The validation of estradiol extraction and analysis from hair

Authors:

Wen Wang, Lotte van Dammen, Shannin N. Moody, Jeff Kiesner, Jenae M. Neiderhiser,
Andrew Dismukes, Junda Zhu, Christos Constantinidis, Elizabeth A Shirtcliff,

Corresponding Author:

Elizabeth A Shirtcliff
Professor
Human Development Family Studies
Virtual Reality Applications Center
Iowa State University

Abstract

Background. Saliva is a popular biospecimen for the measurement of hormones, yet fluctuations in hormone levels limit the extent to which saliva can address focusing on basal or long-term levels. Hair steroid assays return basal hormonal levels by collapsing across short-term hormonal variability, including menstrual cyclicity. Here we sought to validate a hair bioassay methodology that can capture stable estradiol levels from both human and monkey hair samples.

Methods. Three projects were involved to examine hair-saliva correspondence and estradiol stability in hair. *Project 1.* Saliva samples were collected once per week for two cycles in 11 emerging adult women. Hair samples were collected at the end of each menstrual cycle and were segmented by 1 cm for the first 4 cm to reflect the past four serial months' hormone levels. *Project 2.* Hair samples collected from 23 adolescent participants ($M_{\text{age}} = 14.1$, 56.5% female) were cut to three 1.5 cm segments from the scalp end. *Project 3.* Two hair samples were collected from two adjacent skin areas on each monkey ($N = 8$, 75% males). Whole hair samples were sheared and used for assay without segmentation. Hair biospecimens were processed using a double-extraction protocol validated in this study, then assayed using commercially-available enzyme-immuno-assays for estradiol.

Results. *Project 1.* Hair estradiol concentrations were significantly associated with averaged saliva estradiol levels ($r = 0.77$, $p < .05$). Estradiol levels in two consecutive segments were significantly associated (1st vs. 2nd: $r = .63$, $p < .01$; 2nd vs. 3rd: $r = .49$, $p < .05$; 3rd vs. 4th: $r = .53$, $p < .05$). *Project 2.* Estradiol concentrations were significantly correlated between the first two successive hair segments from the scalp end ($r = .69$, $p < .01$). *Project 3.* Estradiol levels in the two hair samples from each monkey were significantly correlated ($r = .66$, $p < .05$).

Discussion. Results suggest hair captures valid and reliable average estradiol concentrations using a double-extraction protocol that is applicable for both human and monkey hair. The measurement of hair provides valuable information on individual differences in average estradiol levels across months. Results also indicate that it is feasible and reliable to collect the first 2-3 centimeters of studies in which basal estradiol levels in the past 2 to 3 months are of interest as a stable hormonal index for different species.

Keywords: Immuno-assay; Hair; Estradiol; Assay validation; Menstrual cycle; Across species.

1. Introduction

Saliva has gained popularity in empirical research in the last decades because sampling is noninvasive and easy-to-conduct (Wren et al., 2015; Wilson, 1993). Salivary hormones have provided opportunities for researchers to explore naturalistic human physiological and psychosocial development (Mernone et al., 2019; Ives et al., 2011; Edelstein et al., 2012; Shirtcliff et al., 2009) by probing momentary fluctuations in hormone levels. These fluctuations are beneficial when acute or short-term body functions are of interest, or hormone levels at particular time point. However, this method may confine the feasibility and utility of saliva as a biospecimen when research questions focus on chronic levels or long-term developmental changes. To fill the need for a reliable method to measure long-term basal hormone levels, researchers have begun to explore the possibility of analyzing noninvasive steroid hormones from hair samples (Wosu et al., 2013; Wang et al., 2019).

As a product of hypothalamic-pituitary-gonadal (HPG) axis and an important member of the estrogen class of hormones, estradiol levels fluctuate following a circadian rhythm and changes rapidly across the female menstrual cycle (Albert et al., 2015; Bao et al., 2003; Steinbeck et al., 2020), such that estradiol increases during the follicular phase, peaks right before ovulation, and again shows rising levels during the mid-luteal phase (Jones & Lopez, 2013). Estradiol may contribute in understanding the mechanisms that play a role in development and health in broad research areas (e.g. reproductive medicine, menopause, psychobiological research, puberty development, sexual development), but remains understudied as a biomarker because it is difficult to obtain stable basal levels.

When long-term estradiol levels are of interest, noninvasive biospecimens such as saliva are problematic because they are heavily influenced by momentary or day-to-day fluctuations, and thus require many data points to provide a reliable and stable estimate of average levels (Schultheiss & Stanton, 2009; Short et al., 2016). Although repeated sampling can be done, it is expensive and requires high abidance to sample collection instructions which may be burdensome for at-home collection. It also necessitates the difficulty and financial burden to store and ship samples frozen and transport without new freeze-thaw cycles (Lewis, 2006; Toone et al., 2013; Granger et al., 2004).

Assaying steroid hormones from hair samples, on the other hand, reduces storage and shipping difficulties because samples can be shipped and stored at room temperature, and also may provide a reliable and stable measure of estradiol for extended time frames.

Previous work has shown that cortisol, dehydroepiandrosterone (DHEA) and testosterone basal levels can be measured reliably in human and animal hair/fur samples (Davenport, et al., 2006; Wosu, et al., 2013; Stalder et al., 2012; Sauvé et al., 2007; Wang et al., 2016). Hair as a biospecimen provides an opportunity to explore basal hormone levels within prior months since hair grows approximately 1 cm per month (Dettenborn et al., 2012; Sharpley et al., 2012; Russell et al., 2012), with the first centimeters of hair nearest the scalp end retrospectively reflecting levels of steroid hormones over the most recent months (LeBeau et al., 2011; Li et al., 2012). Quantitative analysis of cortisol and androgen in hair has been validated and can be used to reflect basal steroid levels (Wang et al., 2019; Pereg et al., 2013; Gao et al., 2013; Wheeler, 2006; Rambaud et al., 2005; Gleixner & Meyer, 1997). Because estradiol is also a steroid hormone which diffuses into biospecimens like saliva via passive diffusion, we expect that basal estradiol can also be detected in hair.

Previous work has shown that estradiol can be extracted from animal hair samples (Rambaud et al., 2005; Hooijerink et al., 2005), but the methods cannot be directly translated to human hair samples for multiple reasons. First, human hair samples are often smaller in terms of weight, compared to animal samples. Second, some purification procedures used for animal hair samples is not necessary for human hair samples. Finally, steroid concentrations in hair are potentially very different in animal hair, compared to human hair. Previously published protocols developed specifically for detecting levels of estradiol in human hair samples vary in the amount of hair used, but also in the type of extraction reagents used to extract estradiol from hair (e.g. hexane, ethyl ether), and in the extraction conditions and assay methods used (e.g. gas chromatography–mass spectrometry, radioimmunoassay) (Choi et al., 2000; Yang et al., 1998). A protocol that is applicable to both human and animal hair samples is currently lacking. With all of the above challenges, it is necessary to generate and validate a protocol to measure estradiol in hair across different species.

In this study, we sought to validate a protocol using an enzyme immunoassay (EIA) method to measure estradiol in hair that can be used for both human and monkey hair. First, assaying estradiol from a hair sample was expected to yield a reliable basal level, collapsing across diurnal and cyclic fluctuations. With this underlying expectation, we examined the feasibility of hair estradiol working as a basal hormone index to eliminate the estradiol level change over time, specifically across menstrual cycle. The validation procedure included examining correspondence across bio-specimens (hair and salivary samples collected in corresponding time period) and assessing stability of hair estradiol in different hair segments. Second, the present protocol was verified using monkey hair samples. The protocol validity was also tested by examining the correspondence of two samples collected at the same time but from different spots on monkeys in order to rule out site-specific effects of hair/fur collection.

2. Methods and results

2.1 Hair estradiol assay protocol

Hair (either segmented or a whole hair sample) was washed twice in high performance liquid chromatography (HPLC)-grade isopropanol with 3-minute repeated inversion using a rotator. The washed hair was dried under forced air for 4-5 hours to ensure complete isopropanol evaporation. For the protocol validation, different quantities of hair were used; 5 mg, 10 mg, 15 mg, and 25 mg of sample. For each quantity, estradiol results were within the assay range of sensitivity and there was a linear increase of concentration for heavier samples ($R^2=.967$). Quantities of 5 mg and 10 mg returned relatively low estradiol concentrations close to the edge assay's range; therefore, 15 mg of sample is the ideal weight for each assayed hair sample. Exactly 15 mg of washed and dried hair was weighed and 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 ball mill for 8 min at 30 Hz. Using ethyl acetate as extraction solution, extraction efficiency of single extraction and double extraction methods were compared. On average, using double extraction returned 51.7% higher estradiol concentration compared with that of single extraction. A unique double-extraction method was therefore applied in final protocol. In the

double-extraction procedure, for the first round of extraction, 1.5 mL of HPLC-grade ethyl acetate was added into the tube with ground hair powder, and the samples were incubated at room temperature with constant inversion using a rotator. After a 24-hour incubation, the tubes were centrifuged and 1 mL of supernatant was transferred to new, clean tubes and stored in a refrigerator at 4°C. For the second round of extraction, another 1 mL of new ethyl acetate was added into the original vial and incubated at room temperature with constant inversion for another overnight incubation period (15-18 hours). Then the original vials were centrifuged and a second 1 mL of supernatant was mixed into supernatant from the first round. The vial (with 2 mL of liquid) was placed under a nitrogen evaporator in a water bath (65°C) for 10-15 min and the extract was reconstituted with 250 µL of kit-provided estradiol assay diluent, vortexed for 1 minute, and assayed immediately. All studies described below used this final protocol using ethyl acetate as extracting reagent with a double extraction process.

2.2 External validation project 1: correspondence between estradiol in saliva and hair

2.2.1 Overview

This project sought to examine the feasibility that hair estradiol may work as a basal hormone index to eliminate estradiol variation over time. In this project, both hair and salivary samples were collected during corresponding time period. The correspondence between hair estradiol level and averaged estradiol concentration of multiple salivary samples was tested. In addition, this project aimed at exploring the stability of estradiol in hair by assaying estradiol from multiple hair segments and examining their correlations.

2.2.2 Methods

Participants and Procedures

Eleven female college students were recruited at the University of Padova in Italy (age 20-24, $M_{age}=21.55$). Female students were eligible if they had a regular menstrual cycle and were not using hormonal contraception. Nearly all of the participants had type II hair in terms of curliness

(Loussouarn et al., 2007). One participant's data was excluded for analysis because of insufficient hair.

The study collection procedures have been reported in a previously published paper (Wang, et al., 2019). In brief, following recruitment and instruction, female students completed an online questionnaire and self-collected, stored and delivered saliva samples to the laboratory. Starting on the first day of their menstrual cycle, participants self-collected weekly saliva samples across two menstrual cycles and delivered the samples to the laboratory by end of each menstrual cycle. Participants visited the lab at the end of each menstrual cycle and one hair sample was collected by a trained research assistant during each visit.

Measures

Demographic Information. Participants' answered questions about age, height, weight, past use of birth control, medical condition, use of medications, etc. For hair sample collection, participants answered questions regarding the use of shampoos, hair dyes, or other hair treatments (see details in Wang et al., 2019).

Saliva Sample Collection. Participants drank a small amount of water and then waited at least 5 minutes before collection. Saliva samples were self-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). The first saliva sample was collected at the first day of a menstrual cycle and the following samples were collected on the same day each week (at a 7-day interval) until the start of next menstrual cycle. This procedure was repeated for the subsequent menstrual cycle. For each collection, participants sealed the polypropylene cryovial, secured it into a small bag, froze the sample in their home freezer and filled out a daily diary questionnaire which included questions about menstrual status. After the final saliva sample was collected, participants self-couriered saliva samples to the local laboratory using freezer-brix to keep

samples frozen. Samples then were shipped overnight on dry ice to the Stress Physiology Investigative Team (SPIT) lab at Iowa State University and stored at -80°C.

Saliva Sample Assay. Samples were brought to room temperature and centrifuged at 3000 rpm for 15 min. The clear supernatant was assayed for estradiol using commercially available enzyme immunoassay kits (Salimetrics, State College, PA). A plate was considered viable if the standard curve $R^2 > 0.996$. All samples were assayed in duplicate and samples were re-assayed if duplicates varied $> 10\%$.

Hair Sample Collection and Segmentation. Hair samples were collected in the lab at the end of each menstrual cycle (two samples across this study) by trained research assistants. Approximately 450 strands were carefully cut as close to the scalp as possible from the posterior vertex area. Hair samples were wrapped, unbent, in a foil sheet and sealed in an envelope to avoid light exposure. Hair samples were stored in a dry and dark environment at room temperature until shipped for assay. Four 1 cm segments were then cut, starting from the scalp end, to roughly approximate the four most recent months of hair growth.

2.2.3 Results

Is there Correspondence between Estradiol Concentration in Hair and Saliva?

For the purpose of providing an external validation of the current hair estradiol protocol, we conducted comparison of estradiol concentrations across the two different bio-specimens: hair and saliva. We examined the correspondence between the estradiol values in the first segment of hair sample (1cm) and the average estradiol values in saliva samples that were collected weekly during the same time period of hair collection (one menstrual cycle, about one month). Pearson Correlation results showed that hair estradiol concentration in the first 1 cm hair segment in the first month was significantly correlated with averaged saliva estradiol level ($r = 0.77$, $p < .05$, see Figure 1). For the second month, hair estradiol concentration (in the 1 cm segment of month 2 sample) was not

correlated with averaged saliva estradiol level ($r=.08$, $p=.82$). The results indicate that a substantial saliva-hair correlation, but it is inconclusive that the correspondence is stable.

We also examined the correspondence between estradiol levels in each single saliva sample and estradiol concentration in hair samples. The estradiol concentration in the first saliva sample (in the first month), which was the sample collected on the first day of menstrual cycle, was correlated with hair estradiol concentration ($r = .687$, $p = .041$). No significant correspondence was found between estradiol concentration in other saliva samples and hair sample (Month 1: 2nd sample: $r = .635$, $p = .066$; 3rd sample: $r = .505$, $p = .202$; 4th sample: $r = .290$, $p = .486$; Month 2: 1st sample: $r = -.036$, $p = .920$; 2nd sample: $r = .154$, $p = .672$; 3rd sample: $r = .112$, $p = .758$; 4th sample: $r = .088$, $p = .809$). However, in month 1, estradiol concentration in the second saliva samples was marginally significant correlated with hair estradiol concentration. And the magnitude of association between single saliva sample and hair sample kept decreasing from the beginning of the menstrual cycle.

Figure 1. Association of Estradiol in Hair and Saliva Samples in Month 1

Is Estradiol Stable in Hair?

The mean hair estradiol concentration of all 1-cm hair segments was 45.96 pg/g (SD = 10.60) with a range of 29.08-79.70 pg/g (segment 1: M = 47.57 pg/g (SD = 12.49); segment 2: M = 47.13 pg/g (SD = 7.53); segment 3: M = 44.87 pg/g (SD = 12.37); segment 4: M = 44.43 pg/g (SD = 9.72)). The

stability of estradiol in hair over time was analyzed by examining correlations between estradiol concentrations in the four 1 cm hair segments. The results showed that estradiol levels in one segment were significantly associated with the levels in the next segment (see Table 2 for details). Estradiol levels in segments that were > 1 cm apart, were not significantly correlated.

Table 2. Correlation of estradiol levels between different hair segments (project 1)

Segments	1st	2nd	3rd	4th
1st	--			
2nd	.63**	--		
3rd	-.03	.49*	--	
4th	-.25	.21	.53*	--

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

2.2.4 Project 1 brief discussion

We found that levels of estradiol in hair correspond with averaged levels of estradiol in repeated saliva samples in the first month of data collection. Additionally, level of salivary estradiol collected on the first day of the first menstrual cycle corresponded with estradiol level in hair. Results were less consistent for the second month of data collection. These results indicate that hair is a promising biospecimen that can be used to measure estradiol levels. Moreover, hair samples could potentially render more reliable results compared to saliva samples, since there is less impact of menstrual variability and unreliable sample collection. Our results are also in line with the typical cyclical change in estradiol, such that estradiol levels are relatively stable at the beginning of the menstrual cycle, which was reflected by the correspondence between hair estradiol levels and the first two salivary estradiol levels of the cycle. After menses, intra-cycle variability of estradiol level starts increasing (Fehring et al., 2006) and it helps to explain the lower correspondence between other saliva samples and hair samples.

Hair estradiol concentrations were significantly correlated in two consecutive segments, suggesting that hair estradiol is stable within two contiguous months, but unstable across longer time frames.

Although the underlying mechanism is still unclear, the photodegradation process of estradiol might be one explanation (Zhang et al., 2007; Lin et al., 2005; Mazellier et al., 2008). Sunlight radiation is able to provoke chemical bonds breaking within organic molecules such as corticosteroids and steroids (Signori, 2004). This also echoes evidence in previous literature that when hair is exposed to more ultraviolet or natural sunlight, steroid hormones concentrations decrease (Wester et al., 2016; Grass et al., 2016).

2.3 External validation project 2: stability of estradiol in hair

2.3.1 Overview

This project aimed to provide further evidence of estradiol stability in hair samples. In this project, hair samples were cut into multiple segments in which hair estradiol was analyzed. The correlation of estradiol concentration between different hair segments was calculated.

2.3.2 Methods

Participants

There were 23 adolescent participants in this study (age 12-16, $M_{age}=14.1$, 56.5% female) who comprise a subgroup from a large, multi-method and longitudinal research project entitled Early Growth and Development Study (EGDS; Leve et al., 2019; Funding: Environmental influences on Child Health Outcomes Program, UH3 OD0233809). Samples and data were from families living throughout the United States who have been participating in the EGDS. All of the samples used in this project were collected by trained interviewers who traveled to the participant's home. Participants with all hair types were recruited and answered questions regarding the use of heat to style hair and time of sunlight exposure every week.

Hair Sample Collection and Segmentation

Hair was carefully cut from the posterior vertex area as close to the scalp as possible. Hair samples were wrapped in a foil sheet and sealed in an envelope in a dry and dark environment at room

temperature until shipped to the Iowa State SPIT lab. In this study, hair was cut by 1.5 cm from the scalp end and the first 4.5 cm were assayed (a total of 3 segments per sample). This segmentation length was determined by the research questions raised by the large EGDS research project.

2.3.3 Results

In this project, estradiol concentrations from three 1.5 cm-segments were analyzed. The mean hair estradiol concentration of all single hair segments was 44.11 pg/g (SD=12.70) with range of 23.22-77.77 pg/g. Similar with project 1, we examined the stability of estradiol in different hair segments. The results showed that estradiol concentrations were significantly correlated between the first two successive hair segments from scalp end (see Table 3 for details).

Table 3. Correlation of estradiol levels between different hair segments (project 2)

Segments	1st	2nd	3rd
1st	--		
2nd	.69**	--	
3rd	.21	.37	--

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

2.3.4 Project 2 brief discussion

The results provide further confirmation that estradiol is stable across the first two 1.5 cm segments, especially in the recent past. The lower non-significant correlations with the distal sections suggest that estradiol is invalid further from the scalp and the levels are not highly stable across several months. Therefore, when targeting estradiol, segmenting hair by 3 cm or less is recommended in order to return reliable basal estradiol concentration, although segmenting for up to 5 cm has been considered acceptable for other steroids (e.g. cortisol, Russell et al., 2012). The recommended 3 cm segmentation balances the needs for reliable samples and increases the feasibility of sample collection quantity, especially for studies with multiple steroids assaying. Segmenting hair sample further than 3 cm from the scalp is not recommended especially for studies in which very short-term basal estradiol

level (less than 3 months) are of interest. Moreover, hair estradiol was analyzed from all types of hair in this project. It provided complementary evidence to project 1 that this EIA protocol is applicable to curly hair styles and it is feasible to segment curly hair to appropriate length.

2.4 External validation project 3: multi-location reliability of estradiol in monkey hair

2.4.1 Overview

This project sought to examine the feasibility of applying the hair estradiol assay protocol to monkey hair. Also, the multi-location reliability of estradiol in monkey hair was tested. Two samples were collected from each monkey and the correlation of estradiol levels was analyzed.

2.4.2 Methods

Study Subjects

In this study, hair samples from eight young rhesus macaque monkeys (age 3.5-4.2 years, $M_{age}=3.9$, 75% males) were assayed for estradiol concentration. Samples were collected at Wake Forest School of Medicine.

Hair Sample Collection and Segmentation

Two hair samples were collected from two different 9 x 9 cm skin areas on each monkey, located adjacent to each other, between the shoulders extending rostrally to the level of the scapula, and mid-back. Whole hair samples (2.5 – 3.5 cm) were obtained by shearing with clippers and were used for assay without segmentation.

2.4.3 Results

The mean estradiol concentration in monkey hair was 29.11 pg/g (SD=6.27) with a range of 18.92-42.48 pg/g. For stability of estradiol across samples from the same subject, results showed that estradiol levels in the two hair samples were significantly correlated ($r=.66$, $p=.027$). The results

suggest that the reported protocol is applicable for monkey hair and estradiol is stable across samples collected from different body parts.

2.4.4 Project 3 brief discussion

The result is encouraging that the protocol is feasible in empirical field across different species to obtain stable basal estradiol level. The significant correspondence between two samples not only indicates the stability of the present hair estradiol protocol, but also suggests the multi-location reliability of estradiol in monkey's hair.

3. General discussion

The present study validated the EIA protocol of measuring estradiol from human and monkey hair. The validation showed high correspondence between basal hair estradiol concentration and averaged weekly saliva estradiol levels across corresponding time period ($r=.77$, $p<.05$). The external validation suggested that hair estradiol works as a cumulative steroid index and estradiol concentration was stable in hair, from one segment to the next and from one sample to the next. Another important finding is that this protocol is applicable not only for human hair, but also for monkey hair. Across the studies, results suggest that the hair estradiol value within the first 2-3 centimeters of scalp hair can be used to examine a long-term basal hormone level over a particular time period retrospectively.

The EIA method described in this protocol allows researchers to extract and assay estradiol from hair samples efficiently. In the estradiol extraction procedure, it provides different and independent method compared with assaying other steroids (Meyer et al., 2014; Wang et al., 2019). Several novel aspects of this estradiol protocol deserve to be highlighted. First, modest 15 mg of hair was determined because the estradiol concentration in the 15 mg of sample is sufficiently high relative to the lower edge of the sensitivity range of estradiol EIA kit. It is exciting that the protocol worked optimally with 15 mg of hair because 15 mg is a relatively small amount of hair (especially for a 3 cm segment) and it is easy for researchers to collect sufficient amount and usually acceptable for participants to consent. Second, a novel part of this protocol is that the double-extraction procedure is

applied to fully extract estradiol from hair samples. If using one round of extraction, estradiol cannot dissolve into ethyl acetate efficiently and completely, in turn influencing the returned hair estradiol concentration. Third, ethyl acetate was determined as the chemical reagent to extract estradiol because estradiol is more soluble in ethyl acetate compared with other commonly used extraction chemicals (e.g. methanol). Notably, ethyl acetate is inexpensive and readily available for research labs, so this extraction is very feasible. Fourth, 250 μL of assay diluent was recommended for reconstitution. Considering the low estradiol concentrations in noninvasive assays (Hampson et al., 2013; Shirtcliff et al., 2000), this amount was determined to maximally increase the concentrations in reconstituted samples; fortunately, this reconstitution is ideal for the sample amount needed by common commercially available estradiol immunoassay kits such as the Salimetrics kit which requires 200 μL of assay diluent for assaying in duplicate.

This study has several important implications. First, similar with other steroids, estradiol can be measured from hair samples, with some special processing procedure (e.g., double extraction) using appropriate extraction reagent (ethyl acetate). Second, concentration of estradiol was found to be low and not very stable in other bio-specimens (e.g. saliva, Toone et al., 2013; Shirtcliff et al., 2000). It is impressing that hair estradiol may return stable long-term hormone concentration across 2-3 months (reflected by the first 2-3 cm of scalp hair) fairly well, although not as well as other hair hormones (Short et al., 2016; Wang et al., 2019). This is very exciting because it opens the possibility to study basal levels of female-predominant hormones, avoiding cyclical fluctuations in these steroids, and without requiring multiple sampling across time (Gandara et al., 2007; Bao et al., 2003). Third, these results contribute to the fields of psychoendocrinological and developmental studies in which the difficulty of obtaining basal estradiol level limits the feasibility and probability to answer some important research questions (Rosner et al., 2013). It is also important to note that hair estradiol assay is lack of clinical reference, which requires blood estradiol level, in clinical research, diagnosis and therapies. Moreover, it is important that this hair estradiol protocol can also be applied across species (e.g. monkeys). Finally, these findings provide support for the validity of this protocol and highlights

the usefulness of hair estradiol in answering boarder research questions that may involve animal models.

Several limitations should be noted. First, the examination of linear dilution and spike-and-recovery (a common step in validation studies), was not conducted in this study due to the limited amount of reconstituted samples. The low estradiol concentration in hair requires to use as less as assay diluent to reconstitute estradiol extracts to maintain a relative high concentration in reconstituted samples (in this study, 200 μ L of sample is needed for assay and 250 μ L of assay diluent is used for reconstitution). Second, female estradiol level is highly impacted by menstrual cycle, therefore, in the validation procedure (first external validation project), only female data was collected and we did not explore the correspondence between hair estradiol value and weekly saliva estradiol levels for males. Future studies may also consider expanding the validation procedure to human males who have much lower estrogen levels. Third, as is common in protocol validation studies, the participant number is small and it might influence the precision of results. Fourth, given the goal of validating hair estradiol protocol using immunoassay, we examined correspondence across bio-specimens and applied it across different species. However, the results were not validated across different techniques (e.g., immunoassay vs. LC/MS). Considering the mass spectrometry technique is another popular methodology in the area of hair biomarker assay, future studies may conduct cross-validation between the two main techniques.

In conclusion, the hair estradiol immunoassay protocol in this present study was validated by examining the correspondence between hair estradiol concentration and averaged saliva estradiol level over a corresponding period. The protocol can be used for both human and monkey hair samples to obtain reliable estimates of estradiol levels across long periods of time with a single biosample. It is also encouraging that this methodology may largely reduce financial burden of related studies by requiring one single hair sample from each participant.

Reference

- Albert, K., Pruessner, J., & Newhouse, P. (2015). Estradiol levels modulate brain activity and negative responses to psychosocial stress across the menstrual cycle. *Psychoneuroendocrinology*, 59, 14-24.
- Bao, A. M., Liu, R. Y., van Someren, E. J., Hofman, M. A., Cao, Y. X., & Zhou, J. N. (2003). Diurnal rhythm of free estradiol during the menstrual cycle. *European Journal of Endocrinology*, 148(2), 227-232.
- Choi, M. H., Kim, K. R., & Chung, B. C. (2000). Determination of estrone and 17 β -estradiol in human hair by gas chromatography–mass spectrometry. *Analyst*, 125(4), 711-714.
- Davenport, M. D., Tiefenbacher, S., Lutz, C. K., Novak, M. A., & Meyer, J. S. (2006). Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *General and comparative endocrinology*, 147(3), 255-261.
- Dettenborn, L., Muhtz, C., Skoluda, N., Stalder, T., Steudte, S., Hinkelmann, K., ... & Otte, C. (2012). Introducing a novel method to assess cumulative steroid concentrations: increased hair cortisol concentrations over 6 months in medicated patients with depression. *Stress*, 15(3), 348-353.
- Edelstein, R. S., Kean, E. L., & Chopik, W. J. (2012). Women with an avoidant attachment style show attenuated estradiol responses to emotionally intimate stimuli. *Hormones and Behavior*, 61(2), 167-175.
- Fehring, R. J., Schneider, M., & Raviele, K. (2006). Variability in the phases of the menstrual cycle. *Journal of Obstetric, Gynecologic & Neonatal Nursing*, 35(3), 376-384.
- Gandara, B. K., Leresche, L., & Mancl, L. (2007). Patterns of salivary estradiol and progesterone across the menstrual cycle. *Annals of the New York Academy of Sciences*, 1098, 446.
- Gao, W., Stalder, T., Foley, P., Rauh, M., Deng, H., & Kirschbaum, C. (2013). Quantitative analysis of steroid hormones in human hair using a column-switching LC–APCI–MS/MS assay. *Journal of Chromatography B*, 928, 1-8.
- Gleixner, A., & Meyer, H. H. (1997). Detection of estradiol and testosterone in hair of cattle by HPL/EIA. *Fresenius' journal of analytical chemistry*, 357(8), 1198-1201.
- Granger, D. A., Shirtcliff, E. A., Booth, A., Kivlighan, K. T., & Schwartz, E. B. (2004). The “trouble” with salivary testosterone. *Psychoneuroendocrinology*, 29(10), 1229-1240.
- Granger, D. A., Shirtcliff, E. A., Zahn-Waxler, C., Usher, B., Klimes-Dougan, B., & Hastings, P. (2003). Salivary testosterone diurnal variation and psychopathology in adolescent males and females: individual differences and developmental effects. *Development and Psychopathology*, 15(2), 431-449.
- Grass, J., Miller, R., Carlitz, E. H., Patrovsky, F., Gao, W., Kirschbaum, C., & Stalder, T. (2016). In vitro influence of light radiation on hair steroid concentrations. *Psychoneuroendocrinology*, 73, 109-116.
- Hampson, E., & Morley, E. E. (2013). Estradiol concentrations and working memory performance in women of reproductive age. *Psychoneuroendocrinology*, 38(12), 2897-2904.
- Hooijerink, H., Lommen, A., Mulder, P. P. J., Van Rhijn, J. A., & Nielen, M. W. F. (2005). Liquid chromatography–electrospray ionisation-mass spectrometry based method for the determination of estradiol benzoate in hair of cattle. *Analytica chimica acta*, 529(1-2), 167-172.
- Ives, S. J., Blegen, M., Coughlin, M. A., Redmond, J., Matthews, T., & Paolone, V. (2011). Salivary estradiol, interleukin-6 production, and the relationship to substrate metabolism during exercise in females. *European journal of applied physiology*, 111(8), 1649-1658.
- Jones, R. E., & Lopez, K. H. (2013). *Human reproductive biology*. Academic Press.
- LeBeau, M. A., Montgomery, M. A., & Brewer, J. D. (2011). The role of variations in growth rate and sample collection on interpreting results of segmental analyses of hair. *Forensic science international*, 210(1-3), 110-116.
- Leve, L. D., Neiderhiser, J. M., Ganiban, J. M., Natsuaki, M. N., Shaw, D. S., & Reiss, D. (2019). The early growth and development study: A dual-family adoption study from birth through adolescence. *Twin Research and Human Genetics*, 22(6), 716-727.
- Lewis, J. G. (2006). Steroid analysis in saliva: an overview. *Clinical Biochemist Reviews*, 27(3), 139.

- Li, J., Xie, Q., Gao, W., Xu, Y., Wang, S., Deng, H., & Lu, Z. (2012). Time course of cortisol loss in hair segments under immersion in hot water. *Clinica chimica acta*, 413(3-4), 434-440.
- Lin, A. Y. C., & Reinhard, M. (2005). Photodegradation of common environmental pharmaceuticals and estrogens in river water. *Environmental Toxicology and Chemistry: An International Journal*, 24(6), 1303-1309.
- Loussouarn, G., Garcel, A. L., Lozano, I., Collaudin, C., Porter, C., Panhard, S., Saint-Léger, D., & de La Mettrie, R. (2007). Worldwide diversity of hair curliness: a new method of assessment. *International journal of dermatology*, 46, 2-6.
- Mazellier, P., Méité, L., & De Laat, J. (2008). Photodegradation of the steroid hormones 17 β -estradiol (E2) and 17 α -ethinylestradiol (EE2) in dilute aqueous solution. *Chemosphere*, 73(8), 1216-1223.
- Mernone, L., Fiacco, S., & Ehlert, U. (2019). Psychobiological Factors of Sexual Functioning in Aging Women—Findings From the Women 40+ Healthy Aging Study. *Frontiers in psychology*, 10, 546.
- Meyer, J., Novak, M., Hamel, A., & Rosenberg, K. (2014). Extraction and analysis of cortisol from human and monkey hair. *Journal of visualized experiments: JoVE*, (83).
- Pereg, D., Chan, J., Russell, E., Berlin, T., Mosseri, M., Seabrook, J. A., Koren, G., & Van Uum, S. (2013). Cortisol and testosterone in hair as biological markers of systolic heart failure. *Psychoneuroendocrinology*, 38(12), 2875-2882.
- Rambaud, L., Bichon, E., Cesbron, N., André, F., & Le Bizec, B. (2005). Study of 17 β -estradiol-3-benzoate, 17 α -methyltestosterone and medroxyprogesterone acetate fixation in bovine hair. *Analytica chimica acta*, 532(2), 165-176.
- Rosner, W., Hankinson, S. E., Sluss, P. M., Vesper, H. W., & Wierman, M. E. (2013). Challenges to the measurement of estradiol: an endocrine society position statement. *The Journal of Clinical Endocrinology & Metabolism*, 98(4), 1376-1387.
- Russell, E., Koren, G., Rieder, M., & Van Uum, S. (2012). Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. *Psychoneuroendocrinology*, 37(5), 589-601.
- Sauvé, B., Koren, G., Walsh, G., Tokmakejian, S., & Van Uum, S. H. (2007). Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clinical & Investigative Medicine*, 30(5), 183-191.
- Schultheiss, O. C., & Stanton, S. J. (2009). Assessment of salivary hormones. *Methods in social neuroscience*, 17.
- Sharpley, C. F., McFarlane, J. R., & Slominski, A. (2012). Stress-linked cortisol concentrations in hair: what we know and what we need to know. *Reviews in the Neurosciences*, 23(1), 111-121.
- Shirtcliff, E. A., Dahl, R. E., & Pollak, S. D. (2009). Pubertal development: correspondence between hormonal and physical development. *Child development*, 80(2), 327-337.
- Shirtcliff, E. A., Granger, D. A., Schwartz, E., & Curran, M. J. (2001). Use of salivary biomarkers in biobehavioral research: cotton-based sample collection methods can interfere with salivary immunoassay results. *Psychoneuroendocrinology*, 26(2), 165-173.
- Shirtcliff, E. A., Granger, D. A., Schwartz, E. B., Curran, M. J., Booth, A., & Overman, W. H. (2000). Assessing estradiol in biobehavioral studies using saliva and blood spots: simple radioimmunoassay protocols, reliability, and comparative validity. *Hormones and behavior*, 38(2), 137-147.
- Signori, V. (2004). Review of the current understanding of the effect of ultraviolet and visible radiation on hair structure and options for photoprotection. *International Journal of Cosmetic Science*, 26(4), 219-219.
- Short, S. J., Stalder, T., Marceau, K., Entringer, S., Moog, N. K., Shirtcliff, E. A., Wadhwa, P. D., & Buss, C. (2016). Correspondence between hair cortisol concentrations and 30-day integrated daily salivary and weekly urinary cortisol measures. *Psychoneuroendocrinology*, 71, 12-18.
- Stalder, T., Steudte, S., Miller, R., Skoluda, N., Dettenborn, L., & Kirschbaum, C. (2012). Intraindividual stability of hair cortisol concentrations. *Psychoneuroendocrinology*, 37(5), 602-610.

- Steinbeck, K. S., Garden, F. L., Cheng, H. L., Luscombe, G. M., & Handelsman, D. J. (2020). Bumpy and smoother pathways of puberty hormone change: A novel way to define gonadal hormone trajectories in adolescents. *Journal of the Endocrine Society*, 4(2), bvz014.
- Toone, R. J., Peacock, O. J., Smith, A. A., Thompson, D., Drawer, S., Cook, C., & Stokes, K. A. (2013). Measurement of steroid hormones in saliva: Effects of sample storage condition. *Scandinavian journal of clinical and laboratory investigation*, 73(8), 615-621.
- Wang, W., Moody, S. N., & Shirtcliff, E. A. (2016). Noninvasive hair assay for sex hormones: Preliminary protocol validation. *Psychoneuroendocrinology*, 71, 45.
- Wang, W., Moody, S. N., Kiesner, J., Appiani, A. T., Robertson, O. C., & Shirtcliff, E. A. (2019). Assay validation of hair androgens across the menstrual cycle. *Psychoneuroendocrinology*, 101, 175-181.
- Wester, V. L., van der Wulp, N. R., Koper, J. W., de Rijke, Y. B., & van Rossum, E. F. (2016). Hair cortisol and cortisone are decreased by natural sunlight. *Psychoneuroendocrinology*, 72, 94-96.
- Wheeler, M. J. (2006). Measurement of androgens. In *Hormone Assays in Biological Fluids* (pp. 197-211). Humana Press.
- Wilson, J. T. (1993). Clinical correlates of drugs in saliva. *Annals of the New York Academy of Sciences*, 694(1), 48-61.
- Wosu, A. C., Valdimarsdóttir, U., Shields, A. E., Williams, D. R., & Williams, M. A. (2013). Correlates of cortisol in human hair: implications for epidemiologic studies on health effects of chronic stress. *Annals of epidemiology*, 23(12), 797-811.
- Wren, M. E., Shirtcliff, E. A., & Drury, S. S. (2015). Not all biofluids are created equal: chewing over salivary diagnostics and the epigenome. *Clin Ther*, 37(3), 529-539. doi: 10.1016/j.clinthera.2015.02.022
- Yang, H. Z., Lan, J., Meng, Y. J., Wan, X. J., & Han, D. W. (1998). A preliminary study of steroid reproductive hormones in human hair. *The Journal of steroid biochemistry and molecular biology*, 67(5), 447-450.
- Zhang, Y., Zhou, J.L., Ning, B. (2007). Photodegradation of estrone and estradiol in water. *Water Res.* 41, 19–26.