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Schenk, Maria

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The relationship between 63 days of 24-h urinary free cortisol and hair cortisol levels in 10 healthy individuals

SL van Ockenburg, HM Schenk, A van der Veen,
EFC van Rossum, IP Kema, JGM Rosmalen

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Abstract

Background
Interest in measuring cortisol in scalp hair is increasing because of its assumed ability to provide a historical timeline of previous systemic levels of cortisol. Yet, it remains uncertain how well hair cortisol represents the total systemic secretion of cortisol over time.

Methods
Ten healthy individuals collected 24-hour urine samples for 63 consecutive days and provided a hair sample at the end of the study period. 24-hour urinary creatinine levels in every urine sample were determined to assess completeness of the samples. Cortisol levels in 24-hour urine samples and in hair were measured with liquid chromatography tandem mass spectrometry. The correlation between urinary cortisol and hair cortisol were calculated using Kendall's tau.

Results
We found a nonsignificant moderate correlation between average urinary cortisol secretion and average hair cortisol concentration r_T=0.422, p=.089.

Conclusions
Hair cortisol concentration correlates small to moderately with 24-hour urinary cortisol concentration over a period of 63 days.

Introduction

Cortisol is a pulsatile secreted steroid hormone that can be measured in different biological specimens such as blood[1], urine[2], saliva[3], nails[4] and scalp hair[5]. Interest in measuring cortisol in scalp hair is on the rise in both stress research[6,7] and clinical practice[8] due to its usefulness as a non-invasive long-term measure of cortisol exposure/production with the unique possibility to create a historical timeline to assess previous systemic levels of cortisol. Conditions associated with altered cortisol production have been studied in relation to hair cortisol concentration (HCC), for example pregnancy, Cushing's disease and Addison's disease[8,9]. These studies show that HCC followed the clinical course of patients' conditions, thus providing indirect evidence for the validity of HCC as a measure of historical levels of cortisol[10].

Because hair cortisol is being increasingly used in clinical studies, researchers started exploring the relationship between HCC and cortisol concentrations in other frequently analyzed biological specimens such as saliva, urine or feces. Animal studies generally show a strong correlation between mean cortisol concentrations in saliva and feces, and HCC ($r = .48$ - $.90$)[11–13]. In human studies, however, only a weak to moderate correlation was found between salivary cortisol concentration and HCC ($r = .06 – .57$)[9,14–20]. The discrepancy in results between animal studies and human studies might be due to the fact that data in human studies were collected over short time period (i.e. a maximum of 6 days). As cortisol levels in other biological specimens than hair are known to have high intra-individual variation over time[21,22] it is only natural that short-term measures of systemic cortisol exposure do not correlate well with a long-term measure of systemic cortisol exposure such as HCC.

In conclusion, to date it is unclear how well HCC corresponds to other important well-known measures of systemic cortisol exposure such as 24-hour free urinary cortisol levels (24-h UFC). Only a study that measures several individuals for an extensive time period can address this question. In the present study, we investigated the correlation between 63 days of 24-h UFC (i.e. an estimate of total cortisol output over the period of two months) and hair cortisol in the corresponding time period. We expect to find higher correlations than in previous studies, as we expect the high day-to-day fluctuations of urinary cortisol levels to "average out" over a longer time span.

Material and methods

Study population

The study was a longitudinal prospective observational study generating time series data of 10 healthy participants who collected 24-h urine samples for 63 consecutive days and donated a hair sample at the end of the study period. They were paid €5 per day of study participation, thus a total of €315 after completion of the entire study period.

Inclusion criteria were being a healthy adult between the ages of 18 and 65 years and being available for 63 consecutive days. Exclusion criteria were any current somatic and/or mental illnesses and medication use other than oral contraceptives or occasional acetaminophen. A total of 11 participants were included in the study. One person discontinued participation in the study due to a major life event after two days. The study protocol was approved by the Medical Ethics Committee of the University Medical Center Groningen (UMCG) in the Netherlands. All participants were given extensive written and oral information about the study's purposes and protocol and had the option to consult an independent physician for additional information. Before enrollment participants gave written informed consent.

Urinary cortisol

Participants collected all urine in two separate containers each day for 63 consecutive days. They were instructed to use the "night container" from the moment they went to bed until the first morning void. The "day container" was for all urine produced after the first morning void until the last void before going to bed. Containers were stored at room temperature until they were collected, every Monday, Wednesday, and Friday. Before processing, the urine containers were weighted on a scale with a precision of 1 gram. Urine samples from the "day container" and the "night container" were aliquoted separately in 2 ml cups with screwcap (Sarstedt, Nümbrecht, Germany) and stored at -80 degrees Celsius.

Urinary cortisol concentrations were measured with isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS). The day urine and night urine were measured in separate wells. To minimize the effects of interrun variability on the data, all urine samples of one participant were analyzed in one lot. Intra- and interrun coefficients of variation for the lower range of cortisol in urine were 2.4% and 7.8% respectively. Intra- and interrun coefficients of variation for the higher range of cortisol in urine were 1.4% and 3.8% respectively. 24-hour urinary free cortisol excretion (24-h UFC) was computed in the following way: (cortisol concentration of night urine X volume of night urine) + (cortisol concentration of day urine X volume of day urine)= 24-h UFC. Then two variables were computed to represent the systemic exposure to cortisol over the course of one month (UFC-1 represents total cortisol secretion of the first month of study and UFC-2 represents total cortisol secretion of the second month of study). UFC-1 and UFC-2 were computed by summing all the 24-h UFC of the respective month for each participant. A graphical display of the two variables UFC-1 and UFC-2 with respect to the time period they represent can be found in figure 1.

Urinary creatinine excretion was used to assess compliance with the sampling protocol. Urinary creatinine was measured with the creatinine plus enzymatic assay on the Roche Modular. The intra-run coefficient of variation was 0.9% and the inter-run coefficient of variation was 2.4%. All samples of one participant were analyzed in one run. Each participant showed considerable day-to-day fluctuations in total creatinine output. As this

variation was either normally distributed around the mean or slightly positively skewed, we assume, in accordance with other studies[23–25] that this concerns mostly natural physiological variation. We considered a sample incomplete if the 24-hour creatinine output was lower than 2 standard deviations (SD's) from the person's own mean. This method allows natural physiological variation in creatinine excretion. It can, however, not detect if a participants that was noncompliant from the start. We used this information to assess and report the quality of the data, but did not exclude samples with low creatinine output in our principal analyses, as we are interested in the total cortisol output over one month. A graphical display of the time series of 24-h creatinine excretion of the 10 healthy individuals in the current study has recently been published elsewhere[6].

Hair cortisol

At the end of the 9 weeks, study participants were asked to provide a hair sample that was cut by the researcher directly over the scalp. Hair samples were then attached to a piece of plain paper indicating the scalp side and the roots side. Subsequently they were stored at room temperature in an envelope. All samples were analyzed in one run approximately one year later. Hair cortisol concentrations were measured at the laboratory of clinical chemistry in the University Medical Center Groningen with online-solid phase extraction (SPE) combined with a fully validated isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) method. On the 'scalp side' two hair samples of 1 cm in length were cut; each 1 cm sample representing the hair growth of approximately one month. Samples were weighted in polypropylene containers to ensure the minimum sample weight of 25 mg required for the laboratory analysis.

Samples were subsequently washed once with dichloromethane. About 50 steel balls were added to the container, together with 50µL deuterated cortisol dissolved in 1450µL methanol. The hair was pulverized using the Ball Mill (Retsch, MM400). The suspension was centrifuged and the supernatant was evaporated to dryness at 45°C using a nitrogen flow. The samples were resuspended in 10% methanol. Subsequently, 50µL was injected onto the online SPE-LC-MS/MS system. For SPE and LC we used an integrated Symbiosis system from Spark-Holland combined with a Xevo TQ MS mass spectrometer from Waters. A reversed phase Phenyl-Hexyl column from Phenomenex was used. The applied LC-MS/MS conditions were adopted and modified from Li et al. (Li et al., 2012) For this method the intra-run coefficients were 9.3% at 3.5 pg/mg, 6.2% at 8.8 pg/mg and 4.3% at 30.3 pg/mg. The inter-run coefficients were 6.1% at 3.4 pg/mg, 5.5% at 8.8 pg/mg and 6.0% at 10.6 pg/mg. The lower limit of quantitation for hair cortisol was 0.70 pg/mg hair. For hair, we use the concentration of cortisol in picogram per milligram (pg/mg) of hair. Two variables were created representing HCC in pg/mg in the first month (HCC-1) of study (distal hair segment) and in the second month (HCC-2) of study (proximal hair segment). A graphical display of the two variables HCC-1 and HCC-2 with respect to the time period they represent can be found in figure 1.

Statistical analyses

First, we assessed the stability of the repeated measures of urinary cortisol and hair cortisol over time by calculating the intra-class correlation (ICC) coefficient for UFC-1 and UFC-2, and HCC-1 and HCC-2 respectively. The ICC was calculated using a two factor mixed effect model assessing absolute agreement[26]. It is known that cortisol levels in hair decrease in the more distal older hair segments, in particular in fragments beyond 3-6 cm, known as the wash-out effect. We therefore also performed a sign test to evaluate if there was a significant difference between the medians of the two hair samples HCC-1 and HCC-1.

Then, to assess the relationship between urinary cortisol and hair cortisol for the first and the second month of study separately, Kendal's Tau correlation coefficients were calculated between UFC-1 and HCC-1, and UFC-2 and HCC-2 respectively. It is known, however, that a more reliable estimate of the between-subject correlation of repeated measures data can be obtained by first computing the mean value of a variable for each individual and then calculate the correlation using those means[27]. Therefore we computed the mean value of urinary cortisol (i.e. UFC-1 + UFC-2/2= mean-UFC) and hair cortisol (i.e. HCC-1 + HCC-2/2= mean-HCC) for each individual and then calculated the Kendall's tau correlation between mean-UFC and mean-HCC. To be sure that lack of compliance to the study protocol did not influence our results, we also conducted a post-hoc analysis in which we calculated the within-individual median 24-h UFC levels of the first ($UFC\text{-}1_{median}$) and second month ($UFC\text{-}2_{median}$) after excluding incomplete urine samples. The median was chosen because 24-h UFC was positively skewed within individuals. Moreover, for post-hoc analysis, we created a sum-score of 24-UFC of day 19 through 49 (UFC-14) creating a variable that represents a shift back in time of 14 days. This variable was used to account for approximately 5 days grow out period (of hair still residing in the hair follicle within the skin) and the residue of hair left behind on the scalp after cutting the sample[28].

Based on the previous results of animal studies[11–13], which covered a significantly longer time span than human studies, we expected to have correlations between hair cortisol and urinary cortisol levels to be $r > 0.7$. With the current sample of 10 individuals, we had a power of 0.80 at an alpha level of 0.05 to detect a significant correlation. All analyses were conducted with SPSS 23.0.

Table 1. Population characteristics

Subject	Sex	Age	UFC-1	UFC-2	HCC-1	HCC-2
1	♂	24	1934.6	2642.1	4.97	4.32
2	♀	58	1017.5	1056.3	2.89	3.80
3	♀	29	2039.2	1981.0	2.58	4.69
4	♀	33	2731.4	3273.5	4.32	6.24
5	♂	39	3598.9	3963.2	9.31	8.15
6	♂	19	3475.4	4061.5	3.87	3.56
7	♀	21	3164.6	3636.6	2.90	3.40
8	♀	21	3559,.5	2827.8	6.37	6.05
9	♀	48	2470.4	2761.5	5.09	4.97
10	♀	22	3142.7	3498.1	5.12	5.93

Age is expressed in years. UFC-1 and UFC-2 are the total output of urinary cortisol for the first and second month respectively expressed in nanomol. HCC-1 and HCC-2 are hair cortisol concentration of the first and the second month expressed in picogram/mg.

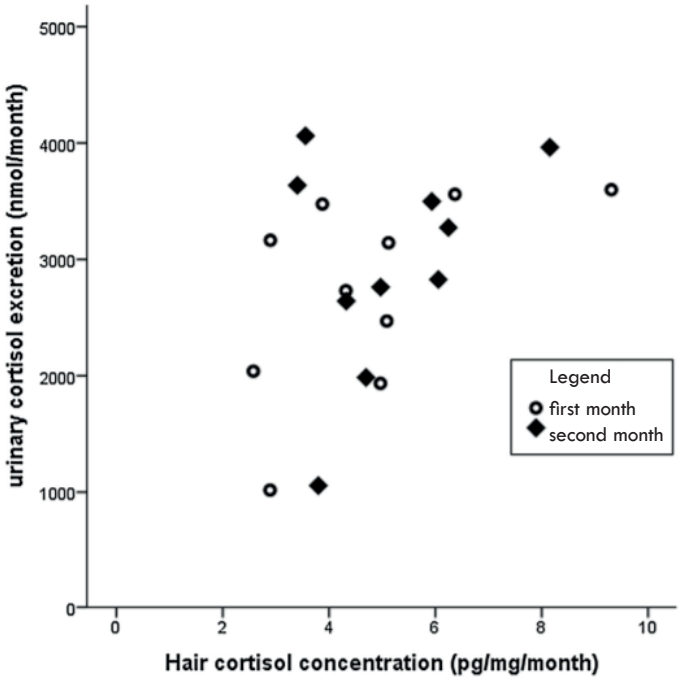

Figure 1. Scatterplot of urinary cortisol excretion and hair cortisol concentration per month

Results

Descriptive data with regard to the study population and cortisol values can be found in table 1.

Completeness of the 24-h urine samples

Urine samples were considered to be incomplete if 24-h creatinine output was smaller than two SD's of a person's own mean. Based on this method, day 23, 24, 28, 29, and 32 for participant 1 were regarded incomplete, leaving 58 complete days. Participant 2 had incomplete samples at day 41 and day 58. For participant 5 only day 63 was regarded incomplete, for participant 6 day 22, for participant 7 day 1 and day 34, for participant 8 day 56, and finally participant 10 had an incomplete sample at day 40. For participant 3, participant 4, and participant 9, all urine samples were judged complete based on their urinary creatinine output. As a sensitivity analysis we also checked the effects of excluding samples of which the 24-h creatinine output was smaller than one SD of a person's own mean. The median cortisol levels of the full dataset, and after removal of incomplete samples based on the 2 SD and 1 SD selection criteria respectively are displayed in a supplemental table (supplement 1).

Test-retest reliability of the repeated measures

The test-retest reliability of urinary cortisol (i.e. the intra-class correlation between UFC-1 and UFC-2) was high with a ICC of 0.926 (95% CI .678 to .982; F=17,021, p<.001). Likewise, the test-retest reliability of hair cortisol (i.e. the intra-class correlation between HCC-1 and HCC-2) was also high with a ICC of 0.895 (95% CI .605 to .973; F=9,686, p<.001). A sign test showed that there was no significant difference between the HCC-1 and HCC-2 (medians 4.74 vs 5.11 pg/mg, p= 1.00), indicating no washout effect.

Correlation between 24-h UFC and HCC

The strength of the between-subject correlation between urinary cortisol and hair cortisol (i.e. UFC-1 and HCC-1) in the first month was moderate (r_T=0.467, p=.060). The strength of the between-subject correlation between urinary cortisol and hair cortisol (i.e. UFC-2 and HCC-2) in the second month was low (r_T=0.200, p=.421). The correlation between the individuals' means of urinary cortisol and hair cortisol over the two months time period (i.e. mean-UFC and mean-HCC) was also moderate (r_T =0.422, p=.089). As a post-hoc analyses, to check if the relatively low correlations that we found could be attributed to incomplete urine samples, we reran the models after excluding possibly incomplete urine samples based on the creatinine output. We did this by first removing all samples that were having a 24-h urinary creatinine output smaller than two SD's or smaller than one SD from a person's own mean. Yet, the results were almost similar to the results of the original analyses, indicating that lack of compliance did not explain the results.

The strength of the between-subject correlation between the median of urinary cortisol

and hair cortisol in the first month (i.e. UFC-1median and HCC-1) was moderate after exclusion of samples with a creatinine output smaller than two SD's (r_T=0.467, p=.060) or smaller than one SD from a person's own mean (r_T=0,467 p=.060). Likewise, the strength of the between-subject correlation between the median of urinary cortisol and hair cortisol in the second month (i.e. UFC-2median and HCC-2) was low after exclusion of samples with a creatinine output smaller than two SD's (r_T=0.244, p=.325) or smaller than one SD from a person's own mean (r_T=0.244, p=.325). Finally, to account for a approximately 5 days grow out period (of hair still residing in the hair follicle within the skin) and the residue of hair left behind on the scalp after cutting the sample we calculated the correlation between UFC-14 and HCC-2. The correlation between urinary cortisol and hair cortisol levels of the second month remained low, even after accounting for the grow out period (r_T=0.289, p=.245).

Discussion

This is the first study that has investigated the relationship between long-term systemic levels of cortisol, as indexed by two months of 24-h UFC, with concurrent HCC in humans. We found that both urinary cortisol and hair cortisol have a high test-retest reliability indicating high reproducibility. Furthermore, we found that the strength of the between-subject correlation between urinary cortisol and HCC was moderate at best. The results need to be interpreted in the light of the strengths and limitations that pertain to this study.

Regarding the test-retest reliability for UFC, the intra-class correlation (0.926), comparing two consecutive months of urinary cortisol output, was exceptionably high compared to what was found in a much larger epidemiological cohort study. In the PREVEND study, stability on a day-to-day basis ranged from 0.69 to 0.72, compared to 0.60 over a two year period29. The difference in findings between our study and the PREVEND study probably arises because we obviated the issue of high day-to-day variability in UFC[30] by creating a measure that spans a much longer time period (i.e. a month), thus averaging out these day to day differences. Similarly to UFC, the test-retest reliability for HCC was also high (0.90) compared to two larger epidemiological studies (0.68 to 0.79)[31]. The fact that the test-retest reliability was so high in this study should also be viewed in the light of the small sample size, which can lead to instability of the estimate, reflected in wide confidence intervals. Another explanation for the high test-retest reliability is that our samples were taken at a closer time interval (i.e. two consecutive months) than in Stalder's study, which sampled at two months and two year interval[31].

The low to moderately strong correlations found in our study are in accordance with the other human studies described below. The other most extensive validation study of HCC in humans found a moderate correlation between six consecutive salivary cortisol diurnal profiles and hair samples collected during the 2nd (r=0.43) and 3rd (r=0.54) trimester of pregnancy respectively[9]. This is very similar to the correlation we found (r=0.42) bet-

ween urinary cortisol and hair cortisol. Considering the enormous day-to-day variability in salivary cortisol levels and the limited sampling time of six days for saliva compared to the hair cortisol sample representing a trimester, the correlations in the pregnancy study are likely to be underestimations. This is also illustrated by the low correlation found between one day of 24-h UFC and HCC (r=0.33) in another study[14]. We have, however, obviated the problem of high intra-individual variability of day-to-day cortisol levels by sampling 24-h urine for 63 consecutive days. Some researchers also investigated whether physiological stress responsiveness – indexed by the area under the curve (AUC) of salivary cortisol obtained during psychological stress testing – would be a better predictor for HCC, but results were either negative[17] or showed a relationship of only moderate size (maximum r=0.3)[18,19] which is equal to what is found under resting conditions. However, since salivary cortisol better reflects bio-available cortisol than urinary cortisol, we could hypothesize that time series of AUCs of saliva cortisol of 63 days would have a stronger correlation with hair cortisol. However, this was beyond the scope of this project.

Several factors of our hair sampling protocol could have led to an underestimation of how well cortisol in scalp hair represents systemic exposure to glucocorticoids, and might explain the often higher correlations found in animal studies compared to human studies. Firstly, we used scissors to cut a strand of hair at the end of the study. We used concaved very fine scissors to cut as closely to the scalp as possible, but this is arguably less precise than shaving participants' hair at the start of the study and at intermittent time points thereafter. Differences between investigators in cutting accuracy are large, and cutting closely to the scalp proves to be difficult even for experienced investigators, leaving an average length of 0.8 cm of hair behind on the scalp[28]. Moreover, the shaving method would have taken into account that growth rates may differ between persons. On the other hand, intermittent shaving may result in partially other locations of the scalp, as human hairs can be thin, and previous shaved scalp areas may be difficult to delimit for a next shaving. In our study, as in many others, the growth rate was assumed to be exactly 1 cm/month which decreases precision of the estimated time period the hair sample represents[28].

Indeed, in a study of Rhesus macaques with a saliva sampling protocol of more than two weeks, that used the "shaving method" to obtain hair samples, the correlation was found to be much higher (r=0.8)[32]. Secondly, in our study hair was sampled almost directly after the collection of the final urine sample. Part of the hair that was formed during the study period resides within the hair follicle in the scalp for approximately 5 days to two weeks before it has grown out long enough to be cut[28]. This means that the time period that the urine samples represent does not fully overlap with the time period the hair samples. To decrease this bias we averaged the cortisol levels of the hair and urine samples of both months. Moreover, we performed a post-hoc analysis in which we shifted the urine samples 14 days back in time, but this improved the correlation between urinary cortisol and hair cortisol only slightly (r=0.289 versus r=0.200) indicating that the above mentioned factors probably play only a moderate role in explaining our results.

A third explanation for the large differences in effect size between human studies and animal studies is that human subjects tend to wash their hair daily or every other day. In experimental studies hair cortisol levels were significantly reduced by washing, with an inverse relationship between number of shampoo washes and the cortisol concentration[33]. Yet, we did not find evidence for this in our study, as the correlation between UFC and HCC was higher in the first month (0.467) of the study than in the second month (0.200), while the hair sample of the second month had actually less exposure to washing, or other environmental factors e.g. UV light, than the sample of the first month of study because hair was sampled at the end of the entire study period. Moreover, HCC-1 and HCC-2 were not significantly different from one another ($p=1.00$). Finally, it is known that serum cortisol is not the only source of HCC, because for instance hair follicles also may produce and secrete cortisol[34]. We believe, however, that the overall contribution of these alternative sources to the total HCC is small as can be deduced from case reports on HCC levels in patients with Addison's disease[34]. We believe, however, that the overall contribution of these alternative sources to the total HCC is small as can be deduced from case reports on HCC levels in patients with Addison's disease[35]. The small correlation between UFC and HCC does not automatically disqualify HCC's usefulness as a marker of long-term systemic cortisol exposure in disease or abnormal physiological states, as in for instance pregnancy and cyclical Cushing HCC does follow a patients' clinical course[8,9].

The results need to be interpreted in the light of some limitations. Our study was powered to detect correlations of 0.7 or larger in accordance with the size of correlations found in animal studies covering a longer time span. The correlation that we found was lower than expected, and did not reach statistical significance, because our study was underpowered. Furthermore, we did not measure certain variables known to influence hair cortisol levels such as the use of hair products or sweat frequency[7]. We do know however, as the investigator visited the participants every other day, that none of the participants have been observed to use hair dye during the study period. Another possible explanation for the only moderate correlation between UFC and HCC is that UFC depends not only on endogenous cortisol production, but also on renal conversion of cortisol to cortisone and on hepatic metabolism. We have tried to account for renal metabolism by creating a composite measure of cortisol and cortisone by summing urinary cortisol with urinary cortisone, and summing hair cortisol with hair cortisone. The correlation between these composite measures was, however, smaller than the relationship between UFC and HCC (supplement 2). Regarding hepatic metabolism, the vast majority of serum cortisol is converted to the metabolites tetrahydrocortisol (THF), allo-tetrahydrocortisol (5α-THF) and tetrahydrocortisone (THE) in the liver, which are excreted in urine[36]. As we did not measure THF, 5α-THF and THE we could not account for the effect of hepatic metabolism. This could potentially lead to underestimation of the real relationship between the level of glucocorticoids in urine and in hair. Yet, our participants were healthy individuals (i.e. free of liver disease) making it less likely that there will be large individual differences in liver metabolism.

The current study has several strengths that need to be mentioned as well. First, we collected data over an extensive time period and our unique dataset represents two months of systemic cortisol exposure. Second, we compared HCC to 24-h UFC, the latter being a reliable and integrated measure of total daily cortisol secretion frequently used in clinical care. Third, all our samples were analyzed by means of LC-MS/MS which is not limited by interferences from cortisol metabolites and conjugates[37]. To conclude, our study showed that there is a moderate nonsignificant correlation between two months of 24-h UFC levels and concurrent HCC in humans.

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Supplement 1

As a sensitivity analysis we also checked the effects of excluding samples of which the 24-h creatinine output was smaller than 1 SD from a person's own mean. Based on this method, day 8, 23, 24, 28, 29, and 32 for participant 1 were regarded incomplete, leaving complete samples on 57 days. Participant 2 had incomplete samples at day 5, 12, 13, 16, 26, 43, 54, 56, 57, and 58, leaving complete samples on 53 days. Participant 3 had incomplete samples at day 2, 25, 26, 31, 43, and 60, leaving complete samples on 57 days. Participant 4 had incomplete samples at day 53, 57, 60, and 63, leaving complete samples on 59 days. For participant 5 only day 33, 35, and 63 were regarded incomplete, leaving 60 complete samples for analysis. For participant 6 day 1, 8, 13, 22, 34, 42, and 52 were deemed incomplete, leaving 56 samples for analysis. Participant 7 had incomplete samples at day 1, 17, 23, 34, 41, 47, and 51, leaving complete samples on 56 days. Participant 8 had incomplete samples at day 12, 19, 25, 26, 54, 56, 57, and 62, leaving 55 complete samples for analysis. Moreover, participant 9 had incomplete samples at day 12, 19, 25, 26, 54, 57, and 62, leaving 56 complete samples for analysis. Finally participant 10 had an incomplete sample at day 40, 43, and 62 leaving complete samples on 60 days.

Supplement 2

Methods

The same analyses as in the original manuscript were repeated for urinary cortisone and hair cortisone, and for a composite measure in which cortisol excretion and cortisone excretion were summed to provide a more integrated glucocorticoid profile.

First, we assessed the stability of the repeated measures of urinary cortisone over time by calculating the intra-class correlation (ICC) coefficient for urinary cortisone excretion in the first month (UFCN-1) and the second month (UFCN-2) and HCC-1 and HCC-2 respectively. The same was done for hair cortisone by calculating the ICC for hair cortisone in the first month (HCCN-1) and the second month (HCCN-2) The ICC was calculated using a two factor mixed effect model assessing absolute agreement ICC(A,k)[1]. It is known that cortisol levels in hair can leach out over time in the more distal older hair segments, known as the wash-out effect. We therefore also performed a sign test to evaluate if there was a significant difference between the medians of the two hair samples HCCN-1 and HCCN-1.

Then, to assess the relationship between urinary cortisone and hair cortisone for the first and the second month of study separately, Kendal's Tau correlation coefficients were calculated between UFCN-1 and HCCN-1, and UFCN-2 and HCCN-2 respectively. It is known, however, that a more reliable estimate of the between-subject correlation of repeated measures data can be obtained by first computing the mean value of a variable for each individual and then calculate the correlation using those means [2]. Therefore we computed the mean value of urinary cortisone (i.e. UFCN-1 + UFCN-2/2= mean-UFCN) and hair cortisone (i.e. HCCN-1 + HCCN-2/2= mean-HCCN) for each individual and then calculated the Kendall's tau correlation between mean-UFCN and mean-HCCN.

To assess if a composite measure of cortisol and cortisone, providing a more complete glucocorticoid profile, would show a larger correlation we created a sum score of urinary cortisol and cortisone for the first (SUM_UFC/N-1) and second month (SUM_UFC/N-2) and hair cortisol and cortisone in the first (SUM_HCC/N-1) and second month (SUM_HCC/N-2) respectively. Kendal's Tau correlation coefficients were calculated between SUM_UFC/N-1 and SUM_HCC/N-1, and SUMUFC/N-2 and SUM_HCC/N-2 respectively. We also computed the mean value of the composite measure of urinary cortisol and cortisone (i.e. SUM_UFC/N-1 + SUM_UFC/N-2/2= mean-UFC/N) and hair cortisol and cortisone (i.e. SUM_HCC/N-1 + SUM_HCC/N-2/2= mean-HCC/N) for each individual and then calculated the Kendall's tau correlation between mean-UFC/N and mean-HCC/N.

Results

Test-retest reliability of the repeated measures

The test-retest reliability of urinary cortisone (i.e. the intra-class correlation between UFCN-1 and UFCN-2) was high with a ICC of 0.975 (95% CI .906 to 0.994; F=42,946,

p<.001). Likewise, the test-retest reliability for hair cortisone (i.e. the intra-class correlation between HCCN-1 and HCCN-2) was high with a ICC of 0.903 (95% CI .187 tot .980; F=21.134, p<.001) but with large confidence intervals. When looking at the test-retest reliability of the composite measure of urinary cortisol and urinary cortisone (i.e. the intra-class correlation between SUM_UFC/N-1 and SUM_UFC/N-2) the ICC was also high 0.973 (95% CI .858 to .994; F=50.631, p<.001). Finally, the test-retest reliability of the composite measure of hair cortisol and hair cortisone (i.e. the intra-class correlation between SUM_HCC/N-1 and SUM_ SUM_HCC/N-2) was high with a ICC of 0.909 (95% CI .315 to .981; F=20.046, p<001). A sign test showed that there was no significant difference between HCCN-1 and HCCN-2 (medians 17,47 vs 21,29 pg/mg, p= .109), indicating no washout effect.

Correlation between 24-h UFCN and HCCN

The analyses above were repeated for urinary cortisone and hair cortisone and showed generally smaller correlations than was the case for urinary cortisol and hair cortisol. The strength of the between-subject correlation between urinary cortisol and hair cortisol (i.e. UFCN-1 and HCCN-1) in the first month was weak (r_T= -0,156 p = .531). The strength of the between-subject correlation between urinary cortisol and hair cortisol (i.e. UFCN-2 and HCCN-2) in the second month was also low and additionally in the opposite direction (r_T=0.111, p = .655). The correlation between the individuals' means of urinary cortisol and hair cortisol over the two months time period (i.e. mean-UFCN and mean-HCCN) was also very weak (r_T =0,067 p = .788).

Correlation between 24-h SUM_UFC/N and SUM_HCC/N

Finally, we tested the correlations between composite measures of urinary cortisol and cortisone with a composites measure of hair cortisol and cortisone, because the composite measures provide a more integrated measure of glucocorticoid excretion. The strength of the between-subject correlation between the composite measure of urinary cortisol+-cortisone and hair cortisol+cortisone (i.e. SUM_UFC/N-1 and SUM_HCC/N-1) in the first month was weak (r_T= 0,111 p = .655). The strength of the between-subject correlation between the composite measure of urinary cortisol/cortisone and hair cortisol/cortisone (i.e. SUM_UFC/N-2 and SUM_HCC/N-2) in the second month was also low (r_T=0.156, p = .531). The correlation between the individuals' means of the composite measures of urinary cortisol/cortisone and hair cortisol/cortisone over the two months time period (i.e. mean-SUM_UFC/N and mean-SUM_HCC/N) was also low (r_T =0,111 p = .655).

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