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Review

Hair cortisol analysis: An update on methodological considerations and clinical applications

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ABSTRACT

Background: Hair cortisol analysis is increasingly being appreciated and applied in both research and medicine, aiding endocrinologists with diagnosis.
Content: We provide an overview of hair cortisol research in general and an update on methodological considerations including the incorporation of cortisol into hair, hair growth rates, and sampling procedures, mincing vs. grinding of samples during preparation for extraction, various extraction protocols, and quantification techniques.

We compare the clinical utility and application of hair cortisol with traditional methods of measurement while acknowledging the limitations of analysis including variations in hair growth parameters. We explore the value of hair cortisol in cases of Cushing syndrome (particularly Cyclical Cushing), Adrenal insufficiency (including Addison's disease), therapy monitoring, cardiovascular disease, stress, and mental illness.
Summary: Hair cortisol provides a unique objective biomarker for the analysis of endogenous cortisol levels for not only clinical diagnostic purposes but also in research. The use of hair cortisol has great potential for advancing patient care.

1. Introduction

Cortisol, the prominent human glucocorticoid hormone, is secreted from the adrenal cortex and plays an important role in normal physiology and disease. Cortisol supports the maintenance of homeostasis by increasing gluconeogenesis, proteolysis and lipolysis to increase glucose levels, as well as modulating immunity and inflammation [1–3]. Endogenous cortisol is regulated by the hypothalamic-pituitary-adrenal (HPA) axis and influenced by both stress and blood glucose levels.

Cortisol is released in a circadian pattern, peaking in the morning followed by a decline throughout the day to a low in the evening. Alcohol consumption, nicotine, food, glucose levels, exercise, blood oxygen levels, and acute injury have been shown to alter cortisol secretion [4]. Adrenal insufficiency (AI) and Cushing syndrome (CS) are a result of insufficient secretion or overproduction of cortisol, respectively. Given the ubiquitous activity of cortisol, effective measurement is critical for facilitating clinical diagnosis and treatment [5].

Circadian cortisol pulsatility makes it difficult to measure long-term cortisol secretion by means of traditional bodily fluid matrices such as saliva, urine, and blood. Since the first report of cortisol measurement in hair in 2004 [6], there has been increasing interest in potential clinical applications. In general, deposition of compounds and their metabolites in hair during growth allows for retrospective quantification and subsequent application of analyses. Thus, the extraction and analysis of these compounds in hair provide an insightful, non-invasive tool for a multitude of research and clinical diagnostic purposes. In this paper we will review methodological aspects of hair cortisol

Abbreviations: CS, Cushing Syndrome; HCC, Hair Cortisol Concentration; CV, Coefficient of Variability; ELISA, enzyme-linked immunosorbent assay; CCS, Cyclical Cushing Syndrome

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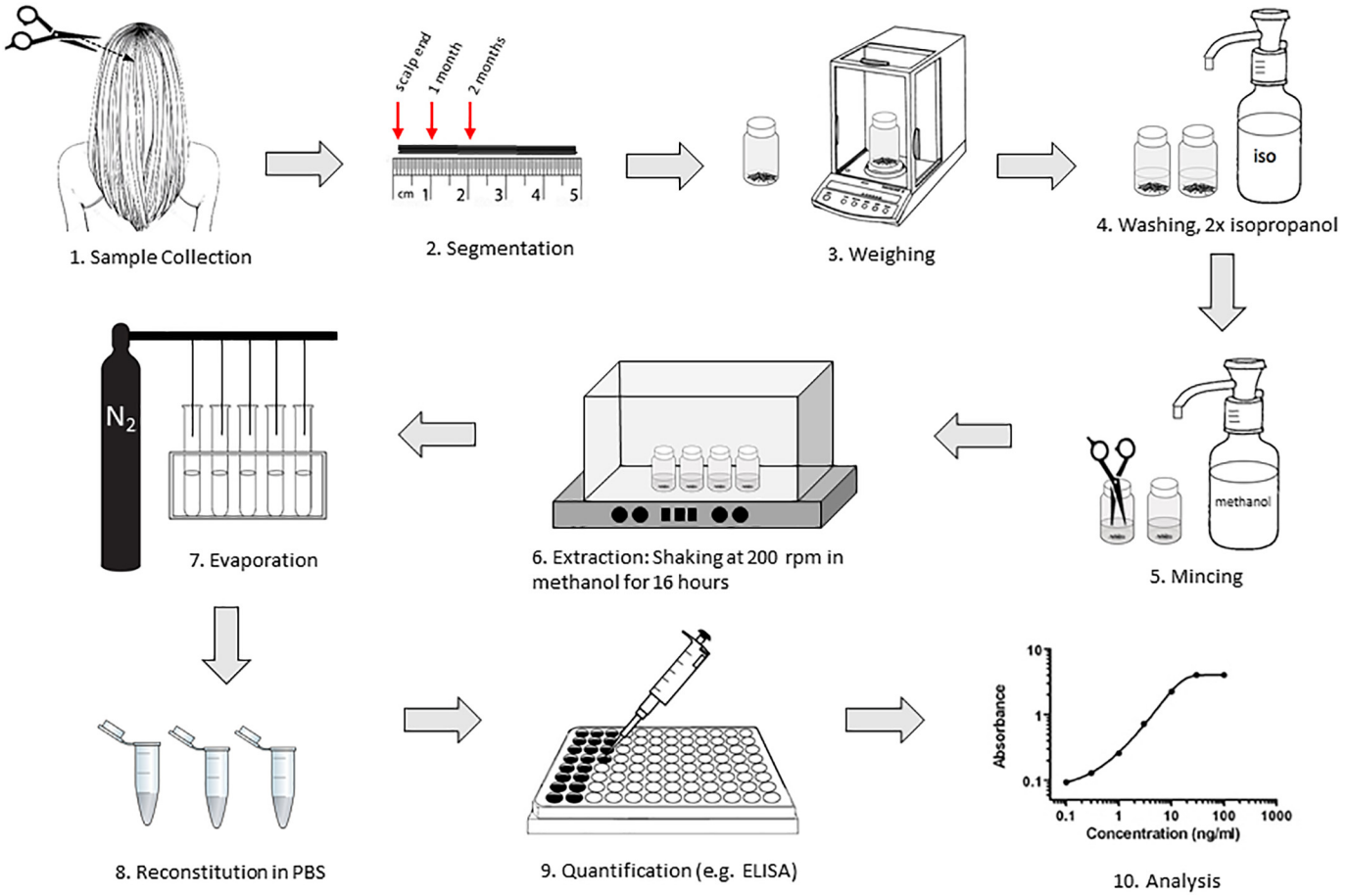

Fig. 1. Standard method for the quantification of hair cortisol concentration.

measurement and describe current knowledge on clinical applications.

2. Methodology and technical aspects of analysis

2.1. Incorporation of cortisol into hair

Incorporation of free cortisol into hair is thought to occur via diffusion from follicular capillaries into the medulla of the hair shaft during growth [7]. Recently, a radiolabelling study was undertaken in rhesus monkeys [8]. After pulse injection of tritium-labeled cortisol into the circulation, the investigators found radiolabelled cortisol and cortisone in hair samples 14 days post-injection [8]. This mechanism is based upon the lipophilicity of cortisol's steroid structure, meaning that cortisol may be deposited into all layers of the hair shaft. As a result, the cortisol that is deposited into growing hair is proportional to the quantity of cortisol in circulation at any given point in time. Cortisol may also be deposited onto the hair shaft via sweat and sebaceous glandular secretions [8,9] as well as exogenous sources. The additional contribution of these sources of cortisol may be mitigated by washing of the hair prior to mincing or grinding with subsequent extraction of cortisol deposited into the medulla [7]. It has been suggested that the hair follicle itself may also produce its own cortisol by means of a local HPA-like pathway [10]. Dermal interconversion of active cortisol and inactive cortisone by the 11β-hydroxysteroid dehydrogenase enzymes may affect the ratio of these moieties as they enter the hair shaft [11]. However, it is unclear whether the contributions from these sources have any clinical significance [7].

2.2. Hair growth

Hair growth occurs in three phases: growth (anagen), cessation (catagen), and rest (telogen) [11]. Historically hair cortisol research has relied on the generally accepted assumption that hair growth occurs at a fairly constant rate of approximately 1 cm per month, with some individualistic and ethnic variability [12–15]. This growth rate has allowed for the assessment of average cortisol levels over time.

As hair cortisol research has advanced it has become apparent that this growth rate is not nearly as constant as previously thought. In a study of ethnic hair growth rates Loussouarn et al. (2001) found that individuals of African descent have a slower growth rate (256 ± 44 μm per day) than Caucasians (396 ± 55 μm per day) [12]. While comparing hair growth parameters in 24 human ethnic groups Loussouarn et al. (2005, 2016) confirmed again that individuals of African descent have slower hair growth than Caucasians, and further reported that individuals with an Asian background have hair that grows faster than both African and Caucasian hair [16,17]. Average hair growth rate and percentage of hair in telogen phase varies among different head regions (i.e., vertex, temporal and occipital) and between genders [12,16]. Similarly, due to the variability of hair growth rates in different regions of the body, hair segments collected from differing regions may not represent equivalent time periods [17].

A recent twin-study by Rietschel et al. (2017) found a hair cortisol concentration (HCC) heritability of 72% with no significant genetic or phenotypic correlation between HCC and three psychological variables [18]. These variations in hair growth parameters may affect the accuracy of chronological segmentation of hair samples as a result of overlapping estimation of cortisol levels from different time periods, ultimately adding variability to standardization of normal HCC values.

The problem of hair growth variability becomes compounded with longer hair samples. Future hair cortisol research should consider both ethic and genetic variation in hair growth patterns during study design.

2.3. Sampling, storage, and segmentation

Sauve et al. found that the relative variability in hair cortisol, as determined by the coefficient of variation (CV), was high (30.5%) when hair was sampled from different regions of the head compared to samples taken from the posterior vertex (CV, 15.3%) [13]. Standardization between laboratories may be accomplished by collecting hair from the posterior vertex of the head, even when exact hair sampling procedures vary slightly between laboratories (Fig. 1).

Hair samples are generally considered stable and capable of being stored at room temperature for extended periods of time [7]. Hair cortisol levels have been measured in archeological adult human specimens from the Nasca Region of Peru (1–1000 CE) [19]. Recent work has shown that HCC decreases with exposure to ultraviolet radiation, meaning that samples should be stored away from UV exposure to allow analysis of samples in the distant future [20].

Although there is some degradation of hair cortisol, analysis is generally only limited by the length of the hair in a sample. Quantification of temporally unique periods of cortisol exposure can be achieved by carefully segmenting hair into sections representing time periods of interest. For example, average cortisol exposure for a 1- or 2-month interval of time can be measured in segments 1- or 2-cm in length, respectively. When working within specific ethic groups for which more specific hair growth rates are known, the length of sample may be adjusted accordingly. Alternatively, in clinical patients for which dates of past interventions such as surgery or initiation of medications are known, hair cortisol can be plotted against multiple timelines to assess the appropriate hair growth for an individual without a priori knowledge of their hair growth rate.

2.4. Weighing and washing

Segments of hair are placed into glass vials and weighed to allow for the determination of hair cortisol per a given mass of hair. A washing step is regularly included to remove contributions of sweat and sebaceous secretions that may be deposited on the surface of hair. Sweat and sebum contains cortisol that may contaminate measurements and washing normalizes any individual differences in hygiene [7]. Exogenous cortisol may also be deposited onto the outer surface of the hair from the use of topical steroids for skin conditions such as psoriasis. Two to three washes with isopropanol for 3 min at room temperature followed by air drying is common practice.

2.5. Mincing versus grinding

Efficient cortisol extraction requires a large surface area for the interaction of the hair medulla and the extraction solvent. The hair extraction surface has been increased by manually mincing the hair, blending the hair, and grinding or pulverizing the hair by means of milling. Particle size reduction has classically been accomplished by mincing the hair into ‘small pieces’ using surgical scissors. Mincing of the sample with scissors is far from being standardized as the size of the minced pieces is determined by how meticulous the technician is in the cutting process. This may potentially lead to variations in the amounts of cortisol extracted. However, intra-laboratory validation of hair cortisol measurement has been shown to have high correlation between results when using the mincing method (r^2: 0.9692) [21]. Alternatively, blenders or mills with zirconium oxide beads and liquid nitrogen cooling (cryomilling) can produce hair particles of ~5 μm in size.

Although grinding can theoretically improve cortisol dissolution and extraction, direct methodological comparison studies are lacking. A 2015 study by Slominski et al. concluded that the mincing of hair was optimal. It was hypothesized that grinding of hair and use of a ball mill sets up researchers for increased possibility for loss of sample while also potentially contributing to the degradation of hair proteins or steroids contained therein [22]. It can also be proposed that the grinding of hair samples may greatly increase the possibility for sample carryover. Chemical degradation from heat produced during milling may also occur [22], however, cryomilling might be able to abrogate this effect. In another study, results from both ball milling and mincing had a high correlation (r^2: 0.947–0.978) indicating that the effect of particle size reduction during cortisol dissolution may not be as important as consistency with the method employed [21].

A minor methodological note is that when these mechanical techniques are employed rather than mincing with scissors, samples are often ground following the washing step but prior to weighing the sample to be analyzed. This is to account for loss of sample.

2.6. Extraction, evaporation, and reconstitution

Cortisol is extracted from minced or ground hair using organic solvents (e.g., methanol, acetone). Typically, cortisol is extracted from 10 mg of hair by adding 1 to 2 ml of methanol to the glass vial in which the hair was washed and weighed. Shaking of the sample on a rocker overnight in methanol at ambient temperature or at 52 °C is the most common method for extracting cortisol from hair. However, data has suggested that a single methanol extraction method may only yield 40–60% with an average of 46% of absolute HCC [22]. A 4-step method that employs alternating methanol and acetone for 15 h at 52 °C and 5 min at room temperature, respectively, repeated twice, has yielded an estimated 98–100% of hair cortisol [22]. Although the effect of extraction efficiency on the clinical utility of the technique is not certain, it can be argued that following a consistent protocol still results in useful comparisons between subjects and controls.

After extraction is complete, the supernatant is transferred to a disposable glass culture tube and then evaporated under nitrogen and heat until completely dry. The nitrogen atmosphere is used to reduce degradation of cortisol due to oxidation through exposure to air. Other groups have effectively employed air drying of the extracts at 4 °C but this method takes longer to achieve complete dryness. It is important to note that air drying of cortisol extracts for LC-MS/MS procedures do not lead to loss of signal or immunoreactivity, and thus nitrogen may not be required. However, no data is available to compare between the efficiency of both methods, something which should be assessed in future research. Samples can then be sealed frozen until measurement until analysis.

Finally, sample residues are re-suspended in 150–250 μL of phosphate buffered saline (PBS, pH 8.0) and vortexed until completely dissolved.

2.7. Measurement of cortisol, ELISA versus LC-MS/MS

Competitive solid-phase enzyme-linked immunosorbent assay (ELISA, also luminescence immunoassay, LIA, and radio immunoassay, RIA) or liquid chromatography-mass spectroscopy (LC-MS/MS) have both been used to measure the concentration of cortisol in hair extracts [21]. The commercially available and commonly used ELISA kits are designed for measurement of cortisol in saliva rather than hair extracts. The reported analytical sensitivities of these kits range between 0.09 and 1.0 ng/ml with variable cross-reactivity to endogenous and exogenous steroids [21].

The use of ELISA has the advantage of low cost, not requiring the use of sophisticated equipment, and analytical capacity for multiple samples in parallel. Results obtained by four of the commonly used commercially available ELISA kits were found to have a strong correlation with the more specific LC-MS/MS method (r^2 ranges from 0.88 to 0.97). Work by Slominski et al. reported a correlation of $r_s = 0.972$ ($p < .0001$) between LC-MS/MS and ELISA with pooled samples [22].

Table 1
Comparison of samples types for cortisol measurement.

Sample	Requirements for samples storage and transportation	Sampling invasiveness	Retrospective cortisol level measurement	Collection method	Time period assessed	Effect of circadian cortisol fluctuation	Dynamic response assessment
Serum	Immediately measured or freezing	Highly invasive	Not possible	Venipuncture	Minutes	Affected	Possible
Saliva	Immediately measured or freezing	May cause discomfort	Not possible	Use of saliva collecting tubes.	Minutes	Affected	Possible
Urine	Immediately measured or freezing	Could be difficult in certain patient populations	Not possible	24 h urine collection	Days	Not affected	Not Possible
Hair	Room temperature/long shelf-life	Painless, Not invasive	Possible	Small samples from the vertex posterior side of the head	Weeks to months	Not affected	Not Possible

However, the agreement between the two methods in terms of absolute values is very low. Russell et al. reported that at low ranges (50–100 ng/ml) ELISA results are 2–3 times higher than the values of LC-MS/MS results, which can be even greater at high values. For this reason, the authors called for use of a correction factor when using immunoassays to calculate LC-MS/MS equivalents and this should be determined for every ELISA kit manufacturer [21]. This work by Russell et al. was completed in 2014, as hair cortisol research analysis has evolved, more research is needed to determine if these differences have transformed.

ELISA methods result in higher absolute values compared to LC-MS/MS likely due to the latter being more specific [21]. In one study, measurements using the two detection methods of pooled hair samples were highly correlated, yet measurement of individual samples showed a low correlation [22]. The authors hypothesized that the amount of protein in extracts may affect the stability of cortisol. Other groups have been able to enhance the detection of cortisol in hair extracts by adding bovine serum albumin (BSA) to the extraction cocktail in order to replicate the matrix for which the assays were developed [23].

ELISA assays are known for having cross-reactivity with other steroids such as cortisone and progesterone. This has the potential to inflate the reported cortisol measurements. However, as discussed by *Slominski* et al. this effect is likely minimal [22]. This study compared ELISA with LC-MS and found that while LC-MS was highly specific, in the opinion of the authors, this method under reported true cortisol levels. LC-MS results in their study were 12% lower than those reported by ELISA. They also looked at the cortisone and progesterone levels and concluded that the lower cortisol levels detected by LC-MS could not be accounted for as a result of cross reactivity of the ELISA test alone. Cross-reactivity of the ELISA test should have only inflated cortisol levels by < 1% [22].

Quantitative hair analysis is known to be more difficult than other matrices due to the solid, heterogeneous solid composition of hair, and insufficient amounts of reference material [24]. In order to ensure quality control in hair analysis, further work should be done to formulate standard practices with regards to sampling, preparation, extraction, reconstitution, and calibration. Calibration and validation between laboratories can be accomplished using the same quality control spiked hair samples. Methods to facilitate heterogeneity and more reproducible results between laboratories should be explored as recommended by the Society of Hair Testing [25].

2.8. Comparison of hair as a matrix to traditional matrices and methods

Circulating cortisol is routinely assessed in saliva, serum and urine, each representative of acute or short-term (i.e. 24 h) cortisol levels. Finger nail clippings have also been proposed as a matrix for tracking cumulative cortisol exposure [26,27], yet no correlation was found between hair and nail cortisol [26]. Importantly, none of these matrices provide information that is representative of the extended period of time that hair is capable of providing. The utility of nail cortisol may be limited by multi-factorial fluctuations in nail growth rates including environmental effects across seasons and climate [28]. Research on nail growth rates has not kept pace with the rate of which nail biomarkers have been used in epidemiological studies [29].

Forensic hair toxicology has been widely utilized in the study of drugs of abuse, pharmaceuticals, and doping agents as well as dietary habits, nutrient levels and exposure to pollutants and toxins [30]. Synthetic glucocorticoids, closely related in structure to cortisol, have also been assayed in hair samples [31–33]. It appears that compounds cannot be assayed in hair if they are metabolized too rapidly to be incorporated into the growing hair, are physiochemically hydrophilic and thus poorly diffusible and/or, are larger than 800 Da [34]. Cortisol and its metabolites are consistently in circulation, are sufficiently hydrophobic and small in size to be incorporated into the hair. Hair uniquely provides the opportunity to assess a long-term biomarker of cortisol exposure.

Traditionally serum, saliva, and urine have been used for the measurement of cortisol to assist with clinical diagnosis. Table 1 compares the traditional forms of cortisol measurement with that of hair. Use of these acute and short-term cortisol biomarkers are currently the standard for screening of endocrinological disorders [5]. Hair cortisol levels appear to be fairly stable over time. Stalder et al. demonstrated strong intra-individual stability of HCC in two clinical studies where hair was first sampled after 1-year, and secondly at 2-month intervals [35]. The results from these time points help to demonstrate the utility of this technique in clinical applications and the estimation of cortisol secretion over long periods of time [35].

2.9. Limitations of hair cortisol analysis

Recent research by the Rotterdam group has shown that natural sunlight and artificial UV radiation can reduce HCC by up to 32% and 50% respectively [20]. Conflicting reports exist regarding the effect, if any, of chemical treatment of hair with dyes and shampoos on HCC [36,37]. A recent study found no association with either frequency of hair washing or hair dying, and cortisol content. The authors noted that the results may be limited due to not differentiating between the types of hair, and that any true effects may have been negated [37]. Similarly, the authors noted that their study only examined the most proximal scalp hair segment. More distally located hair segments may have a more prolonged exposure to the offending agents. Different dyes may have opposing effects resulting in either increases or decreases in cortisol content [36,37]. It is therefore important to consider that the effect of these environmental factors may be greater with increasing distance from the scalp.

Cortisol concentrations are influenced by the frequency of sweating on the scalp and the frequency with which hair is washed. These opposing effects indicate that personal hygiene is a factor worth taking note of when employing this technique [38]. Medications and natural health products that may affect cortisol concentrations are important variables that modulate HCC.

Cultural differences must also be taken into consideration when sampling hair for analysis. Certain cultures practice the act of shielding their skin and hair from the sun, which may result in reduced exposure to UV radiation. Researchers must also be cognisant of cultural practices with regards to the importance of hair for beauty and religious reasons [39]. Hair collection may be frowned upon because of these norms and beliefs [39]. As a result of these culturally based beliefs, hair cortisol data in specific regions of the world, or in specific populations may be lacking or limited to small sample sizes. Developing a positive association with hair sampling within these populations may lead to psychological acceptance of hair sampling, and thus allowing for hair cortisol analysis to be implemented in a more diverse population.

3. Applications of hair cortisol in animals

The utility of hair cortisol has been investigated in a number of animal models with a particular emphasis on behavioral and neurocognitive outcomes from various stressors in wild animals. Importantly, labs with the capacity to measure hair cortisol in human samples, can easily modify their methodology to quantify cortisol in samples from other species. Initial investigation of hair cortisol testing was undertaken in wild male hyrax [40] followed by using hair to capture stress from housing relocation in Rhesus monkeys [41]. Subsequently, hair cortisol was measured in domestic cats and dogs [42], dairy cattle [43], and several species of wild animals [44–47]. More recently, hair cortisol analysis has been utilized to assess stress in the laboratory in non-human primates [17] and rodents (corticosterone) [48,49]. Hair testing in these animals could prove to be invaluable within the developmental and neuroscience realms by providing a long-term & non-invasive measure of stress.

4. Clinical applications of hair cortisol measurement

Assessment of cortisol in hair has recently gained attention as a viable biomarker for diagnosis, prognosis and management of clinical conditions. Studies have focused on Cushing syndrome, the classic example of increased cortisol production, as well as other conditions associated with increased cortisol secretion including severe stress, depression, cardiovascular disease, recent myocardial infraction, Diabetes Mellitus (DM), obesity, and severe chronic pain [50–54]. Cortisol secretion is decreased or absent in Addison's disease, and has also been reported to be decreased in other conditions including chronic pelvic pain, endometriosis, post-traumatic stress disorder (PTSD) and panic disorder [55–58].

4.1. Cushing syndrome

Cushing Syndrome (CS) is characterized by signs and symptoms of exposure to excess glucocorticoids for an extended period of time. For patients with clinical suspicion of CS, the first step is to exclude exogenous causes, usually iatrogenic due to treatment with dexamethasone, prednisone, or other glucocorticoids [59]. The next step is to evaluate if there is indeed endogenous cortisol overproduction. In clinical practice, three tests are currently used for diagnosing CS, these are urine free cortisol (UFC; at least two measurements), late-night salivary cortisol (two measurements), and the 1 mg overnight dexamethasone suppression test (DST) [59–62]. Typically, two concordantly positive tests from two methods are required to diagnose CS. Our group was the first to demonstrate elevated hair cortisol in CS as compared to healthy controls [63]. Since then, several groups have analyzed the performance of the measurement of cortisol in the most proximal hair segment. Using the upper limit of normal for healthy non-obese individuals as cut-off, two HCC cut-off values have been suggested for the diagnosis of CS: Firstly an upper limit of 75.9 ng/g with 86% sensitivity and specificity of 98% (lean individuals) to 93% (individuals with abdominal obesity) [64] (Fig. 2), and secondly, a lower

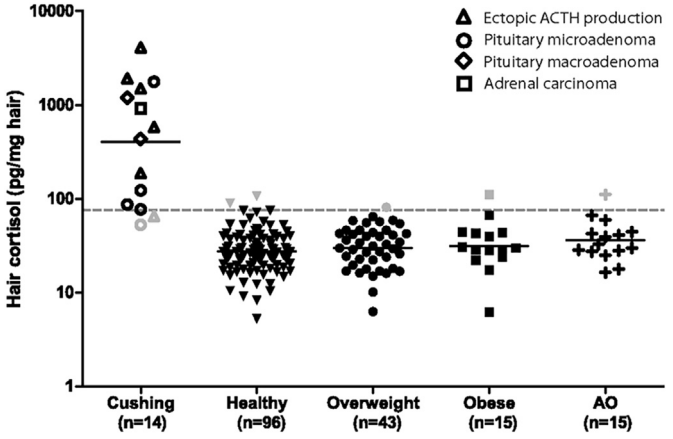

Fig. 2. Hair cortisol levels in patients with CS and healthy overweight and obese controls and individuals with abdominal obesity. The dotted line represents the upper limit of normal hair cortisol levels (75.9 pg/mg hair). The gray symbols represent the individuals with cortisol levels below the upper limit of normal (in case of confirmed CS) or above the upper limit of normal (no CS). The nature of the CS is indicated by the different symbols used for the patients.
From: A Novel Tool in the Diagnosis and Follow-Up of (Cyclic) Cushing's Syndrome: Measurement of Long-Term Cortisol in Scalp Hair. J Clin Endocrinol Metab. 2012;97(10):E1836-E1843. doi:10.1210/jc.2012-1852

cut-off of 31.1 ng/g with 93% sensitivity and 90% specificity [5]. Hodes et al. found higher hair cortisol values in 36 CS patients than in 6 controls, with mean hair cortisol of 266 ± 738.4 and 38.9 ± 25.3 pmol/g in each group, respectively ($P = .003$) [65]. Overall, these results appear to be comparable to commonly used tests.

A unique aspect of hair cortisol measurement is its ability to provide retrospective information about systemic cortisol exposure over months or even years. Several case descriptions show that multiple HCC measurements along the hair shaft provide insight into the timeline of CS development as well as documenting response to treatment [5,63]. Recognizably, this technique has greater utility in patients with longer hair growth as the longer the hair, the more information about past cortisol exposure can be extracted. Absolute values of observed peaks and troughs of HCC in more temporally distant hair segments may be lower than more recent time periods due to the washout discussed above. However, the shape of the curve provides a picture of variable steroidogenesis that can be very useful when used in combination with a patient's clinical history. Clinicians should take washout of cortisol into consideration when making any conclusions about past cortisol levels.

The assessment of cortisol production over time may be particularly useful for evaluation of cyclical CS (CCS). Cyclical CS is a rare condition characterized by recurrent episodes of hypercortisolemia interspersed by episodes of normal or decreased cortisol production [66]. The observed cyclicity can be either regular or irregular in nature, ranging from days to years, making a diagnosis of CCS especially difficult [64]. HCC is able map this fluctuation in cortisol levels over time. A diagnosis of true cyclicity of CS requires evidence of at least 3 peaks and 2 troughs in cortisol levels over time [66]. Fig. 3 demonstrates the cyclical nature of a patient measured in our lab, with a subsequent diagnosis of CCS. Depending on the nature of a patient's condition it may take months to observe these findings with typical laboratory assessments. In fact, a recent study has shown that CS patients have normal cortisol secretion for large periods of time, with one patient having normal late-night salivary cortisol levels in 33/34 samples [67]. HCC has the potential to eliminate weeks to months of daily measurements, allowing for earlier diagnosis and treatment.

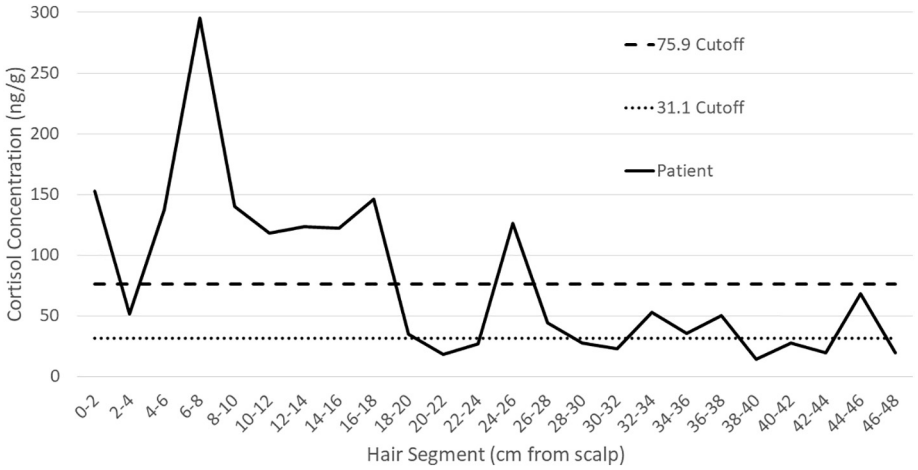

Fig. 3. Hair cortisol analysis of a patient with cyclical Cushing's syndrome with overlapping proposed CS diagnosis cut-off values.

5. Adrenal Insufficiency

Adrenal Insufficiency (AI) is a syndrome resulting from an inadequate ability to produce sufficient cortisol levels. In primary adrenal insufficiency (Addison's Disease) a lack of steroid hormone production is caused by the inability of the adrenal glands themselves to produce enough hormone as a result of genetic factors, hyperplasia or cancer, or autoimmune destruction of the steroid-secreting cells [58]. In secondary and tertiary adrenal insufficiency, reduced steroidogenesis is the result of a lack of signal from the pituitary or hypothalamus to the adrenal cortex to produce more hormone [68]. Adrenal Insufficiency is usually diagnosed by insufficient cortisol response in a short corticotropin stimulation test (250 μg) as the gold standard, or if this isn't possible with low morning serum cortisol and ACTH measurements [69].

For patients with sufficiently long hair, segmental analysis of cortisol production over time provides a unique record of the natural development of auto-immune adrenal insufficiency [70]. Fig. 4 shows the development of AI over a period of more than two years, with the patient gradually progressing from mild chronic AI symptoms toward adrenal crisis, necessitating admission to intensive care. Based on this pattern, hair cortisol measurement may also help in diagnosing AI in patients who have already been started on glucocorticoid treatment, which jeopardizes the ability of saliva and serum cortisol measurements for AI diagnosis.

Further, hair cortisol measurement may be used to monitor treatment of AI with hydrocortisone. Two studies in patients on glucocorticoid replacement therapy demonstrated that HCC was associated with hydrocortisone replacement [71,72]. Interestingly, both studies found that HCC was higher in patients than in controls, and higher in males compared to female patients. Given the association between hair cortisol and BMI, these studies support the notion that glucocorticoid replacement is still resulting in supraphysiologic cortisol levels in a significant number of patients (suboptimal in most patients). Similar results were found in children with congenital adrenal hyperplasia on hydrocortisone replacement who had significantly higher HCC than controls (mean 13.3 vs 8.2 pg/mg, $P = .022$) [73]. However, it is currently not clear if the results of hair cortisol measurements can be used to adjust the dosing of glucocorticoids. Importantly, there is major interindividual variation in HCC between subjects receiving the same hydrocortisone dose. Indeed, there are other factors that can affect glucocorticoid concentrations, including differences in hydrocortisone absorption and metabolism as well as differences in glucocorticoid sensitivity [74]. Unlike other matrices, hair cortisol is thought to incorporate variability in absorption and metabolism because it reflects systemic exposure at the tissue level over longer periods of time.

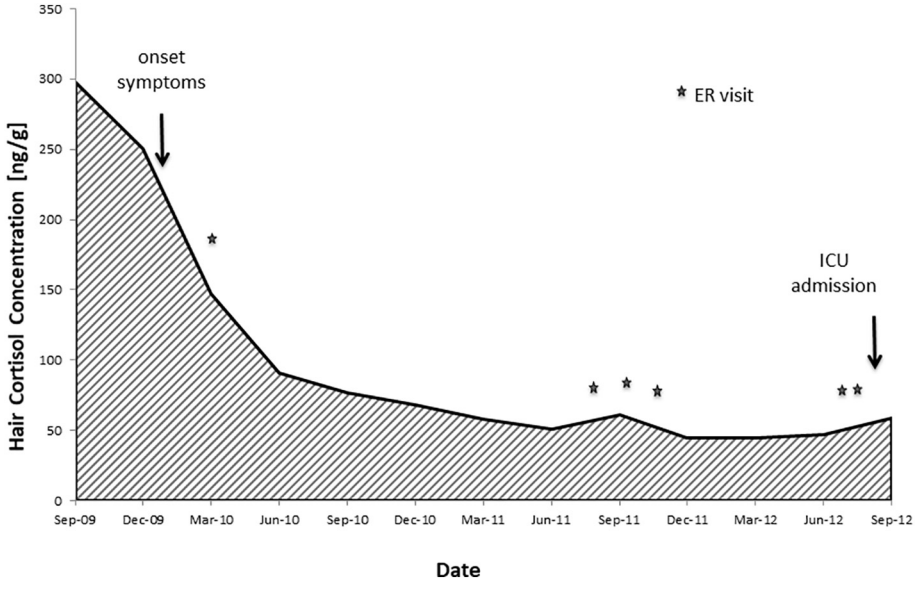

Fig. 4. Hair cortisol concentration in a patient with adrenal Insufficiency over time.
Reprinted from Ibrahim, C. & S. van Uum. Hair analysis of cortisol levels in adrenal insufficiency, Fig. 1. Canadian Medical Association Journal 2014; 186: 1244. © Canadian Medical Association 2014. This work is protected by copyright and the making of this copy was with the permission of the Canadian Medical Association Journal. (www.cmaj.ca) and Access Copyright. Any alteration of its content or further copying in any form whatsoever is strictly prohibited unless otherwise permitted by law.

6. Hair cortisol and cardiovascular disease

High exposure to glucocorticoids is linked to an increase in risk factors for cardiovascular events [75]. Observation of patients with hypercortisolism has shown that obesity, dyslipidemia, glucose intolerance and hypertension can be caused by prolonged exposure to corticosteroids [75]. However, a study on cortisol in serum, salivary, and urine could not show a direct relationship between cortisol levels and cardiovascular diseases [76]. On the other hand, a study using hair cortisol found an association between HCC and cardiovascular events and risk factors [77]. This group found that patients within the highest quartile of HCC had an almost 3-fold greater risk of having had a cardiovascular event in the past, as well as being associated with DM [77]. Further, HCC was found to be higher in patients who suffered recent myocardial infarctions than in patients admitted to the hospital for other reasons [53]. It is important to realize that in this study, the HCC represented cortisol exposure in the two months before the heart attack occurred, suggesting that the higher systemic cortisol levels may have contributed to the development of heart attacks. It is also known that increased cortisol levels are associated with metabolic syndrome indicating a relationship between HCC and cardiometabolic status.

6.1. Hair cortisol analysis as a biomarker of chronic and acute stress

Stress is considered to be a very subjective internal state with many contributing factors, and as such has been difficult to objectively quantify. Measurement of long-term stress has primarily used questionnaires and other self-reporting tools. While often useful, these are limited by their subjective nature and recall bias. This is especially true when working with groups such as children who may be unable to adequately articulate perceived stress to caregivers and researchers [78]. Cortisol has become an important biological correlate of stress. In contrast to questionnaires, the measurement of cortisol in hair results in an objective biomarker to quantify stress.

Hair cortisol analysis is now at the forefront of research addressing the effects of both acute and chronic stress. Endogenous cortisol levels are influenced by a broad range of psychiatric disorders [79]. Stress has been collectively recognized as a premorbid feature associated with several risk factors for numerous chronic disorders [80]. The effect of chronic maternal stress on early child development is one area where hair cortisol can be particularly useful to quantify intrauterine exposure to stress hormones, a factor which is known the effect later development [54]. Hair cortisol has been used as an objective biomarker in studies investigating medical internship [81], the effect of natural disasters [82], and war [83]. Description of all studies on hair cortisol and stress is beyond the scope of this paper. For a more detailed review of stress-related and basic determinants of hair cortisol we refer to the excellent paper by Stalder et al. [4].

7. Mental health

Cortisol plays a role in psychopathology, especially in anxiety and mood disorders. It has also been found that patients with major depression take longer to return to baseline cortisol levels after exposure to stressors [84,85]. However, patients with major depression and coronary artery disease (CAD) did not have significantly ($p = .162$) different HCC levels after rehabilitation period compared to healthy controls [86]. These findings are limited by CAD comorbidity, which is known to increase cortisol levels. In addition, HCC was found to be significantly higher in depressed patients with no major comorbidity compared to healthy controls [87]. Wei et al. measured HCC in 22 first-episodic and 13 recurrent female patients with depression as well as 30 health controls. Only HCC in first-episodic patients was significantly higher compared to healthy controls and cortisol levels did not correlate with Hamilton depression scale or Hamilton anxiety scale scores [88]. In conclusion, altered HPA axis activity in mood disorders can result in increase in chronic levels of cortisol that can be detected through hair cortisol analysis.

Hair cortisol levels do not seem to elevate in patients with bipolar disorders. However, differences have been found in patients experiencing their first episode of depression or mania when older than 30 years, an effect that was not detected by saliva cortisol measurement [64,89]. In this study, the patient population was heterogeneous with other psychiatric co-morbidities, including panic disorders, which might affect cortisol levels to different directions resulting in disguising the true correlation between HCC and each disorder.

Patients with generalized anxiety disorder (GAD) were found to have significantly lower HCC than healthy controls indicating that hypocortisolaemia may be a characteristic of this group of patients (62). Salivary cortisol analysis did not show this difference, which was attributed to acute increase in cortisol levels due to sampling procedure. This clearly demonstrates the advantage of measuring hair cortisol over salivary cortisol.

Many studies assessing acute cortisol levels have not found any correlation between cortisol and PTSD, yet new studies assessing HCC have shown interesting correlations. Luo et al., measured HCC in adolescent females exposed to an earthquake and found that subjects with PTSD had significantly lower HCC than the non-PTSD group 2-, 4-, and 6-months following the earthquake, indicating a blunting of the stress response in PTSD [90]. Another study, found that HCC was decreased in both traumatized PTSD and non-PTSD individuals compared to non-traumatized controls [91]. On the other hand, one study found that individuals with PTSD had higher levels of HCC compared to traumatized-non-PTSD control subjects [92]. These different finding indicate that there may be differential effects on HCC based on the type and frequency of trauma. Similarly, HCC has been used to quantify the psychoneuroendocrinological impacts of war [93] as well as the impact of humanitarian intervention in refugees [83].

8. Post-mortem applications

The utility of hair cortisol analysis extends beyond the use in living populations. One of the major benefits of hair as a biological matrix is that it extends the window of cortisol detection much beyond biological fluids. This technique can be invaluable to anthropological and archeological samples for which no other samples are available [19]. Another potentially useful application would also be in the diagnosis of death by adrenal crisis for which samples were not collected in temporal proximity to the death. In-depth examination of post-mortem hair toxicology is beyond the scope of the current discussion.

9. Discussion and future directions

Hair cortisol analysis provides a perceptive tool permitting the quantification of cortisol levels over time. Work is still needed to optimize and standardize extraction and quantification of cortisol content because of the protocol variations that exist across the globe. Research has shown that complete extraction may not be achieved until the contents of 4 sequential extractions are pooled [22].

The unique capability to capture cortisol exposure in hair lends itself to many potential research opportunities assessing an array of factors that influence the HPA axis in properly controlled studies. However, the same multiplicity of factors that influence the HPA axis also potentially limits the usefulness of this tool. It may be hard to control for all other factors influencing HCC, thus it may be advisable to use this technique in combination with additional assays before drawing any conclusions.

Future research should test the accuracy of ELISA techniques compared with the use of mass spectrometry. Immunoassays provide a relatively easy and inexpensive option for analysis, however this is an indirect measurement based on binding of the assay, whereas MS directly measures the molecule of interest. Prior research comparing the

use of ELISA and MS has highlighted the contrasting differences in both sensitivity and specificity. Techniques need to be developed to better detect hair cortisol using MS to more easily allow for the detection of hair cortisol without the cross reactivity associated with ELISA.

There is little information regarding hair cortisol values in certain populations such as children, where reference ranges are currently non-existent. Reference ranges for children and adolescents undergoing puberty still need to be determined, as we know that during this time endogenous cortisol levels rise from those seen in children to that of adults. More work needs to be done to understand this change as it relates to HCC so that it may be used for interpretation of individual results.

Additional research should address the time from the formation of the hair shaft in the follicle to the eruption of the hair shaft from the skin, which relates individual hair growth rates. There is conflicting research on whether this process ranges from a few days up to 2 weeks [94,95]. What kind of clinical effect this may have is yet to be determined.

10. Conclusion

Hair cortisol analysis is a tool that allows insights for both clinical diagnostic purposes and scientific research, as well as having the potential to play an invaluable role in personalized and preventative medicine. Further studies are required to explore the potential for its use in wider settings.

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