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Assay validation of hair androgens across the menstrual cycle

Wen Wang[a], Shannin N. Moody[a], Jeff Kiesner[b], Anna Tonon Appiani[b], Olivia C. Robertson[c], Elizabeth A. Shirtcliff[a,*]

[a] Iowa State University, United States
[b] University of Padova, Italy
[c] Purdue University, United States

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ABSTRACT

Introduction: Saliva is a common noninvasive biofluid for measuring stress and sex hormones, yet one pressing limitation is that salivary hormones fluctuate momentarily, daily, and (for girls) across the menstrual cycle. Hair steroid assays are thought to provide a cumulative index which collapses across hormonal variability, potentially eliminating the confound of daily and menstrual cyclicity and thereby reflecting individual differences in average hormone levels. Here we seek to validate a hair bioassay methodology and test whether hair androgens accurately measure long-term, stable androgen levels in emerging adult women across two menstrual cycles.
Methods: Hair samples were collected at the end of each menstrual cycle for two cycles, and saliva samples were collected in the morning once per week across two menstrual cycles (N = 11 women). Hair samples were segmented by 1 cm for the first 4 cm to reflect the hormone levels of the past four serial months. Hair samples were assayed using commercially-available enzyme-immuno-assays for testosterone and DHEA.
Results: Hair androgen concentrations were significantly correlated with averaged saliva hormone levels (DHEA: $r = .75$, $p < .05$; Testosterone: $r = .67$, $p < .05$). With respect to hair hormone stability, there were significant correlations for almost all the pairs of two 1 cm hair segments collected in two months that corresponded to the same time period. Hair androgens in one segment were significantly correlated with those in next segment. Regarding salivary androgen stability, the intra-class correlation across the weekly saliva samples indicated that for DHEA 59% of the total variance was within person and 41% was between person; and for testosterone 91% of the total variance was between person, and only 9% within person.
Discussion: Results suggest that a one-time measure of hair provides a valid and reliable estimate of average steroid levels across two months. Moreover, whereas saliva measures of androgen levels capture week-to-week fluctuations in steroids, hair samples provide information on individual differences in average exposure to steroids, across long periods of time, such as months. Results are encouraging that hair DHEA and testosterone reflects the cumulative hormonal concentration and can be used as a stable hormonal index. Results also indicate that it is feasible to collect the first 3–4 centimeters of hair for studies of stable hormone levels.

1. Introduction

Noninvasive measurement of androgens like testosterone and dehydroepiandrosterone (DHEA) in salivary samples has been widely used as an index of hypothalamic-pituitary-gonadal (HPG) axis responses to stress and daily experiences (Kirschbaum and Hellhammer, 1994; Meyer et al., 2014; Karlamangla et al., 2013) and applied to multiple professional fields such as human development, psychobiology and mental health (Adam, 2006; Knorr et al., 2010; Shirtcliff et al., 2009). The popularity of salivary biomarkers research is largely due to its feasibility, noninvasive ease of sampling, point-of-care collection and repeated sampling (Shirtcliff et al., 2015; Wilson, 1993). However, there are two major methodological limitations with salivary biomarker measurement. First, a practical challenge is that samples are recommended to be frozen immediately and during transport which can reduce feasibility, particularly for at-home collection (Lewis, 2006; Toone et al., 2013; Granger et al., 2004). A more pressing limitation is that the momentary or daily fluctuating levels of hormones in many biofluids, including saliva, limit the field's ability to answer questions about chronic or long-term hormone exposure levels, such as average

* Corresponding author at: Human Development Family Studies, Iowa State University, 2361C Palmer Building, Ames, IA 50010, United States.
E-mail addresses: wenwang@iastate.edu (W. Wang), shannin@iastate.edu (S.N. Moody), jeff.kiesner@unipd.it (J. Kiesner), annatononappiani@gmail.com (A. Tonon Appiani), rober511@purdue.edu (O.C. Robertson), birdie@iastate.edu (E.A. Shirtcliff).

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hormone levels during puberty or across a menstrual cycle. Although androgens are relatively stable, levels can change dramatically within a short time, such as in response to exercise (Di Luigi et al., 2006), competition (Gonzalez-Bono et al., 1999), sexual stimuli (Escasa et al., 2011) and emotionally-charged tasks (Schultheiss et al., 2004; Lennartsson et al., 2012) in addition to longer fluctuations such as diurnal variation (Brambilla et al., 2009).

Given these challenges, the repertoire of noninvasive bioassays in psychobiological research has recently broadened to include hair steroid hormone assays. For example, cortisol, a primary stress hormone, has been measured using hair or fur samples (Davenport et al., 2006; Wosu et al., 2013). Such bioassays of steroid hormones are valid (Stalder et al., 2012; Sauvé et al., 2007) and useful within large-scale research settings (Vanaelst et al., 2012). Hair assays are valuable as an index of *basal hormone level* (Dettenborn et al., 2012; Sharpley et al., 2012; Russell et al., 2012) from clippings close to the scalp that reflect the prior 1–3 months of steroid hormones (depending on the length of clippings) (Li et al., 2012). Moreover, hair assays are feasible as several protocols use commercially available enzyme-immuno-assay kits validated for use with saliva and other biological specimens. Given that many steroids diffuse into the hair, other steroids such as testosterone and dehydroepiandrosterone (DHEA) could potentially also be assayed from hair (Chan et al., 2014; Pereg et al., 2013; Gao et al., 2013; Wheeler, 2006; Yang et al., 1998; Rambaud et al., 2005; Gleixner and Meyer, 1997).

An emerging literature has begun to explore methods for quantitative analysis of hair androgens (Thomson et al., 2009; Pereg et al., 2013; Ullmann et al., 2016; Qiao et al., 2017), but these studies have varied regarding targeted hormones, samples resources (human or animals) and assay methods. For example, both enzyme immunoassay (EIA) and mass spectrometry (MS) methods have been applied to detect testosterone in human hair (Pereg et al., 2013; Gao et al., 2013; Chan et al., 2014). Although MS method has been validated and reported (Yang et al., 1998; Rambaud et al., 2005; Choi et al., 2000), MS can be challenging for many labs which do not have access to expensive equipment. Moreover, protocols varied across studies in terms of hair source (human hair vs. animal fur) (Schell et al., 2017; Gao et al., 2013; Kapoor et al., 2014). Therefore, there is a need in the field to establish protocols for human hair androgens using EIA methodology.

In this study, protocol validation procedures for hair androgens were conducted. Beyond validation procedures for hair androgens, the present study sought to empirically test the claim that the hair assays can work as a cumulative index to minimize variation in hormones over time. The premise is that a cumulative index should return stable basal hormone estimates as it collapses across extraneous and day-to-day hormone fluctuations, including across menstrual cycles. Since blood or salivary hormone levels change moment-to-moment (Dabbs, 1990; Dettenborn et al., 2012), it has been challenging to derive stable basal androgen levels as multiple samples per person over a wide span of time is needed to minimize variance in hormone levels over time. In addition, androgens are affected by menstrual cycle. For example, salivary DHEA varies across the menstrual cycle, reaching its lowest point in the luteal phase (Symonds et al., 2004). Moreover, significant physical and psychological changes associated with menstrual cycle have been demonstrated (Kiesner, 2011, 2012; Kiesner and Pastore, 2010; Kiesner and Poulin, 2011). A limitation of such research has been the shortage of hormonal measures that would allow the verification of ovulatory cycles as opposed to anovulatory cycles, and provide a measure of individual differences in basal levels of hormones across women.

To both examine correspondence across bio-specimens and assess stability of both androgen protocols, the current study seeks to validate a protocol using an EIA method to measure two androgens (testosterone and DHEA) in human hair. This validation will be tested by examining the correspondence between hair androgen values and the average values of sex hormones in saliva samples collected weekly during the corresponding time period, as well as across different segments of the hair shaft representing different time frames (months) of exposure. We collected samples across two menstrual cycles in order to calculate stability in both hair and saliva androgens across samples and directly test the premise that hair androgens minimize menstrual cycle-related fluctuations.

2. Methods

2.1. Participants

Eleven female university students at the University of Padova in Italy were involved in this study (age 20–24, mean 21.55). In order to participate, participants were required to have a regular menstrual cycle, and not to use birth control pills or other hormonal contraception or medication. After male students were asked to leave the classroom at the end of a lecture, a detailed explanation of this study was provided orally. Recruitment focused on the importance of having accurate and reliable measures of hormones. Slips of paper were then provided for the eventual enrollment and participation. Nearly all of the participants had 2A-C hair type (wavy). One participant's data was excluded for analysis because she finally did not provide enough hair sample for androgen assays (N = 10).

2.2. Procedure

Following recruitment, female students arrived for an instructional laboratory visit. Participants signed a consent form and were instructed on how to use the online questionnaire, how to self-collect and store frozen saliva samples at home and how to transport samples back to the laboratory. Starting on the first day of their menstrual cycle, participants self-collected weekly saliva samples across one menstrual cycle and then returned to the laboratory for a second visit when a hair sample was collected. This procedure was repeated for a second menstrual cycle and subsequent hair sample so that hair samples were procured at the end of two menstrual cycles, and saliva samples were obtained each week for approximately 8 weeks. Given expected variation in sample duration even for women with regular cycles, the weekly saliva collection did not necessarily result in 4 monthly samples per participant. Salivary and hair samples were assayed for DHEA and testosterone.

2.3. Measures

2.3.1. Demographic information

Participants were asked questions about age, height, weight, past use of birth control, past medical or psychiatric condition, use of medications, etc. Participants also answered questions regarding the use of shampoos, hair dyes, or other hair treatments, that could interfere or interact with the assay procedure (Short et al., 2016).

2.3.2. Daily symptom reports

To measure a wide range of physical, psychological, social, and behavioral changes that are often associated with the menstrual cycle, an online questionnaire was used (http://dpss.psy.unipd.it/quest/prorisip2015demo/) which had been developed and used in prior research (Kiesner, 2011, 2012; Kiesner and Pastore, 2010). Participants were asked to complete the questionnaire at approximately the same time every day. If they skipped one day, they were asked to go back one day and completed the questionnaire also for "yesterday", but they were asked not to recall further back than one day.

2.3.3. Saliva sample collection

Participants were asked to drink a small amount of water and wait five minutes. Saliva samples were collected by participants at home via passive drool upon awakening in the morning when hormone levels are highest following published recommendations (Shirtcliff et al., 2001;

Granger et al., 2003). Participants collected the first saliva sample at the first day of a menstrual cycle and kept collecting same time every week throughout the whole menstrual cycle. They repeated this procedure for the subsequent menstrual cycle. Participants then sealed the cryovial, placed it into a small bag and froze the sample in their home freezer and filled out a daily diary questionnaire which asked about menstrual status. After the final saliva sample was collected, participants self-couriered saliva samples to the laboratory, with samples remaining frozen during transport via freezer-brix. Samples then were shipped overnight on dry ice to the Iowa State SPIT lab and stored at -80℃.

2.3.4. Saliva sample assay

On the day of assay, samples were brought to room temperature and centrifuged at 3000 rpm for 15 min. The clear supernatant was assayed for DHEA and testosterone using commercially available enzyme immunoassay kits (Salimetrics, State College, PA). A plate was considered viable if the standard curve had $R^2 > 0.996$. All samples were tested in duplicate and samples were re-assayed if duplicate test values that varied by more than 10% error.

2.3.5. Hair sample collection

Hair samples were collected at the end of each menstrual cycle (twice across the study) at the University of Padova. To do so, hair was parted along the posterior vertex of the head horizontally in line with the middle of the ears, and a hair band and clips held the hair away from the part to easily isolate the hair sample. Approximately 450 strands were carefully cut close to the scalp, secured with a small band, wrapped unbent in a foil sheet, sealed in an envelope, and labeled. Hair samples were stored in a cool and dry cabinet in the laboratory until shipped to the Iowa State SPIT lab for assay.

2.3.6. Hair sample assay

Hair was segmented by 1 cm (the first 4 cm was used) and was washed twice in high performance liquid chromatography (HPLC)-grade isopropanol with 3 min repeated inversion using a rotator. The washed hair was dried under forced air for 4–5 hours to ensure complete isopropanol evaporation. The steroid extraction method was adapted from previous published studies (Meyer et al., 2014; Pereg et al., 2013). Fifteen milligram of clean hair was placed into a 2 ml microcentrifuge tube together with three 4.9 mm stainless steel beads. Hair was ground to a fine powder using a Retsch ball mill (MM400) for 5 min at 30 Hz. For extraction, HPLC-grade methanol (1.5 ml) was added into the tube and the samples were incubated for 24 h at room temperature with constant inversion using a rotator. After extraction, the tubes were centrifuged and 1 ml of the supernatant was transferred to a new microcentrifuge tube. Methanol was dried down under a stream of nitrogen gas in an evaporator at 50℃ for 20–30 min. The steroid extract was reconstituted with 400 µl assay diluent (vortexing for 30 s). Reconstituted samples were assayed immediately for testosterone using EIA kit which was same as saliva samples. For DHEA, to ensure the concentration fits the EIA kit sensitivity range, one fourth dilution was made prior to assay.

2.4. Measures: protocol validation

Hair sample quantity, linearity of dilution and spike-recovery were examined to validate the protocol for measurement of hair testosterone and DHEA, respectively. Hormones were assayed using 50 mg, 25 mg, 15 mg, 10 mg, and 5 mg of sample. For each quantity, hormones were within the assay range of sensitivity and there was a linear increase in testosterone and DHEA for heavier samples (R^2 = .893 for testosterone; R^2 = .895 for DHEA). Quantities of 50 mg and 25 mg returned DHEA above assay's range. Samples were subsequently assayed with 15 mg and DHEA samples were diluted x4 prior to assay.

Linearity of dilution was determined by assaying serial dilutions of reconstituted 15 mg samples (1/2, 1/4 and 1/8 diluting with assay diluent) and then calculated as [observed-expected/expected]. Spike-recovery was examined by mixing half of a known quantity of hormone and half of the reconstituted hair sample (high and low concentrations) and then calculated as [observed-expected/expected]. High and low concentrations were from Salimetrics enzyme immunoassay kits with concentrations as 398.85 pg/mL and 25. 40 pg/mL respectively for DHEA, 194.24 pg/mL and 8.96 pg/mL respectively for testosterone. For testosterone, samples fell on average 4.05 pg/mL (15.53%) above expected values for linearity and 7.40% for spike-and-recovery. For DHEA, samples fell on average 15.55 pg/mL (14.94%) above expected values for linearity and 13.5% for spike-and-recovery.

3. Results

3.1. Correspondence between androgen concentration in hair and saliva

An important component to final protocol validation is for external validation that compares concentrations across bio-specimens. We examined the correspondence between the androgen values in hair sample and the average values for the sex hormones in saliva samples that were collected repeatedly and regularly during the same time period of hair collection (i.e., the weekly saliva samples across the prior menstrual cycle). Pearson Correlation results showed that hair androgen concentration (the average value of the first and second 1 cm segments of the second hair sample which was collected at the end of the second month) was significantly correlated with averaged saliva sex hormone level (for DHEA, r = 0.65, p = 0.044, see Fig. 1; for testosterone, r = 0.67, p = 0.034, see Fig. 2).

We also examined the correspondence between hair androgen concentration and androgen levels in each single saliva sample in the two menstrual cycles. None of the saliva androgen level was correlated with hair androgen concentration.

3.2. Stability of hormone measures

To measure the stability of hair androgen concentrations, we segmented the first 4 cm of each hair sample by 1 cm. The mean hair DHEA concentration of all single hair segments was 18.25 pg/mg (SD = 7.37) with range of 2.05–41.12 pg/mg. The mean hair testosterone concentration of all single hair segments was 1.27 pg/mg (SD = 0.34) with range of 0.58–2.26 pg/mg. Of primary importance for establishing that 1 cm of hair growth corresponds to one month of hormone exposure, we focus on the correspondence of the first segment of hair (closest to the scalp) in the second sample with the second segment of hair in the first sample which are thought to correspond to the same period of time. For hair testosterone, the first segment of month 1 hair was significantly correlated with the second segment of month 2 hair (r = 0.718,

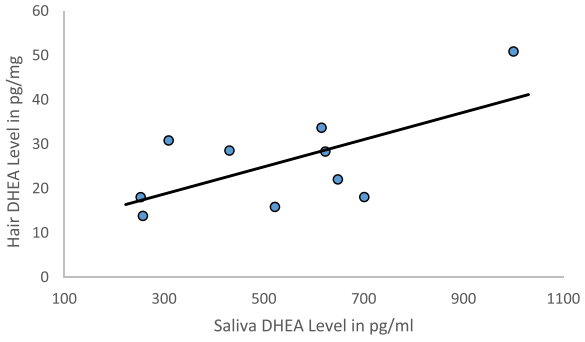

Fig. 1. Association of Hair and Saliva DHEA.
*Y-axis indicates the average DHEA value of the first and second 1 cm segments of the second hair sample which was collected at the end of the second month, x-axis indicates averaged saliva DHEA level across two menstrual cycles.

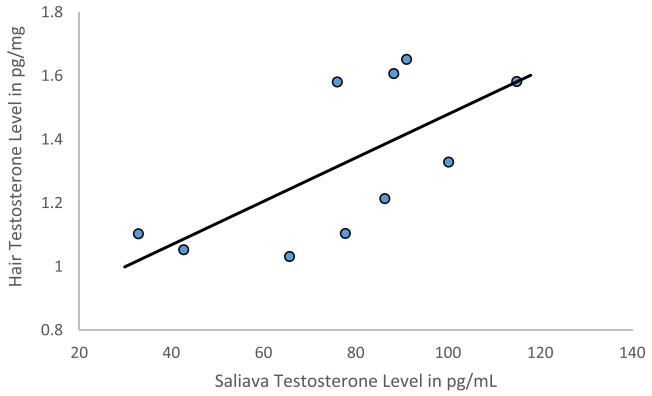

Fig. 2. Association of Hair and Saliva Testosterone.
*Y-axis indicates the average testosterone value of the first and second 1 cm segments of the second hair sample which was collected at the end of the second month, x-axis indicates averaged saliva testosterone level across two menstrual cycles.

Table 1
Correlation between two hair segments in two months that correspond to the same period of time (Testosterone).

Segments	1 st in 2nd month	2nd in 2nd month	3rd in 2nd month	4th in 2nd month
1 st in 1st month	–	.72*	–	–
2nd in 1st month		–	.58+	–
3rd in 1st month			–	.88**
4th in 1st month				–

Notes: ***p < .001, ** p < .01, * p < .05, + p < .10.

Table 2
Correlation between two hair segments in two months that correspond to the same period of time (DHEA).

Segments	1 st in 2nd month	2nd in 2nd month	3rd in 2nd month	4th in 2nd month
1 st in 1st month	–	.47	–	–
2nd in 1st month		–	.21	–
3rd in 1st month			–	.70*
4th in 1st month				–

Notes: ***p < .001, ** p < .01, * p < .05.

p = 0.045). Similarly, the second segment of month 1 hair was marginally correlated with the third segment of month 2 hair (r = 0.584, p = 0.099), and the third segment of month 1 hair was strongly correlated with the fourth segment of month 2 hair (r = 0.875, p = 0.002) (see Table 1). For hair DHEA, the third segment of month 1 hair was found to significantly correlated with the fourth segment of month 2 hair (r = .697, p = .037). Correspondence between the other pairs of hair segments was not significant (see Table 2).

Another method of establishing stability is to examine, within a single hair sample, hair androgen concentrations over time (i.e., across 1 cm segments). To do so, we conducted a series of Pearson Correlations to test correlations between steroid concentration in different hair segments across 4 months (4 segments reflect basal hormone level in the past four months in this study). Tables 3 and 4 demonstrated that hair sex hormone concentrations in all pairs of two successive segments were significantly associated. Moreover, the correlations among the 4 segments showed that the magnitude of association decreased with a greater distance of hair segments, following a general pattern of first-order autoregressive correlation structure. For hair DHEA, the magnitude of associations between the prior first month and the other three decreases from the prior second month (r = .624. p = .004), to the prior third month (r = .507, p = .027), and became non-significant by

Table 3
Correlation of Hair Segments (DHEA).

Segments	1 st	2nd	3rd	4th
1 st	–			
2nd	.62**	–		
3rd	.51*	.54*	–	
4th	.24	.46*	.81***	–

Notes: ***p < .001, ** p < .01, * p < .05.

Table 4
Correlation of Hair Segments (Testosterone).

Segments	1 st	2nd	3rd	4th
1 st	–			
2nd	.88***	–		
3rd	.68**	.81***	–	
4th	.58**	.74***	.94***	–

Notes: ***p < .001, ** p < .01, * p < .05.

the prior fourth month (r = .238, p = .327) (see Table 3). The same magnitude of associations for hair testosterone also decreases from the prior second month (r = .884, p < .000), to the prior third month (r = .682, p = .001), and to the prior fourth month (r = .579, p = .009) (see Table 4).

Regarding the stability in salivary hormones, we calculated the intra-class correlation across the weekly saliva samples to account for the variation in the number of samples per cycle from person to person (range 3–6 weekly samples per cycle). The ICC = .41 for salivary DHEA suggesting that the within-individual monthly variation is 59% of the total, and 41% of the total DHEA variation is between person. The ICC = .91 for saliva testosterone, suggesting that most of the variance in testosterone (91%) is between persons; whereas 9% of the total variance in testosterone is within person. In sum, within each person, hormone levels fluctuate across menstrual cycle. Figs. 3 and 4 illustrate the saliva hormone variability across two menstrual cycles.

4. Discussion

The present study validated the EIA protocol of measuring two androgen hormones, DHEA and testosterone from hair samples by examining the associations between 2-menstrual cycle accumulated hair hormone concentrations and averaged weekly saliva hormone levels across corresponding time period. Our results generally supported validation of the hair androgen immunoassay protocols by returning good expected concentrations following linearity of dilution and recovery of spiked samples, and by demonstrating that androgen levels are within the respective immunoassay's range of sensitivity with modest quantities of hair (15 mg). Moreover, external validation showed high correspondence between hair androgen concentrations and averaged saliva hormone levels across two menstrual cycles (for DHEA, r = .65, p < .05; for testosterone, r = .67, p < .05). Perhaps most importantly for hair androgens, hormone concentrations were stable, from one segment to the next and from one sample to the next (primarily for testosterone), suggesting that hair androgens are indeed returning a cumulative steroid index. Results are encouraging that the hair androgen values can reflect the retrospective hormonal concentrations and can be used as a stable long-term hormonal index over a particular time period.

The premise of the current study was that, much like the demonstrated emerging utility of hair cortisol, a cumulative androgen index would be useful as a measure of basal androgen levels. Due to their nature as sex hormones, we were particularly interested in whether a cumulative index eliminated noise due to menstrual cyclicity. Across two cycles, the ICC in salivary androgen levels was high, although there

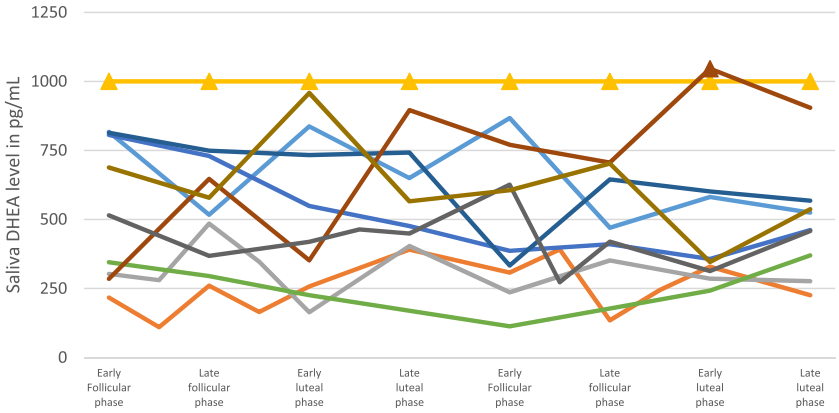

Fig. 3. Monthly Variability of Saliva DHEA. (Samples marked with a triangle are above the assay's level of sensitivity (1000 pg/mL) including one participant with a single sample above 1000 pg/mL and one participant with DHEA consistently above 1000 pg/mL.).

was substantial variability in salivary hormone levels across samples and a clear pattern of luteal and follicular concentrations did not emerge. For DHEA, intra-individual variation across a menstrual cycle accounted for 59% of variability implicating that multiple weekly or daily saliva samples may be necessary to reflect women's basal DHEA. Intra-individual variation accounted for only 9% of the total variability in testosterone concentration across a menstrual cycle. This fits with prior research that women's saliva testosterone level is stable across the menstrual cycle (Liening et al., 2010; Dabbs and de La Rue, 1991). Moreover, results shows androgen level in each single saliva sample was not corresponded with hair androgen level, confirming that single saliva sample cannot provide good measure of basal hormone levels. Results suggest that an average of weekly salivary androgen concentrations returns basal levels, but with reduced feasibility as compared to a one-time easily operable hair collection. The repeated saliva collection and hormone assessment is a huge financial challenge with large participant burden and increased likelihood for noncompliance and failure to return samples across durations of 1–2 months. Attempts to minimize menstrual cycle influences by collecting a salivary sample according to day-count may have little utility as there was not a clear week-to-week pattern in androgen concentrations (see Figs. 3 and 4) and, even within regularly cycling young adult women, there was substantial variation in cycle length both within and across women, rendering it difficult to determine whether a sample was indeed collected in the luteal or follicular phase. Therefore, researchers interested in chronic or basal androgen levels may consider hair androgens a reliable and affordable option.

We revealed that hair samples collected two times with one-month intervals and segmented into 1 cm prior to assay showed impressive correspondence between the two segments that reflected hormone accumulation of a same month for testosterone for all three pairs of hair segments, but for DHEA, it showed one significantly associated pair. Significant correlations for testosterone across 2 samples support the idea that 1-cm of hair corresponds to approximately 1 month of hair growth (LeBeau et al., 2011). Only one significant corresponded pair of segments was found for hair DHEA. Rather than suggest this violates the 1-cm guideline, we suggest this may be a methodological limitation for DHEA. DHEA concentrations can be very high in hair, so much so that reconstituted hair samples require a x4 dilution prior to assay in order to return DHEA results within EIA kit sensitivity. Moreover, the high concentrations may be due to the fact that the hair follicle itself can operate as an endocrine organ (Randall et al., 2000) which may be highly active in a subset of participants. In addition to classical steroidogenic organs (e.g., adrenal glands), skin also contributes to androgen synthesis and is able to produce androgens *de novo*. Specifically in hair follicles, steroid sulfatase in the sebaceous glands and dermal papilla cells catalyze the process of hydrolyzation from DHEA-S to DHEA (Ceruti et al., 2018; Nikolakis et al., 2016). Although speculative, this could help explain the reason why not all the pairs of hair segments across two samples significantly corresponded for DHEA.

We also determined stability in androgen concentrations with a single hair sample as another external validation step and to provide guidelines and recommendations for hair segmentation. Hair steroid concentrations were significantly associated in nearly all of the pairs of two successive segments. The magnitude of association decreased when two segments were further apart and, by extension, reflected androgen

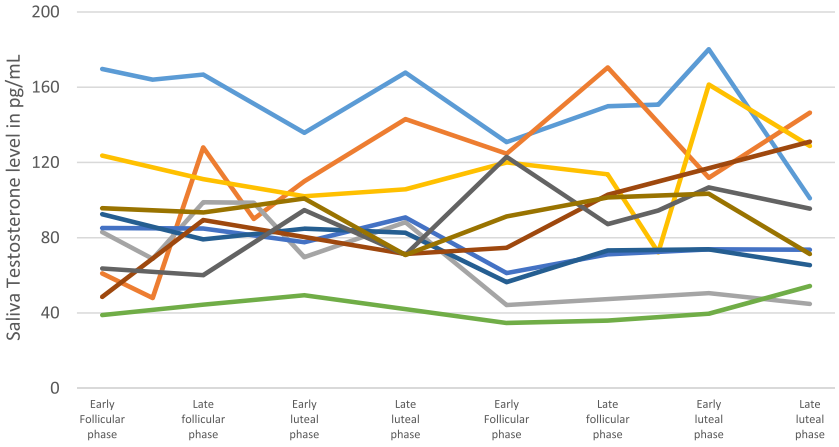

Fig. 4. Monthly Variability of Saliva Testosterone.

concentrations further apart in time. One possible explanation is that samples farther apart were exposed to more external influential factors such as sunlight radiation that might provoke steroid changes in hair (Grass et al., 2016). Despite this, our findings suggest that hair androgens are stable within 2–3 months and continue to return valid results several centimeters out on the hair strand. This may be good news for studies that wish to minimize participant burden as both testosterone and DHEA can be collected from a sample as small as 15 mg which amounts to very little hair if segmented to 2- or 3 cm in length. Moreover, we have developed the "small bits" method (Wang et al., 2016) to minimize the aesthetic burden of providing a hair sample compared with 20–50 mg of hair need in previous literature (Gao et al., 2013; Deshmukh et al., 2012). Segmenting hair sample over 3 cm may not be generally recommended for studies that aim to examine short-term or monthly stable hormone levels. Therefore, it is critical for researchers to collect enough hair strands to return stable concentrations and for clear instructions to be provided to ensure sample fidelity. We are currently recommending that laboratories consider investing in small portable scales that weigh samples down to 0.1 mg levels to measure hair samples accurately.

There are four main limitations in this study despite the discovery of significant correspondence between hair and saliva hormone levels. First, only weekly saliva samples (4–6 samples in one menstrual cycle) were obtained so that the averaged saliva hormone level was based on limited number of samples. Increased number of saliva samples has been approved to return better results from previous study in which daily samples over a month were gained (Short et al., 2016). Second, as is common for validation studies, the participant number is small which might limit precision of the results. Moreover, when considering menstrual cyclicity as a factor for hormone variation, only adult female participants were included which may limit generalizability to populations with higher (e.g., males) or lower androgens (e.g., children). Third, given our goal to validate an immunoassay, we examined correspondence across bio-specimens in the same commercially available salivary immunoassays. Future studies may also consider external validation across techniques (e.g., LC/MS-MS) or with other measures that are correspond with androgens (e.g., gender, neural structure, age, puberty). Fourth, to minimize hair type influences, all participants had similar hair types (2A-2C); it will be important for future studies to examine the influence of hair types across the full range of hair types (1A-4B).

In conclusion, the present study validated hair androgen immunoassay protocol by examining the correspondence between hair hormone concentration and averaged saliva hormone level over a corresponding period. The findings suggested that the novel hair hormone assay methodology can provide estimates of long-term average hormone levels reliably and economically. Especially, this method can be used to reduce the day-to-day variability of testosterone and DHEA levels and contribute to women's health and behavior study.

Conflict of interest

The authors declare they have no conflict of interest of this study.

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