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Methodological Challenges and Clinical Applications of Hair Cortisol Analysis

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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METHODOLOGICAL CHALLENGES AND CLINICAL APPLICATIONS OF HAIR CORTISOL ANALYSIS

(Spine title: Hair Cortisol Analysis: Current Challenges and Applications)

(Thesis format: Monograph)

by

Evan W. Russell

Graduate Program in Physiology & Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
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Methodological Challenges and Clinical Applications of Hair Cortisol Analysis

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Abstract

This thesis examines methodological and clinical aspects of hair cortisol analysis. The methodological study examines the role of sweat as a contributor to hair cortisol concentrations. Hair cortisol analysis is an effective measure of chronic stress. Cortisol is assumed to enter the hair via blood, sebum, and sweat, however the extent to which sweat contributes to hair cortisol content was unknown. This study concluded that human sweat contains cortisol that likely contributes to hair cortisol content. Subjects with prolonged sweating at the time of hair collection may have increased hair cortisol concentrations that cannot be decreased with conventional laboratory washing procedures. Clinically, hair cortisol analysis is explored as a tool to determine if obstructive sleep apnea (OSA) is associated with increased stress. OSA is a common sleep disorder with serious cardiovascular and metabolic co-morbidities that may be mediated by increased cortisol secretion. Recent studies have focused on the ability of continuous positive airway pressure (CPAP) to reduce cortisol secretion in OSA patients, but the results have been mixed and only point measures of cortisol measurement have been used. Hair cortisol analysis presents a means of non-invasively and retrospectively examining cortisol production in these patients. This study examined whether hair cortisol concentrations are increased in OSA patients. Further, the effect of CPAP on hair cortisol concentrations was examined. It was concluded that cortisol secretion may be up-regulated in severe cases of OSA. The psychological stress of OSA may be reduced with CPAP, however physiological stress may remain unchanged after 3 months of treatment.

Keywords

Hair, Cortisol, Sweat, Washing, Obstructive Sleep Apnea

Co-Authorship Statement

Chapters 1, 2, and 7 were written in collaboration with Dr. Gideon Koren, Dr. Michael Rieder, and Dr. Stan Van Uum in the Division of Clinical Pharmacology at the University of Western Ontario. They assisted with the interpretation of results and the revisions process.

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List of Abbreviations

Abbreviation	Expanded
11β-HSD1	11-beta hydroxysteroid dehydrogenase type 1
11β-HSD2	11-beta hydroxysteroid dehydrogenase type 2
ACTH	Adrenocorticotropic hormone
AHI	Apnea hypopnea index
CPAP	Continuous positive airway pressure
CPT	Cold pressor test
CRH	Corticotropin releasing hormone
EEG	Electroencephalogram
ELISA	Enzyme-linked immunosorbent assay
EOG	Electrooculogram
ESS	Epworth sleepiness scale
HPA	Hypothalamic-pituitary-adrenal
LC-MS/MS	Liquid chromatograph tandem mass spectrometry
OSA	Obstructive sleep apnea
PBS	Phosphate buffered saline
PSS	Perceived stress scale

PTSD	Post traumatic stress disorder
RIA	Radioimmunoassay

Chapter 1: Introduction

Parts of this chapter have been published:

Russell E, Koren G, Rieder M, Van Uum S. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. *Psychoneuroendocrinology* 2012. 37:589-601.

1.1 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 et al. 2000b;Dimsdale ,2008a;Appels ,1990;Dimsdale ,2008b;Appels et al. 2000a), 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 1,134 adults aged 18 and up, and 1,136 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.

1.2 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 (Goldstein ,1995). 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, cortisol will be focused on as the primary biomarker of stress.

During times when an organism undergoes physiologic duress, cortisol acts to mobilize energy stores and modulate the immune system. Cortisol is a steroid hormone produced by the cortices of the adrenal glands in response to stress (be it physiological or psychological). The signal for cortisol production arises in the hypothalamus that releases corticotropin releasing hormone (CRH) onto the anterior pituitary, which in turn releases adrenocorticotropic hormone (ACTH) into circulation to act upon the adrenal glands (Miller et al., 2007). Once released, 90% of cortisol circulates in the blood in an inactive protein bound

fraction, and the remaining 10% of free cortisol is physiologically active (Lacroix et al., 2001). This system is subject to negative feedback; when sufficiently high, cortisol levels act to inhibit the system both at the level of the anterior pituitary and the hypothalamus (Henley et al., 2005).

Cortisol is a derivative of a sequence of biochemical reactions that modify cholesterol. Cortisol is converted into its inactivate metabolite, cortisone, via 11-beta hydroxysteroid dehydrogenase 2 (11β-HSD2). Cortisone can then be conjugated in the liver, making it soluble and capable of excretion via the kidneys. Cortisol has a mean half-life of 66 minutes (Weitzman et al., 1971). It should be noted that 11-beta hydroxysteroid dehydrogenase 1 (11β-HSD1) opposes the action of 11β-HSD2, and therefore a significant portion of cortisone produced is converted back to cortisol (Kacsoh, 2000). The Michaelis-Menten constant of 11β-HSD1 is in the μM range, whereas it is in the nM range for 11β-HSD2 (Hammer & Stewart, 2006). Thus, 11β-HSD2 has an approximately 3-fold greater binding affinity for cortisol compared to that of 11β-HSD1 for cortisone.

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. 1983a). Salivary cortisol concentrations correlate well with serum concentrations (Vining et al. 1983a;Aardal and Holm ,1995a;Aardal and Holm ,1995b;Vining et al. 1983b). The reference ranges of normal serum cortisol concentrations are considered 200-800 nmol/L at 0800h and <300 nmol/L at 2200h (Aardal & 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. The reference ranges of salivary cortisol concentrations are considered to be 3.5-27 nmol/L at 0800h and <6 nmol/L at 2200h (Aardal & Holm, 1995). A similar strategy is employed when urine is used; 24-hour 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.

1.3 Hair Cortisol Analysis

1.3.1 *Development*

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 1cm/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.* using hair from wild hyraxes (small herbivorous mammals) (Koren et al. 2002). In this study, the hyraxes were briefly captured so that 7 to 20mg of hair could be plucked. Cortisol was then extracted from the hair with 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 to this 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).

Sauvé and colleagues were the first to use an ELISA protocol similar to the one used by Koren *et al*. in 39 non-obese human subjects (Sauve et al. 2007). Hair cortisol concentrations were not normally distributed, but after log transformation a reference range of 1.7-153.2pg/mg was established, with a median concentration of 46.1pg/mg. The hair cortisol concentrations obtained were compared against those obtained from saliva, serum, and 24 hour urine collections. A positive correlation was observed between cortisol concentrations in hair and in 24 hour 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 hour 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 macques, in contrast, there was found a significant correlation between the cortisol concentration in saliva and that in hair ($r=0.797$, $P<0.001$)(Davenport et al. 2006). Bennett et al.

measured hair cortisol in dogs, and found that it was significantly correlated with cortisol in saliva ($r=0.48$; $P<0.001$) (Bennett and Hayssen ,2010).

Cirimele and colleagues were the first to examine whether glucocorticoids could be detected in human hair (Cirimele et al. 2000). 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.* showed that in hair samples of 44 subjects a mean hair cortisol concentration of 18pg/mg, ranging from 5-91pg/mg, could be detected (Raul et al. 2004). 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).

Of note, in addition to glucocorticoids, a variety of other steroid hormones including estradiol, progesterone, and testosterone are known to incorporate into hair (Yang et al., 1999).

1.3.2 *Advantages*

There are various advantages to using cortisol in hair as a biomarker of chronic stress. Hair has a fairly predictable growth rate of approximately 1cm per month. Therefore the most proximal 1cm segment to the scalp approximates the last month's cortisol production, the second most proximal 1cm 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 ~1cm 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 sample represents approximately one 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 et al. 2011). The median cortisol concentration was 69.5pg/mg (36-158pg/mg) and the median cortisone concentration was 65pg/mg (32-133pg/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.

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 hours; 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

1.3.3 *Lab Procedure*

Overall, the methods used for measurement of cortisol in hair are very similar, with some variations in procedures among 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 3cm 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.

Chapter 2: Literature Review

Parts of this chapter have been published:

Russell E, Koren G, Rieder M, Van Uum S. Hair cortisol as a biological marker of chronic stress: Current status, future directions and unanswered questions. *Psychoneuroendocrinology* 2012. 37:589-601.

2.1 Emergence of Hair Cortisol Analysis to Detect Clinical or Stress-Mediated Changes in Cortisol

2.1.1 *Observational Studies:*

Kirschbaum and colleagues investigated if the well-established increase in cortisol production in the third trimester of pregnancy could be detected using hair cortisol analysis (Kirschbaum et al. 2009a). 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 ($t(1,120)=4.77$; $P<0.001$) (Kirschbaum et al. 2009b). 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 controls (51.99 ± 43.30 vs. 13.98 ± 10.63, 16.55 ± 12.59pg/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* 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 ($P<0.0001$) (Manenschijn et al. 2011b). In aggregate, these studies support the notion that cortisol in hair provides a reflection of long-term systemic cortisol exposure.

2.1.2 *Intervention Studies*

The first experimental animal study that demonstrated that hair cortisol can change following an intervention was published by Davenport *et al (Davenport et al. 2006).* They studied rhesus macaques that had recently undergone relocation from their original housing environment, and were displaying behavioral 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±7.5pg/mg, respectively; $P<0.001$), and one 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 their 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 behavior following relocation and had lower hair cortisol both before and after the placement in the large. In infants raised by peers only, hair cortisol before separation were 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 behavior 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* investigated the relation between hair cortisol and novelty seeking behavior (Laudenslager et al. 2011). 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 behavior was correlated with hair cortisol as a tool to assess long-term 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* examining the heritability of HPA activity in response to a stressor (Fairbanks et al. 2011). 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*. (Davenport et al. 2006). The added facet of this 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.* involved patients with Cushing's Syndrome (Thomson et al. 2010). 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 and colleagues, 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).

2.2 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 (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.

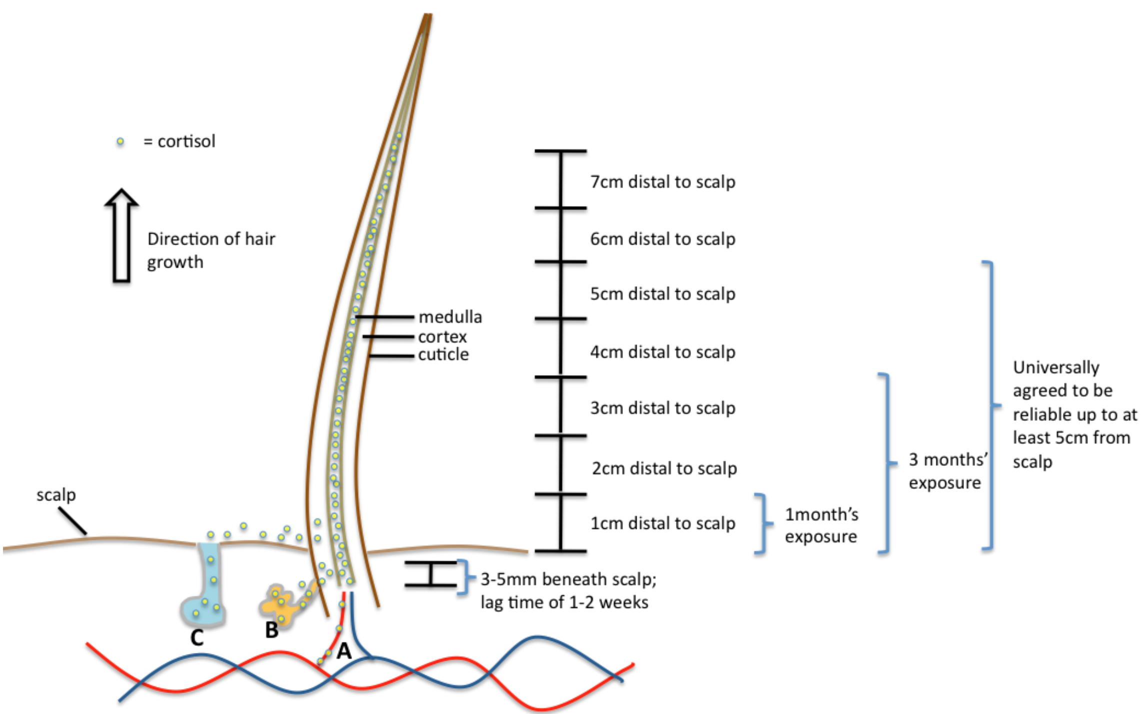

Fig 1. Proposed mechanisms of cortisol incorporation into hair via blood (A), sebum (B), and sweat (C).

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 demonstrated that hair follicles contain a functional equivalent of the HPA axis and can synthesize cortisol after stimulation by Corticotrophin-Releasing Hormone (CRH) (Ito et al. 2005). This was supported by a study by Sharpley *et al*. 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 one minute (Sharpley et al. 2009). 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 that 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 minutes. 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 minutes. 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 might 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. Finally, very high hair cortisol concentrations were found in a patient receiving treatment with high dose hydrocortisone (Thomson et al. 2010). In this 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. Moreover, 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 color or cosmetic treatments on hair cortisol concentrations is another frequently debated topic. With respect to natural hair colour, neither Sauvé and colleagues nor Manenschijn *et al.* have detected significant differences in cortisol levels due to natural coloration (Sauve et al. 2007;Manenschijn et al. 2011b). In dogs, Bennet et al. 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 ($r=0.47$; $P=0.001$) (Bennett and Hayssen ,2010). This was the first study to suggest that melanin content could impact how cortisol is sequestered into hair. Sauvé et al. demonstrated that

cosmetic treatments such as hair dying have been shown to significantly decrease hair cortisol levels relative to controls ($P<0.036$) (Sauve et al. 2007). A similar trend that failed to reach significance was noted by Manenschijn *et al.* (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 isopropranolol wash 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 known what effect, if any, frequency of hair washing has on hair cortisol concentrations. In rhesus macaques, Hamel *et al.* have shown that repeated washing with either shampoo or water decreased hair cortisol concentration (Hamel et al. 2011). 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 10ml addition of the solution, inversion for 45 seconds, 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, hair that had been washed with shampoo 30 times had significantly less cortisol than that which had been only washed 10 times ($P<0.005$) in all four pools. 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 1cm/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±59μm/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.* examined hair cortisol concentrations along the hair shafts of 9 nulliparous women (Kirschbaum et al. 2009a). Hair samples of at least 18cm length were collected from each woman and divided into six 3cm 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 the hair integrity. Thus, they suggested using only the most proximal 1-6cm of hair to reliably estimate systemic cortisol concentrations. Gao *et al.* found similar results with segmental analysis of 5 subjects (Gao et al. 2010). The first five 1cm segments experienced an average linear hair cortisol decline of -2.7±0.3pg/cm ($\beta=-0.98$; $P<0.01$). In contrast, several studies have contradicted these results. Thomson *et al.*, recruiting 9 healthy control subjects with hair lengths ranging from 10-14cm, could not confirm the previous studies (Thomson et al. 2010). Hair

samples were segmented into 1cm 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±46ng/g); one participant did demonstrate a significant decrease ($P<0.05$) over time. Similarly, Manenschijn *et al.* could not show time-dependent changes (Manenschijn et al. 2011b). A group of 28 women provided hair samples at least 18cm in length, and these were then segmented into six 3cm 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 2.3-4.3cm 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 6cm distal from the scalp. While further studies are needed to resolve this controversy there is a wide consensus that the first 5-6cm 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 and colleagues who obtained hair samples ranging in length from 6 to 21cm from 10 ancient Peruvian mummies, dating from AD550-1532 (Webb et al.

2011). 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±35ng/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.

2.3 Hair Cortisol Analysis in Clinical Sources of Stress

While hair is still a relatively new means of measuring cortisol, several human studies have shown 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 and colleagues sought to examine whether higher cortisol concentrations might predict spontaneous pre-term birth (Kramer et al. 2009). 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 9cm 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.* compared hair cortisol concentrations of infants requiring a stay in a NICU with those of healthy infants born at term (Yamada et al. 2007). The cortisol concentrations of the NICU group were significantly higher than the control (2.06±2.05 vs. 0.11±0.42nmol/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.* who recruited adult patients who were using opioids to help control severe chronic non-cancer pain and compared them to non-obese controls (Van Uum et al. 2008). 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. 46.1pg/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.* investigated the role of chronic stress as measured by hair cortisol, in the development of an acute myocardial infarction (AMI) (Pereg et al. 2011). 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. 224.9ng/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*. to assess its potential to predict depressive symptoms in patients suffering from coronary artery disease (CAD), a population far more at risk of depression (Dowlati et al. 2010). Depressive symptoms were demonstrated in 34 of the 121 patients with CAD. The most proximal 3cm 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, might 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 and colleagues predicted that since higher

cortisol levels are associated with immunosuppression, the long-term carriers of *Staphylococcus aureus* would likely have the highest hair cortisol levels (Manenschijn et al. 2011a). Of 72 healthy subjects, 38 were non-carriers, 10 were intermittent carriers, and 24 were long-term carriers of *Staphylococcus 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 *Staphylococcus aureus* colonization.

Van Rossum and colleagues employed hair cortisol analysis to investigate patients with bipolar disorder, a condition in which HPA dysregulation may play an etiological role (van Rossum et al. 2011). The most proximal 3cm 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 (31.84pg/mg vs. 28.18pg/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.

2.4 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 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.* examined whether hair cortisol analysis could fill this void (Gow et al. 2011). 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[57.7-14790]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.* supported the finding that hair cortisol concentrations can be used to monitor hydrocortisone replacement in patients (Manenschijn et al. 2011b). 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.

2.5 Hair Cortisol Analysis to Identify Psycho-social Sources of Stress

Financial and work-related concerns are subjectively the most common causes of social stress experienced by individuals today. Dettenborn and colleagues used hair cortisol analysis to rate levels of psychological stress (Dettenborn et al. 2010). Individuals who had been unemployed for at least one 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 Stres*s (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=0071; $P<0.05$ 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 and colleagues 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 (Steudte et al. 2011b). 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 9cm hair samples from 15 patients with GAD and 15 age-, gender, and lifestyle-matched controls. These samples were then divided into 3cm 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 3cm 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*. (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 3cm 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) that, 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.

2.6 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. Trier Social Stress Test) 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 additional 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!

Chapter 3: The Focus of the Present Work

3.1 Methodological Challenges

While hair cortisol analysis continues to prove to be a useful means of monitoring cortisol exposure as a marker of chronic stress, some methodological questions remain unanswered. Even though the literature on hair cortisol measurement is growing quickly, there are still gaps in the knowledge that need to be addressed. First, 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 cortical layers, and if hair cortisol is a reflection of total and/or free cortisol exposure. The paradigm for cortisol incorporation into hair has assumed that cortisol enters the medulla of the hair shaft via blood, and therefore hair analysis would reflect an integral of serum concentrations over time. It has also been postulated that sebaceous and eccrine secretions may contribute to hair cortisol concentrations, but supporting evidence is lacking (Pragst et al., 2006). It is presently unknown what, if any, cortisol concentrations are found in human sweat. Since individuals have various levels perspiration, personal hygiene, and hair washing frequencies, it would be important to assess to what extent sweat affects hair cortisol concentrations.

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, and 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.

3.2 Clinical Uses

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.

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. An excellent candidate condition to apply hair cortisol analysis to is obstructive sleep apnea (OSA).

3.2.1 Obstructive Sleep Apnea Background

Obstructive sleep apnea is a type of sleep-disordered breathing that is characterized by frequent decreases (hypopneas) or cessations (apneas) of breathing during the course of sleep. The hyponeas and apneas are the result of a collapse of the upper airway that is due to a lack of neuromotor tone in the tongue and/or airway dilator muscles (Dempsey et al., 2010). It is estimated that the prevalence of OSA is 3-7% and 2-5% in adult men and women, respectively (Punjabi 2008). OSA is largely underdiagnosed with up to 82% of men and 93% of women having undiagnosed OSA (Young et al., 1997). The prevalence increases dramatically to 41% in patients with a BMI in excess of 28. Other risk factors include mandibular insufficiency, abnormalities in the soft tissue of the

upper airway, tonsillar and adenoid hypertrophy, and smoking (Young et al., 2004).

Untreated OSA has been independently identified as a risk factor for a number of comorbid conditions. Associated sequelae include nighttime awakenings, excessive daytime somnolence, increased cardiovascular morbidity and mortality, and increased insulin resistance resulting in higher risks for metabolic syndrome and type II diabetes (Kezirian et al., 2010; Levy et al., 2009). Additionally, untreated OSA can complicate anesthetization, making intubations more difficult and respiratory depression more pronounced under anesthesia (Park et al., 2011).

3.2.2 Diagnosis of Obstructive Sleep Apnea

Investigation of OSA is usually initiated if a patient presents with snoring. Daytime somnolence is another common complaint, and is quantified using the Epworth Sleepiness Scale (ESS), an 8 point questionnaire which assesses the patient's tendency to fall asleep at inappropriate times of the day (Appendix X). Higher ESS scores indicate a higher degree of daytime somnolence (Johns, 1991). The presence of snoring, daytime somnolence and risk factors (e.g. obesity) present the impetus to send a patient for an overnight sleep student (polysomnography).

Polysomnography is the standard test for diagnosing OSA and other forms of sleep-disordered breathing. During a polysomnogram heart rate, respiratory rate, and oxygen saturations are monitored. An electroencephalogram (EEG)

and electrooculogram (EOG) are used to discern the stages of non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. The EEG is additionally used to monitor the presence of alpha, theta, and beta wave frequencies, and disturbances in these wave frequencies provide a means of assessing how many times a patient arouses from sleep. Additionally, nasal pressure, esophageal pressure, and intercostal and abdominal muscle movements are recorded to aid in discerning between obstructive sleep apnea and central sleep apnea. Finally, the combined number of apneas and hypopneas per hour are measured, generating a measure known as the apnea-hypopnea index (AHI). The severity of OSA is categorized based on AHI, with AHI scores less than 5 indicating the absence of OSA, 5-15 indicating mild OSA, 15-30 indicating moderate OSA, and scores greater than 30 indicating severe OSA (Patil, 2010).

3.2.3 Treatment of Obstructive Sleep Apnea

Addressing modifiable risk factors is a universal treatment approach used for all severities of OSA. Examples include exercise and healthy nutrition to promote weight loss, cessation of smoking, and decreased alcohol consumption. Modifying the sleep position from a supine position to a side position may also decrease the extent of upper airway obstruction. Changes to medications that may promote weight gain or respiratory depression may also be discussed (Tuomilehto et al., 2009).

For patients who are unable to make the necessary lifestyle changes, or who require more aggressive treatment, continuous positive airway pressure (CPAP) is the standard of care. CPAP machines are equipped with a mask that covers the nose and mouth of the patient. Air travels through the mask in the direction of the patient's upper airway, and the pressure generated maintains a patent airway (Parks et al., 2011). Proper use of CPAP can significantly improve OSA, and decrease its associated morbidity and mortality. Monitoring patient compliance is crucial, as it is highly variable. The noise emitted by the machine, the pressure used, and the type of mask used may all need to be personalized to the patient's preferences to enhance adherence (Shapiro & Shapiro, 2010). A recent study by Sharma et al. (2011) demonstrated that 3 months of CPAP significantly improved the metabolic profile of individuals with metabolic syndrome when compared to a sham treatment. Individuals on CPAP had significantly reduced systolic blood pressure, diastolic blood pressure, low density lipoprotein, triglycerides, and glycated hemoglobin. Additionally, the symptoms of metabolic syndrome reversed in 13% of the participants on CPAP, compared to just 1% undergoing the sham therapy.

Another treatment option is the use of an oral appliance, a device that advances the mandible forward, reducing the chance of an obstruction. Oral appliances can significantly reduce AHI of a patient with OSA. While not as efficacious as CPAP, they can serve as a primary treatment for patients with mild OSA, or be a complement to CPAP for moderate to severe cases (Hoffstein, 2007).

Upper airway surgeries that remove or manipulate the tissues that may contribute to the airway obstruction, such as uvulopalatopharyngoplasty, maxillary-mandibular advancement, tonsillectomies, adenoidectomies, and septoplasty exist for patients with clear anatomical lesions. The effectiveness of these procedures is highly variable depending on the patient population and the measure of success applied (Parks et al., 2011).

3.2.4 Cortisol Secretion in Patients with Obstructive Sleep Apnea

Several studies have hypothesized that the metabolic effects of sleep apnea may be mediated by increased cortisol secretion. In this model, the frequent sudden arousals during sleep activate the HPA axis result in increased cortisol secretion at night, at a time when cortisol secretion normally is very low (Trakada et al., 2007). As cortisol overproduction is associated with hypertension, metabolic syndrome and diabetes mellitus, cortisol is an important candidate mediator for the metabolic and cardiovascular effects of OSA (Lam et al., 2009).

The interest in the relation between OSA and increased cortisol production has lead to studies investigating the possibility that therapeutic interventions such as CPAP can decrease cortisol production. A recent study by Carneiro et al. (2008) examined 16 obese men with OSA and found that morning salivary cortisol concentrations were significantly less suppressed (P=0.012) by dexamethasone – pointing to increased cortisol production - than obese controls. Nine of the men were then placed on CPAP, and following a 3 month period the

endogenous cortisol production was more sensitive to suppression by dexamethasone, indicating lower, more physiologic level of activity of the HPA axis that now was comparable to the obese controls (Carneiro et al., 2008). Another study examined ACTH and cortisol production in 10 patients before and after being placed on CPAP. Patients were monitored for 24 hours, with blood samples taken at 10-minute intervals. After 3 months of CPAP patients had significantly reduced total serum ACTH ($P<0.001$) and total serum cortisol concentrations ($P<0.001$) (Henley et al., 2009). Schmoller et al. (2009) also examined the effect that a CPAP intervention had on cortisol production. Thirty-eight patients were recruited and 6 salivary cortisol samples were collected throughout the course of the day before placement on CPAP, and another 6 after a 3-month period on CPAP. The results were mixed, with the morning cortisol concentrations being significantly increased after CPAP ($P=0.044$), while evening cortisol concentrations were significantly reduced ($P=0.038$), overall indicating a change towards the normal diurnal cortisol rhythm.

Not all studies have indicated reduced cortisol concentrations after using CPAP. A placebo controlled study of 101 male OSA patients sought to determine if serum cortisol concentrations were affected by nasal CPAP. Following a 4-week period, the change in serum cortisol concentrations of the placebo-controlled group was not significantly different from the treatment group. Additionally the study failed to detect an association between serum cortisol and OSA severity (as measured by oxygen desaturations)(Meston et al., 2003). Tasali et al. (2011) investigated the use of CPAP to improve the cardiac profile in

9 obese women with polycystic ovary syndrome who were diagnosed with OSA. One of the hormone markers investigated was cortisol. Twenty-four hour blood monitoring, with 10-30 minute collection intervals, was done for one day before placement on CPAP and one day following an 8-week trial. Following the CPAP trial, total serum cortisol concentrations were not significantly changed.

While these studies garnered valuable pathophysiological information about OSA, the mixed results and small sample sizes make interpretation challenging. Additionally, the serum collection was quite invasive, and both serum and salivary cortisol concentrations only represent a single point in time. A larger study, using a matrix, such as hair, may present a means of elucidating the role of the HPA axis in OSA, and if it can be modulated with CPAP therapy.

Chapter 4: Hypotheses

There are two overall hypotheses in the present study. Firstly, I hypothesized that the cortisol content of sweat will affect hair cortisol concentrations. Secondly, I hypothesized that hair cortisol analysis can aid in elucidating the role of HPA activity and chronic stress in obstructive sleep apnea.

4.1 Specific Objectives

1. To determine the concentration of cortisol in sweat and its correlation with systemic exposure, as evidenced by salivary cortisol.

2. To determine if hair that is more thoroughly coated with a hydrocortisone solution with a sweat-like cortisol concentration could appreciably increase the total cortisol concentration measured in hair.

3. To determine if isopropanol washing effectively normalizes hair cortisol concentrations of hair samples which have been immersed in a sweat-like hydrocortisone solution.

4. To collect hair samples from patients with OSA and determine if hair cortisol concentrations are related to clinical indicators of OSA severity.

5. To determine what effect placing OSA patients on CPAP has on their hair cortisol concentrations and levels of perceived stress.

Chapter 5: Methods

5.1 The Detection of Cortisol in Human Sweat—Implications for Measurement of Cortisol in Hair

5.1.1 *Ethical considerations*

The study protocol was approved by the University of Western Ontario Health Sciences Research Ethics Board (REB #17930E). Each subject gave written informed consent.

5.1.2 Subjects

Athletes, both recreational and varsity, could be included if they were 18 years of age and older. Subjects were excluded if they used oral, parenteral, or topical glucocorticoids, if they had been diagnosed with Cushing's Syndrome, and if they were pregnant. We obtained information on age, smoking status, and alcohol consumption, and measured height and weight and calculated body mass index.

Saliva and sweat samples were collected immediately following a period of vigorous exercise, lasting 15-60 minutes. Saliva samples were collected using the Alpco Diagnostics Saliv-Saver™ Collection Device. The cotton swab was placed under the tongue, allowing it to saturate, and then placing it in a 5ml salivette. Sweat samples were collected using another Alpco Diagnostics Saliv-Saver™ Collection Device, but this time the cotton swab was rubbed over their scalp hair and necks, allowing it to saturate, and then pacing it in a 5ml salivette.

The salivettes were then centrifuged at 2218 g for 5 minutes, and 1ml of the supernatant was then pipetted into a 1.5ml Eppendorf tube. Samples were stored at -20°C until analysis.

5.1.3 Hydrocortisone Immersion and Hair Wash Experiments

To attempt to simulate the potential effects of hair exposure to sweat for an extended period of time, a hydrocortisone solution was constituted with a concentration of 50 ng/ml, a concentration consistent with low-mid range of sweat cortisol concentrations that were observed. To accomplish this, 276µl of a hydrocortisone standard was dissolved in 100ml of phosphate buffered solution. The concentration of this solution was confirmed by analyzing it on an ELISA as per the method described below. Residual hair samples (n=14) from past studies were used. All of these hair samples had been collected from the vertex posterior of the scalp as per our standard protocol; these subjects had not been sweating before hair collection. The most proximal 3 cm of hair were segmented and then subjected to one of five conditions: a control condition in which hair was washed then analyzed, and four treatment conditions in which hair samples where placed in 2 ml of the 50 ng/ml hydrocortisone solution for either 12 or 24 hours, allowed to dry for at least 12 hours, after which they were either washed prior to analysis or analyzed without a wash.

Hair washing involved placing each hair sample in a scintillation vial, adding 3 ml of isopropranol, incubating it at room temperature at 0.28 g for 3 minutes and decanting the isopropanol. This procedure was repeated once and

then hair samples were allowed to air dry at room temperature for at least 12 hours.

5.1.4 Effect of Duration of Hydrocortisone Immersion

Residual hair samples (n=7) from past studies were used. All of these hair samples had been collected from the vertex posterior of the scalp as per our standard protocol. The most proximal 3 cm of hair were segmented and then subjected to one of five conditions: the control hair sample was briefly immersed in the 50ng/ml hydrocortisone solution for approximately 2 seconds and then immediately removed from the solution, and the other hair samples were immersed for 15, 30, 60, and 120 minutes, respectively. Following the conditions hair samples were allowed to dry for at least 12 hours, washed, and then analyzed.

5.1.5 Measurement of Cortisol in Saliva and Sweat

Sweat and saliva samples were analyzed as per the commercially available salivary ELISA from Alpco Diagnostics (Salem, NH, USA). The intra-assay and inter-day coefficients of variation were determined to be 10.31% and 4.13%, respectively. The kit reported a sensitivity of 1.0ng/ml.

5.1.6 Hair Cortisol Analysis

Hair cortisol analysis was performed in accordance with a protocol our laboratory has established (Sauvé et al. 2007, Thomson et al. 2008). Briefly, 10-15 mg of each hair sample was placed in a scintillation vial, 1 ml of methanol was

added, and the hair segments were minced with surgical scissors until the hair became granular in appearance. The vials were sealed and incubated at 50°C for 16 hours at 0.28 g. The methanol solution containing cortisol extracted from the hair was then pipetted into a 5 ml test tube, placed on a hot plate at 50°C and evaporated under a stream of nitrogen gas. The remaining residue was then reconstituted with 250 µl of phosphate buffered saline. These reconstituted samples were analyzed as per the commercially available salivary enzyme linked immunoassay kit from Alpco Diagnostics (Salem, NH, USA). The kit reported a sensitivity of 1.0ng/ml.

5.1.7 Statistical Analysis

Results are presented as mean±SD unless indicated otherwise. Data was assessed for normality using Kolmogorov-Smirnov test; non-normally distributed data was log-transformed before statistical analysis. Correlation coefficients between different parameters were calculated with Pearson's correlation coefficient for normally distributed data,. A repeated measures ANOVA with a post-hoc Bonferroni test was used to examine if differences existed among the control and treatment conditions for the hydrocortisone immersion experiments. GraphPad Prism version 4.0b was used for all statistical analyses (GraphPad Software Inc. La Jolla, CA, USA), a *p* value below 0.05 was considered statistically significant.

5.2 Hair Cortisol Concentrations in Patients with Obstructive Sleep Apnea

5.2.1 Ethical considerations

The study protocol was approved by the University of Western Ontario Health Sciences Research Ethics Board (REB #17340E). Each subject gave written informed consent prior to enrolment.

5.2.2 Subjects

The subjects recruited were patients who had been referred to the Sleep Clinic at South Street Hospital (London, Ontario) to investigate possible OSA, but had not yet been placed on CPAP. Subjects could be included if they were 18 years of age or older, and had undergone a polysomnogram within the last 4 months. Subjects were excluded if they indicated a medical history that is known to affect HPA axis functioning. This included the use of oral or parenteral glucocorticoids in the past 3 months, the use of topical glucocorticoids in the past 1-month, diagnosis with Cushing Syndrome, heart or renal failure, and pregnancy. Additionally, any subject unwilling or unable to provide a 2cm sample of hair was excluded.

Subjects were informed that if they elected to be placed on CPAP, a second assessment would be done at their 3-month follow-up appointment if they consented. For the follow-up visit the same exclusion criteria applied.

5.2.3 Physical Exam Characteristics and Medical History

Consult visit:

For each subject we recorded the age, sex, height and weight (to calculate BMI), neck circumference, blood pressure, and heart rate. Additionally, daily alcohol consumption, smoking status, and “pack years” (number cigarette packs smoked per day multiplied by the number of years smoking) were recorded from each subject, as both alcohol consumption and smoking are known to alter HPA axis activity.

Past medical history information was gathered from each subject’s hospital chart. The current and past diagnoses were noted, as well as the surgical history. All of the current medications and dosages were recorded for each subject.

Follow-up visit:

We recorded weight and blood pressure, and any changes to alcohol consumption, smoking status, current diagnoses, and medication use. Finally, we inquired about any major psychosocial stressors such as a death in the family, loss of employment, and others.

5.2.4 Sleep Information Recorded

Consult visit: The most recent polysomnogram information was obtained for each subject. The polysomnogram had to be obtained within the past 4 months

to be considered a close representation of the subject's current status. The number of arousals from sleep per hour, total sleep time, and AHI were obtained. The time spent in NREM and REM sleep, and the percentage of time spent below a 90% oxygen saturation for both NREM and REM sleep was also recorded. This allowed for a calculation of the total time spent in a hypoxemic state during the polysomnogram. Additionally, the ESS score from the day of the sleep study was obtained.

Follow-up visit:

A subjective assessment of how well CPAP was working was obtained from each subject. Additionally, each subject's reported use of his/her CPAP machine was noted. This included the number of nights per week, and the number of hours per night. For subjects that had brought their CPAP machines with them, sleep information was downloaded off of the machine's memory card, if capable. This information included the subject's compliance, as measured by the percentage of days used for longer than 4 hours, the average or median daily usage, and the AHI while using CPAP.

5.2.5 Stress Measurements

Consult visit: A hair sample of approximately 4-5 millimeters in diameter was obtained from the base of the hair shaft at the vertex posterior of each subject as per our standard protocol. The sample was cut with surgical scissors and secured on a hair collection page with non-tack tape. An arrow was used to indicate the scalp end of the hair. This hair sample was stored in an envelope at

room temperature until analysis for hair cortisol. The surgical scissors were cleaned with isopropanol between subjects as a means of preventing cross-contamination.

Each subject was asked to complete the PSS, a validated questionnaire assessing perceived stress over the last 1 month of his/her life. The PSS scores were tallied upon completion.

Follow-up visit: A second hair sample and a second PSS were obtained in the same manner as the consult visit.

5.2.6 Hair Cortisol Analysis

The hair samples were removed from the hair collection page and sectioned using a utility knife. The most proximal 2 centimeters of hair, representing the most recent 2 months of cortisol secretion were cut. The segments were placed in a 5 ml scintillation vial, weighed and 10-15 mg of hair was used. The segments were then washed. The wash consisted of a 3 ml of isopropanol immersion, incubation at room temperature, shaking at 100 RPM for 3 minutes, followed by decanting. This process was repeated once, and then the hair samples were dried for at least 5 hours in a fume hood.

The remainder of hair cortisol analysis was performed in accordance with a protocol our laboratory has established (Sauvé et al. 2007, Thomson et al. 2008). Briefly, 1 ml of methanol was added to the scintillation vial, and the hair segments were minced with surgical scissors until the hair became granular in

appearance. The vials were sealed and incubated at 50°C for 16 hours, rotating at 100 RPM. The methanol solution containing cortisol extracted from the hair was then pipetted into a 5 ml test tube, placed on a hot plate at 50°C and evaporated under a stream of nitrogen gas. The remaining residue was then reconstituted with 250 µl of PBS. These reconstituted samples were analyzed as per the commercially available salivary enzyme linked immunoassay kit from Alpco Diagnostics (Salem, NH, USA). The kit reported a sensitivity of 1.0ng/ml. In accordance with past studies, hair cortisol concentrations in excess of 1,500 ng/g were excluded on the basis of possible glucocorticoid contamination (Thomson et al., 2009).

5.2.7 Statistical Analysis

Results are presented as mean±SD unless indicated otherwise. Analysis of data obtained from the consult visit was performed by comparing the hair cortisol concentrations of each subject with the various parameters obtained from the polysomnogram. Both linear and nonlinear regressions were used to try to model hair cortisol concentrations with AHI, ESS, arousals per hour, and total hypoxemic time. The hair cortisol concentrations of patients with mild, moderate, and severe OSA were compared using a 1-way ANOVA with post-hoc Bonferroni tests. Pair two-tailed t-tests were used to compare the AHI and PSS scores of subjects below and after placement on CPAP. GraphPad Prism version 4.0b was used for all statistical analyses (GraphPad Software Inc. La Jolla, CA, USA); a *p* value at or below 0.05 was considered statistically significant.

Chapter 6: Results

6.1 The Detection of Cortisol in Human Sweat—Implications for Measurement of Cortisol in Hair

We recruited 17 subjects; their characteristics are displayed in Table 2. Subjects included varsity rowers, recreational soccer players, and recreational long distance runners, and had done intensive exercise making them sweat profusely for at least 10 minutes.

Table 2: Athlete characteristics*

Age	25±9.3 (19-58)
Gender (M:F)	12 (71%) : 5 (29%)
BMI	24.2±2.94
Smokers	0
Alcohol drinks per day (median [range])	0 [0-3]
Subjects taking prescription drugs	0
Time of sampling (median [range])	10:40h [07:40-22:00h]

*Results are presented as mean±SD unless indicated otherwise

Sweat cortisol concentrations were 74.62±41.51ng/ml (mean±SD), ranging from 8.16-141.7ng/ml. The concentration of sweat cortisol determined was dependent on the time of day collected, with the highest concentrations being found in morning samples and lower concentrations in evening samples.

Sweat cortisol concentrations were significantly correlated with the log transformed time of day (r=0.66, $p<0.01$)(Fig. 2). Sweat cortisol concentrations were significantly correlated with salivary cortisol concentrations (r=0.55, $p<0.05$) (Fig. 3).

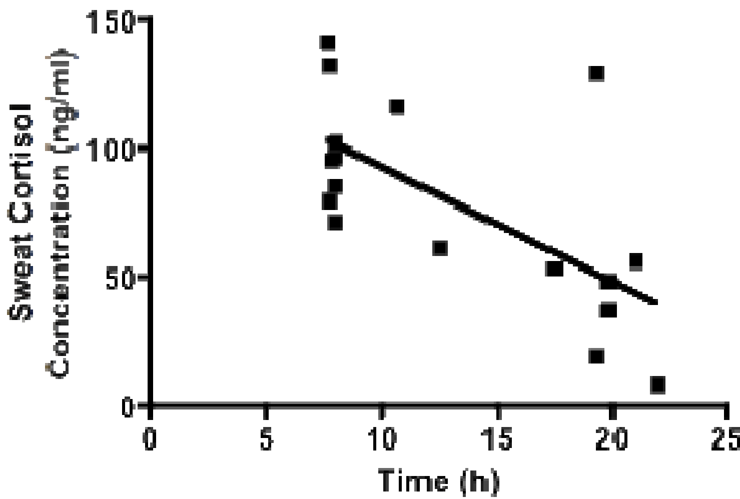

Figure 2: The association between sweat cortisol concentrations and the time of day (r=0.66, $p<0.01$); n=17. When analyzing with linear regression, time of day values were log corrected.

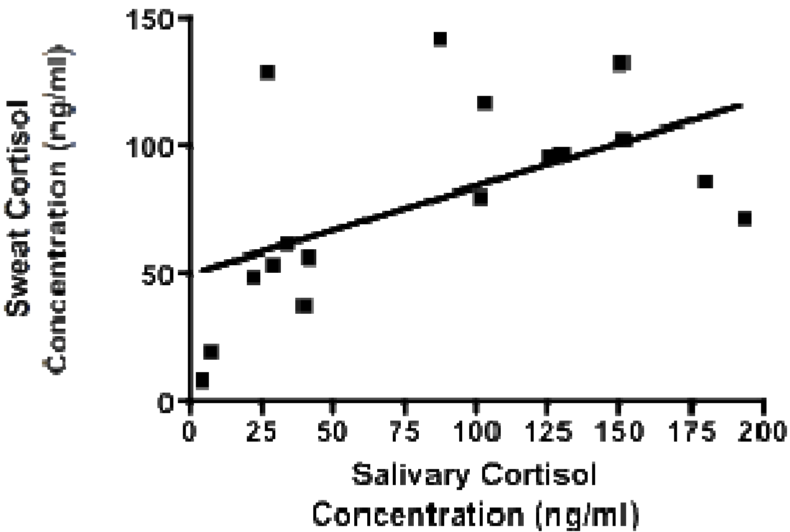

Figure 3: The association between sweat cortisol concentrations and salivary cortisol concentrations (r=0.55, $p<0.05$); n=17.

Twelve and 24-hour hair exposure to the cortisol containing solution resulted in increased hair cortisol content ($p<0.001$) (Fig. 4). Post-hoc analysis identified that the control group had a significantly lower hair cortisol concentration ($p<0.001$) when compared to all of the treatment conditions, but none of the treatment conditions differed significantly from each other. Longer incubation periods were shown to significantly increase hair cortisol content ($p<0.001$). Post-hoc analysis identified that hair cortisol concentrations for hair samples that had incubated for 60 and 120 minutes were significantly increased from the control concentration ($p<0.01$ and $p<0.001$, respectively). Further, hair samples incubated for 120 minutes had significantly increased cortisol relative to those that had only incubated for 15 and 30 minutes ($p<0.001$) (Fig. 5).

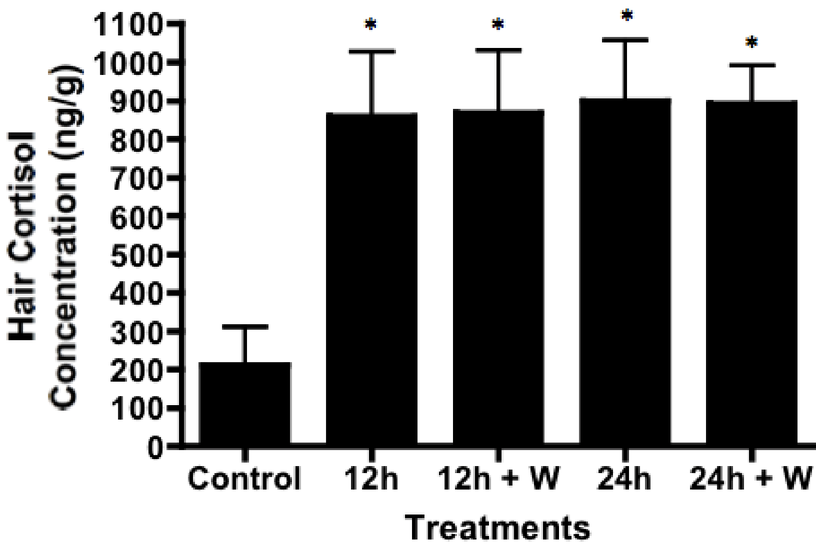

Figure 4: Effect of prolonged hair immersion for 12 or 24 hours in a 50ng/ml hydrocortisone PBS solution (mimicking sweat containing cortisol) and washing on hair cortisol concentrations. W: pre-analysis wash; * $p<0.001$ compared to control; n=14. Data displayed as mean±SD.

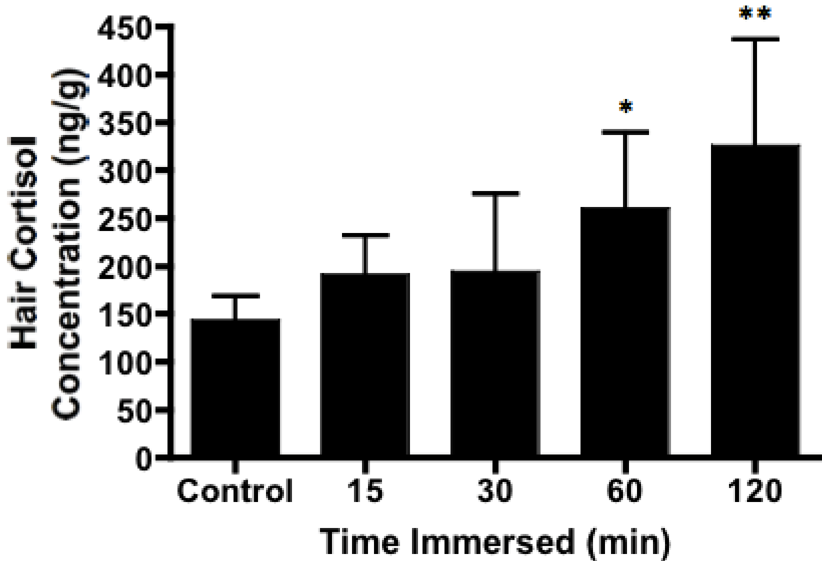

Figure 5: Effect of immersion time on hair cortisol concentrations. Hair samples were immersed a 50ng/ml hydrocortisone PBS solution for 15-120 minutes, followed by an isopropanol wash; n=7. Data displayed as mean±SD.

* $p<0.01$, ** $p<0.001$ for hair cortisol compared to control.

6.2 Hair Cortisol Concentrations in Patients with Obstructive Sleep Apnea

6.2.1 Results from Consult Visit

Ninety-three subjects were recruited; their characteristics are displayed in Table 3. Of the subjects recruited, two were excluded, one of the basis of prednisone use, and another because an insufficient amount of hair was obtained.

Table 3: Obstructive sleep apnea patient physical exam characteristics and medical history (n=91)*

Age	55±11 years
Gender (M:F)	56 (61%) : 35 (39%)
BMI	35.4±7.3 kg/m^2
Mean Arterial Pressure	98±10 mmHg
Neck circumference	42.9±5.1 cm
Smokers	14 (15%)
Alcohol drinks per day (median [range])	0 [0-3.5]
Subjects taking prescription drugs	78 (86%)
Comorbidities	
Hypertension	45 (49%)
Hyperlipidemia	23 (25%)
Type II Diabetes	14 (15%)
Past Myocardial Infarction	7 (8%)
Chronic Obstructive Pulmonary Disease	5 (5%)
Hypothyroidism	6 (7%)
Gastroesophageal Reflux Disease	9 (10%)
Arthritis	5 (5%)
Fibromyalgia	7 (8%)
Depression	14 (15%)
Anxiety	8 (9%)

*Results are presented as mean±SD unless indicated otherwise

The sleep parameters collected at the time of each subject's initial polyosomnogram are displayed in Table 4. The mean PSS score was 17±7.9. The median hair cortisol concentration was 191.1 ng/g (range 66.2—1152 ng/g).

Table 4: Summary of the obstructive sleep apnea patient physical exam characteristics and medical history (n=91)*

Total sleep time	332.2±62.3 min
NREM time	278.3±50.5 min
REM time (median [range])	52 [0—153] min
Total hypoxemic time (median [range])	12.1 [0—260] min
Arousals/h (median [range])	27.2 [4.6—98.4]
AHI (median [range])	31.0 [8.1—147.2]
OSA Severity	
Mild	15 (17%)
Moderate	24 (28%)
Severe	47 (55%)
ESS Score (median [range])	10 [1—23]

*Results are presented as mean±SD unless indicated otherwise

When examining how the parameters taken during the polysomnogram correlated with hair cortisol concentrations, the following relationships were observed. AHI did not have a significant linear or nonlinear association with hair cortisol concentrations (Fig. 6). A one-way ANOVA comparison OSA severity subgroups with hair cortisol concentrations was also not significant (p=0.31) (Fig. 7). However, the grouped hair cortisol concentrations of moderate and severe OSA patients were almost significantly increased (p=0.056) when compared to

mild OSA patients (one-tailed Mann Whitney test) (Fig. 8). Total hypoxemic time was significantly associated with hair cortisol concentrations (r^2=0.063; p<0.05) when an exponential decay model was applied (Fig. 9). A trend (p=0.1010) towards a positive linear correlation between arousals per hour and hair cortisol concentrations was noted (Fig. 10). Finally, no significant associations were observed when comparing PSS or ESS scores with hair cortisol concentrations.

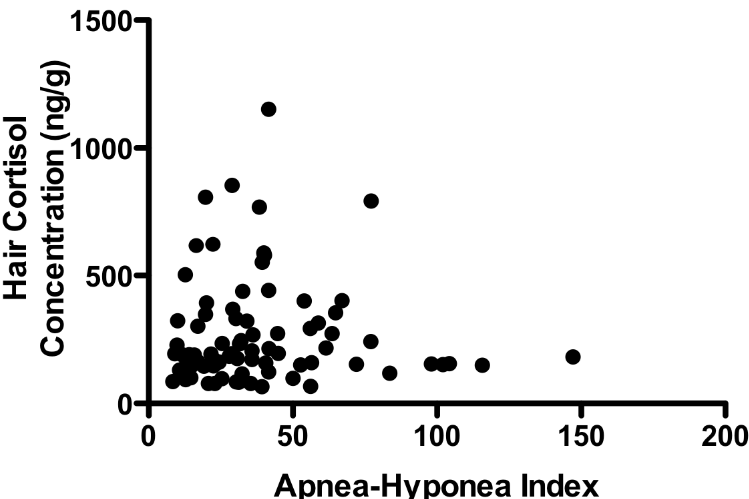

Figure 6: Hair cortisol concentrations with respect to AHI; n=80. Data displayed as mean±SD.

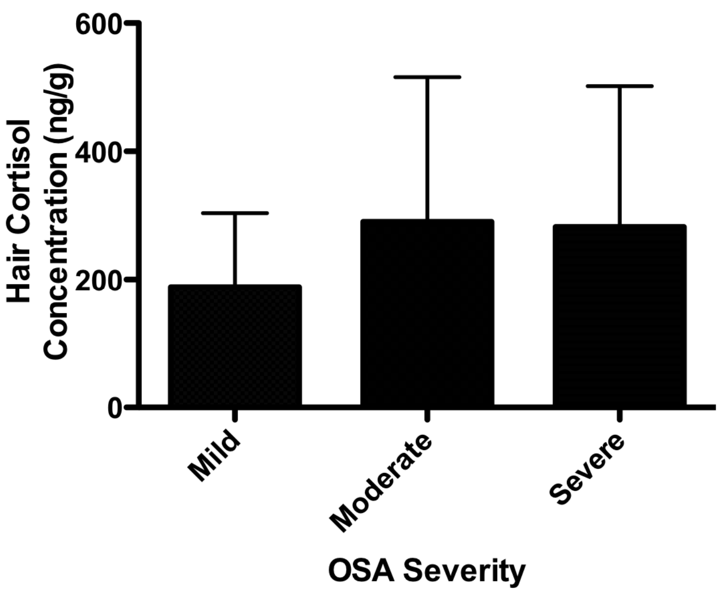

Figure 7: Hair cortisol concentrations in patients with mild, moderate and severe OSA (p=0.31); n=80. Data displayed as mean±SD.

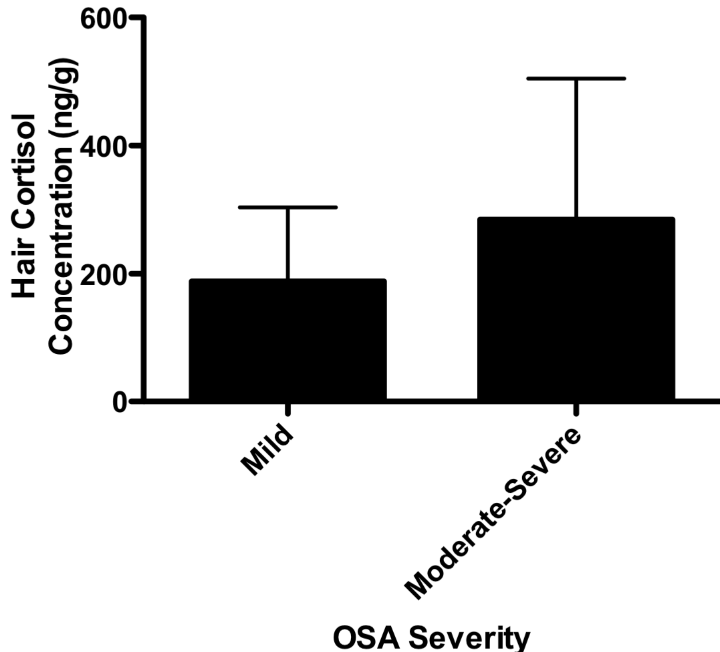

Figure 8: Hair cortisol concentrations of grouped moderate and severe OSA patients trend towards being significantly higher than mild OSA patients (p=0.056); n=80. Data displayed as mean±SD.

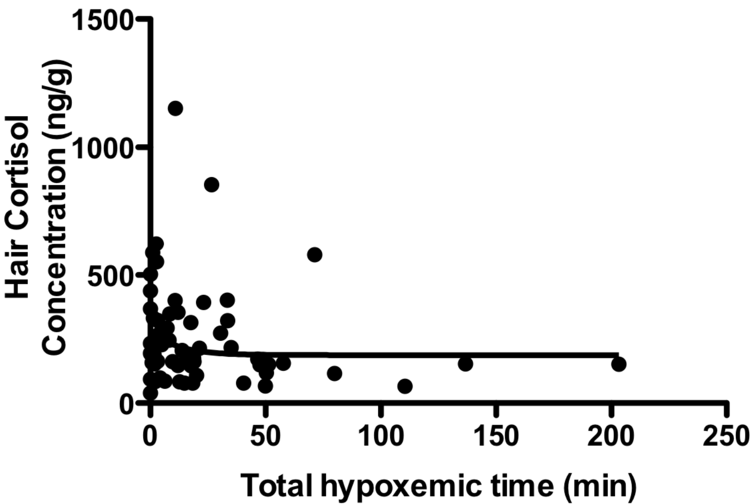

Figure 9: Total hypoxemic time follows an exponential decay pattern when compared with hair cortisol concentrations (r^2=0.063; p<0.05); n=80.

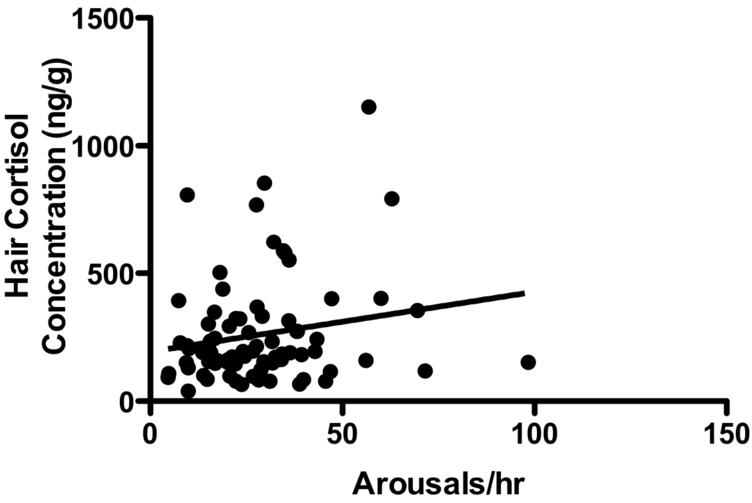

Figure 10: The number of arousals per hour trends towards a weak significant positive linear correlation with hair cortisol concentrations (r^2=0.036; p=0.1010); n=80.

6.2.2 Results from Intervention

Second hair samples and PSS were collected from 31 of the patients who returned to the sleep clinic after 3 months of CPAP. A two-tailed Wilcoxon

matched pairs test did not detect a significant difference in hair cortisol concentrations after placement on CPAP (p=0.69) (Fig. 11). A paired two-tailed t-test detected a significant decrease in perceived stress following placement on CPAP (p<0.001) (Fig. 12).

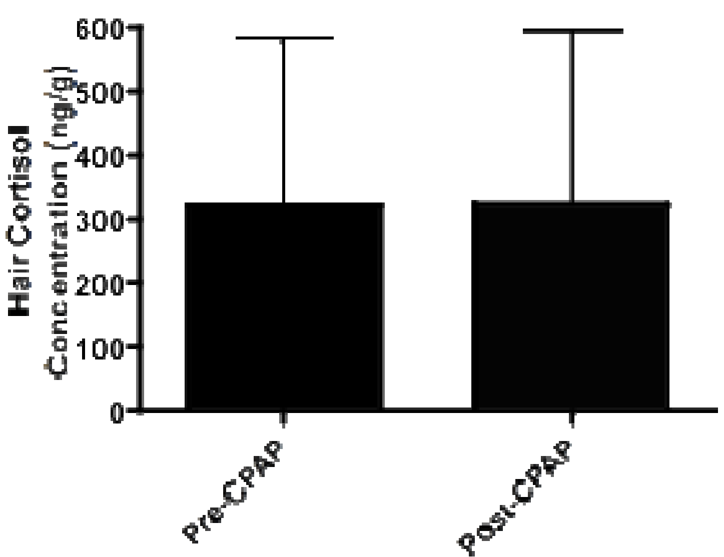

Figure 11: No significant differences are observed in hair cortisol concentrations before and after placement on CPAP (p=0.69); n=31. Data displayed as mean±SD.

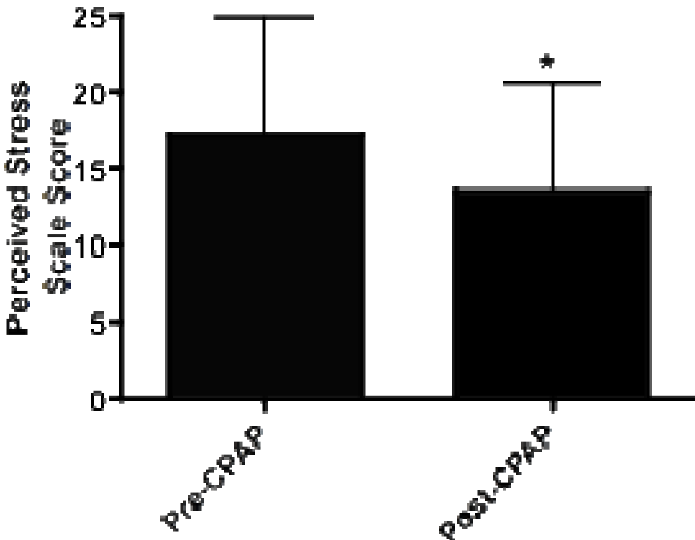

Figure 12: Levels of perceived stress are significantly reduced following a 3 month placement on CPAP (*p*<0.001); n=31. Data displayed as mean±SD.

Of the 31 patients who returned for a follow-up appointment after CPAP, only 21 had either remembered to bring their CPAP machine to the appointment or had a machine capable of downloading sleep information off of it. A sub-group analysis of these patients was performed, examining their pre- and post-CPAP AHI scores, hair cortisol concentrations, and PSS scores. The mean duration of CPAP use was 5 hours and 49 minutes per night. A one-tailed Wilcoxon matched pairs test revealed that the AHI scores of these patients were significantly reduced when placed on CPAP (*p*<0.0001) (Fig. 13). Similar to the entire group of follow-up patients, hair cortisol concentrations were not significantly changed (*p*=0.78)(Fig. 14) and perceived stress was significantly reduced (*p*<0.001) (Fig. 15). Additionally, when comparing the change in perceived stress with the change in AHI, a significant positive linear association was found (*p*=0.0164) (Fig. 16). No significant correlations were found when

comparing either the change in AHI with the change in hair cortisol concentrations, or the change in perceived stress with the change in hair cortisol concentrations.

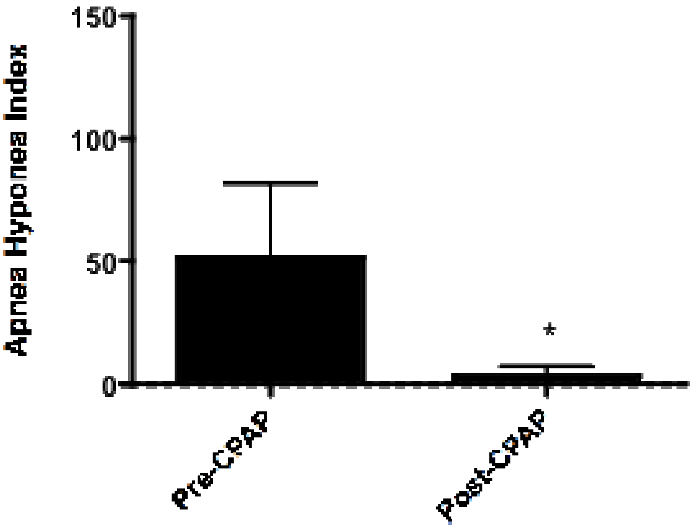

Figure 13: Patient AHI values are significantly reduced after placement on CPAP ($p<0.0001$); n=21. Data displayed as mean±SD.

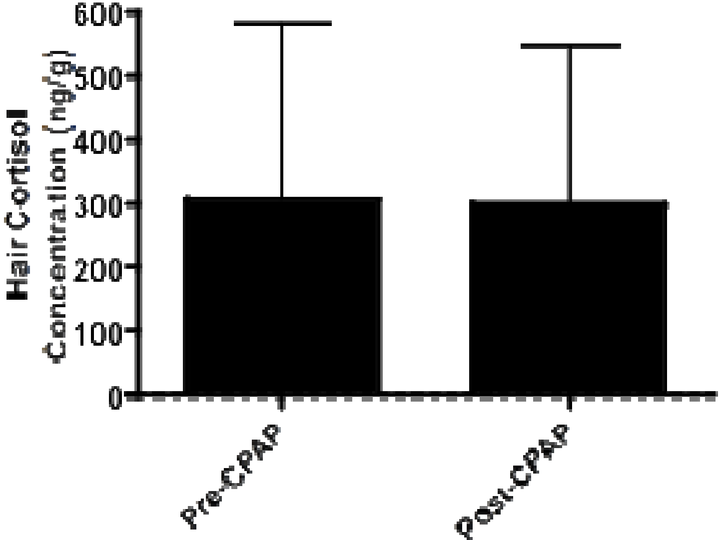

Figure 14: Subgroup analysis of patients who had a follow-up AHI value. Hair cortisol concentrations are not significantly different after placement on CPAP ($p=0.78$); n=21. Data displayed as mean±SD.

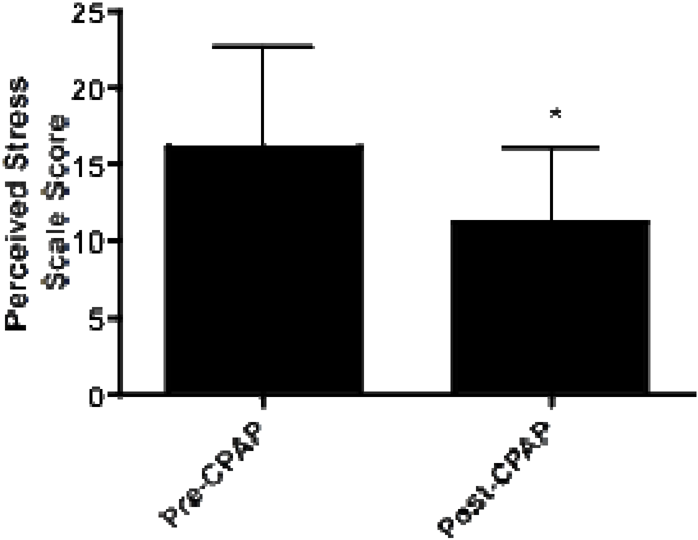

Figure 15: Subgroup analysis of patients who had a follow-up AHI value. Levels of perceived stress are significantly reduced after placement on CPAP (p=0.0009); n=21. Data displayed as mean±SD.

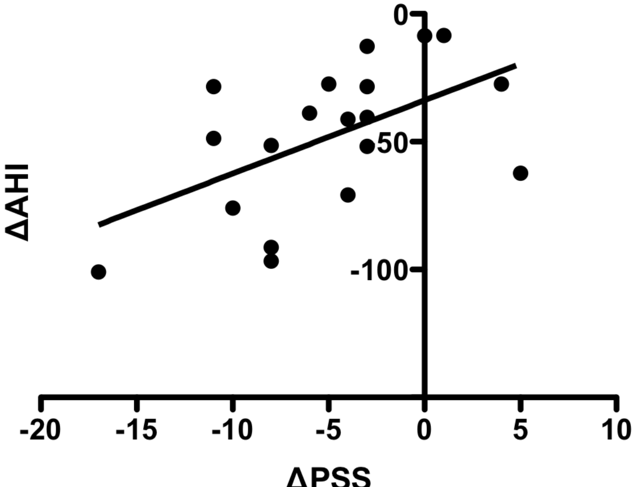

Figure 16: The change in AHI is significantly positively correlated with the change in perceived stress (p=0.0164); n=21.

Chapter 7: Discussion

7.1 The Detection of Cortisol in Human Sweat—Implications for Measurement of Cortisol in Hair

To our knowledge this is the first report to date that has employed an ELISA to detect cortisol in sweat. The presence of a quantifiable amount of cortisol in sweat lends support to the proposal that some of the hair cortisol content in hair is of eccrine origin. Similar to salivary cortisol concentrations, which are known to represent the free, unbound fraction of cortisol in the blood (Jessop & Turner-Cobb, 2008), it is plausible that sweat cortisol concentrations also represent the free fraction of hormone. Further, the correlation suggests that cortisol in sweat, like in saliva reflects acute HPA activity (Kudielka et al. 2008).

The hydrocortisone immersion experiments underscore the need to record personal hygiene habits (e.g. how recently a subject has washed his/her hair, how frequently and for how long the subject sweat extensively) when conducting studies that use hair cortisol analysis. The results demonstrate that when hair is exposed to sweat cortisol concentrations well within the range of concentrations that were observed clinically hair cortisol content is substantially increased ($p<0.001$). This increase does not appear to be dependent on the duration of exposure at durations of longer than 12 hours. Importantly, the standard wash procedure used to remove external contaminants proved ineffective in removing the cortisol from the hair that was added from the hydrocortisone solution. Thus,

if hair samples were collected from subjects in whom hair hygiene varied significantly, washing would likely fail to normalize the concentrations and present a significant confounder. These results contrast a study by Hamel et al. (2011) in which repeatedly exposing hair samples to shampoo washes decreased the hair cortisol concentrations. A possible reason for this contrast is that surfactants are potentially more effective than alcohol washes at removing sweat and sebum from hair.

When hair is exposed to hydrocortisone for shorter periods of time, more in line with real-life duration of exposure of hair to sweat, relationship between exposure time and hair cortisol content becomes more apparent. When exposed to hydrocortisone for just 60 minutes, the hair cortisol concentrations are significantly increased from a brief control exposure ($p<0.01$). Thus, if hair samples were collected when a subject had been sweating for 60 minutes, it is possible the hair cortisol concentration determined would be increased than if the individual was not sweating. A previous paper found increased hair cortisol concentrations in athletes compared to control subjects. In light of the current findings it is conceivable that some of this increase in hair cortisol content may be due to prolonged sweating (Skoluda et al., 2011).

A limitation to this study was that only perspiration induced through intensive exercise was examined. Cortisol concentrations are known to increase during periods of physical stress such as exercise (Shojaei et al. 2011), therefore it is possible that the sweat cortisol levels were temporarily elevated in our treatment conditions. However, increased cortisol concentrations have been

noted in cases of passive hyperthermia (Jimenez et al., 2007). Further, in conditions such as acute myocardial infarctions or fever, where diaphoresis is a common symptom, elevated cortisol concentrations are noted as well (Vallance et al., 1978). Thus, most conditions that induce sweating are likely to be associated with elevated cortisol concentrations.

Recently, 11B-HSD 1 has been shown to be expressed in the skin (Tiganescu et al. 2011). This expression was significantly correlated with age and with photo-exposure. This finding suggests that cortisol may be produced in excess at the level of the dermis and epidermis. Therefore, sweat cortisol concentrations may be proportionally higher than what could be expected systemically. While this may be true of sweat, it does not likely translate to a proportionally higher hair cortisol concentration as past studies have demonstrated that changes hair cortisol concentrations closely mirror the changes in serum cortisol concentration in patients receiving a successful intervention for Cushing Syndrome (Thomson et al., 2009).

Additionally, it would have been preferable to examine any inter-day variations in sweat cortisol concentrations to try to establish its stability over time. Another limitation to this study is that multiple sweat collections were not performed on the same individual at multiple time points. This design would have being capable of more robustly demonstrating if a diurnal variation occurs for sweat cortisol concentrations. Future studies should also examine *in vivo* cases of sweating and observe if similar increases in hair cortisol concentrations are observed.

In previous studies both our group and others have shown that the patterns of change in hair cortisol, when segmented to reflect month-by-month exposure, closely follow the changes measure in blood and saliva. This was commonly interpreted that most of the hair load of cortisol enters the shaft from the blood. Our new observation, that sweat contains cortisol in levels reflecting systemic concentrations, and that sweat cortisol is not easily washable from the hair shaft, indicate that at least some of the measured cortisol in hair may stem from sweat. Because sweat cortisol significantly correlates with systemic levels of the hormone, sweat-derived cortisol is not likely to disrupt the correlation between hair cortisol and blood.

7.2 Hair Cortisol Concentrations in Patients with Obstructive Sleep Apnea

Initially the apparent downregulation of HPA activity, as shown by hair cortisol concentrations, in response to an increased AHI or increased total hypoxemic time is somewhat paradoxical. In both *in vitro* and *in vivo* models, a hypoxemic environment has been demonstrated to down regulate the expression of 11β-HSD2 (Heiniger et al., 2003). In this event, cortisol excretion in the form of cortisone is reduced, and an increase in circulating cortisol would be expected.

Not all studies have linked a hypoxic environment with an increase in cortisol. A recent study that used healthy volunteers compared the effects of 5 hours of intermittent hypoxia on serum cortisol, but failed to detect any change in serum cortisol concentrations (Louis & Punjabi, 2009). In a study of 14 healthy men subjected to a hypoxic condition that reduced their oxygen saturation to

75%, their serum cortisol was not significantly different from the normoxic control condition (Oltmanns *et al.*, 2004). Likewise, Benso *et al.* (2007) studied 9 mountaineers at sea level and at 5,200m after summiting Mt Everest 7 weeks later, and the chronically hypoxic environment did not significantly affect serum cortisol concentrations.

HPA downregulation is also observed in a number of other clinical examples of severe chronic stress. The hallmark example is PTSD. As mentioned earlier, cortisol is thought to be initially increased in the case of PTSD, but as the stressor becomes less salient and the duration from the initial stressor becomes more temporally distant, cortisol secretion has been shown to decline below normal levels—suggesting negative feedback overregulation. In addition to PTSD, chronic pain (Gaab *et al.*, 2005), rheumatoid arthritis and asthma (Heim *et al.*, 1999; Riedel *et al.*, 2002) have been associated with hypocortisolism. Finally, recently hair cortisol analysis has been used to indentify HPA downregulation in cases of temporomandibular joint disorder and in boys that were born pre-term (unpublished findings).

In the follow-up patient sample, in spite of significant clinical improvement, the hair cortisol changes were not significantly different. When interpreting the effect that CPAP had on hair cortisol concentrations, the number of patients with comorbid conditions known to be associated with increased cortisol secretion. Hypertension (Martins *et al.*, 2012), type II diabetes, (Reynolds et al., 2010), and depression (Zunszain *et al.*, 2011) were all common comorbidities of patients in this study, and their association with hypercortisolemia may have masked any

change that CPAP had on cortisol secretion. Additionally, the noted reduction in perceived stress may suggest that psychological stress is more rapidly responsive to clinical improvement, whereas physiological changes in stress may require a longer duration before improvement is observed. However, a limitation to this study is that the participants were not blinded to the research question of if hair cortisol and physiologic stress are altered by being placed on CPAP. Therefore, they may have been biased, whether consciously or subconsciously, to report reduced psychological stress at the follow-up appointment.

Chapter 8: Conclusions

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. Developing a complete knowledge of how sweat and sebum contribute to total hair cortisol content will be useful when interpreting results. As observed in this project, sweat cortisol may significantly increase hair cortisol content even when attempts are made to normalize it with the use of isopropanol washes. Additionally, hair cortisol analysis is continuing to provide new insight of how HPA functioning is altered in disease states. Specifically, hair cortisol analysis may help discern the complex changes cortisol secretion, such as in the case of OSA.

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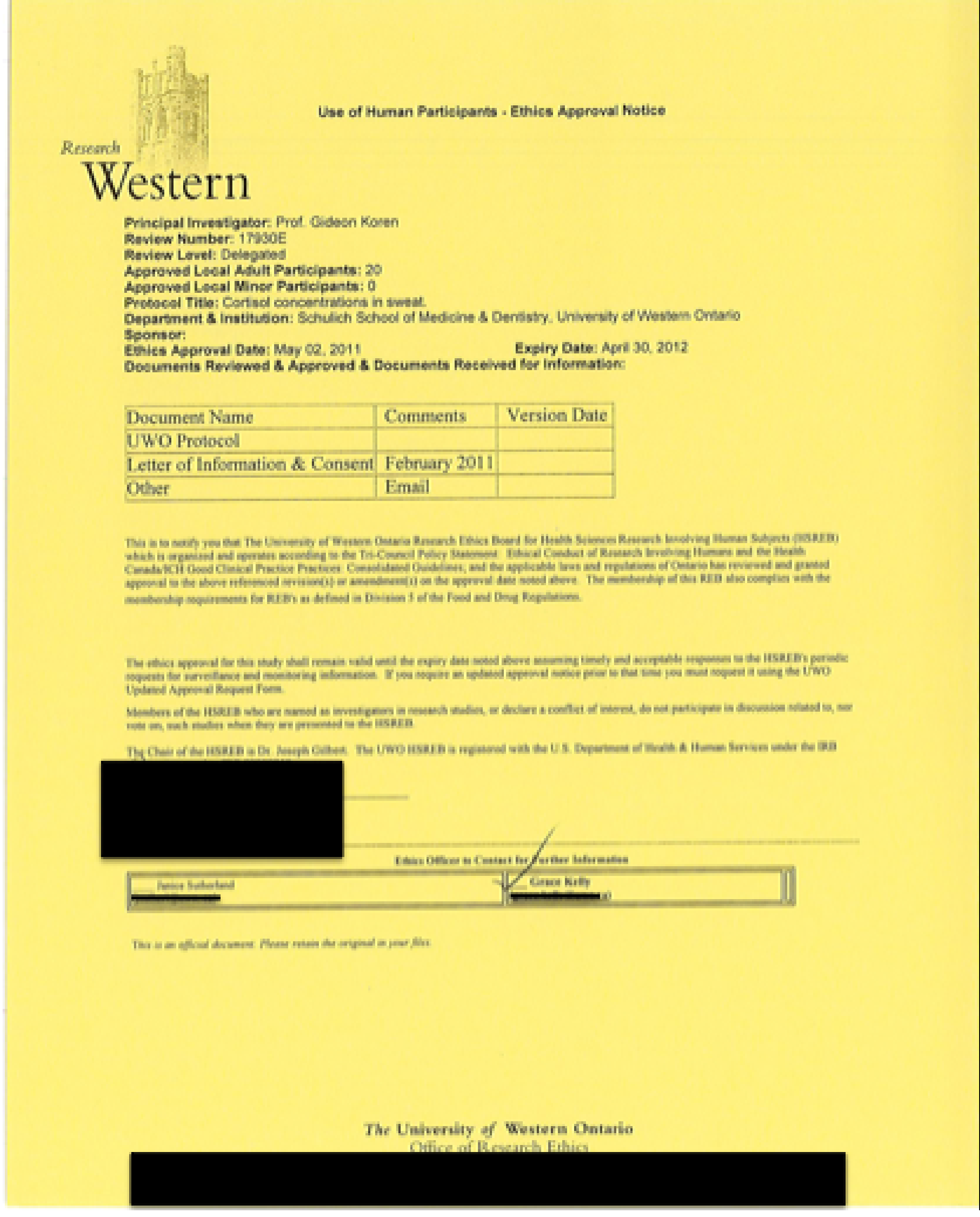
Appendix A: UWO ethics approval for sweat study



Research Western

Use of Human Participants - Ethics Approval Notice

Principal Investigator: Prof. Gideon Koren
Review Number: 17930E
Review Level: Delegated
Approved Local Adult Participants: 20
Approved Local Minor Participants: 0
Protocol Title: Cortisol concentrations in sweat.
Department & Institution: Schulich School of Medicine & Dentistry, University of Western Ontario
Sponsor:
Ethics Approval Date: May 02, 2011 **Expiry Date:** April 30, 2012
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
UWO Protocol		
Letter of Information & Consent	February 2011	
Other	Email	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The UWO HSREB is registered with the U.S. Department of Health & Human Services under the IRB

Ethics Officer to Contact for Further Information

Janice Sutherland	Grace Kelly

This is an official document. Please retain the original in your files.

The University of Western Ontario
Office of Research Ethics

Appendix B: UWO ethics approval for OSA study

Office of Research Ethics

The University of Western Ontario

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. S. Van Uum
Review Level: Expedited
Review Number: 17340E
Revision Number: 1
Review Date: January 20, 2011
Approved Local # of Participants: 100
Protocol Title: Hair Cortisol levels in patients with obstructive sleep apnea
Department and Institution: Medicine-Dept of, St. Joseph's Health Care London
Sponsor:
Ethics Approval Date: January 28, 2011
Expiry Date: April 30, 2012
Documents Reviewed and Approved: Revised participant recruitment. Letter of Information and Consent (December 2010).
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

Ethics Officer to Contact for Further Information			
☐ Janice Sutherland	☐ Elizabeth Wambolt	☑ Grace Kelly	

This is an official document. Please retain the original in your files.

cc: ORE File
LHRI

UWO HSREB Ethics Approval - Revision
V 2008-07-01 (rptApprovalNoticeHSREB_REV)

17340E

Page 1 of 1

Appendix C: Perceived Stress Scale

Perceived Stress Scale

The questions in this scale ask you about your thoughts and feelings **during the last month.** In each case, you will be asked to indicate by *circling how often* you felt or thought a certain way.

Participant Code:______________ Date:___________________________
Age:_____________
Gender (circle) M F

0= Never 1= Almost Never 2= Sometimes 3= Fairly Often 4= Very Often

1. In the last month, how often have you been upset because of something that happened unexpectedly?	0 1 2 3 4
2. In the last month, how often have you felt that you were unable to control the important things in your life?	0 1 2 3 4
3. In the last month, how often have you felt nervous or, "stressed?"	0 1 2 3 4
4. In the last month, how often have you felt confident about your ability to handle your personal problems?	0 1 2 3 4
5. In the last month, how often have you felt that things were going your way?	0 1 2 3 4
6. In the last month, how often have you found that you could not cope with all the things that you had to do?	0 1 2 3 4
7. In the last month, how often have you been able to control irritations in your life?	0 1 2 3 4
8. In the last month, how often have you felt that you were on top of things?	0 1 2 3 4
9. In the last month, how often have you been angered because of things that were outside of your control?	0 1 2 3 4
10. In the last month, how often have you felt that difficulties were pilling up so high that you could not overcome them?	0 1 2 3 4

Appendix D: Epworth Sleepiness Scale

Directions: How likely are you to doze off or fall asleep in the following situations, in contrast to feeling just tired. This refers to your usual way of life in recent times. Even if you have not done some of these things recently, try to work out how they would have affected you. Use the following scale to rate your chance of dozing in each situation. Circle the correct number to indicate your chance of dosing for each item below.

0= would *never* doze
1= *slight* chance of dozing
2= *moderate* chance of dozing
3= *high* chance of dozing

1. Sitting and reading	0 1 2 3
2. Watching TV	0 1 2 3
3. Sitting, inactive in a public place (e.g., a theatre or a meeting)	0 1 2 3
4. As a passenger in a car for an hour without a break	0 1 2 3
5. Lying down to rest in the afternoon when circumstances permit	0 1 2 3
6. Sitting and talking to someone	0 1 2 3
7. Sitting quietly after a lunch without alcohol	0 1 2 3
8. In a car, while stopped for a few minutes in the traffic	0 1 2 3

Appendix E: Information collection page for sweat study

Information Collection Page—Sweat Study

Participant Code: ____________ Sex: ______________

Date: ______________ Time: _________________

Pre-collection checklist:

- Taking any oral, parenteral glucocorticoids in past 3 months? (Y/N; if yes, please provide the name of the medication)
- Used any hydrocortisone containing creams in past month?
- Currently pregnant? (Y/N/unsure)
- Height: ______________ Weight: ______________
- Smoking Status (Y/N; if yes, how many years of smoking, and how many packs/day?):
- Alcohol consumption (standard alcoholic drinks/day): _____________________

Appendix F: Information collection page for OSA study

Checklist for Participants at the Sleep Clinic

Participant Code: ____________________

<table>
<tr><td></td><td colspan="3">Letter of information and consent read and signed.</td></tr>
<tr><td></td><td colspan="3">Participant has a signed copy of the letter of information and consent.</td></tr>
<tr><td></td><td colspan="3">Participants are familiar with exclusion criteria:
• Oral, parenteral glucocorticoids in past 3 months
• Cushing’s Syndrome
• Use of hydocortisone-containing creams in past month
• Major systemic disease
• Pregnancy
• Unwilling/Unable to donate 2cm sample</td></tr>
<tr><td></td><td colspan="3">Perceived Stress Scale has been completed by the participant.</td></tr>
<tr><td></td><td colspan="3">Hair sample collected and place in envelope.</td></tr>
<tr><td></td><td colspan="3">Age: _________ Gender: ___________ Height: ________ Weight: _________

Neck circumference: __________________ BP: ________ HR: _________</td></tr>
<tr><td></td><td>Current medical conditions:</td><td>Current medications:</td><td>Smoking Status:

Alcohol Consumption:</td></tr>
<tr><td></td><td>Apnea-Hypopnea Index (AHI):</td><td>Epworth Sleepiness Scale (ESS):</td><td>Arousals/h:</td></tr>
<tr><td></td><td colspan="3">NREM time: ____________% NREM time <90% O2 sat: ______________</td></tr>
<tr><td></td><td colspan="3">REM time: __________ % REM time <90% O2 sat: ________________</td></tr>
</table>

Curriculum Vitae

EVAN RUSSELL

Education

University of Western Ontario, London, Ontario **2010-present**
M.Sc. (candidate) Physiology & Pharmacology
Proposed Thesis: Methodological Challenges and Clinical Applications of Hair Cortisol Analysis
Expected Defense: April 2012

University of Western Ontario, London, Ontario **2005-2010**
Bachelor of Medical Sciences (Honors)
Specialization: Medical Sciences

Teaching Experience

Physiology 3130Y Teaching Assistant **September-April 2010/2011, 2011/2012**

- Prepare third year undergraduate students to conduct physiological experiments that provide a practical demonstration of principles learned in lecture.
- Teach students by providing tutorials, offering technical assistance throughout the laboratory, and grading their papers and presentations.

Presentations

- Poster Presentation, "The Detection of Cortisol in Sweat: Implications on Hair Analysis", London Research Day, University of Western Ontario, London, Ontario, Canada, March 2012

- Poster Presentation, "Hair Cortisol Levels in Patients with Obstructive Sleep Apnea", Canadian Society of Pharmacology and Therapeutics annual meeting 2009, Montreal, QC, Canada, May 2011

- Poster Presentation, "The Detection of Cortisol in Sweat: Implications on Hair Analysis", Charles W. Gowdey Lecture and Research Day, University of Western Ontario, London, Ontario, Canada, November 2011

Publications

Papers

- Russell E, Koren G, Rieder M, Van Uum S. (2012). Hair cortisol as a biological marker of chronic stress-Current status, future directions, and unanswered questions. *Psychoneuroendocrinology*. 37: 589-601.

- Sanders A, Slade G, Lambert C, Russell E, Koren G, Van Uum S. (2012). Hair cortisol concentrations in patients with temporomandibular joint disorder. *Submitted to the Journal of Dental Hygiene*.

- Russell E, Koren G, Rieder M, Van Uum S. (2012). The detection of cortisol in human sweat: Implications for measurement of cortisol in hair. *In preparation.*

- Russell E, Koren G, Rieder M, Van Uum S. (2012). Hair cortisol concentrations in patients with obstructive sleep apnea. *In preparation.*

- Russell E, Koren L, Koren G, Rieder M, Van Uum S. (2012). Validation of the Alpco Diagnostics Salivary ELISA for use in hair cortisol analysis with liquid chromatography-mass spectroscopy. *In preparation.*

- Grunau RE, Cepeda IL, Chau CMY, Brummelte S, Russell E, Koren G, Synnes A, Van Uum S, Gosse G, Miller SP, Weinberg J. (2012). Hair cortisol levels suggest HPA downregulation at age 7 years in boys born very preterm. *In preparation*.

Abstracts

- Russell E, Koren G, Rieder M, Van Uum S. (2011). Hair cortisol concentrations in patients with obstructive sleep apnea. In: Multidisciplinary Approaches to Modern Therapeutics, May 24-27, 2011, pp. 132-133.

- McClure HH, Shortt JW, Eddy JM, Van Uum SHM, Russell E, Koren G, Snodgrass JJ. (2011). The meaning of a lock: Cortisol from hair and psychosocial stress among incarcerated mothers in Oregon. *Accepted for publication in Human Biology Association 2012 Annual Meeting conference program.*

- Grunau RE, Cepeda IL, Chau CMY, Brummelte S, Russell E, Koren G, Synnes A, Van Uum S, Gosse G, Miller SP, Weinberg J. (2011). Hair cortisol levels suggest HPA downregulation at age 7 years in boys born very preterm. *Submitted for publication in the Pediatric Academic Societies 2012 Annual Meeting conference program.*

Academic Awards/Funding

- Ontario Graduate Scholarship 2011/2012
- Western Graduate Research Scholarship 2010/2011, 2011/2012

- Dean’s Honor List 2009, 2010