Original Research Article

A Longitudinal Evaluation of the Relationship Between First Morning Urinary and Salivary Cortisol

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Abstract: Cortisol is one of the most frequently used stress biomarkers in humans. Urine and saliva are the matrices of choice to longitudinally monitor cortisol levels. Salivary and urinary cortisol are often discussed as though they provide similar information. However, the relationship between "free" cortisol levels in urine (nonconjugated) and saliva (non-protein-bound) has yet to be properly evaluated using naturalistic designs.

Objectives: To investigate the longitudinal relationship between salivary cortisol (SC) and first morning urinary cortisol (FMUC), and to compare the advantages and disadvantages of these matrices in assessing longitudinal changes in cortisol secretion using naturalistic designs.

Methods: Cortisol levels from 31 healthy, Kakchiquel Mayan women in Guatemala were compared in one first morning urine (FMU) and four saliva specimens collected daily across three alternate days. Linear mixed-effect regression models including fixed and random effects were used to analyze the repeated-measures data.

Results: FMUC levels (16.04–242.18 ng/ml) were higher than SC levels (0.21–5.16 ng/ml). A small but statistically significant relationship was found between FMUC and SC (each 1 ng/ml increase in FMUC predicted a 0.1% increase in SC; $P < 0.05$).

Conclusions: Nonconjugated FMUC levels are related to non-protein-bound SC levels collected throughout the day. FMU presents several advantages over saliva for the longitudinal assessment of cortisol in naturalistic studies. Cortisol levels are about 53-fold higher in FMU than in saliva, which makes between- and within-individual variation easier to detect, and FMUC levels are less likely to be affected by confounders than diurnal SC levels. Am. J. Hum. Biol. 25:351–358, 2013. © 2013 Wiley Periodicals, Inc.

Physiologic stress, resulting from any combination of energetic, immune or psychosocial challenges, has been linked to a variety of negative developmental and health outcomes, widely ranging from metabolic syndrome and neuroendrocrine disorders to inflammatory disease and reproductive suppression (Brudasca and Cucuianu, 2011; De Vriendt et al., 2009; Miller et al., 2002; Nepomnaschy et al., 2004, 2006; Sebert et al., 2011). Consequently, there is keen interest in the development of methods to accurately assess physiologic stress levels. One of the most frequently used biomarkers of physiologic stress is cortisol, a glucocorticoid secreted by the adrenal cortex into the blood stream in response to the activation of the hypothalamic-pituitary-adrenal axis (HPAA) or "stress" axis (Altemus et al., 2001; Hruschka et al., 2005; Kanaley and Hartman, 2002; Miller and O'Callaghan, 2002; Padgett and Glaser, 2003; Pollard, 1995, 1997).

The majority of cortisol in circulation is bound to carrier proteins, but under normal, nonstressed conditions, approximately 5–10% of all cortisol circulating in blood is "free" (i.e. not bound to proteins) (Beisel et al., 1964a,b; Daughaday et al., 1956; Gatti et al., 2009). The non-protein-bound cortisol is the active form of the hormone. It is also the form that can be excreted into urine and saliva as carrier protein-bound cortisol is generally too large to pass from circulation through the epithelial cells of the salivary glands and the glomerulus of the nephron in the kidneys (Beisel et al., 1964a,b; Chu and Ekins, 1988; Daughaday and Bremer, 1955; Daughaday et al., 1956; Hellhammer et al., 2009; Levine et al., 2007; Lindholm, 1973; Riad-Fahmy et al., 1982; Schedl et al., 1959). Therefore, both urinary and salivary cortisol (SC) reflect a proportion of the active, "free" non-protein-bound cortisol in circulation. While SC is always "free" in that it is not bound to carrier proteins, urinary cortisol can either be "free" (nonconjugated) cortisol or conjugated to sulfonide or glucuronide groups. Throughout this article, to avoid confusion, we refer to **free cortisol in circulation and saliva as "non-protein-bound"** cortisol and to **free cortisol in urine as nonconjugated cortisol**.

Although cortisol levels can be assessed in a variety of matrices, urine and saliva are favored over other matrices such as cerebrospinal fluid or serum, especially in studies based on naturalistic, longitudinal designs involving the collection of multiple biospecimens per participant (Gatti and De Palo, 2011; Nepomnaschy et al., 2011, 2012; Papacosta and Nassis, 2011; Plenis and Baczek, 2010; Wedekind et al., 2008). Urine and saliva present several advantages including less-invasive, simpler and less-onerous collection protocols than other matrices. In addition, urine and saliva lend themselves to self-collection (by participants), thereby reducing the anticipation

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TABLE 1. Comparisons of matrices for the quantification of cortisol

Matrix	Collection	Values affected by	Compatible with	References
A. Blood	Highly invasive Technician required	Food consumption and physical activity Circadian cortisol secretion profiles Anxiety provoked by the imminence of the prick	Experimental, short-term, pre-post challenge sampling	Lilliecreutz et al., 2011; Nager et al., 2010
B. Saliva	Minimally invasive Technician or self-collected	Food consumption and physical activity Circadian cortisol secretion profiles	B_i: Experimental, short-term, pre-post challenge sampling	Aschoff, 1981; Broderick et al., 2004; Clow et al., 2004; Kudielka et al., 2003; Plenis and Baczek, 2010; Touitou and Haus, 2000; Wust et al., 2000b
		Failure to follow collection protocol by participant (loose adhesion to collection schedule, sub-reporting of food consumption or physical activity)	B_{ii}: Monitoring cortisol awakening response (CAR)	
C. Urine	Minimally invasive Technician or self-collected	Water consumption Midnight voids	Longitudinal, follow-up, intra- and inter-individual comparisons	Nepomnaschy et al., 2011, 2012; Wedekind et al., 2008

anxiety generated by finger pricks or more invasive procedures, which may affect circulating levels of cortisol (Lilliecreutz et al., 2011; Nager et al., 2010; Sarkar et al., 2006) (Table 1).

Interestingly, cortisol results from urinary and salivary specimens are frequently discussed as though these two matrices provide the same information regarding HPAA function (Jerjes et al., 2006a; Neary et al., 2002; Putignano et al., 2003). The assumption is that levels of free, nonconjugated cortisol evaluated in urine and free, non-protein-bound cortisol in saliva are both related to the levels of free, non-protein-bound cortisol in circulation (Beisel et al., 1964a,b; Daughaday et al., 1956; Lindholm, 1973; Schedl et al., 1959). However, the relationship between free, nonconjugated first-morning urinary cortisol (FMUC) and free, non-protein-bound SC levels in natural (as opposed to experimental) conditions has yet to be properly evaluated.

Existing studies comparing nonconjugated urinary cortisol versus non-protein-bound SC have used cross-sectional designs and are often based on clinical populations. These studies offer disparate results; while some find significant associations between cortisol levels in both matrices (Neary et al., 2002; Putignano et al., 2003), others do not (Hurwitz Eller et al., 2001; Jerjes et al., 2006b; Kidambi et al., 2007; Putignano et al., 2001). These inconsistencies highlight the need to learn more about the relationship between urinary and SC levels and properly evaluate the information they provide about the functioning of the HPAA.

Here we present a longitudinal analysis of the relationship between nonconjugated cortisol levels quantified in first morning urine (FMU) specimens and non-protein-bound cortisol levels quantified in saliva specimens collected throughout the day, on each of three alternate days, from a group of healthy Kakchiquel Mayan women. We complement this analysis with a comparison of the advantages and disadvantages associated with the use of urine versus saliva specimens to assess cortisol levels in longitudinal studies using naturalistic designs.

METHODS

Study population

The collection of data and specimens analyzed in this article took place between July 30 and August 4, 2001, in a rural Kaqchikel Mayan community located in the southwest highlands of Guatemala. At the time this community was composed of 1,159 inhabitants. All women matching the following inclusion profile were invited to participate: parous, living with a co-resident male partner, not pregnant, not using any form of chemical contraceptive method, and having had their last parturition at least 6 months prior to the onset of the study (Nepomnaschy et al., 2004, 2006). Fifty-one women volunteered to participate in this specific study. Of those 51 women, a subset of 32 women provided one urine and four saliva samples over a week. All women in this subset were experiencing regular ovarian cycles and breastfeeding their youngest child. Potential daily stressors in this population include negative energetic balance (due to their often poor diet and high levels of physical activity), immune challenges (including common urinary and upper respiratory tract infections), and psychosocial stressors (such as interpersonal conflicts with members of their social networks) (Nepomnaschy, 2005).

Sampling schedule, collection, storage, and transportation protocols

One FMU and four saliva specimens were collected every other day for a total of 3 days, completing a total of three urine and 12 saliva specimens per participant. This sampling design helped us account for within-individual, day-to-day variation in free cortisol levels (Nepomnaschy et al., 2011). Participants collected their first urinary voids upon waking in clean, dry, inert plastic containers provided by our research team the night prior to the collection day. Local female research assistants visited participants at their homes to collect bio-specimens four times a day: FMU and saliva in the early morning (T1) and only saliva in the afternoon (T2), evening (T3), and night (T4). To stimulate the production of saliva, participants chewed half a stick of Wrigley's spearmint gum and were asked to swallow the sugar. Next, participants spat into 5 ml inert tubes with pop lids. Specimens were transported in coolers filled with ice to our field laboratory. Specimens were then aliquoted into 2 ml cryo-vials. Sodium azide was added into the saliva samples to prevent bacterial growth (final concentration 0.025%). Within 4 h of being produced, all samples were stored at -10°C in the field. Within 6 months samples were transported on dry ice from the field to our laboratories and then archived at -80°C until analysis. This research was approved by the Research Ethics Boards of Simon Fraser

University (previous phases of this study were approved by Ethics committees at The University of Michigan and the USA's National Institutes of Environmental Health).

Quantification of free cortisol levels in urine and saliva

Hormonal analyses were conducted at the Maternal and Child Health Laboratory at Simon Fraser University. SC was quantified using a high-sensitivity enzyme immunoassay kit (Salimetrics, State College, PA). Intra- and inter-assay CVs were 4.0% and 4.4%, respectively, and the lower limit of detection of the SC assay was <0.003 μg/dl. First morning urinary cortisol (FMUC) was quantified using an immunoassay array (Quansys Biosciences, Logan, UT), validated in-house (Salvante et al., 2012). Intra- and inter-assay CVs were 2.7% and 3.6%, respectively, and the lower limit of detection for urinary free cortisol was 0.5 ng/ml. To control for dilution effects, urinary free cortisol concentrations were corrected by specific gravity (Miller et al., 2004; White et al., 2010). All samples were run in duplicate. All data points for which the coefficient of variation between duplicates was >12% were rerun. We detected a particular SC value from one participant that was approximately 10-fold higher than our samples' average. Given that this outlier was most likely due to a recording or measurement error, it was excluded from the statistical analysis to avoid biasing our results. We also detected a particular participant whose SC values fell outside three standard deviations from our samples' average. A diagnostic of the participant's condition leading to such high levels of cortisol in all of her saliva samples is not possible with the information we have at this time. To avoid the potential biases due to measurement error, the data from this participant was excluded from the analysis, resulting in our final sample size of 31 participants.

Statistical analysis

In an effort to normalize the distribution of the data and reduce the influence of outliers, free cortisol concentrations were log-transformed. Analysis for repeated-measures data was conducted to characterize diurnal changes in SC and to detect possible associations between SC and FMUC by using linear mixed-effect regression models that included both fixed and random effects. SC was considered to be the response of interest, and the explanatory variables that we considered included time of the day (discrete taking values T1, T2, T3, and T4) and FMUC. The intercept was assumed to be random and individual-specific and the coefficients of time had both a fixed and an individual-specific random component. The effect of FMUC was treated as fixed. The specification of the individual specific random effects in the model allows individual deviations from the mean diurnal trend of SC, and also accounts for the correlation among the repeated SC measures within an individual. The source of variability in SC is thus partitioned as within- and between-individual variance based on the mixed effect model. The between-individual variance varies over time due to the random trend, and the within-individual variance is assumed to be heterogeneous across individuals. This model provides a more sophisticated characterization of SC measures across the day and also more accurate estimates of the association between FMUC and SC. The fit of competing models was assessed using likelihood ratio tests.

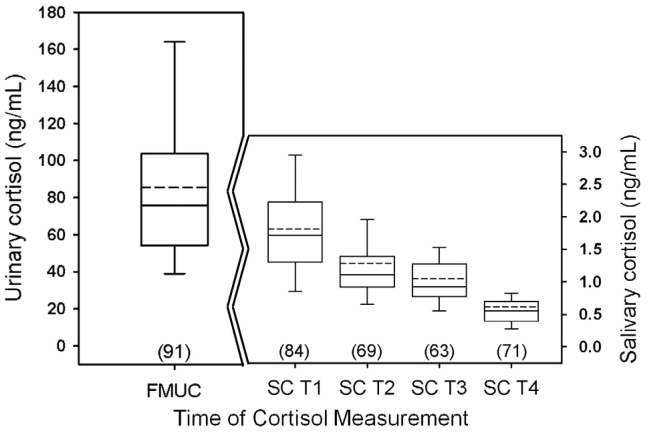

Fig. 1. FMUC and diurnal SC across the day. Cortisol measured in FMU was 53-fold higher than cortisol measured in saliva throughout the day. Diurnal SC profile shows the expected circadian pattern, peaking in the early morning and declining throughout the day and into the evening. The dashed line indicates the mean, and the central solid line indicates the median. The lower and upper edges of the box indicate the 25th and 75th percentiles, respectively. The lower and upper error bars indicate the 5th and 95th percentiles, respectively. Sample sizes for each cortisol measurement are shown in parentheses.

To explore the relationship between FMUC and individual SC measures for each day, we analyzed the correlations between FMUC and SC at each time point (T1 through T4) and each day (Day 1 to Day 3) separately. For each time point we also ran a linear regression model using SC as the response variable and FMUC, day and their interaction as predictors to assess the extent of day-to-day variability in the relationship between FMUC and individual SC measures. In addition, the potential association between variation in FMUC and the diurnal change in SC between time T1 and T4 was examined by using linear mixed effect models. The diurnal change in SC has also been used as a biomarker of stress (Gex-Fabry et al., 2012; Kraemer et al., 2006).

RESULTS

Free cortisol levels were higher in urine (FMUC range = 16.04–242.18 ng/ml) than in saliva (SC range = 0.21–5.16 ng/ml) (Fig. 1). There was a significant diurnal change in the mean SC ($P < 0.0001$; Table 2) with a peak in the early morning followed by a monotonous decline through the day (Fig. 1). This agrees with observed circadian patterns of SC reported by previous studies (Hucklebridge et al., 1998; Laakso et al., 1994; Shimada et al., 1995; Touitou and Haus, 2000; Touitou et al., 1983). The variance components for the individual specific random effects of time were tested to be significant based on likelihood ratio test, which implies that the shape of the diurnal cortisol trend varies among individuals.

When individual specific effects were taken into account, a small but statistically significant positive association between FMUC and average SC profiles emerged (beta estimate = 0.000486; $P = 0.0095$; Table 2). This means that for every increase in a unit of urinary free cortisol, there was an associated 0.1% increase in SC

TABLE 2. Salivary cortisol declines throughout the day, and first morning urinary cortisol levels are positively associated with SC levels across the day

Fixed effects	Numerator degrees of freedom	Denominator degrees of freedon	*F*-value			*P*-value
Time	3	116	97.94			<0.0001
FMUC	1	160	6.90			0.0095
Effect	Logged estimate	Standard error	Estimate	DF	*t*-value	*P*-value
SC T1	−0.7979	0.02552	$10^{-0.7979} = 0.16$ μg cortisol/dl saliva	116	−31.26	<0.0001
SC ΔT2	−0.1891	0.02735	$(10^{-0.1891} - 1) \times 100\% = 35.3\%$ decrease from T1	116	−6.91	<0.0001
SC ΔT3	−0.2584	0.03417	$(10^{-0.2584} - 1) \times 100\% = 44.8\%$ decrease from T1	116	−7.56	<0.0001
SC ΔT4	−0.5319	0.03125	$(10^{-0.5319} - 1) \times 100\% = 70.6\%$ decrease from T1	116	−17.02	<0.0001

Linear mixed effect model assumed that random fluctuations in average SC varied across individuals and that individuals differentially deviated from the population average SC level at each time point, i.e., individuals differed in the shape of their diurnal SC profiles. Time of saliva specimen collection within a day (time) and FMUC levels were both predictors of SC levels. Diurnal changes in SC throughout the day are described as SC at T1 and changes in SC from T1 to T2 (ΔT2), T1 to T3 (ΔT3), and T1 to T4 (Δ T4).

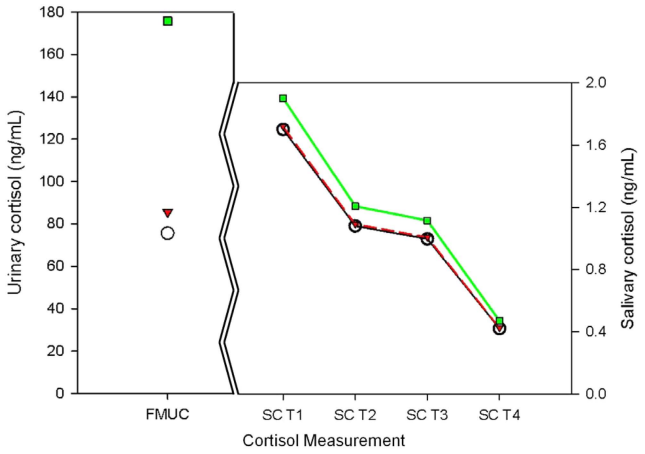

Fig. 2. Unit changes in FMUC and the associated changes in diurnal SC profiles. Black empty circles represent the initial values of FMUC and SC throughout the day for one participant. Each unit increase in FMUC (1 ng/ml) is associated with a 0.1% increase in SC across all four sampling time points (T1, T2, T3, and T4). Therefore, a 10-unit increase in FMUC (10 ng/ml), which is equivalent to a 13.2% increase in FMUC, is associated with a 1.1% increase in SC across the day (red triangles). Similarly, a 100-unit increase in FMUC (100 ng/ml), which is equivalent to a 132.3% increase in FMUC, is associated with an 11.8% increase in SC throughout the day (green squares). [Color figure can be viewed in the online version of this article, which is available at wileyonlinelibrary.com.]

$((10^{0.000486} - 1) \times 100\% = 0.1\%)$ (Fig. 2), and this relationship was constant throughout the day.

FMUC levels were not significantly associated with individual SC, regardless of sampling day or the time of day SC was measured (all $P > 0.05$). The relationship between FMUC and SC did not vary across days (FMUC cortisol by day interaction: $P > 0.3$ for all four time points). In addition, despite the common use of diurnal SC slope as a biomarker of stress (Gex-Fabry et al., 2012; Kraemer et al., 2006), we did not find a significant association between variation in FMUC levels and daytime SC slope between T1 and T4 ($P = 0.4063$).

DISCUSSION

We found a small, but significant, positive association between FMUC levels and diurnal SC profiles in our sample of healthy Mayan women. Each 1 ng/ml increase in FMUC was associated with a monotonous 0.1% increase in the cortisol levels measured in all four salivary specimens collected across the day ($P < 0.05$). As expected, based on previous studies, cortisol was several-fold higher in urinary than salivary specimens (Jerjes et al., 2006a; Neary et al., 2002; Putignano et al., 2003). The small magnitude of the association between FMUC and SC levels, and the large difference in the absolute cortisol levels observed between these two matrices may be explained by: (a) the fact that FMUC is a marker of nocturnal HPAA activity, while SC reflects diurnal HPAA activity, and (b) the fact that FMUC represents the amount of cortisol accumulated in the bladder over a period of several hours, while SC reflects cortisol secreted just prior to sample collection.

Night and day

Nocturnal versus diurnal cortisol production: The most obvious difference between the information provided by FMUC and SC measures corresponds to the different periods of HPAA activity they reflect (Beisel et al., 1964b; Gröschl, 2008; Lewis, 2006; Lindholm, 1973; Schedl et al., 1959). While FMUC reflects nocturnal HPAA activity, SC reflects that axis' diurnal function. HPAA activity is comparatively low during nighttime sleep (Hucklebridge et al., 1998; Laakso et al., 1994; Shimada et al., 1995; Touitou and Haus, 2000; Touitou et al., 1983). Cortisol secretion increases towards the end of the night, peaking approximately 30–45 min after awakening and declining across the day until it reaches its nocturnal nadir (Hucklebridge et al., 1998; Laakso et al., 1994; Shimada et al., 1995; Touitou and Haus, 2000; Touitou et al., 1983). The weakness of the association between FMUC and SC may then be partially explained by the differences between the low nocturnal HPAA activity and the more dynamic HPAA activity patterns during the day.

Nocturnal versus diurnal exposure to confounders: In addition to nocturnal versus diurnal dissimilarities in basal HPAA activity patterns, it is necessary to consider differences in exposure to stimuli that can affect cortisol profiles. During the night most individuals in our population are asleep and were, therefore, less likely to be exposed to stimuli that can activate their HPAA. In

contrast, during the day our participants were typically exposed to a variety of stimuli such as physical activities or the ingestion of food that can cause ephemeral changes in cortisol secretion (Follenius et al., 1987; Kudielka et al., 2009; Lovallo, 2006; Mello, 2010; Papadimitriou and Priftis, 2009; Pollard, 1995; Young et al., 2001, 2004). These routine activities and their short-term effects on cortisol levels do not usually represent the variable of interest (i.e., physiologic stress) and, thus, are often considered "confounding" factors. While not completely free from the effects of these confounders (Reiches, 2012), nocturnal HPAA activity is less likely to be affected by said stimuli than diurnal HPAA activity. Thus, differences in cortisol secretion observed between FMUC and SC may also be partially explained by the different levels of exposure to confounders during those two periods.

Cortisol kinetics and differences in time elapsed between excretion and measurement

Cortisol is excreted into urine and saliva through different pathways. The non-protein-bound cortisol circulating in blood is a lipophilic molecule that diffuses across capillary membranes of the kidney into the glomerular filtrate, traveling through the proximate convoluted tubule, then Henle's loop and the distal convoluted tubule wherein it can be reabsorbed back into circulation (Beisel et al., 1964a,b; Daughaday et al., 1956; Lindholm, 1973; Schedl et al., 1959). Circulating, non-protein-bound cortisol, on the other hand, diffuses passively across the epithelial cells and the basement membrane of the capillaries surrounding the salivary glands, directly into saliva (Beisel et al., 1964a,b; Daughaday et al., 1956; Gröschl, 2008; Jerjes et al., 2006b; Levine et al., 2007; Lewis, 2006; Lindholm, 1973; Morineau et al., 1997). Despite these differences, in both cases the excretion process takes approximately 10–20 minutes (Aardal-Eriksson et al., 1998; Beisel et al., 1964a,b; Daughaday et al., 1956; Jerjes et al., 2006b; Kudielka and Wust, 2010; Lindholm, 1973; Morineau et al., 1997).

In terms of measuring cortisol, however, the relevant difference between these two processes is that while saliva is secreted continuously into the oral cavity, urine accumulates in the bladder. Overnight, urine will commonly be held there for several hours until it is eliminated in the first morning void (Jerjes et al., 2006a). Consequently, non-protein-bound cortisol can be measured in saliva immediately after it is excreted and reflects HPAA activity with a delay of about 10–20 minutes, while nonconjugated cortisol measured in FMU specimens accumulates in the bladder for several hours and reflects HPAA activity during the period between the last urinary void before an individual retires for the day and her FMU void (Jerjes et al., 2006a). These differences between matrices in cortisol accumulation prior to sampling may help explain why the absolute levels of nonconjugated urinary cortisol we observed in this study were over 50-fold higher than non-protein-bound cortisol levels in saliva.

To summarize, there are pronounced differences in: (a) the periods of HPAA activity represented by cortisol as measured in FMU and diurnal saliva samples, (b) diurnal versus nocturnal exposure to confounders, (c) the metabolic pathways involved in cortisol excretion and, more importantly, (d) the period over which cortisol accumulates in each of these two matrices prior to sampling. Combinations of within- and between-individual variation in any of these processes (e.g. excretion rates, amount of time elapsed between voids, etc.) could, at least in part, explain the small magnitude of the association between FMUC and SC (Fenske, 2006; Shi et al., 2008).

Monitoring longitudinal variation in physiologic stress levels: saliva or urine?

The small size of the association between FMUC and diurnal SC and the differences in time elapsed between excretion and measurement between those two matrices highlight the importance of an under-discussed issue: which matrix provides the most advantages when assessing longitudinal variation in physiologic stress levels in naturalistic conditions: saliva or urine?

Saliva: Given the relatively short time elapsed between adrenal secretion of non-protein-bound cortisol, its excretion in saliva and the time in which the sample can be collected (approximately 10–20 minutes) (Aardal-Eriksson et al., 1998; Buske-Kirschbaum et al., 1997; Kudielka et al., 2004; Kudielka and Wust, 2010; Petroski et al., 2012), saliva provides an appropriate matrix to detect rapid changes in physiologic stress levels. Consequently, saliva is often used in "experimental" designs in which specimens can be easily collected "before" (to establish a cortisol baseline) and "after" programmed experimental challenges (Table 1B$_i$) (Buske-Kirschbaum et al., 1997; Kudielka et al., 2004; Petroski et al., 2012). However, when the goal is to understand the effect of naturally-occurring challenges or evaluate longitudinal changes in HPAA functioning, saliva presents a number of limitations. First, the timing of natural challenges is difficult to predict and, therefore, researchers cannot plan the collection of salivary specimens before and after these challenges. Second, as HPAA activity follows a diurnal circadian pattern, to evaluate within- and between-participant variability in cortisol levels, salivary specimens should be collected from all participants at the same time. In naturalistic, longitudinal studies with large sample sizes, this task is very difficult to achieve. To do so would require a one-to-one correspondence between the number of participants and the number of field assistants. Alternatively, researchers can rely on participants to collect their own salivary specimens following an established protocol. Yet, compliance with the collection protocol tends to be low as in longitudinal studies with repeated measurements participants often forget the protocol and consume food or engage in physical activities prior to specimen collection, or fail to collect specimens at the right time (Broderick et al., 2004; Clow et al., 2004; DeSantis et al., 2010; Fabian et al., 2009; Jacobs et al., 2005; Kudielka et al., 2003; Thorn et al., 2006; Yehuda et al., 2003), thereby introducing sampling error (Table 1B). Third, in addition to its circadian profile, HPAA activity also follows an ultradian rhythm with pulses of cortisol being secreted every 1–3 h throughout the day (8–26 peaks per 24-h period) (Follenius et al., 1987; McMaster et al., 2011; Veldhuis et al., 1990; Young et al., 2004). Thus, the timing of sample collection relative to these pulses could also influence cortisol levels measured in saliva samples and, therefore, introduce "statistical noise" (Clements, in press).

Nonetheless, saliva is still the most commonly used matrix to assess physiologic stress. To distinguish between the contributions of circadian variation and diurnal confounds to variation in SC levels from those of stressful life events, researchers attempt to control for a number of covariates such as collection time, waking time, activity level and food consumption prior to specimen collection, timing relative to stressor, etc. Controlling for all those confounders is, however, not an easy task when studying individuals as they carry out their everyday regular activities. To address this problem, Lovallo and colleagues (2010) propose the use of a "resting control day." Applying this method they found that pre- to post-stress SC levels were 54% larger on a stress day than on a resting control day. If establishing a resting control day is possible, this method does help circumvent some of the noise introduced by diurnal confounders. However, asking participants to rest for a day in populations that depend on their regular activities to obtain their basic sustenance, such as agro-pastoralists, hunter-gatherers and low socioeconomic status laborers living in industrial populations, may be difficult or inappropriate. Furthermore, in some cases the between- and within-individual variation in daily activities would also affect the utility of a single resting day as a reference for basal HPAA activity.

An alternative solution to the problem presented by diurnal confounders can be the use of "cortisol awakening response" (CAR) (Fabian et al., 2009; Geiss et al., 1997; Kumari et al., 2009; Pruessner et al., 1997; Schulz et al., 1998; Wust et al., 2000a,b) (Table $1B_{ii}$). CAR captures the increase in circulating cortisol levels upon waking and its peak approximately 30 min later (Aschoff, 1981; Touitou and Haus, 2000; Touitou et al., 1983). CAR patterns are relatively stable within individuals across days (Pruessner et al., 1997; Schulz et al., 1998; Wust et al., 2000b). Within individual CAR departures have been linked to physiologic stress including fatigue (Kumari et al., 2009), pain (Fabian et al., 2009; Geiss et al., 1997), chronic social stress, concerns, lack of social recognition (Wust et al., 2000a); burnout and work overload (Pruessner et al., 1999; Schulz et al., 1998). However, again, CAR requires the self-collection of saliva by participants outside of laboratory situations. Failing to adhere to the collection time even by a few minutes can dramatically change CAR's slope and create spurious differences between compliant and noncompliant participants (Table 1B) (Broderick et al., 2004; Clow et al., 2004; DeSantis et al., 2010; Fabian et al., 2009; Jacobs et al., 2005; Kudielka et al., 2003; Thorn et al., 2006; Yehuda et al., 2003). Another alternative is to evaluate the diurnal SC slope between waking and evening (Gex-Fabry et al., 2012; Kraemer et al., 2006). This method presents similar problems to CAR (Table 1B) and, in our analyses, we found no evidence of a relationship between the diurnal SC slope and FMUC levels.

Urine: Contrasting with the HPAA "snapshot" provided by SC measurements, FMUC offers an integrated measure of nocturnal HPAA activity. As such FMUC is less susceptible to perturbation by variation introduced by the pulsatile nature of cortisol secretion. In addition, FMUC's nocturnal nature reduces the impact of diurnal confounding factors that strongly influence diurnal SC profiles (Table 1C). FMUC has been previously associated to self-reported stress (Nepomnaschy, 2005). An analysis of a subset of data collected from the same population showed that day-to-day variation in the participants' daily life concerns was associated with FMUC. Self-reports of daily concerns (e.g., health problems faced by the individuals themselves or members of their extended family, interpersonal conflicts, work overloads, and economic concerns) from the previous 2 days were accurate predictors of increases in each woman's first morning cortisol levels compared to their FMUC levels following days when they reported no concerns (Nepomnaschy, 2005).

As FMUC provides an integrative measurement of cortisol excretion between two voids, the timing of collection of the specimen is less critical than in the case of SC. What is important when assessing FMUC is controlling for voids taking place during the night and ensuring that the specimen collected is the first urinary void produced upon awakening. FMUC lends itself more easily to participants' self collection than saliva as urination is one of the first activities individuals perform upon awakening and, therefore, there are no concerns about the potential effects of physical activity or food consumption that tend to affect SC. It is important to note, however, that as with saliva, in order to interpret the meaning of a single cortisol measurement, it is necessary to know each individual's baseline levels (Nepomnaschy et al., 2012).

Urine or saliva? Given the time periods of HPAA activity represented by FMU and diurnal saliva, their vulnerability to the effects of diurnal confounders, their rate of accumulation in each matrix and their differences in the time elapsed between adrenal excretion and sample collection, saliva appears to be the more appropriate matrix for evaluating the effects of known, specific, experimental stressors, while FMU appears to be more appropriate for evaluating an individual's overall physiologic stress load across several days (Table 1).

Finally, FMU presents one extra advantage over saliva as a matrix to assess cortisol. As the concentration of free cortisol in FMU is about 53-fold higher than in diurnal saliva specimens, and every unit of increase in FMUC is mirrored by a mere 0.1 % change in SC, changes in HPAA function are more easily detectable in FMU than in diurnal saliva specimens.

CONCLUDING REMARKS

To our knowledge, this is the first study to investigate the relationships between nonconjugated FMUC and non-protein-bound diurnal SC in a healthy population using a longitudinal, naturalistic design. The implementation of our longitudinal study design on an ethnically, chronologically, and socially homogenous population of women helped reduce the effect of potential confounders such as those introduced by inter-individual differences in ethnicity, age, sex, diet, and physical activities on cortisol secretion and excretion. As our analyses are based on data collected from nominally healthy women, they contribute to building foundational knowledge regarding normative free cortisol excretion through urine and saliva and their relationships, which should serve as a reference for future studies focused in assessing physiologic stress levels.

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