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REVIEW

Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions

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Summary The detrimental effects of stress on human health are being increasingly recognized. There is a critical need for the establishment of a biomarker that accurately measures its intensity and course over time. Such a biomarker would allow monitoring of stress, increase understanding of its pathophysiology and may help identify appropriate and successful management strategies. Whereas saliva and urine cortisol capture real-time levels, hair cortisol analysis presents a complementary means of monitoring stress, capturing systemic cortisol exposure over longer periods of time. This novel approach for cortisol quantification is being increasingly used to identify the effects of stress in a variety of pathological situations, from chronic pain to acute myocardial infarctions. Because of its ability to provide a long-term, month-by-month measure of systemic cortisol exposure, hair cortisol analysis is becoming a useful tool, capable of answering clinical questions that could previously not be answered by other tests. In this paper we review the development, current status, limitations and outstanding questions regarding the use of hair cortisol as a biomarker of chronic stress.

Contents

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1. The role of stress in health

The term *stress* refers to the body's nonspecific adaptive response to try to adapt to a perturbation. The source of the stress, the stressor, may be actual or perceived, and can be psychological or physiological (Selye, 1950). The sum of physiological effort to compensate for the perturbations caused by a stressor is defined as the *allostatic load* (McEwen, 1998). Allostatic load can be quantified through measurement of physical changes such as blood pressure, heart rate, waist-hip-ratio, and body fat percentage, or through biochemical concentrations of various substances, including cortisol, catecholamines, high-density lipoprotein (HDL), total cholesterol:HDL ratio, triglycerides, glycosylated haemoglobin, glucose levels, C-reactive protein, fibrinogen, D-dimer, and tumour-necrosis-factor-alpha (Bellingrath et al., 2008). Initially, the physiologic changes induced by the stress response serve an adaptive role as the body attempts to maintain homeostasis in spite of the stressor, but a sustained increase in allostatic load is associated with a host of deleterious consequences. These may include the development or exacerbation of mental health disorders (Kessler et al., 1985; Brady and Sinha, 2005; Kim et al., 2007; Lee et al., 2010), hypertension (Esler et al., 2008), an increased risk for cardiovascular disease (Appels, 1990; Appels et al., 2000; Dimsdale, 2008), obesity (Vicennati et al., 2009), type 2 diabetes (Pouwer et al., 2010), exacerbation of chronic obstructive pulmonary disease (Andenaes et al., 2004) or asthma (Sandberg et al., 2004), exacerbation of skin conditions such as psoriasis (Malhotra and Mehta, 2008), an increased risk of ulcerative colitis (Mawdsley et al., 2006), reduced fertility (Ebbesen et al., 2009) and poor pregnancy outcome (Latendresse, 2009).

A recent study by the American Psychological Association sought to quantify the extent to which subjective stress is present in North America. The authors interviewed 1134 adults aged 18 and up, and 1136 children between the ages of 8 and 17 from 8 cities across the continental United States. The study included both interviews and self-reported stress (using a Likert grading scale), and found that the majority of Americans have moderate to high levels of stress. The three most commonly cited sources of stress were money, work, and economy-related. Children were more likely to cite money as a significant source of stress. Perhaps most concerning is that almost half (44%) of the participants reported that their stress level had *increased* over the past 5 years (Anderson et al., 2011). In light of the above-mentioned significant sequelae associated with stress, this study underscores the need of finding an objective means of quantifying the degree of chronic stress. Such a biomarker would potentially help facilitate earlier detection, could help identify individuals most at risk for deleterious health outcomes, and hopefully help gauge effective methods to mitigate stress.

2. Lack of a marker for chronic stress

Biomarkers of *acute* stress have been well established and primarily assess catecholamine release. Goldstein's (1995) paper provides a thorough review of the way in which sympathetic responses can be quantified during acute stress. In contrast, finding a ''gold standard'' biomarker for *chronic* stress has proved challenging given its complex etiology and the highly individual manifestations.

Glucocorticoids are commonly used as biomarkers of stress. In humans, non-human primates and many larger mammals cortisol is the most common glucocorticoid, while in other vertebrates including rodents, corticosterone is the primary stress hormone. As there are only very few studies on corticosterone in hair, this paper will focus on cortisol as a biomarker of stress.

During times when an organism undergoes physiologic duress, cortisol acts to mobilize energy stores and modulate the immune system. Despite its well-recognized role in stress in both animals and humans, the ability of cortisol to reflect stress levels over long periods of time has been limited. This is largely due to the nature of the traditional matrices in which cortisol has been sampled. To date, the majority of studies have investigated cortisol responses using samples of serum, saliva, or urine. The most commonly used assays to detect cortisol in these samples are radioimmunoassays (RIAs), liquid chromatography—mass spectrometry (LC—MS/MS) and enzyme-linked immunosorbent assays (ELISA) (Gatti et al., 2009).

Both saliva and serum samples provide a measurement of the cortisol concentration at a single point in time. They can therefore be used to test acute changes, but are subject to major physiological daily fluctuations, making the assessment of overall long-term systemic cortisol exposure difficult. In healthy individuals, plasma cortisol levels peak in the early morning, and gradually decrease thereafter. Hence, a single measurement cannot reflect the integral of systemic

exposure. To help overcome this challenge, most contemporary studies obtain multiple salivary samples from the time of waking until sleep, but this is experimentally complex, the compliance of individual participants with the sampling schedule may vary (Yehuda et al., 2004), and this methodology is difficult to apply to larger populations. In addition, measuring cortisol in serum samples assesses total serum cortisol that includes both protein-bound and bioactive (free) cortisol. Consequently, total serum cortisol is affected by changes in levels of cortisol-binding globulin (e.g. by birth control pills or pregnancy) that can result in increases in total cortisol concentration measured, even though there is no increase in stress or free cortisol concentrations. In addition, the act of obtaining a sample via venipuncture could by itself be a source of stress and increase cortisol (Vining et al., 1983). Salivary cortisol concentrations correlate well with serum concentrations (Vining et al., 1983; Aardal and Holm, 1995). In contrast to serum cortisol, salivary cortisol reflects free (unbound) cortisol and is collected by a less-invasive method. However, salivary cortisol concentrations still fluctuate significantly throughout the course of the day. A similar strategy is employed when urine is used; 24-h urine collections provide an integral of the free cortisol concentrations through the day, thus overcoming the issue of its diurnal rhythm (Burch, 1982). However, the collection is labor intensive for participants, and cannot be used in cases of chronic renal failure or dialysis.

3. The development of cortisol detection in hair

Hair analysis has been used for decades to monitor exposure to exogenous compounds, with particular emphasis on detecting drugs of abuse (Gaillard et al., 1999). Because head hair grows at an average of 1 cm/month, assessment of drugs in hair can reflect changes in drug exposure over time (Wennig, 2000). More recently there has been a growing interest in quantifying *endogenously* produced compounds such as cortisol in hair (Gow et al., 2010).

One of the earliest studies examining whether cortisol could be detected in hair was performed by Koren et al. (2002) using hair from wild hyraxes (small herbivorous mammals). In this study, the hyraxes were briefly captured so that 7—20 mg of hair could be plucked. Cortisol was then extracted from the hair using methanol, followed by analysis on a modified salivary ELISA. Cortisol was found in detectable ranges in the hair, and a significant positive correlation was found between hair cortisol concentration and social ranking of the hyraxes. A follow-up study used hair cortisol analysis to support the 'stress of dominance' hypothesis. In the most dominant group of hyraxes, higher hair cortisol concentrations were predictive of greater social dominance (Koren et al., 2008).

Sauve et al. (2007) were the first to use an ELISA protocol similar to the one used by Koren et al. in 39 non-obese human subjects. Hair cortisol concentrations were not normally distributed, but after log transformation a reference range of 1.7—153.2 pg/mg was established, with a median concentration of 46.1 pg/mg. The hair cortisol concentrations obtained were compared against those obtained from saliva, serum, and 24-h urine collections. A positive correlation was observed between cortisol concentrations in hair and in 24-h urine ($r = 0.333$, $P < 0.04$), but no significant correlation was found when comparing cortisol in hair with salivary or serum cortisol. The authors postulated that this lack of correlation was due to the differences in time period assessed by the various matrices: saliva and serum provide information on brief point measures, whereas the total 24-h urine collection provided an integral of production more close to the integral of production that hair analysis captured. Additionally, this study reported that sampling from the vertex posterior region of the scalp proved optimal because it had the lowest intra-individual coefficient of variation for cortisol concentration. In rhesus macaques, in contrast, there was a significant correlation between the cortisol concentration in saliva and that in hair ($r = 0.797$, $P < 0.001$) (Davenport et al., 2006). Bennett and Hayssen (2010) measured hair cortisol in dogs, and found that it was significantly correlated with cortisol in saliva ($r = 0.48$; $P < 0.001$).

Cirimele et al. (2000) were the first to examine whether glucocorticoids could be detected in human hair. Hair samples were taken from a deceased man who had been receiving prednisone for sarcoidosis, patients receiving prednisone after kidney transplantation, and patients receiving beclomethazone for asthma. The glucocorticoids were extracted with incubation in a Sorenson buffer. Using high performance liquid chromatography—ion spray mass spectrometry, a total of ten glucocorticoids, including cortisol and cortisone, were detected in hair. A follow-up study by Raul et al. (2004) showed that in hair samples of 44 subjects a mean hair cortisol concentration of 18 pg/mg, ranging from 5 to 91 pg/mg, could be detected. Cortisone was also quantified in this study, and, interestingly, cortisone concentrations in hair were higher than cortisol concentrations, unlike plasma in which concentrations of cortisol are significantly higher than of cortisone. It was postulated that this might be due to increased activity of 11β-hydroxysteroid dehydrogenase type 2 (the enzyme responsible for converting cortisol to inactive cortisone) in the hair bulb (Tiganescu et al., 2011).

4. Advantages of hair cortisol analysis

There are various advantages to using cortisol in hair as a biomarker of chronic stress. Hair has a fairly predictable growth rate of approximately 1 cm/month. Therefore the most proximal 1 cm segment to the scalp approximates the last month's cortisol production, the second most proximal 1 cm segment approximates the production during the month before that and so on (Wennig, 2000). This phenomenon enables researchers to retrospectively examine cortisol production at the times when a stressor was most salient, without needing to take a sample right at that time. Alternatively, it can provide a baseline cortisol assessment for a time period during which the stress had not yet occurred. This was demonstrated in a study in rhesus macaques in which hair samples for cortisol were obtained both at baseline and after a major stressful event (relocation to a new habitat) (Davenport et al., 2006). The sample can be collected non-invasively by simply cutting a ~1 cm diameter sample of hair at the base of the vertex posterior of the head. This eliminates the risk that the sampling itself may have an impact upon cortisol production. Furthermore, as each centimeter

Table 1 A comparison of properties of the various matrices for cortisol measurement.

Property	Serum	Saliva	Urine	Hair
Subjective level of invasiveness associated with sample collection	High	Low	Moderate	Low
Cortisol affected by stress of sampling procedure?	Possibly	Possibly	Possibly	No
Storage requirements	Spinning and refrigeration followed by freezing	Refrigeration or freezing	Refrigeration or freezing	Room temperature; stable for years
Time periods of cortisol production represented	Single point measure	Single point measure	12—24 h; integral of exposure	Months to years; integral of exposure
Affected by changes in cortisol binding globulin?	Yes; total cortisol measured	No; only free cortisol measured	No; only free cortisol measured	No; only free cortisol measured
Clinically relevant reference ranges established?	Yes	Yes	Yes	No

sample represents approximately 1 month's worth of cortisol production, the issue of intra- and inter-day cortisol fluctuations is mitigated. Finally, unlike the bodily fluids that require special storage conditions prior to analysis, hair samples are easily transported and stored in envelopes or vials at room temperature (Gow et al., 2010). A summary of the different properties of existing matrices for cortisol measurement is presented in Table 1.

Another matrix that may be capable of providing cumulative cortisol exposure is fingernails. Recently a pilot study was performed to determine whether cortisol and dehydroepiandrosterone (DHEA) could be detected in fingernails (Warnock et al., 2010). Using a methanol extraction and ELISA, both cortisol and DHEA were detected in fingernails from 33 university students. During times of exam stress the ratio of cortisol over DHEA was found to be significantly increased compared to baseline levels obtained at the beginning of the school year. One of the limitations that the authors noted was how fingernail growth is known to change depending on environmental factors (e.g. seasonal changes) and differences in personal behaviour (e.g. nail-biting habits). Thus, controlling for such variables would be important for fingernail cortisol concentrations to accurately reflect time periods of interest. Another study used a Sorenson buffer extraction and ultra-performance mass spectrometry analysis to investigate whether cortisol, cortisone, and DHEA could be detected in fingernails (Ben Khelil et al., 2011). The median cortisol concentration was 69.5 pg/mg (36—158 pg/mg) and the median cortisone concentration was 65 pg/mg (32—133 pg/mg). Further studies are required to validate fingernails as a reliable matrix, but they may present an alternative for cumulative cortisol measurement when hair analysis is not possible, such as in cases of balding or cultural objections against hair sample collection.

5. Hair cortisol analysis

Overall, the methods used for measurement of cortisol in hair are very similar, with some variations in procedures amongst laboratories. Briefly, to extract cortisol from hair, the sample is carefully sectioned into segment lengths that will approximate the time period of interest (e.g. the most proximal 3 cm for the last 3 months of cortisol production). Then, the hair is finely minced with scissors or ground with a ball mill, and incubated in a solvent such as methanol. The resulting solution is evaporated to dryness, and then reconstituted in a solution such as phosphate buffered saline (Sauve et al., 2007). Following the extraction, ELISA, RIA, or LC—MS/MS have all been used for cortisol quantification (Gow et al., 2010). Presently, there can be significant interassay variability in the commercially available immunoassays. The immediate implication is that researchers in this field must try to perform all tests of a particular protocol using the same batch of cortisol immunoassay, using internal positive controls as standards, and preferably using assays that have low interassay variability.

6. Emergence of hair cortisol analysis to detect clinical or stress-mediated changes in cortisol

6.1. Observational studies

Kirschbaum et al. (2009) investigated if the well-established increase in cortisol production in the third trimester of pregnancy could be detected using hair cortisol analysis. Mothers who had recently given birth provided hair samples that were divided into section lengths corresponding to their first, second, and third trimesters. These samples were then paired against nulliparous women and the hair cortisol concentrations were compared. The cortisol concentrations in the section of hair corresponding to the third trimester were significantly higher when compared to earlier trimesters and when compared to non-pregnant controls ($t(1,120) = 4.77$; $P < 0.001$). While this study was appropriately designed to assess the effect of pregnancy on hair cortisol content, an effect of repeated hair washings on hair cortisol along the hair shaft (when comparing sections obtained from a single hair sample) cannot be ruled out. This limitation was not present in the study by D'Anna-Hernandez et al. (2011) who took hair samples at the end of each trimester of pregnancy and compared hair cortisol changes throughout pregnancy and the postpartum period. Hair cortisol concentrations were found to be significantly elevated in the third trimester

relative to the first trimester ($t = 4.1$; $P < 0.001$) and decreased again in the postpartum period ($t = 2.9$; $P = 0.004$).

Stalder et al. (2010) investigated hair cortisol concentrations in alcohol-dependent subjects in acute withdrawal compared to long-term abstinent alcoholics and a control group of matched non-alcoholic subjects. The study rationale was that alcoholics in acute withdrawal have been documented to be hypercortisolemic. Indeed, the samples from the alcoholics in acute withdrawal revealed significantly higher cortisol concentrations when compared to both the abstinent alcoholics and the controls (51.99 ± 43.30 vs. 13.98 ± 10.63, 16.55 ± 12.59 pg/mg, respectively; $P < 0.001$).

Finally, hair cortisol content was also significantly increased in patients with Cushing's syndrome, a condition characterized by the endogenous overproduction of cortisol (Thomson et al., 2010). Similarly, Manenschijn et al. (2011b) also found significantly elevated hair cortisol concentrations in a group of 9 patients with Cushing's syndrome as compared to 195 non-Cushing's controls ($P < 0.0001$). In aggregate, these studies support the notion that cortisol in hair provides a reflection of long-term systemic cortisol exposure.

6.2. Intervention studies

The first experimental animal study that demonstrated that hair cortisol can change following an intervention was published by Davenport et al. (2006). They studied rhesus macaques that had recently undergone relocation from their original housing environment, and were displaying behavioural characteristics of increased stress. Three hair samples were obtained: one representing the 13 weeks before the relocation, one representing the 14 weeks immediately after relocation, and one taken a year after the relocation. A significant increase in cortisol concentration was observed in the 14-week post-relocation samples compared to baseline levels (129.6 ± 15.5 vs. 81.1 ± 7.5 pg/mg, respectively; $P < 0.001$), and 1 year following the re-location the cortisol levels approximated the pre-relocation levels again. These results documented not only that stress is associated with increased hair cortisol concentrations, but also that hair cortisol content responds dynamically to changes in cortisol over time.

Two recent studies in rhesus macaques studied the effect of maternal separation on hair cortisol levels of the infants. In the first study, the macaques were reared from birth onwards by either their mothers and peers, just their peers, or by surrogate peers. At 8 months of age, all macaque groups were placed in a large common social environment. Compared to the other two groups, macaques that were raised by their mothers exhibited reduced anxious behaviour following relocation and had lower hair cortisol both before and after the placement in the large social environment. In infants raised by peers only, hair cortisol before separation was positively correlated with later composite anxiety (Dettmer et al., 2011). In the second study, peer-raised infant macaques had *lower* hair cortisol (measured at 18 month age) than those raised by their mothers. The peer-raised macaques demonstrated abnormal stress-response and decreased socialization behaviour at 3 years of age (Feng et al., 2011). Thus, the direction of the HPA axis activity (hyper- or hyposecretion) may vary in relation to age, anxiety and social situation.

Of note, short-term increases of cortisol following brief stressors cannot be detected by measurement of cortisol in hair. This is illustrated by a recent study in caribou and reindeer that received a single injection of ACTH. This did not affect hair cortisol, suggesting that hair cortisol is not sensitive enough to detect minor or short-lived alterations of the HPA axis (Ashley et al., 2011).

Laudenslager et al. (2011) investigated the relation between hair cortisol and novelty seeking behaviour. They used a standardized test to determine the response of female vervets to a potentially dangerous foreign object. This generated a Novelty Seeking Score, with higher scores indicating a greater tendency to explore the object. Next, novelty-seeking behaviour was correlated with hair cortisol as a tool to assess long-term hypothalamic pituitary adrenal (HPA) axis activity. Vervets with low hair cortisol concentrations had significantly higher Novelty Seeking Scores than vervets with high cortisol concentrations ($P < 0.01$). These results suggest that a dampened HPA response may be associated with more bold behaviour and that consistently elevated HPA activity may inhibit the ability or willingness to examine novel challenging circumstances. This study demonstrates the ability of hair cortisol analysis to be used as a tool to expand the animal paradigm of human novelty seeking behaviour.

Work on the same vervets has been continued by Fairbanks et al. (2011) examining the heritability of HPA activity in response to a stressor. The 226 female vervets used were living in a colony of 16 multigeneration, matrilineal social groups. Hair cortisol analysis was used to measure cortisol concentrations before and after relocation to another housing facility across the country. This process was thought to be stress-inducing because of the required multiple anesthetizations, transportation, and interaction with unfamiliar staff. Pre-move hair samples were collected to establish a baseline, and post-move hair samples were taken 25–29 weeks after the relocation, a study design similar to the rhesus macaque study by Davenport et al. (2006). The added facet of the vervets study was that the pedigree of individual vervets was delineated with the use of microsatellite markers to determine paternal and maternal contributors to each vervet's DNA. As expected, mean cortisol concentrations were significantly elevated post-move (27% increase; $P < 0.001$). Additionally, when examining genetic influences in hair cortisol levels, there was a significant concordance in both the baseline ($h^2 = 0.13$; $P < 0.001$) and the post-move ($h^2 = 0.13$; $P < 0.001$) cortisol concentrations amongst vervets with similar genetic complements (Fairbanks et al., 2011). This study validated results from previous studies indicating the stress of moving and more importantly demonstrated a genetic influence governing hair cortisol concentrations. This may prompt human studies to determine the extent to which hair cortisol concentrations are governed by genetics, independent of environmental factors.

The ability of hair to effectively detect changes in cortisol concentrations has been convincingly demonstrated in several proof-of-concept human studies. One such study by Thomson et al. (2010) involved patients with Cushing's syndrome. In this study, hair samples were obtained from patients at the time of first presentation in the clinic. When

analyzed month by month, a steady increase in cortisol concentration was observed up until the point of presentation, consistent with clinical symptoms of increasing cortisol exposure. Following a successful surgical intervention to correct the condition another hair sample was obtained, and the cortisol content of those samples was significantly reduced. This was corroborated by Manenschijn et al., who took hair samples during the clinical course of a patient with Cushing's disease (hypercortisolism caused by a pituitary ACTH producing adenoma). Hair cortisol concentrations were elevated initially, and showed a marked decline following a corrective surgery (Manenschijn et al., 2011b).

7. Methodological challenges in hair cortisol analysis

From its inception as a tool to monitor stress and cortisol concentrations there have been some persistent questions about the nature of hair cortisol analysis and the underlying (patho-)physiology.

A frequently raised question is the mechanism by which cortisol enters the hair. Several mechanisms have been proposed (see Fig. 1).

The most commonly suggested hypothesis is based upon the complex multi-compartment model that has been used to explain drug incorporation in hair (Boumba et al., 2006). Cortisol is thought to enter hair primarily at the level of the medulla of the hair shaft via passive diffusion from blood. In this scenario hair cortisol would be hypothesized to reflect the integrated free cortisol fraction rather than the total cortisol concentration in serum. Additional cortisol may coat the outer cuticle from sebaceous and eccrine secretions (Pragst and Balikova, 2006; Raul et al., 2004). However, to date no studies have been conducted to confirm that cortisol is present in sebum or sweat.

There has been some discussion as to whether the cortisol found in hair is representative of systemic concentrations. Most authors assume that hair cortisol content is representative of systemic levels. However, local cortisol production may participate as well, particularly as Ito et al. (2005) demonstrated that hair follicles contain a functional equivalent of the HPA axis and can synthesize cortisol after stimulation by corticotrophin-releasing hormone (CRH). This was supported by a study by Sharpley et al. (2009) in which three subjects were subjected to a cold pressor test (CPT), in which their hand was immersed into a container of ice water for 1 min. Six minutes prior to the test, baseline levels were obtained by taking a saliva sample (meant to represent central HPA activity), a hair sample from the wrist which was going to be immersed (meant to represent peripheral HPA activity), and a hair sample from the opposite leg (meant to represent the control for peripheral HPA activity). Immediately following the test, samples were collected again from the aforementioned areas, and 6 additional samples were collected from each participant over the following 30 min. Of interest, the hair cortisol concentrations for the wrist that underwent the CPT were markedly elevated from the baseline immediately following the CPT in each of the 3 participants. These concentrations then proceeded to decrease over the course of the next 30 min. No changes were observed in the hair samples taken from the opposite leg which did not experience the CPT. Salivary cortisol levels did not appear to correlate with the hair measures. The authors postulated that this may demonstrate a peripheral HPA activity influencing hair cortisol levels in a transitory way, independent of central HPA activity. A caveat for this study is that a control experiment assessing the effect of non-stressing (e.g. room or body temperature) immersion in water was lacking. Additionally, the hair examined was from the participants' arms, and compared it with hair from their legs—hair from these locations is differently regulated than scalp hair. Further, the number of participants was small and the experiment still requires confirmation. Very high hair cortisol concentrations were found in a patient receiving treatment with high dose hydrocortisone (Thomson et al., 2010). In this

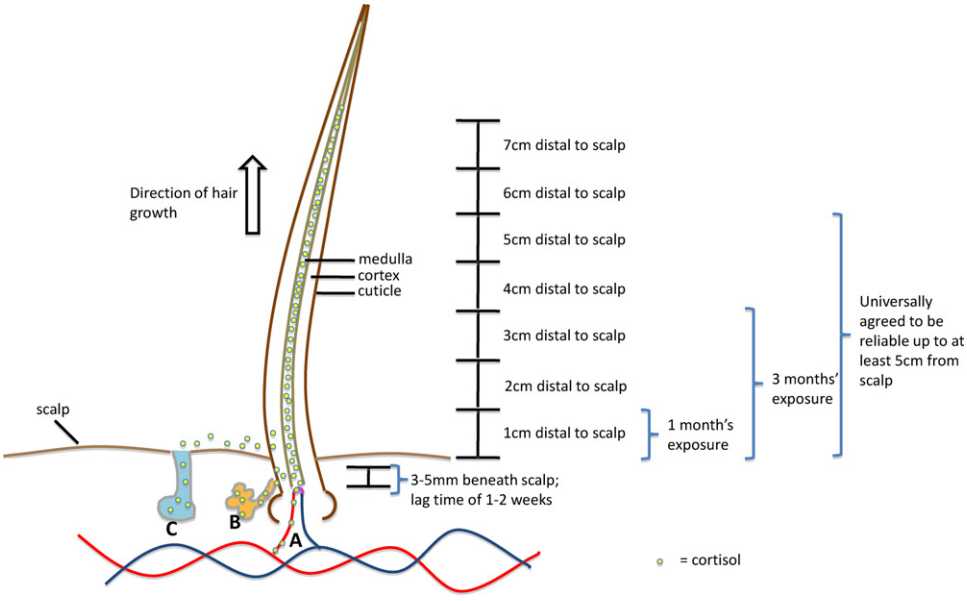

Figure 1 Proposed mechanisms for incorporation of cortisol into hair via blood (A), sebum (B), and sweat (C).

clinical scenario the CRH production is suppressed so that the hair bulb HPA unit would not be stimulated by CRH and thus produce none or very little cortisol. Finally, recent data from a patient with primary adrenal insufficiency, with elevated ACTH, showed low hair cortisol levels, supporting the evidence that cortisol levels detected in hair are primarily from central HPA activity (Manenschijn et al., 2011b).

The effect of natural hair colour or cosmetic treatments on hair cortisol concentrations is another frequently debated topic. With respect to natural hair colour, neither Sauve et al. nor Manenschijn et al. have detected significant differences in cortisol levels due to natural colouration (Sauve et al., 2007; Manenschijn et al., 2011b). In dogs, Bennett and Hayssen (2010) found that hair pigmentation has a significant impact on cortisol concentration, with black (eumelanin), agouti (mixed melanin content), and yellow (pheomelanin) hairs showing an ascending hair cortisol concentration ($r = 0.47$; $P = 0.001$). This was the first study to suggest that melanin content could impact how cortisol is sequestered into hair. Sauve et al. (2007) demonstrated that cosmetic treatments such as hair dying significantly decrease hair cortisol levels relative to controls ($P < 0.036$). A similar trend that failed to reach significance was noted by Manenschijn et al. (2011b). A possible mechanism for this decrease could be that cosmetic treatments such as bleaching may increase the porosity of hair, allowing more cortisol to be leached out (Boumba et al., 2006), or that they add weight to the hair causing a dilution-like effect.

Wildlife studies using point measures of cortisol to examine the relationship between basal cortisol concentrations and organic pollutants have frequently suffered from the confounding effect of the stress *induced* by the chase to anesthetize the animal. As hair cortisol measurement can potentially overcome this confounder, two studies measured hair cortisol in bears to determine factors that affect hair cortisol content. Hair cortisol was found in both studies, and isopropanol washes eliminated any contribution from contamination. These studies did not find any affect of age or hair colour on hair cortisol in bears. In grizzly bears, hair cortisol content was affected by body hair type (with lowest hair cortisol variability for guard hair), body region and capture method (Macbeth et al., 2010). Interestingly, female polar bears had higher hair cortisol than male polar bears (Bechshoft et al., 2011), while in grizzly bears such a difference was not found. This may perhaps be explained by a difference in pregnancy states, as hair cortisol in humans and primates increases with pregnancy, but information on pregnancy state was not provided in these studies.

Since personal hygiene is quite variable from person to person, it is important to know what effect, if any, frequency of hair washing has on hair cortisol concentrations. In rhesus macaques, Hamel et al. (2011) have shown that repeated washing with either shampoo or water decreased hair cortisol concentration. Hair samples from 20 different macaques were collected, and hair shavings were divided into 4 different pools of hair with hair from 5 different macaques contributing to each pool. Hair samples from each pool were placed in separate test tubes and subjected to 20 water washes and 10, 20, and 30 shampoo washes with a 10% shampoo solution. A wash consisted of a 10 ml addition of the solution, inversion for 45 s, and decanting. For the test tubes with shampoo additional water washes were used to remove any residual shampoo. Upon analysis, non-washed control samples had significantly more cortisol than any of the wash treatments in all 4 pools ($P < 0.001$). Additionally, all hair that had been washed with shampoo 30 times had significantly less cortisol than that which had been only washed 10 times ($P < 0.005$). This finding implies that information on the frequency of hair washing should be collected when conducting hair cortisol analysis studies. A follow-up study to replicate these results in humans would be prudent, since macaques and humans have very different baseline levels of hygiene.

Though the mean hair growth rate averages about 1 cm/month, variations in hair growth profile do exist, and may in certain circumstances need to be accounted for. Hair growth varies on different regions of the scalp, but consistent sampling from the vertex posterior should overcome intra-scalp differences. When sampling from different populations, taking note of the ethnic background will be important, as African, Asian, and Caucasian individuals have different hair growth rates (288 ± 51, 421 ± 53, 371 ± 59 μm/day, respectively) (Loussouarn et al., 2005). In addition, it is possible that clinical conditions affect hair growth rate, as suggested by a study in Angora goats in whom hyperthyroidism resulted in increased mohair fiber growth (Puchala et al., 2001).

Maybe the most contentious methodological debate surrounding hair cortisol analysis pertains to whether cortisol concentrations remain constant along the length of the hair shaft. Kirschbaum et al. (2009) examined hair cortisol concentrations along the hair shafts of 9 nulliparous women. Hair samples of at least 18 cm length were collected from each woman and divided into six 3 cm sections. In the first segments, cortisol concentrations decreased continuously ($P < 0.0001$), 30–40% each time, reaching an asymptotic-like level in the final two most distal segments. The authors suggested that this decrease was likely due to a leaching effect, where more distal hair segments had experienced greater environmental damage, compromising hair integrity. Thus, they suggested using only the most proximal 1–6 cm of hair can reliably estimate systemic cortisol concentrations. Gao et al. (2010) found similar results with segmental analysis of 5 subjects. The first five 1 cm segments experienced an average linear hair cortisol decline of -2.7 ± 0.3 pg/cm ($\beta = -0.98$; $P < 0.01$). In contrast, several studies have contradicted these results. Thomson et al. (2010) recruited 9 healthy control subjects with hair lengths ranging from 10 to 14 cm, and could not confirm the previous studies. Hair samples were segmented into 1 cm sections and of the 9 healthy controls, 8 had no significant differences in cortisol concentration along the length of the shaft (mean for all sections 147 ± 46 ng/g); one participant did demonstrate a significant decrease ($P < 0.05$) over time. Similarly, Manenschijn et al. (2011b) could not show time-dependent changes. A group of 28 women provided hair samples at least 18 cm in length, and these were then segmented into six 3 cm sections. No significant differences in hair cortisol concentration were observed in consecutive segments ($P = 0.249$). Adding to the argument that cortisol does not naturally vary in distribution along the length of the hair shaft, both a study in rhesus macaques (Davenport et al., 2006) and a study in dogs (Bennett and Hayssen, 2010) found no significant differences between the most proximal half and the most distal half of the hair shaft. However, these animal studies may not

necessarily translate to the human condition, as the hair length and the hygiene in the animals may be considerably different from humans. The dog hair length of 2.3—4.3 cm is shorter than in many human samples, and the argument surrounding a significant decrease in hair cortisol concentration is most often applied to segments beyond 6 cm distal from the scalp. While further studies are needed to resolve this controversy there is a wide consensus that the first 5—6 cm of hair away from a person's scalp can reliably reflect HPA activity.

Finally, the question of stability of cortisol over longer durations of time has also arisen. This question was effectively addressed by a study performed by Webb et al. (2011) who obtained hair samples ranging in length from 6 to 21 cm from 10 ancient Peruvian mummies, dating from AD550 to 1532. Segmental analysis was performed on the hair samples provided, and while variation was observed from segment to segment, proximal and distal segments were not significantly different. The mean cortisol concentration determined was 281 ± 35 ng/g, similar to those measured by the same laboratory in healthy volunteers today, demonstrating that cortisol can even be extracted from hair over thousands of years old.

8. Hair cortisol analysis in clinical sources of stress

While hair is still a relatively new means of measuring cortisol, several human studies have showed its ability to identify important pathophysiological sources of stress.

Kalra et al. were the first to correlate cortisol levels in hair with self-reported stress using the *Perceived Stress Scale* (PSS), a validated self-report questionnaire of an individual's stress level over the past month (Cohen et al., 1983). In that study, 25 healthy pregnant women were assessed around the time of late first trimester to early second trimester (Kalra et al., 2007). They provided a hair sample representing the last month's worth of cortisol production and also filled out a PSS. There was a significant correlation between hair cortisol concentration and PSS score ($r_s = 0.47$; $P < 0.05$). The concordance between cortisol as a measure of stress and a self-report measure indicated that hair cortisol analysis might provide a good assessment of an individual's chronic stress level.

Kramer et al. (2009) sought to examine whether higher cortisol concentrations might predict spontaneous pre-term birth. Levels of maternal CRH are reportedly elevated in such cases, so it could be expected that cortisol concentrations in the hair might be found in higher concentrations as well. The case group consisted of 207 women who spontaneously began labor prior to 37 weeks in their pregnancy, and the control group consisted of 444 women who initiated labor at term. Hair samples were obtained (most proximal 9 cm to the scalp), a PSS was completed, and a pregnancy-related anxiety assessment was performed. Hair cortisol concentrations of the case and control groups were not significantly different, nor were hair cortisol concentrations significantly associated with PSS scores or anxiety assessments. This study demonstrates how in some instances hair cortisol analysis on its own may not be able to detect stress in cases where the etiology of prematurity is multifactorial and where every woman experiences elevated cortisol production in late pregnancy, irrespective of stress.

A stay in the neonatal intensive care unit (NICU) is thought to be stressful in infants because they endure a series of diagnostic and/or therapeutic procedures that are often painful and highly stressful. To examine whether this stress could be quantified, Yamada et al. (2007) compared hair cortisol concentrations of infants requiring a stay in a NICU with those of healthy infants born at term. The cortisol concentrations of the NICU group were significantly higher than the control (2.06 ± 2.05 vs. 0.11 ± 0.42 nmol/g, respectively; $P = 0.004$). Of note, the infants in the NICU group were born at younger gestational age than the control group, which could create a potential confounding effect.

Another study looking to capture the stress effects of chronic pain using hair analysis was performed by Van Uum et al. (2008) who recruited adult patients who were using opioids to help control severe chronic non-cancer pain and compared them to non-obese controls. A hair sample was obtained in both groups, and the PSS questionnaire completed. The patients with chronic pain had significantly higher mean hair cortisol concentrations than the controls (83.1 vs. 46.1 pg/mg) and their PSS scores indicated significantly more stress relative to the controls as well ($P < 0.001$). This lends powerful support to the notion that hair cortisol concentrations can provide an indication as to an individual's perceived stress level.

Recently, Pereg et al. (2011) investigated the role of chronic stress as measured by hair cortisol, in the development of an acute myocardial infarction (AMI). As chronic psychosocial stressors (e.g. financial concerns, marital stress, job stress) are frequently listed as risk factors for AMIs, the authors hypothesized that hair cortisol analysis could potentially be a useful tool to quantify these stressors. Hair samples representing the past 3 months of cortisol production were obtained from patients within 2 days of admission to a hospital for chest pain. The study group consisted of 56 patients who had a confirmed AMI, with a control group consisting of 56 patients in whom chest pain was attributed to other causes. Median cortisol concentration of the AMI group was significantly higher than that of the control group (295.3 vs. 224.9 ng/g; $P = 0.006$). In logistic regression, accounting for age, lipid status, smoking, and other predictors, hair cortisol was the strongest predictor of AMI, followed by BMI. It should be emphasized that the hair cortisol measurement reflected the 3 months *before* the heart attack, and not the stress caused by the heart attack. Thus, this suggests that chronic stress plays a causative role in the pathophysiology of AMI. This type of information cannot usually be obtained using other matrices, except as part of a prospective study, as illustrated by the predictive effect of urinary cortisol excretion on cardiovascular morbidity and mortality (Vogelzangs et al., 2010).

Hair cortisol analysis was also used by Dowlati et al. (2010) to assess its potential to predict depressive symptoms in patients suffering from coronary artery disease (CAD), a population far more at risk of depression. Depressive symptoms were demonstrated in 34 of the 121 patients with CAD. The most proximal 3 cm of hair were obtained from each patient and hair cortisol concentrations were determined. When comparing depressed and non-depressed CAD patients, no significant difference in hair cortisol concentration was observed between these two groups. It was postulated that the general psychosocial stress associated

with CAD, irrespective of the presence of depression, may have masked any actual differences in cortisol production between these two groups.

Hair cortisol analysis has been used to determine to what extent chronic stress is related to the nasal carriage of *Staphylococcus aureus*, a common bacterial infection. Manenschijn et al. (2011a) predicted that since higher cortisol levels are associated with immunosuppression, the long-term carriers of *S. aureus* would likely have the highest hair cortisol levels. Of 72 healthy subjects, 38 were non-carriers, 10 were intermittent carriers, and 24 were long-term carriers of *S. aureus*. An analysis of hair segments representing the last 3 months of cortisol exposure, no significant differences were detected amongst the 3 groups of carriers ($F_{(2,66)} = 0.425$; $P = 0.638$). The authors concluded that higher cortisol levels might not play a significant role in the degree of *S. aureus* colonization.

Van Rossum et al. (2011) employed hair cortisol analysis to investigate patients with bipolar disorder, a condition in which HPA dysregulation may play an etiological role. The most proximal 3 cm of hair was collected from 100 bipolar patients and 195 healthy controls and hair cortisol concentrations were determined. There were no significant differences between bipolar patients and healthy controls (31.84 pg/mg vs. 28.18 pg/mg; $P = 0.23$). However, when the bipolar group was split into subgroups in which onset occurred before or after the age of 30, significant differences were noted. Patients with bipolar disorder diagnosed after 30 had significantly higher cortisol concentrations than those diagnosed before 30 or the healthy controls ($P = 0.004$). This led the researchers to suggest that HPA dysregulation may be relevant in older onset patients, and this maybe represent a different disease entity altogether.

9. Hair cortisol analysis for monitoring of glucocorticoid replacement therapy

In addition to its role in monitoring stress, hair cortisol analysis is being explored as a means of monitoring the treatment of patients with adrenal insufficiency (AI). Patients with AI suffer from inadequate cortisol production, either due to disease of the adrenal gland itself ('primary') or due to an inadequate production of adrenocorticotropic hormone (ACTH), the hormone secreted from the pituitary gland stimulating adrenal cortisol secretion. Since patients do not produce sufficient glucocorticoid to sustain normal homeostasis, a replacement therapy, often hydrocortisone, is required to ensure physiological well-being and survival. Because there are currently no effective measures to monitor long-term glucocorticoid exposure in these patients, Gow et al. (2011) examined whether hair cortisol analysis could fill this void. A total of 93 patients with AI were recruited, and their partners were used as controls. Median hair cortisol concentrations were increased in AI patients (230.7 [22.7—1377] ng/g) as compared to controls (184.7 [57.7—14,790] ng/g), but the difference failed to reach statistical significance ($P = 0.08$). Further, in the female subgroup there was a significant positive correlation between daily dose of hydrocortisone (mg/day) and hair cortisol concentrations ($r = 0.28$; $P = 0.01$). These results indicate that patients with AI are often overtreated with glucocorticoid replacement, potentially making them more prone to comorbidities associated with long-term glucocorticoid exposure, such as changes in glucose and lipid metabolism, clinically important osteoporosis, and neuropsychiatric effects.

Similarly, a study by Manenschijn et al. (2011b) supported the finding that hair cortisol concentrations can be used to monitor hydrocortisone replacement in patients. They recorded a case of a patient developing adrenal insufficiency who became hypocortisolemic and required glucocorticoid replacement therapy. Hair samples taken at time points along this patient's clinical course showed gradually decreasing hair cortisol concentrations before the intervention, with concentrations increasing after start of hydrocortisone treatment. Overall, these studies indicate that not only can hair cortisol analysis be a useful tool in detecting and monitoring stress, but also may play an important role in the monitoring and management of diseases of the pituitary—adrenal axis.

10. Hair cortisol analysis to identify psychosocial sources of stress

Financial and work-related concerns are subjectively the most common causes of social stress experienced by individuals today. Dettenborn et al. (2010) used hair cortisol analysis to rate levels of psychological stress. Individuals who had been unemployed for at least 1 year were compared with currently employed control subjects. All participants provided a hair sample and rated their level of chronic stress with the *Trier Inventory for the Assessment of Chronic Stress* (TICS) and a PSS. Cortisol concentrations in hair segments representing the most recent 3 months and the most recent 3—6 months were significantly higher in the unemployed group ($\eta^2 = 0.071$; $P < 0.05$ and $\eta^2 = 0.085$; $P < 0.05$ respectively). Additionally, the unemployed group reported significantly higher levels of worry on the TICS subscale ($P < 0.01$) and had significantly higher scores on the PSS ($P < 0.01$). In this study, hair cortisol analysis was shown to be a powerful tool in measuring chronic stress resulting from a common psychological stressor, further indicating it effectiveness as a biomarker of chronic stress.

Steudte et al. (2011b) recently investigated hair cortisol concentrations in patients with Generalized Anxiety Disorder (GAD), a condition marked by excessive worry and anxiety regarding a variety of life problems. These symptoms are thought to be stressful in nature, but the literature examining its effect on the HPA axis is mixed, with some studies suggesting an overactive HPA axis and others finding no aberrations to the HPA axis. The researchers collected 9 cm hair samples from 15 patients with GAD and 15 age-, gender-, and lifestyle-matched controls. These samples were then divided into 3 cm segments and analyzed for cortisol content. Additionally, all participants completed a PSS. Interestingly, despite the GAD group having significantly higher mean PSS scores than the control group ($P < 0.001$), their mean hair cortisol content in the two most proximal 3 cm segments of hair was significantly *lower* than in the control subjects ($P < 0.01$). This is a new finding to this area of research, and suggests that while GAD patients have higher perceived stress, they may actually be hypocortisolemic. This could be the result of down regulation of the HPA axis with chronic anxiety, as evident in the recent meta-analysis

(Miller et al., 2007). These studies highlight how hair cortisol analysis may provide new insight and perspectives to support or challenge mechanistic notions on stress response in various conditions, and underscores the need to expand this body of research.

The unique ability of hair cortisol analysis to examine temporally distant psychological stressors has been perhaps best exemplified with a recent paper by Steudte et al. (2011a). Ugandan civil war survivors, many of whom had been severely traumatized within the past year, were examined. Traumatized participants were ranked on a Clinician-Administered Posttraumatic Stress Disorder Scale to delineate those who had posttraumatic stress disorder (PTSD). Sociodemographic background information known to affect cortisol production including age, sex, body mass index, smoking status and alcohol consumption was also collected. In addition, a four point PSS, Hopkins Symptom Checklist for depression and Mini-International Neuropsychiatric Interview for suicidal ideation were all conducted. The specifics of the traumatic events that each participant had experienced were recorded, and the most proximal 3 cm of hair were used to represent cortisol production over the past 15 weeks. In total 10 PTSD patients and 17 controls were included for the analysis. Traumatized patients with PTSD were found to have significantly higher hair cortisol concentrations than their paired traumatized controls without PTSD ($F_{(1,25)} = 5.35$; $P < 0.05$; $\eta p^2 = 0.18$). In addition, in both groups the number of lifetime traumatic events was positively correlated with hair cortisol concentration ($r = 0.41$; $P < 0.05$).

These findings are significant because this was the first time that hair cortisol analysis was used to investigate patients with PTSD, and the results stood in stark contrast to most other studies that have found PTSD to be frequently associated with hypocortisolism (Mason et al., 1986; Yehuda et al., 1996; King et al., 2001). Some have postulated that this aberrant finding about a disorder that is clearly stress-mediated may have been an artifact of sampling procedure. Previously the only matrices used to examine cortisol concentrations in patients with PTSD were serum, saliva, or urine—all of which only provide a very narrow window into a patient's cortisol production profile. Steudte's results are consistent with the systematic review by Miller et al. (2007) who, in a meta-analysis of 107 studies on chronic stress, have shown that an important factor influencing cortisol output is the time that has elapsed since the stressful incident occurred. When the source of the stress was ongoing or had been relatively recent, cortisol concentrations appear to be elevated, but when the source of the stress is temporally distant, lower cortisol concentrations are observed. In the past, acquiring samples at the time that the stressful event (such as the Ugandan civil war) occurred has often not been logistically or ethically feasible. Hair cortisol analysis has introduced a new tool to complement current methods for cortisol quantification and may provide a means to overcome some limitations of the established methods. Thus it may reinforce a potential paradigm shift suggesting that patients with PTSD may actually have started out with hypercortisolemia and have transitioned to hypocortisolemia.

11. Limitations

Hair is an exciting new matrix able to provide long-term retrospective measures of cumulative cortisol secretion. However, several challenges need to be considered when applying this novel biomarker of stress. For instance, because psychologically based measures of stress have only been validated for relatively short periods of days to weeks, they cannot serve as a gold standard against emerging methods such as hair cortisol analysis which measures stress levels occurring several months ago. This may explain some of the observed inconsistencies in correlations between hair cortisol levels and psychological tests. The heterogeneity in the types of psychological tests (e.g. PSS vs. MIVE) also presents a challenge when trying to directly compare studies from different research groups. Additionally, in subjects who may be exposed externally to corticosteroids in the form of lotions or creams, external contamination of the hair shaft will preclude analysis. It is important to remember that this matrix is not capable of measuring acute changes in stress, and therefore if such changes need to be observed in addition to chronic stress, the other matrices for cortisol measurement should also be used. Finally, in subjects who are unable or unwilling to provide a hair sample, analysis is not possible—simply put, one cannot measure cortisol in hair if one has no hair!

12. Outstanding questions and areas for future research

Even though the literature on hair cortisol measurement is growing quickly, there are still many outstanding questions and gaps in our knowledge that need to be addressed. First of all, the mechanism of cortisol incorporation in hair needs to be clarified. Further studies need to determine if and to what extent hair cortisol originates from blood, eccrine and/or sebaceous sources, if this is different for medullary versus outer layers, and if hair cortisol is a reflection of total and/or free cortisol exposure.

Secondly, it is important to obtain more specific information on the extent to which hair cortisol varies along the hair shaft, and which factors determine this. This needs to include assessment of the effect of hair washing, both while the hair is in situ, and during sample preparation before measuring hair cortisol. The degree to which washing procedures are required to negate the effects of external contamination (e.g. by blood or saliva) needs to be determined, particularly for animal studies. Furthermore, there is a dearth of knowledge on the effect of factors such as ethnicity, age, sex, and seasonal influences on hair cortisol content.

Immunoassays are commonly used to measure saliva, blood, urine, and hair cortisol concentrations. These methods, while sensitive to changes, are presently subject to interassay variability, precluding a unified definition of physiologic ranges of levels. Development of LC—MS/MS technology is likely to overcome this issue in the future.

As described previously, the effect of maternal separation in animals, and of psychological factors in both humans and animals on hair cortisol can vary in both direction and extent. This is a major area in need of increased understanding before this can be applied in clinical settings.

With respect to application for patient care, the studies on the utility of hair cortisol measurement in diagnosis and treatment of Cushing's disease and adrenal insufficiency need to be expanded and confirmed. In addition, the effect

of other disease states, including malignancies, metabolic and sleep disorders, is currently unknown.

Finally, the majority of human studies using hair cortisol analysis as a chronic biomarker of stress are associative. While initially such studies are important to try to identify some novel sources of stress, they cannot confirm causation or rectification of stress levels. Rather, intervention studies are needed to understand what initiates cortisol release, and, more importantly, if and to what extent hair cortisol (as a reflection of systemic cortisol exposure) can be changed. To date, only the relocation studies involving rhesus macaques and vervets, and the studies following the disease course of Cushing's or adrenal insufficiency in response to treatment have been true intervention studies. A greater emphasis on long-term intervention studies will expand the utility of this novel biomarker.

It is conceivable that hair cortisol analysis will continue to be adopted as a useful tool for measuring chronic stress in many clinical and experimental situations. Currently it is not available in most laboratories, even though the methodology and technology are relatively simple and straightforward, thus allowing a seamless integration with existing biomonitoring techniques. It is foreseeable that its ability to assess systemic cortisol concentrations will be integrated into clinical practice as a measure of chronic stress, as well as monitoring for systemic exposure to cortisol in Cushing's syndrome and in Adrenal Insufficiency for potential deleterious health effects associated with glucocorticoid therapies.

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