Research article

Measuring Earwax Cortisol Concentration using a non-stressful sampling method

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ABSTRACT

Background: "Short-term" samples are not the most appropriate for reflecting chronic cortisol concentration. Although hair is used for reflecting the systemic level of this hormone, its use as a "long-term" measure appears clinically problematic. Local and systemic stress and non-stress related factors may release cortisol that is accumulated in hair. Non-stressful earwax sampling methods may provide a more accurate specimen to measure chronic cortisol concentration.
Methods: Earwax from both ears of 37 controls were extracted using a clinical procedure commonly associated with local pain. One month later, earwax from the left ear side was extracted using the same procedure, and earwax from the right ear side was more comfortably obtained, using a novel earwax self-sampling device. Participants also provided one centimetre of hair that represented the retrospective month of cortisol output, and one serum sample that reflected the effect of systemic stressors on cortisol levels. Earwax (ECC), Hair (HCC) and Serum (SCC) Cortisol Concentration were correlated and compared. Confounders' effect on cortisol levels were studied.
Results: The highest levels of cortisol concentration were found in serum, and the lowest in hair ($p < 0.01$). Left-ECC was larger than Right-ECC ($p = 0.03$). Right-ECC was the only sample unaffected by confounders (all $p > 0.05$). A Pearson correlation showed that Right-ECC and HCC samples were moderately correlated between them ($r = 0.39$; $p = 0.03$).
Conclusions: The self-sampling device did not increase cortisol locally. It provided the cortisol level that was least likely to be affected by confounding factors over the previous month. ECC using the novel device might constitute another accurate, but more suitable and affordable specimen for measuring chronic cortisol concentration.

1. Introduction

Currently, measuring chronic cortisol level is a difficult task. Not only because this hormone has a strong circadian rhythm (Bhagwagar, 2003; Bhagwagar et al., 2005), but also several common variables, such as food intake (Legler et al., 1982), nicotine (Pomerleau and Pomerleau, 1990), alcohol (Gianoulakis et al., 2003), physical exercise (Hill et al., 2008) and systemic stressors (Kirschbaum et al., 1993) can affect its output. These covariates have meant that most available specimens, such as serum or saliva, are not entirely representative of the long-term systemic level of cortisol.

At the beginning of this century, the hair specimen started being used for measuring long-term cortisol levels (Cirimele et al., 2000). It has been postulated that hair specimens provide an accurate index of the average systemic cortisol output over the long-term (Short et al., 2016a; Sugaya et al., 2020). It has been found that hair accumulates the hormone, without being affected by previous covariates capable of increasing cortisol output from the adrenal glands (Short et al., 2016a; Sugaya et al., 2020). However, this postulate has several limitations. Systemic stressors, such as the venepuncture procedure that activates the Sympathetic Nervous System (SNS) (Aslan et al., 1981) might have an effect

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on hair follicles. This hair structure is innervated by autonomic fibres (Gibbons and Freeman, 2012) with conduction velocities that cover nearly all the myelinated fibre range from the slowest (A-δ) to the fastest (A-α) (Brown and Brown, 1981). These findings suggest that hair cortisol levels may also be affected by acute influences in some degree. Furthermore, some studies also indicate that local stressors locally increased cortisol production in epidermal and dermal skin cells, as well as in hair follicles (Arck et al., 2006; Sharpley et al., 2009, 2010a,b,c).

Nonetheless, the methods described by Sharpley et al. (2009, 2010a, b,c) have been criticized by some authors (Russell, 2012; Stalder and Kirschbaum, 2012), claiming that a potential influence of local stressors over the total HCC requires further investigation. Although the local stress response, such as the local pain potentially caused when using a syringe designed for removing impacted earwax from ears, such as the Reiner-Alexander one (Memel et al., 2002a) is not fully understood (Hedenberg-Magnusson et al., 2006), local stressors, throughout a stimulation of nociceptive receptors have little or no interaction with the SNS (Elam et al., 1999; Mense, 1986). The neurobiology of the local stress response has been explained differently. Th_1 cytokines seem to be involved in that circumscribed response. They can be secreted, after the envenomation caused by one specific spider, and the local pain caused by its venom is reduced by Th_1 cytokines blockade (Zanchet et al., 2004). On the other hand, local increases in Th_1 cytokine levels, within the context of a limited inflammatory response, increase cortisol locally (Hardy et al., 2016; Rook et al., 2000). This circumscribed production of cortisol is explained by the action of the 11β-Hydroxysteroid Dehydrogenase Type 1 enzyme (11-HSD-1), which catalyses cortisone to cortisol (Hardy et al., 2016), rather than the SNS effect. Ultimately, non-stress related local factors might also influence the total HCC. The amount of cortisol within hair follicles, and therefore, inside the future hair-free segment, depends upon local metabolic variables that affect hair state (Terao and Katayama, 2016). Hair collection protocols suggest cutting the keratinized tissue from the posterior scalp vertex, as less hair growth variability is observed in this area (Pragst and Balikova, 2006). All these potential influences, including systemic, as well as local stressful and non-stressful factors, might imply that hair is not as good as has been argued for reflecting the average systemic concentration of cortisol over the long-term.

Indeed, most correlation coefficients between hair and varying numbers of short-term specimens have been quite modest (D'Anna--Hernandez et al., 2011; Sauvé et al., 2007; van Holland et al., 2012; Xie et al., 2011). However, these results might improve if future hair validation studies were adequately conducted, also measuring the nightly cortisol level. To date, most of those studies have been done correlating Hair Cortisol Concentration (HCC) with a single sample, or an aggregation of several daily cortisol samples. A proper validation study should correlate HCC with another specimen capable of accumulating cortisol for long periods, such as urine collection, or with several time-points of short-term specimens taken over 24 h. Those studies that have associated HCC with urinary cortisol (collection over several hours) have shown different results; whereas Sauvé et al. (2007) found a moderate correlation, Short et al. (2016b) did not find any.

Collecting hair specimens exhibit some practical limitations that prevent their widespread clinical use. The posterior scalp vertex area is the affected region in those who have begun losing their hair from type IV baldness, which affects up to 40 % of men and 10% of women after 40 years of age (Hamilton, 1951). This figure does not include the percentage of people that cannot provide the sample because they do not have the minimum required length of hair (at least 1 cm). One recent study showed that up to 30% of its participants could not, or were not willing to provide hair samples for various reasons (Fischer et al., 2016). Furthermore, because of the variation in hair consistency, there can be variable accuracy with which hair samples are cut to the required length. The available methods for analysing hair are much slower compared to non-keratinized tissues. Whereas analysing a singular saliva sample takes a few hours on average, the analysis of one hair sample can take more

than a day (Biomarker Analysis Laboratory, Personal communication, 2017). This extended duration may explain why its cost of analysis can be up to 44.3% higher than when analysing one short-term cortisol sample, such as saliva.

Confounding variables may also affect cortisol levels in hair. Males may exhibit higher HCC than females (Garcia-Leon et al., 2018; Vanaelst et al., 2012). This keratinized tissue, when affected by external factors, such as UV radiation or the use of cleaning products, may suffer a potential washout effect. This effect is said to be absent in the first four cm below the root hair (Dettenborn et al., 2010). However, it is unknown whether a washout effect affects the cortisol level when the specimen has newly emerged from the scalp. It is not clear whether some cosmetic treatments, such as dying, affect HCC (Manenschijn et al., 2011; Sauvé et al., 2007). All these issues hinder the widespread clinical use of hair cortisol analysis. Therefore, there is still a need for finding a more accurate, suitable, and affordable specimen to ascertain the long-term systemic level of cortisol over different periods.

Other potential biological samples may address that need, of which cerumen, or earwax, is one. This oily secretion is delivered into the auditory canal by the apocrine and sebum glands of the ear; namely the ceruminous glands (Montagna, 1955). Analogies with another natural wax (honeycomb) suggests that earwax might store different substances over the long-term. Honey is preserved at room temperature, without being consumed by microorganisms because of its bacteriostatic properties (Fratini et al., 2016). These properties are shared with human wax (Ghanem, 2011; Stoeckelhuber et al., 2006). These results imply that earwax might accurately reflect the systemic level of cortisol, because compound analyses of other samples, such as saliva, can be affected by bacterial contamination (Chiappin et al., 2007; Nurkka et al., 2003). The strong hydrophobic features of the human wax, which protects it from a washout effect and UV radiation, also reinforce this assumption. Furthermore, conversely to the hair follicle innervation, systemic stressors might not alter the amount of cortisol stored by earwax, as these glands are not innervated (Bende, 1981).

To date, cortisol levels have not previously been measured using earwax samples. Earwax has not even been extracted using non-stressful methods, an explicit cortisol confounding variable, or using safe self-extraction methods. Cotton swabs should not be used, not only because of the hazard that they can mean for ears - they are the leading cause of eardrum perforation and external otitis (Nussinovitch et al., 2004) - but also because they would not be a reliable method for self-sampling earwax. Their removal effect depends on users, rather than a standard application. Measuring cortisol levels that truly reflect its chronic systemic level is of the utmost importance. Several endocrinological diseases, such as Addison's disease and Cushing syndrome, are characterised by chronic cortisol output alterations. Furthermore, psychiatric conditions, such as the major depressive disorder has also been associated with chronic cortisol level alterations (Herane-Vives et al., 2017a).

In this study we compared and correlated Earwax Cortisol Concentration (ECC) with HCC and Serum Cortisol Concentration (SCC) in samples that were collected during the effect of endogenous (morning) and exogenous (venepuncture and after the intake of one standardized meal) systemic stressors in a sample of 37 healthy participants. Despite the aforementioned drawbacks, we used hair as a gold-standard specimen for measuring chronic cortisol output because, in medicine, the gold-standard test is usually the most feasible, rather than the most suitable (Versi, 1992). We could not compare earwax with urine collection, another potential test for measuring chronic cortisol level, because that sample was designed for measuring the average of cortisol over shorter periods, e.g. 24 hrs; which was not the aim of this study. ECC from the left ear side was obtained using the Reiner-Alexander syringe, and ECC from the right ear side was obtained using a novel self-sampling device. This device was designed to not harm the ear. It has a break that does not allow inserting the device beyond the end of the external auditory canal. It is made of plastic, and has removable sampling tips of cellulose sponge, which are impregnated with a specific concentration

(50%) of a mineral oil solution that has Magnesium Chloride (MgCl$_2$) (Figure 1). In a previous pilot study, we demonstrated that those tips were the most reliable and effective ones for sampling earwax from healthy ears. Users also appraised its use as much more comfortable than the Reiner-Alexander syringe clinical method for earwax extraction (Herane-Vives, 2020). Apart from the local pain potentially triggered by this clinical method (Memel et al., 2002a), its poor evaluation might be explained by the fact that people usually appraise self-applications as more comfortable than those practised by clinicians (Mochizuki et al., 2014; Pata et al., 2003; Terao and Katayama, 2016). Thus, all these results suggest that the novel device might not constitute a stressful method for sampling earwax from healthy ears, either systemically or locally.

Therefore, based on the number of working hours needed for extracting cortisol from keratinized and blood tissues, we hypothesized that: 1) The time needed for analysing cortisol from a wax, such as cerumen, would be less than hair, but more than serum. According to a potential circumscribed cortisol production in response to local stressors, it would be expected that: 2) The Left-ECC would be larger than the Right-ECC. Following some preliminary evidence suggesting that a local cortisol production would be small in comparison to the systemic cortisol output, 3) SCC would exhibit the largest absolute level in comparison to earwax and hair specimens. Based on the previous finding, implying human wax has properties suitable for storing substances, and ceruminous gland immunity to acute influences, 4) HCC would correlate with Right-ECC; but only moderately, due to some acute influences over HCC. However, based on wax, as well as hair properties for accumulating substances, as opposed to short-term samples, 5) SCC would not correlate with HCC and ECC and, 6) Due to the discomfort and potential pain that the clinical earwax sampling method can cause, conversely to the self-sampling one, Right- and Left-ECC would not correlate with each other.

2. Methods

Participants were recruited from staff and student volunteers of Universidad Católica del Norte (UCN) in Coquimbo, Chile, and from its catchment area. We used public and internal advertisements to recruit participants. The same clinical researcher assessed all participants. The sample was comprised by thirty-seven healthy participants (20 female), the mean age was 29.9 years, and the mean Body Mass Index (BMI) was 25.6 kg/m^2.

All participants were recruited during a southern hemisphere winter (between 6th of July and 3rd of August 2018). It was found that the triglyceride composition of earwax varies by season (Cipriani et al., 1990). We excluded people of Asian ethnicity and people with intellectual disabilities, due to their differences in earwax composition and quantity, respectively (Cipriani et al., 1990; Crandell and Roeser, 1993). Participants were required to be free of medical illnesses, including psychiatric and ear pathologies, such as impacted earwax or a perforated eardrum,

for at least one month. Participants also had to be free from any medication use for at least one month prior to the study. Participants were also excluded if they reported during the previous month and the one that the study lasted any illicit substance use or were exposed to any severe physical (illnesses) or emotional stressors, as defined in by the DSM-III definition (Pichot, 1986). DSM-IV and V editions do not provide lists of stressors, grouped by their severity.

This research is a validation study that had two interviews, conducted one month apart. A baseline (day = 1) and a follow-up (day = 30). During the baseline assessment, participants had an external ear examination with an otoscope and a comprehensive clinical interview to rule out the presence of any medical illness, including any ear pathologies. Sociodemographic data were also recorded during this assessment. Once participants were included in the study, both outer ear sides were cleaned using the Reiner-Alexander syringe (Reference Test 1). This clinical method is the standard clinical method to effectively and safely remove earwax from outer ears (Clegg et al., 2010). It is the traditional method used by clinicians for removing impacted earwax. Participants were instructed to avoid using cotton swabs or the use of any other external ear cleaning method during the follow-up period. This instruction allowed us to collect a standardized amount of secreted earwax 30 days after the baseline visit (the follow-up assessment). It has previously been found that 3–8 mg of earwax represents four weeks of earwax production in the healthy ear (Cipriani et al., 1986).

The clinical research assistant (S.E) was trained in the use of the Reiner-Alexander syringe by an Ear-Nose-Throat (ENT) specialist doctor in May 2018. Before cleaning both ears, the external auditory canal was examined using an otoscope to rule out the presence of any external ear pathology, such as impacted earwax or a perforated eardrum. The Reiner-Alexander syringe slowly injected 50 cc of tap water at 37 °C inside the external ear canal. The process of syringing creates a sensation of mild pressure in the ear as the warm water from the syringe flushes the wax out. The expelled water and the obtained earwax secretion were collected in a kidney basin. The earwax solution was dried using the displacement method of N$_2$ at 25 °C. Since the amount of earwax does not differ between ear sides (Cipriani et al., 1986), we could conduct a prospective case-control, rather than a cross-sectional research study. Thus, at the follow-up assessment, left ear cortisol samples were obtained using the Reiner-Alexander syringe [controls], and the right one was obtained using the earwax self-sampling external ear device [cases] (Index Test). During the follow-up visit, earwax samples from the left ear were again collected, using the clinical method, whereas participants self-collected earwax samples from their right ears, using the self-sampling device, according to the manufacturer instructions (www.trears.com). Each tip of the self-sampling device was weighed before its use, using a highly precise digital analytical balance. It was not needed to dry the earwax samples obtained by the self-sampling device since it uses a dry method of extraction, which bypassed that stage before analysis. The four
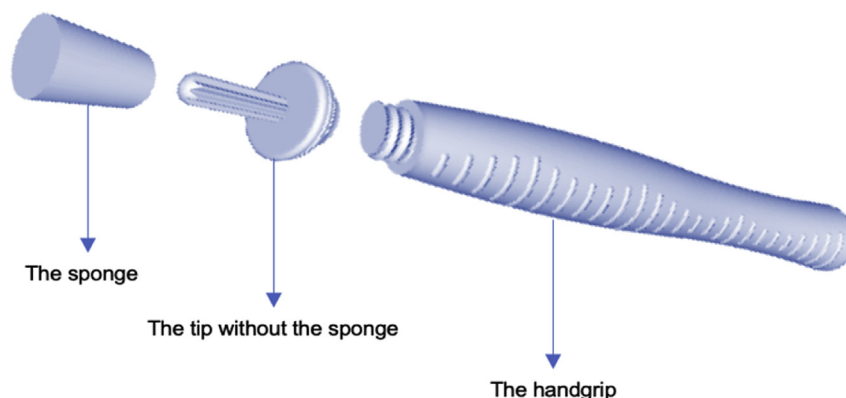

The sponge

The tip without the sponge

The handgrip

Figure 1. The Self-Sampling Earwax Device (Trears Ltd. Copyrights 2020).

labelled earwax samples were stored at 4 °C until they were weighted, using the same digital analytical balance again. All participants provided a morning serum test (Reference Test 2) and 1 cm hair sample (Reference Test 3). ECC, HCC and SCC were obtained under the effect of endogenous [Morning; (Time, {Mean $\pm$ SD = 10:53 $\pm$ 00:44})] and exogenous systemic stressors (Venepuncture and 2 h after consuming a standardized liquid meal, 236 ml of Ensure Avance®). Cortisol concentration was extracted from earwax by using the organic fraction (see earwax analysis). The local ethics committee of UCN, Coquimbo, Chile approved the research and written informed consent was obtained from all participants. Participants were not compensated for taking part in the research.

We chose a longitudinal pilot study because this study is intended for investigating about: 1) Novel research questions, 2) Feasibility and suitability of novel methods, 3) Managing potential limitations of the method that may appear during the conduction of the pilot study and 4) Projecting future more extensive clinical studies (Hertzog, 2008; Johanson and Brooks, 2010). Conversely to high test-retest reliability (Intra-Reliability), moderate/low Inter-Reliability are ordinarily found when two different instruments - or samples - are correlated with one another (0.30–0.40) (Nunnally, 1967). We calculated that a sample size of 35 participants *per* group would be sufficient to be 95% sure that any moderate association (R = 0.40), according to Cohen (2013) is truly significant, within a range between 0.18 and 0.58. A sample size of 40 would provide us with a confidence interval (95%), ranging from 0.20 to 0.57 (Hertzog, 2008).

Data were checked for normality using the Kolmogorov-Smirnov statistical test, as well as graphical methods, such as histograms and Quantile-Quantile normally plots (Q-Q norm Plots), which are the most commonly used and effective diagnostic tool for checking normality of the data (Mohd Razali and Bee Wah, 2010). While Right-ECC (p = 0.30) and HCC (p = 0.34) values were normally distributed, SCC and Left-ECC were not (both p < 0.05). Therefore, we used the Wilcoxon matched-pairs signed-ranks test for comparing Left-ECC and Right-ECC and the Kruskal-Wallis test for comparing Right-ECC, Left-ECC, HCC and SCC. Dunn's test was used for *post hoc* multiple comparisons. Pearson and Spearman correlations were used to determine the association between HCC and Right-ECC, and Spearman correlation was used for all the other pair of associations. Single linear regression analysis was used to determine the association between each biological and psychological covariates and HCC and Right-ECC. Single Generalized Linear Models (GLMs) with a Gamma distribution and a *log-link* function were used to determine the association between each covariate and SCC and Left-ECC. The level of significance was set at p $\leq$ 0.05 (two-tailed).

2.1. Earwax cortisol analysis

The collected earwax samples were resuspended using 500 μl Phosphate-Buffered Saline (PBS) to homogenize them into one solution. Then, 500 μl of diethyl ether was added to each sample and stirred for one minute using a vortex. The emulsion produced was then stored at -20 °C for 2 h. Thereafter, the liquid fraction (the organic fraction) was transferred to a new labelled tube of 5 ml. The solution was dried using a displacement method with N_2. Dried samples were again resuspended in 500 μl of PBS. Cortisol levels were then measured from the final solution using a colorimetric competitive enzyme immunoassay ELISA technique, according to manufacturer instructions (cortisone cross-reactivity=<0.1%) (Enzo Life Sciences, Farmingdale, NY, USA).

100 μl of cortisol standard solutions and 100 μl of the organic fraction were added to wells covered with a plate that contained an antibody. 50 μl of conjugated cortisol was then covalently bound to alkaline phosphatase and bound to 50 μl of a monoclonal antibody against mouse cortisol. The resulting solution was incubated and stirred for 2 h in order to the cortisol of the sample and the standards compete for the conjugated cortisol with an antibody. Following this step, each well was washed and 200 μl of p-nitrophenol-phosphate (pNpp) was added. Then,

the solution was incubated for another hour, this time without stirring. Finally, 50 μl of enzymatic detection solution was added to each well. Absorbance was read at 405 nm using a microplate reader (NovoStar).

2.2. Hair samples extraction

The same clinical research assistant (S.O) collected hair samples from each participant. The presence and frequency of any biological confounders and any procedures potentially affecting hair cortisol levels were measured. Information regarding any include cosmetic treatments (dyeing, bleaching, permanent straightening or waving) and the frequency of hair washing were collected. Hair samples were taken from the vertex at the back of the head and were cut with cleaned scissors as close to the scalp as possible. For this study, four locks of hair were required from different places at the posterior vertex. Each sample needed to be the approximate thickness of a rubber band of 1 cm. One cm of the sampled hair, measured from the hair end to the scalp surface was cut from each lock. This represents approximately one month of hair growth, equivalent to a 1-month retrospective assessment of cortisol production. The total weight of the four 1 cm segments of each lock is approximately equivalent to 25–50 mg of hair. Once collected, hair samples were stored at room temperature in the dark in a sealed container.

2.3. Hair cortisol analysis

Prior to analysis, hair samples were washed with 1 ml of isopropanol to remove any external contaminants. The isopropanol was removed from the vial and the hair was dried in a clean air environment for 48 h. Once fully dried, five ceramic balls were added to each tube and the hair samples were ground into a powder using a MPbio Fast Prep machine (MP Biomedicals, LLC). To extract the cortisol, 1.75 ml of methanol was added to each sample and the samples were incubated for 20 h whilst rotating constantly.

The hair sample, methanol and ceramic balls were decanted into a polypropylene tube (Sarstedt AG & Co, Germany) separating the ceramic balls from the rest of the mixture. The tube was centrifuged at 3000 Relative Centrifugal Force (x g) to separate the ground hair and methanol. The 1.25 ml of clear methanol supernatant was then decanted into a 2 ml polypropylene cryovial. The methanol was then removed using a vacuum centrifuge (Scan Speed 40, Labgene) and the tubes were frozen at -80 °C until their use for the cortisol ELISA. A commercially available, competitive ELISA was used for measuring cortisol (cortisone cross-reactivity = % 0.13). Samples were thawed and reconstituted with 0.125 ml of Salimetrics cortisol assay diluent and the samples were then assayed in accordance with the manufacturer's protocol. The results were expressed as picograms of cortisol per milligram of hair. All hair samples were analysed at Biomarker Analysis Laboratory at Anglia Ruskin University, Cambridge, UK (www.anglia.ac.uk) (Albermann and Musshoff, 2012).

2.4. Blood samples

All blood samples, for every participant, were taken in the morning [mean, (S.D) = 10:53, (00:44)]. Blood samples were taken from the cephalic vein using a 3 cc syringe without using any anticoagulants by the same research assistant (S.O). The blood sample was taken 2 h after consuming a standardized liquid meal, 236 ml of Ensure Avance®.

2.5. Serum cortisol analysis

Blood samples were coagulated after being stored at 4 °C for 24-h. It allows the separation of the serum fraction, without extraction from the clotting factors, such as fibrinogen. Following the separation, serum samples were centrifugated (1000 x g) for 20 min at 4 °C. The resulting

pellet was separated from the solution. The solution was recollected in labelled tubes of 2 ml. These tubes were stored -20 °C until their content was analysed. SCC was analysed using ELISA assays (cortisone cross-reactivity= <0.1%) (Enzo Life Sciences, Farmingdale, NY, USA).

3. Results

Overall, the sample was comprised of a semi-homogenous group of young people, mainly females (54%). A minority of participants were exposed to severe hassles or life events (Table 1). The sample had an average weight, BMI, and waist circumference measurements on average (Table 2).

3.1. Time needed to analyse Right-ECC, Left-ECC, SCC and HCC based estimations

Serum samples needed the fewest number of working hours for analysing cortisol (4:20 h). The time needed to analyse HCC (31:57 h) was two times greater than the time needed to analysed Left-ECC (15:20 h), and four times greater than the time needed to analyse Right-ECC (7:37 h) (Table 3).

Table 1. Socio-demographic and questionnaire results.

Variable		Results
N: Female (%)		20; (54)
Age (Years), Mean (SD)		29.9, (1.4)
Civil status: single (yes), N (%)		32; (87)
Under or postgraduate studies N, (%)		16; (43)
Ethnicity	Mixed race, n (%)	36, (97)
	White N (%)	1, (3)
Alcohol	Yes[δ], n (%)	10, (27)
	Units φ mean, (SD)	1.3; (0.5)
Tobacco (yes), n (%)		9, (24)
Contraceptive pill (yes), n (%)		9, (53)
Medical or psychiatric comorbidity, n (%)		0, (0)
Hair washing frequency (week)		4.9, (0.3)
Cosmetic treatment [Ω], n (%) yes		1, (3)
Medication[&], n (%)		0, (0)
Perceived Stress Scale (PSS), Mean (SD)		22.6, (1.1)
Life events score (RLCQ), Mean (SD)		141.2, (20.8)
History of severe life events (RLCQ) (last month), N (%)		10, (27)
Number of Hassles (last month), Mean (SD)		16.7, (1.7)
Severity index of hassles, Mean (SD)		22.9, (2.8)
Subjects under increased number of hassles (>25) (last month), N (%)		9; (24)
Subjects having problems dealing with their hassles (last month), N (%)		1, (2.7)

δ: at least one-unit last week &: any medication, including psychotropic and steroidal medication. φ: One alcohol unit is measured as 10ml or 8g of pure alcohol. This equals one 25ml single measure of whisky (Alcohol by volume [ABV] 40%), or a third of a pint of beer (ABV 5–6%) or half a standard (175ml) glass of red wine (ABV 12%). Ω: dyeing, bleaching, permanent straightening or waving. RLCQ; Recent Life Change Questionnaire, PSS: Perceived Stress Scale, BMI: Body Mass Index.

3.2. Cortisol level comparisons using different specimens

Hair samples exhibited the smallest cortisol concentration [Mean, (SD): 9.7, (3.8) pg/mg, Median: 9.7 pg/mg Interquartile Range (IQR): 7.6; 10.9 pg/mg] and serum the largest [Mean, (SD): 3874.9, (2136.1) pg/ml; Median: 3825.3 pg/ml; IQR: 2934.9, 3911.4 pg/ml] (p < 0.01) (Table 5). Even though the *post hoc* test showed no difference between Left-ECC [Mean, (SD): 218.7, (192.4) pg/mg, Median: 169.1 pg/mg IQR: 123.7; 214.4 pg/mg] and Right-ECC samples [Mean, (SD): 155.9, (110.7) pg/mg, Median: 124.7 pg/mg IQR: 79.8; 200.3 pg/mg] because of the large SCC and small HCC, a pair comparison between both ECCs did reveal that Left-ECC was significantly larger than Right-ECC (p = 0.03).

3.3. Correlations between cortisol samples

While Right-ECC only correlated with Left-ECC at trend level (R = 0.31; p = 0.06), Right-ECC showed a significant, but moderate association with HCC (R = 0.39, p = 0.03). However, that result was not replicated when Spearman correlation test was used (R = 0.22, p = 0.17). No other significant association was observed among cortisol samples (Table 4 and Figure 2).

3.4. Effect of covariates on Right-ECC, Left-ECC, HCC and SCC

Male participants showed an increased HCC compared to females (p < 0.01). Smoking increased SCC (p < 0.01). Alcohol consumption, contraceptives and cosmetic hair products reduced Left-ECC (all p < 0.05). Right-ECC was not affected by any confounder (Table 5).

4. Discussion

ECC analysis using the earwax self-sampling device was more efficient than the analytic process for measuring HCC and ECC, using a standard clinical syringing method. Regardless of the type of earwax extraction method used, cortisol was significantly more concentrated in earwax than hair, although serum samples contained more cortisol than both earwax specimens. ECC using the clinical method was significantly larger than when the novel device was used. Conversely to serum and hair, earwax sampling, using the self-sampling device, provided the cortisol level that was the least likely to be affected by confounding factors over the previous month. However, some covariates did affect ECC when the clinical method was used. Both ECC samples only showed a clear tendency, when their values were correlated. Right-ECC was significantly, but moderately associated with HCC. SCC did not correlate with any other cortisol sample.

Although SCC analysis required fewer working hours compared to both ECC and HCC, it is well-known that a single blood sample does not provide useful information about chronic systemic cortisol levels. Moreover, repeated short-term samples are required for measuring longer-term levels, a process that may become quite demanding for patients. ECC analysis, using either the novel device or the clinical method, was significantly more efficient than analysing HCC. Unlike with hair, earwax samples did not require processing before analysing cortisol levels. Hair samples needed to be incubated for several hours before extracting the hormone. This fact suggests that ECC analysis, using the earwax self-sampling device, may be more expedited, and therefore, potentially more affordable than HCC analysis. The novel device, compared to the Reiner-Alexander syringe, significantly reduced the time needed to analyse ECC. The extraction using the earwax self-sampling device uses a dry method of extraction which bypassed the need to dry samples before analysis, a typical step of conventional water-based methods. Therefore, the self-sampling earwax device resulted in a faster and potentially more economical ECC analysis.

A limitation of these results is that hair cortisol analytic time was obtained from a personal communication, kindly provided by the Biomarker Analysis Laboratory. However, they estimated that value for

Table 2. Anthropometric results.

Variable		Q1	Median	Mean, (SD)	Q3
Height (cm) Mean, (SD)	Whole sample	160	167	166.7, (1.4)	173
	Female	157	160	161.6 (1.8)	166
	Male	168	173	172.7, (1.3)	176
Weight (kg) Mean, (SD)	Whole sample	62	72	72.5, (2.5)	78
	Female	57.5	65.5	64.6, (2.0)	72
	Male	72	75	81.8, (3.9)	95
BMI (Kg/m^2), Mean, (SD)	Whole sample	23.3	24.9	25.6, (0.6)	26.7
	Female	22.8	24.6	24.2, (0.6)	25.5
	Male	24.1	25.4	27.2, (1.1)	31.2
Waist circumference (cm), Mean, (SD)	Whole sample	77	86	85.9, (2.4)	95
	Female	70.5	78	78.8, (2.3)	87
	Male	88	93	94.4, (3.4)	102

Table 4. Correlations between different cortisol samples.

Sample	Right-ECC (pg/mg)				
	R	P-value	Left-ECC (pg/mg)		
			R	P-value	HCC (pg/mg)
Left-ECC (pg/mg)	0.31$^\varphi$	0.06	R	P-value	
HCC (pg/mg)	0.39$^\delta$/0.22$^\varphi$	0.03*/0.17	0.01$^\varphi$	0.92	**R** **P-value**
SCC (pg/ml)	-0.17$^\varphi$	0.27	0.18$^\varphi$	0.27	0.19$^\varphi$ 0.31

*:$p<0.05$ was significant. δ: R value was calculated using Pearson correlation. φ: R value was calculated using Spearman Correlation. The Right−ECC was obtained using an earwax self-sampling device and the Left−ECC was using a clinical syringing method (The Reiner−Alexander syringe).

us, but did not conduct a research study specifically underpinning that result, as opposed to values we calculated for ECC analysis. Nevertheless, our own previous experience analysing hair cortisol at the Affective Disorders Laboratory at the Bethlem Royal Hospital lab in London also found this to be an extended analysis. Thus, we believe that it is improbable that the vast difference found for analysing and processing both samples in the study could have been found by chance.

The use of the earwax self-sampling device appears to be less locally stressful than the clinical method, not only because its users recently appraised it as more comfortable (Herane-Vives et al., 2020a,b), but also because Left-ECC was larger than Right-ECC. These results suggest that the syringing method, frequently associated with local pain (Memel et al., 2002b) might trigger a local stress response, increasing Th$_1$ cytokine levels throughout the activation of nociceptive receptors (Rook et al., 2000). Thus, our results imply, in agreement with those of Sharpley et al. (2009, 2010a,b,c), that a local cortisol production is largely independent of the systemic cortisol production, based on Bende (1981) histological study that showed that the ceruminous glands are not innervated.

The Left-ECC, possibly under the effect of a local stressor, did not correlate with SCC - a sample that was taken under the effect of endogenous (during the morning) and exogenous (venepuncture procedure and after the intake of one standardized meal) systemic stressors. Then, ECC might not be affected by systemic stressors (Sharpley et al., 2009, 2012),

corroborating again the results of Bende (1981). The fact that Left-ECC and HCC did not correlate suggests that cutting hair with scissors might not represent a local stressful condition for hair follicles or the Left-ECC was influenced by the effect of systemic stressors to some degree. However, this effect seems unlikely since, as said, the ceruminous glands are not innervated. Moreover, even if the syringe caused a systemic, rather than local discomfort, that would be explained by the stimulation of trigeminal nerve receptors located in the external auditory meatus (Racich, 2005), where the ceruminous glands are not located (Perry and Nichols, 1956). Even though there are some afferent nerve fibres underneath the skin, where the ceruminous glands are indeed located, the information conveyed by them is of an itch, rather than pain sensation. Itching, as opposed to pain, is conducted by unmyelinated C-fibres, which are featured by the lowest conduction velocity (Mochizuki et al., 2014). It can be up to 80 times slower than the slowest (A-δ) myelinated fibres of the hair follicle and up to 240 times slower than the fastest A-α of the same structure (Iwata et al., 2017). Thus, that sensory information (local itching), if triggered, might constitute the slowest potential systemic influence.

One limitation of our study is that after meals peaks of cortisol usually appear one hour later in plasma, on average (Legler et al., 1982). The morning blood sample of this study was taken two hours after consuming a standardized liquid meal. Another limitation was that we did not record the awakening time of our participants. Although serum samples were taken during the morning, when the endogenous peak of cortisol occurs, the morning peak of cortisol typically occurs between 20′ and 30′ after awakening – a variable not controlled here. Thus, we cannot guarantee
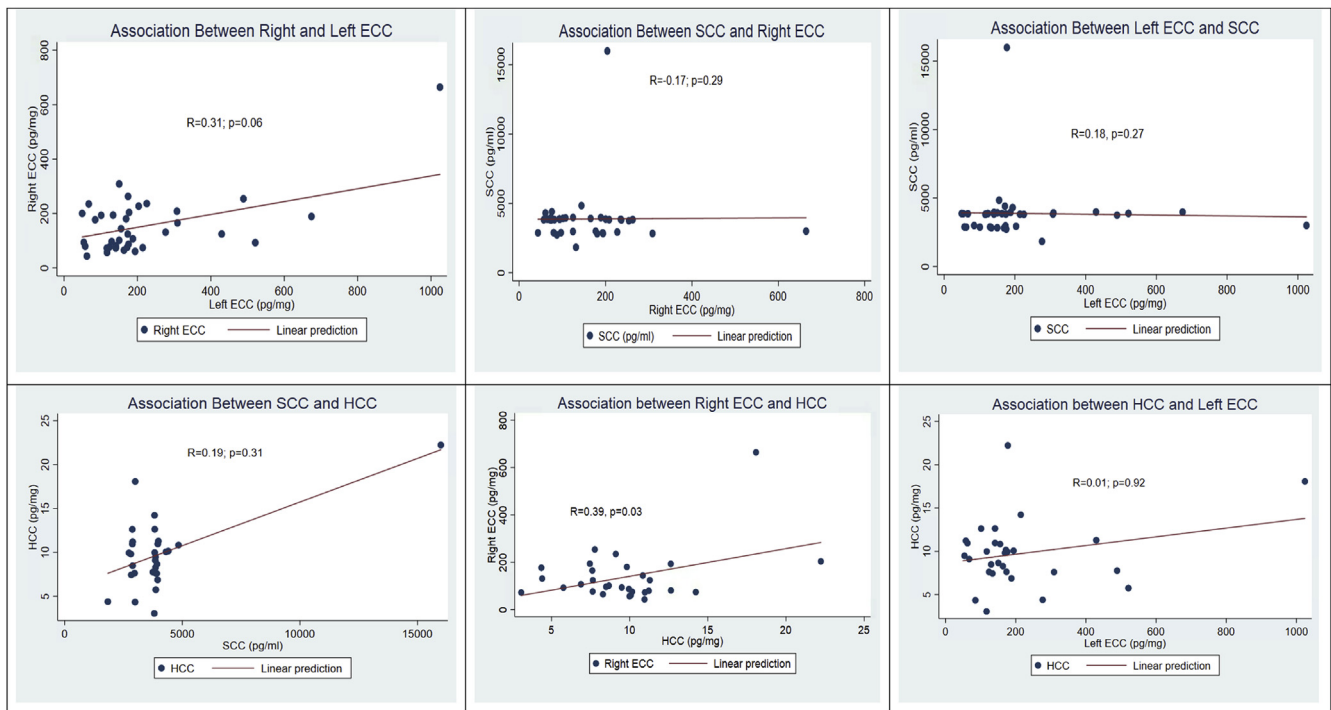
Table 3. Time needed to analyse right and Left-ECC, HCC and SCC.

Processing steps

Step	SCC (Hours)	Step	Left ECC (hours)	Step	Right ECC (hours)	Step	HCC$^\Psi$ (hours)
Centrifugation	00:20	N/A	00:00	N/A	00:00	Technical Time	0:26
						Incubation	24:00
						Rotating evaporator	3:00
(A) Total Processing Time	**00:20**	**(A) Total Processing Time**	**00:00**	**(A) Total Processing Time**	**00:00**	**(A) Total Processing Time**	**27:26**

Analytic Steps

Step	SCC (hours)	Step	Left ECC (hours)	Step	Right ECC (hours)	Step	HCC$^\Psi$ (hours)
Pre-extraction drying of the sample with N$_2$	N/A	Pre-extraction drying of the sample with N$_2$	08:30	Pre-extraction drying of the sample with N$_2$	00:47	Technical time	0:06
Extraction of the sample using an organic solvent	N/A	Extraction of the sample using an organic solvent	02:10	Extraction of the sample using an organic solvent	02:10	Centrifugation	0:25
Post-extraction drying of the sample with N$_2$	N/A	Post-extraction drying of the sample with N$_2$	00:40	Post-extraction drying of the sample with N$_2$	00:40	Robot Time	4:00
Protocol of cortisol extraction	04:00	Protocol of cortisol extraction	04:00	Protocol of cortisol extraction	04:00		
(B) Total analysis Time	**04:00**	**(B) Total analysis time**	**15:20**	**(B) Total analysis time:**	**07:37**	**(B) Total analysis time**	**4:31**
Total time: **(A + B)**	**04:20**	Total time: **(A + B)**	**15:20**	Total time: **(A + B)**	**07:37**	Total time: **(A + B)**	**31:57**

ψ: These values were obtained thanks to the courtesy of Biomarker Analysis Laboratory quotation at Anglia Ruskin Enterprise (2017), Cambridge; N/A: Non applicable.

Figure 2. Single linear and generalized linear models.

Table 5. Single Linear Regression Model between Different Biological and Psychological Variables and Cortisol Levels using Hair and Earwax Samples.

Sample Variable	Right-ECC[δ] (pg/mg)			Left-ECC[φ] (pg/mg)			HCC[δ] (pg/mg)			SCC[φ] (pg/ml)		
	β	p-value	CI	β	p-value	CI	β	p-value	CI	β	p-value	CI
Age	0.6	0.78	-3.8; 6.2	-4.0	0.21	-10.3; 2.3	0.1	0.48	-0.1; 0.2	-43.9	0.14	-103.5; 15.4
Sex	53.8	0.14	-19.0; 126.7	72.8	0.25	-53.2; 198.9	3.7	<0.01*	1.1; 6.3	593.8	0.42	-780.5; 1858.1
Alcohol (unit) ▽	-2.3	0.73	-16.0; 11.4	-12.5	0.03*	-23.0; -1.9	0.1	0.60	-0.4; 0.6	197.3	0.22	-122.8; 517.5
Tobacco usage (yes)	-26.0	0.54	-112.5; 60.8	43.9	0.60	-119.5; 207.2	3.0	0.06	-0.2; 6.3	1800.7	0.01*	317.8; 3283.6
BMI (Kg/cm²)	4.3	0.37	-5.4; 14.0	0.83	0.92	-17.37; 19.1	0.3	0.12	-0.1; 0.7	-24.6	0.78	-207.8; 157.9
Waist circumference (cm)	0.8	0.53	-1.8; 3.4	1.5	0.60	-4.0; 6.9	0.1	0.17	<-0.1, 0.2	-20.6	0.36	-65.2; 24.0
Anti-conceptive pill	-8.6	0.82	-87.8; 70.6	-154.0	0.01*	-274.8; -33.2	<0.1	0.98	-2.8; 2.9	259.9	0.42	-371.6; 891.4
Hair washing frequency	15.1	0.19	-7.9; 38.1	-0.6	0.97	-36.1; 35.0	0.6	0.15	-0.2; 1.5	235.7	0.21	-135.7; 607.2
Cosmetic treatment	81.4	0.47	147.9; 310.7	-155.8	0.02*	-286.4; -25.2	-6.3	0.87	-8.8; 7.5	-24.5	0.99	-4303.5; 4254.5
PSS	-1.0	0.72	-6.9; 4.8	-2.3	0.59	-10.8; 6.2	<-0.1	0.98	-0.3; 0.2	-8.3	0.88	-121.5; 105.1
Number of Hassles	-0.2	0.91	-3.8; 3.4	-3.8	0.21	-9.9; 2.2	<-0.1	0.60	-0.2; 0.1	-0.1	0.99	-69.9; 69.7
Severity of Hassles	-0.6	0.58	-2.7; 1.6	-1.9	0.17	-4.8; 0.8	<-0.1	0.78	-0.1; 0.1	-3.04	0.88	-44.3; 38.3
RLCQ	-0.1	0.51	-0.4; 0.2	<0.1	0.94	-0.6; 0.7	<-0.1	0.88	<-0.1; <0.1	2.9	0.37	-3.6; 9.4
Severe RLCQ	-17.5	0.43	-62.7; 27.6	0.9	0.98	-81.0; 82.9	<-0.1	0.96	-1.7; 1.7	587.7	0.11	-126.8; 1302.2

PSS: Perceived Stress Scale. RLCQ: Recent Life Event Questionnaire. ▽: One alcohol unit is measured as 10ml or 8g of pure alcohol. This equals one 25ml single measure of whisky (Alcohol by volume [ABV] 40%), or a third of a pint of beer (ABV 5–6%) or half a standard (175ml) glass of red wine (ABV 12%). *: p<0.05 was significant. δ: p value was calculated using Linear Regression Models. φ: p-value was calculated using Generalized Linear Models (GLMs) Right−ECC was obtained using an earwax self-sampling device and Left−ECC was using a clinical syringing method (The Reiner−Alexander syringe).

that SCC completely represented the effect of that systemic stressor. Despite this, it would be expected that SCC was indeed under the effect of acute systemic influences, because of the venepuncture procedure at least. One meta-analysis demonstrated that venepuncture procedures trigger adrenal responses in about 47% of people (Weckesser et al., 2014). Therefore, our results suggest that earwax is very unlikely to be affected by the effect of systemic stressors.

The observed association between HCC and Right-ECC implies that earwax cortisol extraction using the novel device might also be suitable for measuring cortisol levels over the long-term. However, we only found a moderate/low size correlation. This result may be explained by the possibility that the hair and earwax samples did not represent the same period. We recently showed that the earwax self-sampling device was significantly more efficient than the Reiner-Alexander syringe at removing earwax from healthy external ears (Herane-Vives, 2020). This finding suggests that some residual amount of earwax may have been left by the Reiner-Alexander syringing at the baseline cleaning, which later could have been extracted by the novel device during the follow-up assessment. This result implies that the follow-up earwax sample extracted by the earwax self-sampling device may have also contained residual earwax, and thus only predominantly, not exclusively, represented the ECC of the last month. This residual amount of earwax may also represent the average of cortisol reactivity, rather than exclusive output. Whereas we were able to assess if our participants have had any severe physical (illnesses) or emotional stressor during the month that the study lasted, we could not do the same before their study entry. Those stressors are capable of increasing cortisol level for long periods. Such stressors are not uncommon - getting a fever, for instance, due to a cold - and so could have affected the cortisol level of the right-earwax sample. By contrast, HCC only represented the month that the study lasted. More extensive studies should aim to investigate more accurately the same period of cortisol concentration, correlating HCC with a follow-up ECC sample that was obtained after a baseline extraction procedure that also used the earwax self-sampling device.

The moderate correlation between HCC and Right-ECC might also be explained, at least in part, by the effect of systemic stressors over HCC. It is not entirely clear whether sweat glands, which are innervated, may also secrete cortisol, which would accumulate inside the hair (Sharpley, 2012). What it is clear, however, is that the hair follicle (Gibbons and Freeman, 2012) and sebaceous glands, which also deliver a certain amount of cortisol to inside the hair shaft (Sharpley, 2012), are influenced by fine networks of nerve fibres (Okumura, 1967). These acute influences, which are under central nervous system control, may also affect the hair growth cycle. There is growing evidence that indicates that the neurohormones and neurotransmitters, which are released during the stress response also affect the hair growth cycle (Botchkarev, 2003; Paus et al., 1997; Stephens, 1986). The autonomic nervous system and hypothalamus control the piloerection mechanism. Their actions are part of the stress response (acute fear). Other stimuli, such as narcotic abstinence syndrome, and cold, are also capable of triggering this response (Stephens, 1986). These results may explain why Boesch et al. (2015) found that HCC was negatively correlated with air temperature. Furthermore, Xie et al. (2011) found that both a single sample and the aggregation of several saliva samples that were taken under the effect of an endogenous stressor, such as the peak of cortisol between 20′ and 30′ after awakening did correlate with 1 cm of hair cortisol. Thus, it appears difficult to completely rule out the role of the systemic stress response on the total HCC. ECC, using the novel device may correlate strongly with other long-term cortisol samples, such as 24h urine collection, fingernails or the continuous measurement of cortisol (Herane-Vives et al., 2017b; Izawa et al., 2015). Future more extensive studies may confirm the use of earwax cortisol for measuring the average cortisol concentration, correlating its level with those other long-term cortisol samples.

Analysis using the Spearman test did not corroborate the results obtained using Pearson correlational analysis, most likely because of the limited size of our sample. Although our results supported the use of a parametric test, future larger studies should validate the use of earwax for reflecting the chronic systemic level of cortisol. Drawbacks of using hair for accurately reflecting the systemic average chronic cortisol concentration, e.g. washout effect, hair follicle innervation and a complex multi-compartment model for incorporating different substances within this specimen suggest that, if future studies do not corroborate our results, it would be likely explained by hair, rather than earwax limitations for storing cortisol over long-term. This presumption is reinforced by the result of one of our most recent studies. We recently found 49% larger earwax glucose correlation coefficient (R = 0.84, p < 0.01) for reflecting chronic glycaemia over a month than the observed one, when Glycated Haemoglobin (HbA$_{1c}$) (R = 0.35; p = 0.03), was related to the same glycaemic level (Herane-Vives et al., 2020a,b). HbA$_{1c}$, conversely to hair, has been widely and undeniable validated for reflecting chronic glucose levels. Therefore, the evidence suggests that earwax is capable of storing other reactive substances, such as cortisol, given that it did particularly well at reflecting the systemic level of sugar that also varies significantly along the day (e.g. fasting v/s postprandial glucose levels). Overall, these results suggest that ECC, using non-locally stressful methods of sampling, as the self-sampling device showed to be in this study, might more accurately reflect the chronic systemic level of cortisol than hair.

We corroborated previous results which indicate that smoking increased SCC (Gossain et al., 1986). ECC using the novel device may provide a more stable hormone level than HCC. We did not find that Right-ECC was affected by any covariates that are known by their adrenal effect. Males have raised HCC in comparison to females, a result that had already been reported elsewhere (Garcia-Leon et al., 2018; Vanaelst et al., 2012). However, some covariates did affect earwax cortisol production under the effect of local stressors, such as the use of the Reiner-Alexander syringe. Unexpectedly, hair cosmetic product, contraceptives, and alcohol decreased Left-ECC. While hair cosmetic products may not have a clinical significance in terms of ECC, the role of combined oral contraceptives and alcohol needs further exploration. Contraceptive medications and alcohol usually increased, rather than decreased, systemic cortisol level (Meulenberg et al., 1987; Thayer et al., 2006). These controversial results require further investigation, as part of more extensive studies, and emphasises the need for validating earwax properties for storing different substances over the long-term.

In comparison to earwax and hair, serum exhibited the largest cortisol concentration, likely because the adrenal glands directly deliver all cortisol secretion into that fluid in a noncompartmental model. Hair accumulated smaller amount of cortisol in comparison to earwax. This result may be explained because cortisol is indirectly delivered into the hair shaft by a multi-compartment model that it is not yet entirely understood (Boumba, 2006; Henderson, 1993a,b). Cortisol in the ear, however, is directly secreted into the external auditory canal by the ceruminous glands, a simple single-compartment model. It seems unlikely that steroid-metabolizing enzymes might explain the large cortisol difference using earwax and hair samples. The hair multi-compartment model is constituted by systemic circulation, as well as many glands, such as eccrine, sebaceous and apocrine, which deliver cortisol into this specimen. Even though cortisol/cortisone ratio in plasma (Vogeser et al., 2001), as well as in sweat are >1 (Jenkins et al., 1969), the overall cortisol/cortisone ratio in hair is <1 (Raul et al., 2004). It is quite possible that sebaceous and apocrine glands mainly deliver these steroids into hair. Then, cortisone difference between earwax and hair samples might be even larger than the cortisol one found here. Earwax cortisol does not directly come from systemic circulation nor sweat glands. This fact means that cortisol/cortisone ratio in earwax would also be <1, as hair. Even though this is a very likely assumption, future studies should measure cortisol, together with other steroids, such as cortisone using earwax. This point is also important, because, even though cortisone cross-reactivity with cortisol is minimal using Enzo Life Sciences

(Farmingdale, NY, USA) (<0.1%) and Salimetrics (LLC, USA) (% 0.13) kits, none of them were specifically designed for measuring steroids in hair and earwax samples.

Another limitation of this study is related to HCC comparison with SCC and ECC. Although it was not the central hypothesis under study, and this study used the same sample of participants, HCC is not strictly comparable with ECC/SCC results, because these two latter specimens were analysed in two different laboratories. Clark et al. (1998) demonstrated significant bias ratios, up to 1.2, between 5 different immuno-assays in controls undergoing a standard corticotrophin test (Vieta and Gasto, 1997). We found a Right-ECC/HCC ratio of 14.3, a Left-ECC/HCC of 22.6 and an SCC/HCC of 399.5. Aside from these extensive figures, both laboratories used ELISA techniques. These figures strongly suggest that methodological differences in laboratory assays might not explain our values. This is also reinforced by the fact that our earwax protocol did not considerably affect the cortisol assay, and it only measured 6.3% less cortisol than when it was used for measuring cortisol level using serum samples.

ECC using the novel device may be measured in patients with stress-related and affective disorders. The ability to discriminate between the average level of cortisol over weeks may become vital for psychiatrists. Indeed, earwax may contribute to investigating the antidepressant effect on mood. Overall, this usually appears after weeks, rather than months (Tanum and Malt, 1996). This clinical observation suggests that the actual antidepressant effect in terms of long-term cortisol level alterations may not be accurately distinguished using hair samples, considering that no less than 1 cm of hair (1 month) has been used for analysing its cortisol level. Conversely, a baseline earwax cleaning, using the self-sampling device might allow measuring chronic cortisol levels over different time frames, including weeks.

5. Conclusions

Stressors having a circumscribed local effect may be different from those that cause a systemic response. Whereas the clinical earwax removal method may trigger a local stress response, the use of the novel self-sampling earwax does not appear to do the same locally, nor systemically. Therefore, the use of this device might provide another specimen to accurately reflect the systemic level of the chronic cortisol concentration. Earwax samples, using the novel device, may be more practical and easier to analyse than hair. Future studies should sample this specimen using this device to measure cortisol levels in cortisol-related conditions, such as Addison's disease, Cushing syndrome and depression.

Declarations

Author contribution statement

A. Herane-Vives: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

L. Ortega and S. Espinoza: Performed the experiments.

R. Sandoval: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

A.H. Young, A. Cleare and A. Hayes: Analyzed and interpreted the data; Wrote the paper.

J. Benohr: Contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare the following conflict of interests:

A. Herane-Vives, has received travel grants from Janssen-Cilag.

A. Cleare has in the last three years received honoraria for speaking from Lundbeck; honoraria for consulting from Livanova, Lundbeck and Janssen; sponsorship for conference attendance from Janssen; and research grant support from the Medical Research Council (UK), Wellcome Trust (UK), the National Institute for Health Research (UK) and Protexin Probiotics.

A.H. Young has given paid lectures and sits on advisory boards for all major pharmaceutical companies with drugs used in affective and related disorders.

Additional information

No additional information is available for this paper.

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