.84 and 0.78 (Figure 4). By contrast, in girls, the correlation of salivary DHEA and OE2 with individual components of PDS and the PDS derived pubertal categories was much weaker (Supplementary Table 7) and the correlation between PDS composite score and salivary DHEA and OE2 was r=0.21 and r=0.18, respectively (p>.05).

Stability analysis

A summary of stability analysis results and post-hoc estimation of degradation kinetics is available in supplementary Table 8. Salivary T, A4, 17-OHP, 11-KT and 11-OHA4 collected from the 16 male volunteers showed a significant effect of storage time (for T: p<0.001; A4: p<0.001; 17-OHP: p<0.017; 11-OHA4: p<0.001; 11-KT: p<0.001). Mean annual degradation as estimated from mixed effects SFO exponential decline models 6.7% for salivary T (95% credible interval of [3.9, 9.4]); for A4, annual degradation 5.8% [3.0, 8.5]); for 17-OHP, annual degradation was estimated to be 7.0% [2.5, 11]; for 11-OHA4, 26% per year [7.6, 42]; for 11-OHA4, 21% per year [16, 25]). Salivary DHEA, which was collected from both male and female volunteers, and salivary OE2, which was collected from females only, showed significant accumulation over the time period ($p < 0.001$ and $p = 0.003$ respectively). Post-hoc Bayesian modelling of annual accumulation of DHEA as mixed effects linear model yielded annual accumulation of +32.5% [17.5, 46.3]. Mean annual accumulation of OE2 was estimated to be +1.81% [-14.8, +17.3].

Discussion

In the current study we have analysed the relationship between salivary sex steroids and age as well as pubertal development. To date, this is the largest study to report a wide range of pubertal sex steroid concentrations in saliva using LC-MS/MS assay in a heterogeneous group of healthy adolescents. Moreover, this study also analysed salivary sex steroid concentrations in relation to pubertal stage using PDS self-report. We observed discriminatory performance of salivary androgens to detect marked voice-change depended on the type of androgen and the time of saliva collection. Lastly, the study examined the long-term stability of these steroids.

As expected in boys, salivary T concentrations gradually increased with advancing age during pubertal years. However, compared with previous reports that measured salivary T levels with LC-MS/MS method, for participants that are that are the same age, T concentrations in the current study were lower (18). When compared with saliva samples collected only before 11:00 AM, the difference in age-matched T concentrations with previous studies was reduced emphasizing the importance of specimen collection time. Salivary androgens exhibit diurnal rhythms (5, 8, 16, 34, 35) and the magnitude of the fall in T across the day depends on the age and stage of pubertal development(11, 34, 36, 37). The current study found that, in boys, salivary T concentration showed a very high correlation with salivary A4. This strong correlation between the two androgens in saliva can be explained, first, by A4 being the last precursor before conversion to testosterone by 17β-hydroxysteroid dehydrogenase (17βHSD) in the testes, and second, by the expression of 17βHSD in salivary glands (38, 39). In salivary gland, 17βHSD favour an oxidative reaction which convert T to A4 locally(40). This may also account for the higher A4 concentrations compared to T in saliva. Unlike salivary T, salivary A4 concentration did not show a marked difference before and after 11:00 AM; this difference between the two androgens has not been described before in adolescents and suggests that salivary A4 may have a greater utility when there are concerns regarding the timing of sampling.

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We have demonstrated that, in the current study, the salivary T and A4 concentrations in boys gradually increased in line with the composite PDS score as well as PDS-derived pubertal categories. Until now, there has been limited information on the correlation of self-reported measures of pubertal development and salivary T concentration (13, 14, 41). Self-reported pubertal assessment by PDS has been recommended by several groups for pubertal assessment in population studies (23, 26, 42). The PDS can also be converted into pubertal categories which are supposed to align to Tanner-like stages (24-26, 43). Although the absolute agreement between professional Tanner Stage assessment and PDS and PDS-derived pubertal category has been reported to be low (23-25, 42), one study found that the absolute agreement between these two measurements rose from low to substantial when combining both Tanner stages and PDS-derived pubertal categories into a PDS score consisting of three broad categories; pre/early (Tanner 1-2), pubertal (Tanner3) and late/postpubertal (Tanner 4-5) (42).

The current study shows that the composite PDS score had a high correlation with morning salivary T concentration, whereas voice changes, facial, axillary, and pubic hair development categories on PDS self-report had a moderate degree of correlation with salivary T concentrations. These correlations with salivary T were almost universally attenuated when analysis was restricted to samples collected after 11:00 AM. On the other hand, salivary A4 concentrations had a moderate degree of correlation with PDS scores, irrespective of the timing of the sample. The current study identified that the voice changes category in the PDS self-report showed the strongest correlation with morning salivary T concentration compared to other categorical variables of puberty development, thus highlighting the reliability of this self-reported marker of puberty in boys in estimating the timing of puberty in population studies(44). Furthermore, the ROC curves analysis showed that whenever salivary androgens (T and A4) were within (lower) adult reference values (13-15), there was a high probability that the boys had marked voice

change, a sign of late puberty. The discriminatory performance was greater for salivary T in the morning; for salivary A4 the relationship did not change when samples were collected later in the day. Although concerns have been raised about using saliva assays to represent unbound, biologically active steroid levels due to bias from possible blood contamination (41, 45), this source of bias is reported to be rare in older children and adolescents and unlikely to significantly affect inferences (46). Taking this into consideration, our findings suggest that salivary T and A4 can be reliable biomarkers for pubertal development.

A significant relationship between pubertal development using PDS self-report and salivary DHEA concentration was not detected in either sex, contrary to a previous report (23). This discrepancy between the previous report and the present study might be due to a difference in the age range of the population studied in the former, which ranged between 9 and 14 years. In addition, in girls, we found only negligible correlations between breast development, menarcheal status, and salivary OE2 concentrations. This may have been due to the fact that over 75% of the girls were post-menarcheal and the timing of the sampling had not been standardised in relation to the menstrual period. Furthermore it is possible that the OE2 assay, itself, did not reach a sufficient level of sensitivity. The 11-oxygenated C19 (11oxC19) adrenal-derived androgens have been considered as clinically important in various human conditions including 21-OH deficiency CAH, polycystic ovarian syndrome, premature adrenarche and castration-resistant prostate cancer (47). Although 11OHA4 has minimal androgenic activity, its downstream metabolite, 11-KT has equivocal evidence on its potency to androgen receptor either lower potency(48) or equivalent potency compared to its parent steroid, testosterone (49, 50). The current study demonstrated negligible to low positive degree correlations between salivary 11OHA4, 11-KT concentration and pubertal development categories from PDS self-report in boys.

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Lastly, in the stability analysis following prolonged storage at -20°C without preservatives of salivary androgens in the current study, 11-OHA4 and 11-KT showed considerable degradation, with an estimated annual degradation of 21% and 26% respectively. Although these two steroids have previously been reported to be stable overnight at 4°C (51), we are unaware of other studies investigating long-term stability. Salivary T, A4, and 17-OHP were estimated to have a maximum annual degradation of 7%. Previous studies have reported conflicting findings for the stability of salivary T in long-term storage at this temperature and the discrepancy may have arisen as previous research groups have relied on immunoassays rather than tandem mass spectrometry (41, 52, 53). The former method has been shown to inflate T concentration estimates when T is low (less than 10 pg/mL), as is often the case in pre-pubertal boys and women (54). The extent of reduction in concentration is much smaller than the inter-individual variation of salivary T and A4 collected at any one timepoint; therefore, despite measurable instability, we believe T and A4 have utility as a marker for puberty, even when stored for long periods. Previous studies have found no measurable degradation of steroid compounds in saliva when stored at -80°C (41, 52), so it is possible that storage at -80°C may be necessary for 11-OHA4 and 11-KT but this requires further study. For OE2 and DHEA, there was a significant effect of storage time under assumptions of F-test, which assumed for each steroid, all samples showed the same rate of concentration change. Once inter-subject heterogeneity of concentration change with storage time was considered using post-hoc linear mixed effects modelling, OE2 was estimated to have a small annual mean increase (<2%), with a large credible interval. This was most likely due to large observed heterogeneity in within-subject trajectories of OE2 concentration. The post-hoc estimation of mean increase of salivary DHEA was over 30%. One possible explanation is possible blood contamination in saliva samples from adult volunteers used for the stability analysis; this has been reported to interfere with quantitative immunoassay based assessment of T, DHEA and OE2 (41, 45, 55). However, the likelihood of this is very low as the same observation was not observed with the salivary T measurements. Furthermore, blood contamination in children and

young adolescents is reported to be rare and unlikely to affect salivary hormone concentrations (46); similar low prevalences of contamination can be expected in samples collected from SCAMP participants. An alternate explanation may be due to evaporation or sublimation of water from saliva samples; this was not considered in the analysis. One method to address this would be to measure sodium (Na^+) concentration, which has been used as a proxy for the 'evaporation constant' (56). Assuming a constant Na^+ concentration in the samples initially, one can calculate the loss of water according to the increase in Na^+ concentration during storage, and correct analyte concentrations for samples stored over a long period.

Although collection of samples at different times of the day posed difficulties in interpretation of the data, subgroup analysis of the samples collected in the morning revealed that if saliva samples can be collected in the morning, testosterone is the best choice to represent puberty in boys. However, if the study is unable to collect saliva samples in the morning, then androstenedione may be a more attractive choice for representing puberty.

The current study did not include a correlation of the salivary samples to serum samples. However, several previous studies have demonstrated a good correlation between salivary and serum androgens(4, 57). The use of self-reported measures of puberty can be considered another limitation of the study as it may not be as accurate as Tanner staging by an experienced observer (23).

In conclusion, in boys aged 11-16 years, we have demonstrated that salivary T and A4 represent valid non-invasive biomarkers that can be used as indicators for pubertal development in population studies. Furthermore, studies that rely on measuring salivary steroids on samples stored for long periods need to carefully consider the likelihood of degradation, particularly for 11-oxygenated androgens.

Conflict of Interests

None

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Declaration

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Legend to Figures

Figure 1. Structure of SCAMP cohort data relevant to this study. 'Bio-Zone' describes school collections where non-invasive biological samples (urine and saliva) and anthropometric measurements were collected. T1 and T2 relate to periods spanning first wave (between March 2015 and July 2016) and second wave (between February 2017 and July 2018) of Bio-Zone data collection.

Figure 2. Scatter plot demonstrating the distribution of A) salivary testosterone and B) androstenedione concentrations in boys. The solid lines represent median, the dash lines represent estimated ±1 and ±2 z-scores.

Figure 3. Boxplots showing salivary concentrations of A) testosterone and B) androstenedione collected from boys before and after 11:00 AM, stratified by age. P-values for Wilcoxon-Mann–Whitney U test comparing of each steroid distributions between time periods. *Wilcoxon-Mann-Whitney U test not performed. For testosterone from within 11-12 age band, this was because majority of samples were left-censored, i.e. below the detection limit (<5 pmol/L). No samples were collected before 11AM from participants aged 15-16.

Figure 4. Receiver operating characteristic (ROC) curves for salivary T and A4 concentrations in each specimen collection time group for predicting marked voice change in boys: (A) ROC curve for salivary T concentrations collected at any time of the day, before 11:00 AM, and after 11:00 AM. (B) ROC curve for salivary A4 concentrations collected at any time of the day, before 11:00 AM, and after 11:00 AM. AUC is reported in brackets. Black dots and values indicate the optimal cut-off value with the boot-strapped maximum Youden index. Bootstrapped estimates for optimum cut-offs for salivary T collected at any time

of day were 76% and 74% for sensitivity and specificity respectively, 84% and 89% for samples collected before 11:00 AM, and 75% and 66% for samples collected after 11:00 AM. Bootstrapped estimates for optimum cut-offs for salivary A4 collected at any time of day were 71% and 74% for sensitivity and specificity respectively, 83% and 72% for samples collected before 11:00 AM, and 61% and 79% for samples collected after 11:00 AM.

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Table 1. Percentile for salivary testosterone and androstenedione concentrations in boys aged 11-16 years.

Age (yrs)	N	Salivary Testosterone (pmol/L)					Salivary Androstenedione (pmol/L)				
		Percentile					*Percentile*				
		2.5	*16*	*50*	*84*	*97.5*	*2.5*	*16*	*50*	*84*	*97.5*
11-12	125	3.5	3.5	6.0	28.5	75.3	20.1	29.0	51.0	78.2	159.1
12-13	479	3.5	3.5	11.0	62.5	161.0	21.0	36.0	62.0	108.0	184.0
13-14	119	4.9	16.7	66.0	166.8	255.4	31.5	56.0	110.0	185.2	245.1
14-15	407	3.5	35.0	107.0	195.0	352.1	24.0	78.9	138.0	198.0	297.1
15-16	37	7.6	43.8	128.0	196.9	337.5	23.5	55.8	154.0	225.2	274.6
11-16	1167	3.5	3.5	41.0	148.0	278.8	21.0	40.0	87.0	167.0	251.7

Table 2. Correlations between salivary androgens and self-reported pubertal development in boys: Data are presented as Spearman correlation coefficient.

Pubertal Measure	Collected at any time						Before 11 AM						After 11 AM					
	T	A4	17-OHP	11-OHA4	11-KT	DHEA	T	A4	17-OHP	11-OHA4	11-KT	DHEA	T	A4	17-OHP	11-OHA4	11-KT	DHEA
N	148	148	144	145	145	155	58	58	58	56	57	62	85	85	81	84	83	88
Individual PDS components																		
Axillary hair	0.52**	0.44**	0.34**	0.24**	0.35**	0.11	0.53**	0.37**	0.39**	0.25	0.40**	0.08	0.52**	0.46**	0.29**	0.19	0.23*	0.17
Growth	0.32**	0.23**	0.21*	0.12	0.13	0.07	0.37**	0.24	0.28*	-0.01	0.06	0.04	0.32**	0.22*	0.2	0.14	0.11	0.08
Facial hair	0.56**	0.42**	0.36**	0.1	0.21**	0.1	0.63**	0.40**	0.46**	0.19	0.28*	0.07	0.49**	0.42**	0.26*	0.01	0.13	0.15
Pubic hair	0.39**	0.34**	0.22**	0.17*	0.29**	-0.02	0.53**	0.44**	0.42**	0.25	0.40**	0.02	0.30**	0.30**	0.12	0.13	0.2	0.02
Skin changes	0.18*	0.18*	0.02	0.05	0.15	0.07	0.26*	0.29*	0.14	0.06	0.09	0.03	0.16	0.09	-0.04	-0.05	0.05	0.12
Voice changes	0.57**	0.46**	0.42**	0.25**	0.36**	0.24**	0.67**	0.44**	0.50**	0.32*	0.51**	0.34**	0.48**	0.41**	0.34**	0.14	0.2	0.14
Composite PDS score	0.64**	0.51**	0.39**	0.23**	0.36**	0.13	0.75**	0.53**	0.57**	0.27*	0.45**	0.14	0.57**	0.45**	0.29**	0.11	0.19	0.16
PDS-derived pubertal categories	0.63**	0.50**	0.45**	0.19*	0.36**	0.15*	0.69**	0.46**	0.57**	0.24*	0.42**	0.25**	0.58**	0.52**	0.39**	0.14	0.23**	0.12

* and ** indicate statistically significant correlation coefficients where $p<0.05$ and $p<0.01$ respectively. Composite PDS score were the mean of all 6 pubertal domain scores. The PDS-derived pubertal category in boys are derived from the sum of voice change, facial hair growth and body hair growth category from PDS self-report: Prepubertal: 3, Early pubertal: 4-5 (no 3-point responses), Midpubertal: 6-8 (no 4-point responses), Late pubertal: 9-11 and Post pubertal: 12. In girls, the puberty category scores used body hair growth, breast development and menarche status as follows: Prepubertal: 2 and no menarche, Early pubertal; 3 and no menarche, Midpubertal: > 3 and no menarche, Late pubertal < 7 and menarche, and Post pubertal: 8 and menarche. Body hair growth in the present study was derived from the average scores between axillary hair and pubic hair development category in PDS self-report, rounded to the nearest integer.

Abbreviations: PDS, Pubertal Development Scale; T, testosterone; A4, androstenedione; 17-OHP, 17-hydroxyprogesterone; 11-OHA4, 11β-hydroxyandrostenedione; 11-KT, 11-ketotestosterone; DHEA, dehydroepiandrosterone.

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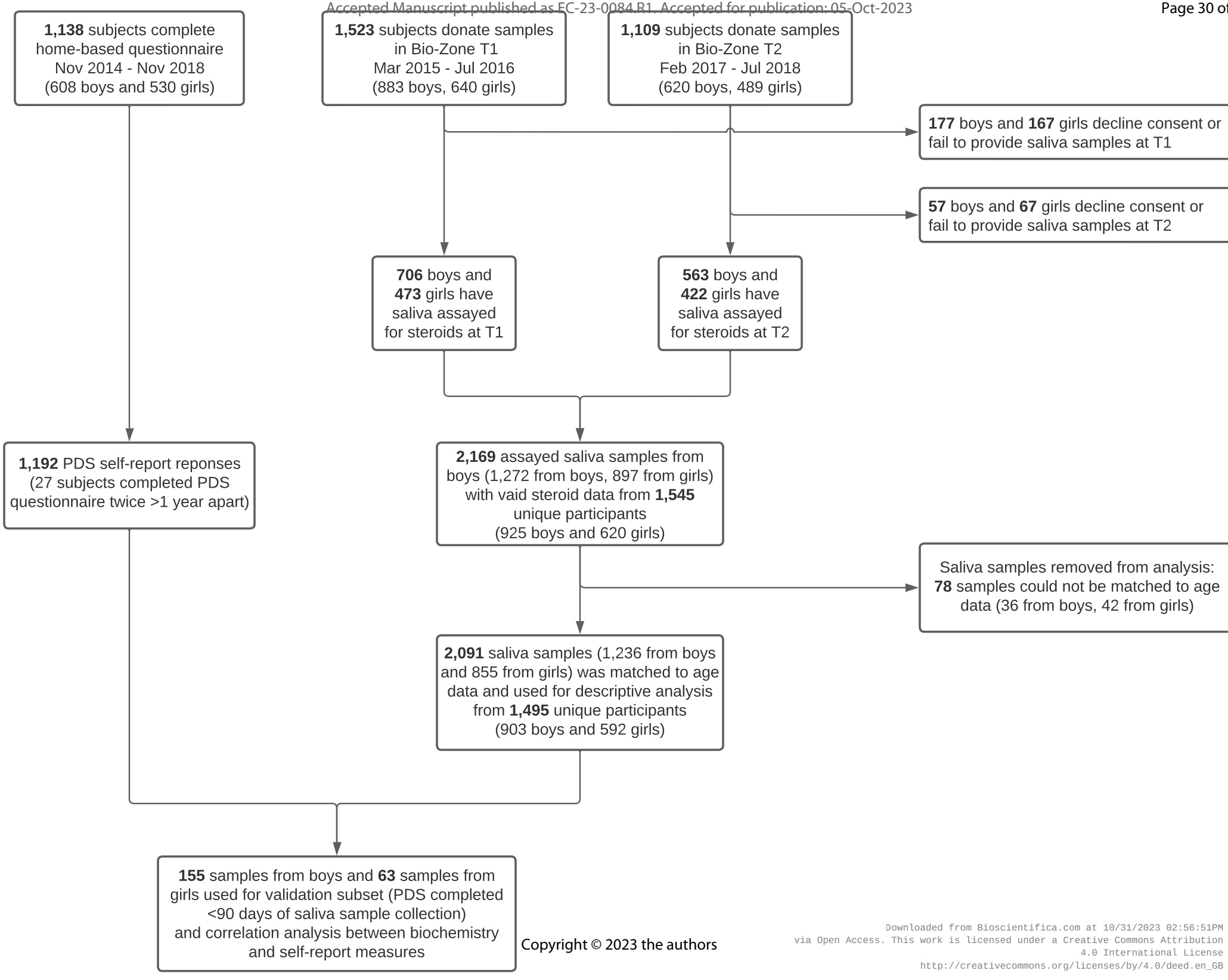
Table 3. Means, standard deviation and percentiles of salivary testosterone and androstenedione (pmol/L) by Composite Pubertal Development Scale (PDS) score in boys.

		Salivary Testosterone (pmol/L)							Salivary Androstenedione (pmol/L)						
Composite PDS Score	N	Mean	SD	2.5%	16%	50%	84%	97.5%	Mean	SD	2.5%	16%	50%	84%	97.5%
1 - 1.49	17	13.9	17.1	3.5	3.5	6.0	29.0	41.6	59.1	35.3	24.6	37.7	51.0	71.6	79.8
1.5 - 1.99	27	40.8	42.6	3.5	3.9	19.0	84.5	98.6	85.6	53.4	29.6	38.3	59.0	157.1	166.6
2 - 2.49	45	90.0	72.9	7.1	16.2	73.0	173.4	180.6	115.9	49.1	42.2	66.1	113.0	162.0	177.6
2.5 - 2.99	35	126.4	74.6	18.2	70.9	119.0	188.4	194.0	148.5	60.8	49.7	88.5	138.0	226.1	231.6
3 - 3.49	21	182.1	133.6	13.8	48.6	158.0	328.2	355.0	153.7	97.3	29.0	78.2	134.0	243.8	332.0

Two participants had composite PDS Score more than 3.5 are not displayed in the table.



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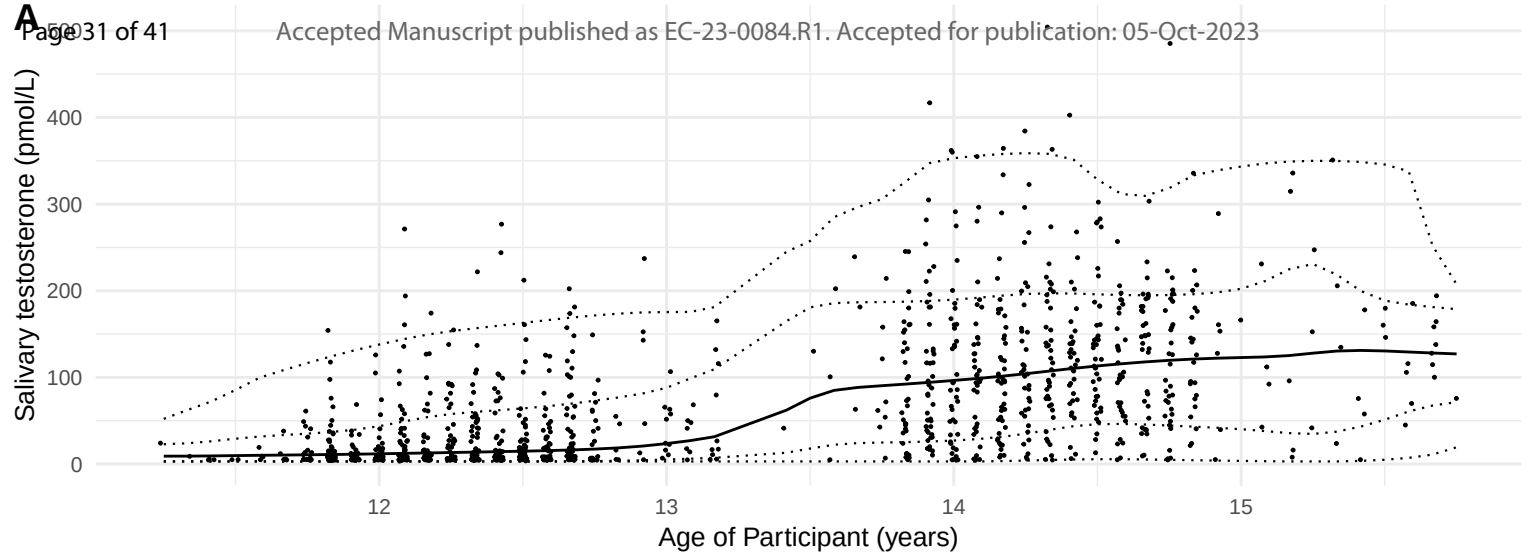

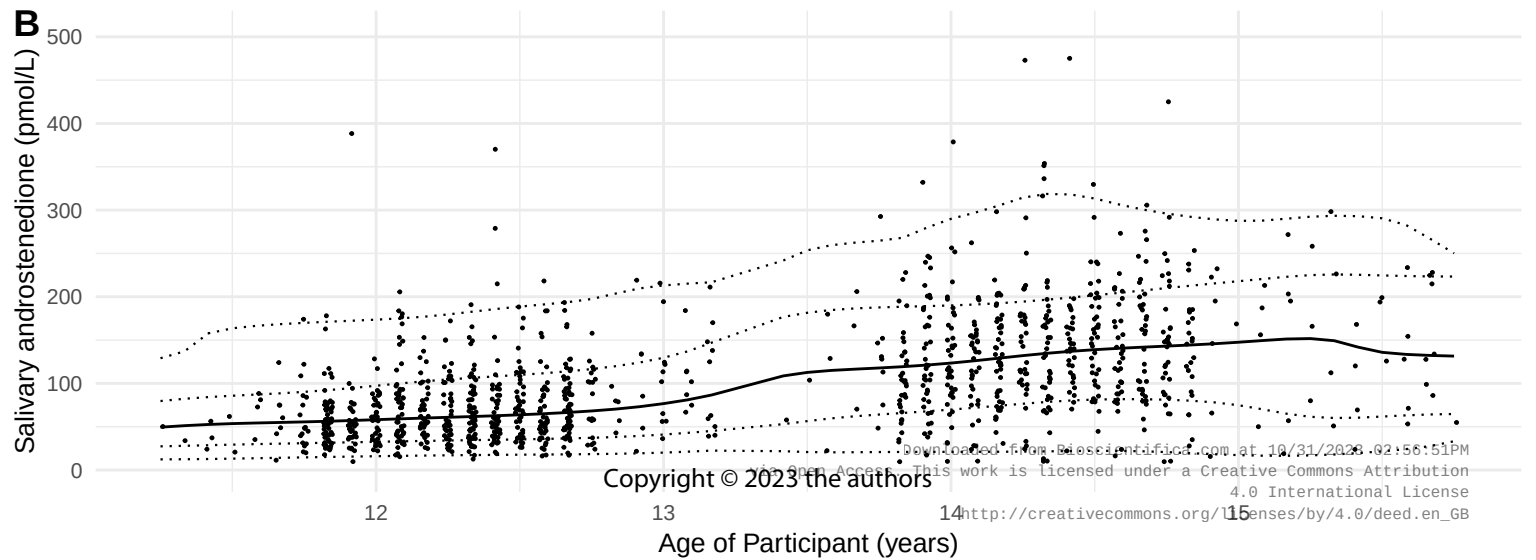

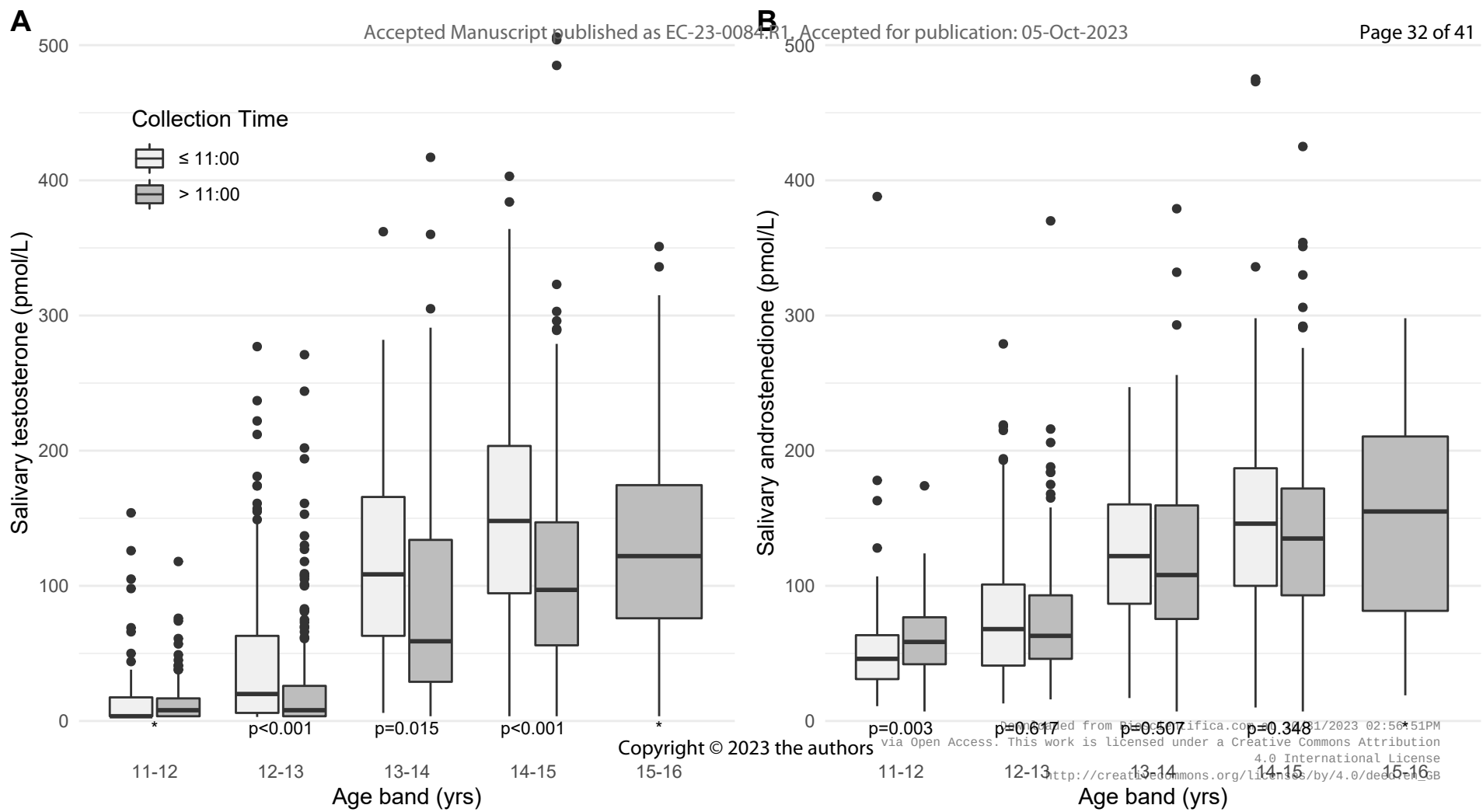

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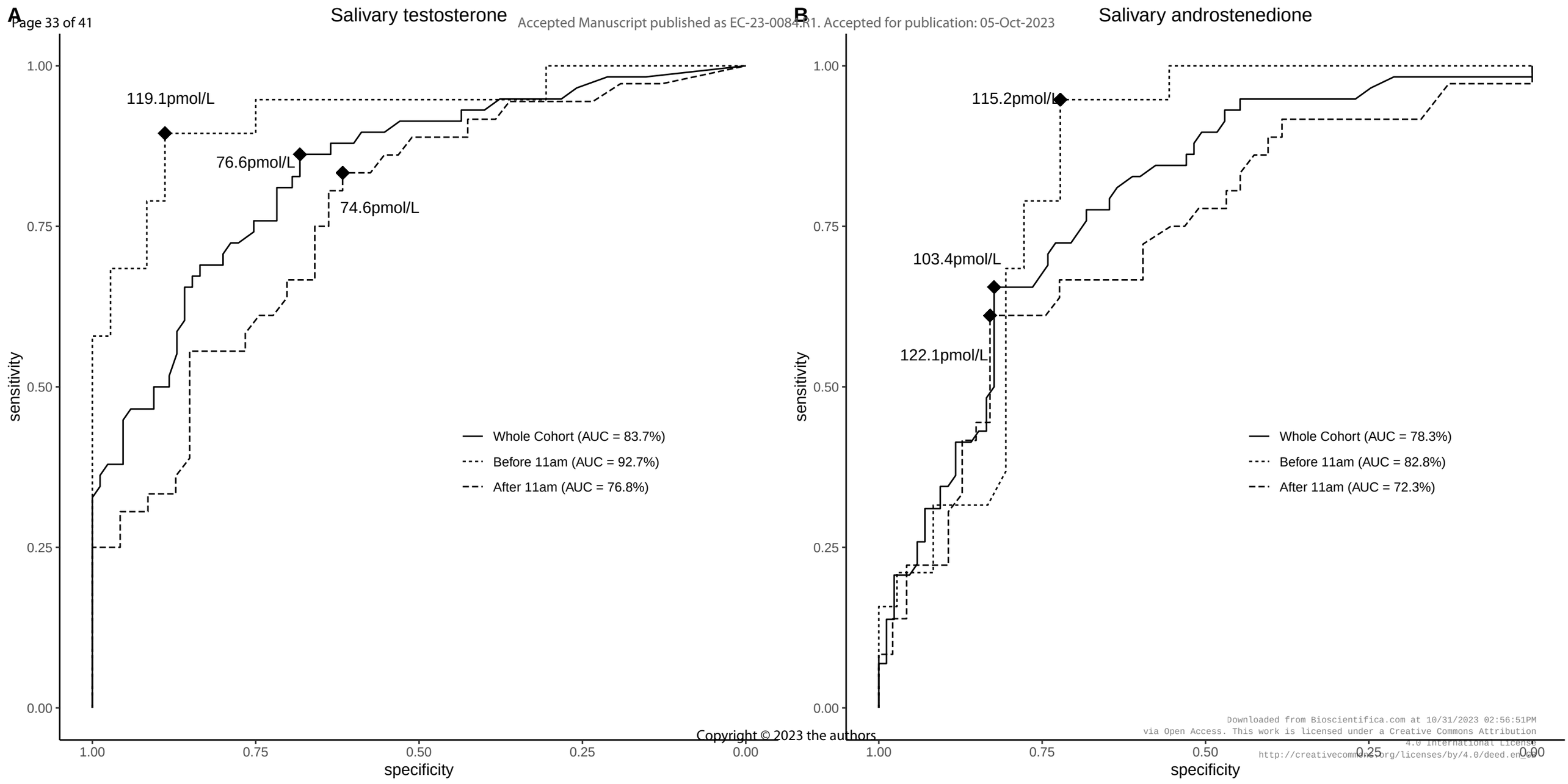

Supplementary tables

Supplementary Table 1. Percentile for salivary 17-OHP, 11-OHA4, 11-KT and DHEA concentrations from saliva samples collected at any time of the day in boys aged 11-16 years.

Androgens	Age	N	Mean	Percentile				
				2.5	16	50	84	97.5
17-OHP (pmol/L)	11-12	122	23.8	9.2	9.2	19.0	33.6	60.0
	12-13	473	26.6	9.2	9.2	20.0	39.0	83.2
	13-14	115	42.0	9.2	13.1	29.0	68.8	151.5
	14-15	393	40.9	9.2	17.0	34.0	64.0	111.0
	15-16	36	41.9	9.2	18.0	32.0	61.6	94.0
	11-16	1139	33.3	9.2	9.2	25.0	52.9	103.1
11-OHA4 (pmol/L)	11-12	124	206.4	50.2	85.4	163.5	283.6	454.3
	12-13	477	210.2	45.9	92.0	172.0	300.8	517.5
	13-14	118	252.9	60.0	115.1	216.5	376	650.1
	14-15	405	277.1	35.4	114	239.0	430.8	719.5
	15-16	37	230.2	35.4	61.0	200.0	347.4	778.8
	11-16	1161	238.1	35.4	97.0	195.0	352.4	649.0
11-KT (pmol/L)	11-12	121	128.4	34.0	59.0	115.0	193.6	343.0
	12-13	471	137.0	27.8	62.5	122.0	204.8	323.5
	13-14	118	197.4	40.8	96.0	179.0	279.8	461.1
	14-15	398	219.5	24.9	107.6	192.0	317.5	558.5
	15-16	37	178.1	38.6	80.8	154.0	271.7	373.7
	11-16	1145	172.3	27.6	71.0	148.0	259.0	457.2
DHEA (pmol/L)	11-12	137	784.4	12.3	37.4	294.0	844.0	4544
	12-13	518	819.7	12.3	102.0	377.5	1221.6	4587.2
	13-14	126	555.7	44.2	150.5	413.0	899.0	1996.2
	14-15	418	721.8	19.0	165.8	422.0	924.3	2652.2
	15-16	37	1139.5	49.4	160.2	676.0	2199.5	3673.9
	11-16	1236	765.3	12.3	122.0	394.0	1051.0	3290.6

Abbreviations: 17-OHP, 17-hydroxyprogesterone; 11-OHA4, 11β-hydroxyandrostenedione; 11-KT, 11-ketotestosterone; DHEA, dehydroepiandrosterone.

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Supplementary Table 2. Percentile for salivary T, A4, 17-OHP, 11-OHA4, 11-KT and DHEA concentrations from saliva samples collected before 11:00 AM in boys aged 11-16 years.

Androgens	Age	N	Median	Percentile				
				2.5	16	50	84	97.5
Testosterone	11-12	59	16.2	3.5	3.5	5.0	25.4	84.9
(pmol/L)	12-13	221	40.6	3.5	3.5	19.0	89.4	174.0
	13-14	37	109.7	11.4	25.2	101.0	184.6	248.7
	14-15	109	161.9	10.5	61.2	148.0	237.2	370.0
	15-16	0	-	-	-	-	-	-
	11-16	426	74.2	3.5	3.5	35.5	158.0	282.4
Androstenedione	11-12	59	56.1	18.9	25.0	46.0	71.7	171.2
(pmol/L)	12-13	221	78.9	22.0	34.0.	61.0	115.6	192.0
	13-14	37	121.4	30.5	55.0	117.0	181.0	222.7
	14-15	109	144.9	24.0	70.2	146.0	203.7	309.4
	15-16	0	-	-	-	-	-	-
	11-16	426	96.3	21	34	80.5	163	228.5
17-OHP	11-12	58	20.2	9.2	9.2	17.0	27.9	53.2
(pmol/L)	12-13	220	28.6	9.2	9.2	23.0	43.9	93.5
	13-14	35	47.3	9.2	20.2	36.0	73.1	173.0
	14-15	106	50.4	9.2	18.0	45.5	72.0	113.0
	15-16	0	-	-	-	-	-	-
	11-16	419	34.5	9.2	9.2	26.0	54.0	111.6
11-OHA4	11-12	59	164.3	39.6	71.4	147.0	262.1	400.8
(pmol/L)	12-13	219	208.6	40.7	81.4	155.0	297.2	510.3
	13-14	36	284.8	34.9	106.2	222.5	478.2	763.2
	14-15	108	255.7	34.2	93.0	224.0	444.6	575.6
	15-16	0	-	-	-	-	-	-
	11-16	422	221.0	33.7	82.2	165.0	333.9	607.5
11-KT	11-12	59	101.1	32.0	53.5	82.0	151.6	227.6
(pmol/L)	12-13	218	121.0	25.0	50.5	97.0.	197.0	310.1
	13-14	36	171.9	25.6	76.2	171.5	239.2	456.6
	14-15	107	183.7	21.2	74.6	162.0	262.4	404.9
	15-16	0	-	-	-	-	-	-
	11-16	420	138.6	24.5	54.0	117.0.	218.9	370.5
DHEA	11-12	66	1006.9	12.3	26.0	334.0	911.8	6692.5
(pmol/L)	12-13	245	877.6	12.3	92.2	384.0	1240.8	4530.0
	13-14	39	667.2	42.4	136.2	445.0	1069.6	2812.1
	14-15	112	870.4	26.8	158.2	410.5	924.2	2748.7
	15-16	0	-	-	-	-	-	-
	11-16	462	876.6	12.3	91.3	402	1067.7	4669.7

Abbreviations: T, testosterone; A4, androstenedione; 17-OHP, 17-hydroxyprogesterone; 11-OHA4, 11β-hydroxyandrostenedione; 11-KT, 11-ketotestosterone; DHEA, dehydroepiandrosterone.

Supplementary Table 3. Percentile for salivary T, A4, 17-OHP, 11-OHA4, 11-KT and DHEA concentrations from saliva samples collected after 11:00 AM in boys aged 11-16 years.

Androgens	Age	N	Median	Percentile				
				2.5	16	50	84	97.5
Testosterone	11-12	66	15.0	3.5	3.5	6.5	32.2	66.6
(pmol/L)	12-13	258	23.7	3.5	3.5	8.0	41.9	134.0
	13-14	81	81.6	3.5	12.0	51.0	156.2	254.0
	14-15	289	108.6	4.0	34.2	96.0	177.9	290.8
	15-16	35	135.2	7.3	43.2	128.0	200.7	338.2
	11-16	729	68.4	3.5	3.5	41.0	141.5	270.2
Androstenedione	11-12	66	62.9	25.1	39.5	55.5	80.6	122.8
(pmol/L)	12-13	258	71.0	20.9	37.0	63.0	100.9	166.7
	13-14	81	117.2	36.0	56.0	104.0	187.6	245.0
	14-15	289	139.0	28.0	79.2	132.0	190.9	291.8
	15-16	35	149.3	23.2	55.2	156.0	225.6	275.9
	11-16	729	106.1	22.0	46	90.0	169.5	257.6
17-OHP	11-12	64	27.1	9.2	10.9	23.5	35.8	76.6
(pmol/L)	12-13	253	24.9	9.2	9.2	19.0	36.0	80.4
	13-14	79	39.6	9.2	13.0	25.0.	67.6	150.0
	14-15	278	37.0	9.2	17.0	30.0	58.0	101.1
	15-16	34	42.7	9.2	17.9	32.0	62.9	99.2
	11-16	708	32.4	9.2	13.0	25.0	49.0	101.3
11-OHA4	11-12	65	244.6	72.6	96.8	182.0	300.3	883.6
(pmol/L)	12-13	258	211.5	53.4	111.5	183.0	305.9	495.9
	13-14	81	239.6	81.0	116.0	207.0	353.4	564
	14-15	288	283.2	50.4	132.0	242.5	422.1	757.7
	15-16	35	235.8	35.4	59.5	212.0	357.4	789.2
	11-16	727	247.2	52.2	114.0	207.0	353.8	699.2
11-KT	11-12	62	154.4	57.0	76.3	139.0	212.3	372.0
(pmol/L)	12-13	253	150.8	34.0	77.2	144.0	217.8	337.0
	13-14	81	208.6	69.0	103.0	190.0	307.0	460.0
	14-15	282	233.0	38.3	119.2	200.0	335.2	564.8
	15-16	35	181.3	37.9	80.2	166.0	288.7	379.5
	11-16	713	191.7	39.6	89.0	166.0	279.1	500.2
DHEA	11-12	71	577.5	12.3	70.0	279.0	707.2	3514.3
(pmol/L)	12-13	273	767.8	12.3	103.8	364.0	1209.4	3601.6
	13-14	86	503.0	47.8	161.8	392.5	820.2	1864.8
	14-15	297	663.8	41.8	172.2	429.0	916.1	2425.6
	15-16	35	1199.8	90.6	235.1	754.0	2272.1	3795.3
	11-16	762	699.5	12.3	136.3	391.5	1023.1	3218.3

Abbreviations: T, testosterone; A4, androstenedione; 17-OHP, 17-hydroxyprogesterone; 11-OHA4, 11β-hydroxyandrostenedione; 11-KT, 11-ketotestosterone; DHEA, dehydroepiandrosterone.

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Supplementary Table 4. Percentile for salivary oestradiol and DHEA concentrations from saliva samples collected at any time of the day, before and after 11:00 AM in girls aged 11-16 years.

	Age	N	Mean	Percentile				
				2.5	16	50	84	97.5
Oestradiol (All time) (pmol/L)	11-12	65	3.4	2.3	0.3	1.2	3.0	5.3
	12-13	357	3.6	2.0	0.5	1.7	3.2	5.4
	13-14	130	4.1	1.7	1.0	2.6	3.9	5.7
	14-15	292	3.9	2.0	1.0	1.9	3.7	5.6
	15-16	17	4.7	3.2	1.4	2.0	4.7	6.5
	11-16	861	3.8	2.0	0.7	1.9	3.4	5.6
Oestradiol (Before 11 AM) (pmol/L)	11-12	26	2.9	1.8	0.7	1.4	2.4	4.1
	12-13	143	4.0	2.2	0.8	1.9	3.8	6.5
	13-14	62	4.3	2.0	1.2	2.4	4.0	6.3
	14-15	113	3.8	1.6	1.2	1.9	3.6	5.2
	15-16	0	-	-	-	-	-	-
	11-16	344	3.9	2	0.8	1.9	3.7	5.9
Oestradiol (After 11 AM) (pmol/L)	11-12	39	3.6	2.5	0.3	0.9	3.4	5.7
	12-13	214	3.3	1.8	0.4	1.6	3.0	4.7
	13-14	58	4.0	1.5	1.2	2.7	3.8	5.5
	14-15	159	4.0	2.2	1.0	1.9	3.8	6.0
	15-16	17	4.7	3.2	1.4	2.0	4.7	6.5
	11-16	487	3.7	2.1	0.4	1.8	3.3	5.5
DHEA (All time) (pmol/L)	11-12	65	445.4	408.1	38	115.6	314	762.4
	12-13	357	644.5	835.4	35.4	146.4	395	1052.1
	13-14	130	516.0	608.2	71	166.0	355	761.4
	14-15	292	654.9	780.9	50.9	162.6	438	1019.4
	15-16	17	888.1	593.4	281.4	378.8	684	1528.2
	11-16	861	618.4	758.8	46.0	154.0	402	955.0
DHEA (Before 11 AM) (pmol/L)	11-12	26	397.4	377	35.9	111.0	296	716.0
	12-13	143	658.8	936.6	66.0	148.5	407	1025.5
	13-14	62	524.0	651.4	71.0	166.0	335.5	761.2
	14-15	113	747.8	948.5	48.6	147.6	465	1107.4
	15-16	0	-	-	-	-	-	-
	11-16	344	644.0	868.1	55.2	148.0	389.5	979.7
DHEA (After 11 AM) (pmol/L)	11-12	39	477.4	429.4	64.7	132.8	333.0	764.8
	12-13	214	634.9	762.5	25.9	144.2	385.5	1048.6
	13-14	58	512.2	609.7	46.2	175.7	370.0	736.8
	14-15	159	585.4	654.6	53.8	195.1	424.0	919.9
	15-16	17	888.1	593.4	281.4	378.8	684.0	1528.2
	11-16	487	600.4	685.1	24.9	161.9	395.0	937.5

Abbreviations: DHEA, dehydroepiandrosterone.

Supplementary Table 5. Median concentrations of salivary steroids collected in boys stratified by one-year age band and two-sided Wilcoxon-Mann-Whitney test.

		Collected before 11 AM		Collected after 11 AM		
	Age Band	N	Median Concentration pmol/L	N	Median Concentration pmol/L	*p* value
T	11-12	59	5	66	6	*
	12-13	221	19	258	8	**<0.001**
	13-14	37	101	81	51	**0.015**
	14-15	109	148	289	96	**<0.001**
	15-16	0	-	35	128	-
A4	11-12	59	46	66	56	**0.003**
	12-13	221	61	258	63	0.671
	13-14	37	117	81	104	0.507
	14-15	109	146	289	132	0.348
	15-16	0	-	35	156	-
17-OHP	11-12	58	17	64	24	**0.016**
	12-13	220	23	253	19	0.068
	13-14	35	36	79	25	0.084
	14-15	106	46	278	30	**<0.001**
	15-16	0	-	34	32	-
11-KT	11-12	59	82	62	139	**<0.001**
	12-13	218	97	253	144	**<0.001**
	13-14	36	172	81	190	0.078
	14-15	107	162	282	200	**0.001**
	15-16	0	-	35	166	-
11-OHA4	11-12	59	147	65	182	**0.028**
	12-13	219	155	258	183	**0.001**
	13-14	36	222	81	207	0.761
	14-15	108	224	288	242	0.092
	15-16	0	-	35	212	-
DHEA	11-12	66	334	71	279	0.578
	12-13	245	384	273	364	0.870
	13-14	39	445	86	392	0.633
	14-15	112	410	297	429	0.817
	15-16	0	-	35	754	-

Boldface p-values are <0.05. * For T from within 11-12 age band, this was because majority of samples were left-censored, i.e. below the detection limit (<5 pmol/L). Abbreviations: T, testosterone; A4, androstenedione; 17-OHP, 17-hydroxyprogesterone; 11-OHA4, 11β-hydroxyandrostenedione; 11-KT, 11-ketotestosterone; DHEA, dehydroepiandrosterone

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Supplementary Table 6. Median concentrations of salivary steroids collected in girls stratified by one-year age band and two-sided Wilcoxon-Mann-Whitney test.

		Collected before 11 AM		Collected after 11 AM		
	Age Band	N	Median Concentration pmol/L	N	Median Concentration pmol/L	*p* value
OE2	11-12	26	2.4	39	3.4	0.239
	12-13	143	3.8	214	3.0	**0.002**
	13-14	62	4.0	58	3.8	0.735
	14-15	113	3.6	159	3.8	0.947
	15-16	0	-	17	4.7	-
DHEA	11-12	26	296	39	333	0.466
	12-13	143	407	214	385.5	0.466
	13-14	62	335.5	58	370	0.948
	14-15	113	465	159	424	0.480
	15-16	0	-	17	684	-

Boldface p-values are <0.05. Abbreviations: OE2, 17β-Oestradiol; DHEA, dehydroepiandrosterone

Supplementary Table 7 Correlation between salivary steroids from girls and self-reported pubertal development on PDS self-report.

Pubertal Measure	Collected at any time		Before 11 AM		After 11 AM	
	OE2	DHEA	OE2	DHEA	OE2	DHEA
N	63	63	33	33	29	29
Individual PDS component						
Axillary hair	0.32*	0.25	0.22	0.30	0.39*	0.14
Breast development	0.02	0.24	-0.21	0.24	0.27	0.26
Growth	0.00	0.08	-0.15	-0.09	0.23	0.42*
Menarche	-0.03	0.08	-0.24	-0.04	0.25	0.22
Pubic hair	0.16	0.19	0.07	0.13	0.24	0.24
Skin changes	0.12	0.07	0.12	0.06	0.04	0.08
Composite PDS score	0.18	0.21	0.02	0.17	0.36	0.28
PDS-derived pubertal categories	0.05	0.12	-0.13	0.11	0.26	0.14

Data are presented as Spearman correlation coefficient. * and ** indicate statistically significant correlation coefficients where $p<0.05$ and $p<0.01$ respectively (no p-value was below 0.01). Composite PDS score were the mean of all 6 pubertal domain scores. The PDS-derived pubertal category used body hair growth, breast development and menarche status as follows: Prepubertal: 2 and no menarche, Early pubertal; 3 and no menarche, Midpubertal: > 3 and no menarche, Late pubertal < 7 and menarche, and Post pubertal: 8 and menarche. Body hair growth in the present study was derived from the average scores between axillary hair and pubic hair development category in PDS self-report, rounded to the nearest integer.

Abbreviations; PDS, Pubertal Development Scale; OE2, Oestradiol; DHEA, dehydroepiandrosterone

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Supplementary Table 8. Stability analysis results and post-hoc estimation of degradation kinetics from adult volunteers. T, A4, 17-OHP, 11-OHA4, 11-KT were collected from males only; OE2 was collected from female only; DHEA was collected from both sexes.

	Test for stability				Kinetics: estimated parameter posterior mean [95% credible interval]			
	Df_n	Df_d	F-statistic	p-value	Model	Rate constant k / yr^{-1}	Half-life $t_{1/2}$ / yr	Annual change in concentration / %
T	1	77	28.68	<0.001	SFO	0.069 [0.040, 0.098]	10.00 [7.06, 17.3]	-6.7 [-9.4, -3.9]
A4	1	77	22.19	<0.001	SFO	0.060 [0.031, 0.089]	11.60 [7.76, 22.7]	-5.8 [-8.5, -3.0,]
17-OHP	1	76	5.94	0.017	SFO	0.072 [0.025, 0.119]	9.59 [5.82, 27.7]	-7.0 [-11.0, -2.5]
11-OHA4	1	77	50.45	<0.001	SFO	0.301 [0.079, 0.547]	3.01 [2.37, 4.05]	-21.0 [-25.0, -16.0]
11-KT	1	77	8.50	0.005	SFO	0.230 [0.171, 0.293]	2.30 [1.27, 8.80]	-26.0 [-42.0, -7.6]
DHEA	1	127	51.7	<0.001	LM	-	-	+32.5 [+17.5, +46.3]
OE2	1	89	8.88	0.004	LM	-	-	+1.81 [-14.8, +17.3]

Abbreviations: T, Testosterone; A4, Androstenedione; 17-OHP, 17-hydroxyprogesterone; 11-OHA4, 11β-hydroxyandrostenedione; 11-KT, 11-ketotestosterone; DHEA, dehydroepiandrosterone; OE2, 17β-Oestradiol; Df_n, degrees of freedom of F-statistic numerator; Df_d, degrees of freedom of F-statistic denominator; SFO, single-first order kinetic model where degradation rate is assumed proportional to steroid concentration; LM, linear model where percentage analyte accumulation rate is assumed constant with respect to time.