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Applications of Hair as a Record of Systemic Exposure to Cortisol

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Abstract

Hair cortisol content (HCC) is a novel biomarker that uniquely captures retrospective systemic cortisol exposure. This thesis aimed to assess how different hair growth rates effect HCC timelines in Cushing's and Addison's patients, investigate the relationship between HCC and age, puberty, sex and BMI in healthy children and adolescents, and assess novel methods to improve cortisol extraction and recovery. Retrospective HCC timelines derived from a 0.75 cm/month growth rate best matched 50% of patients' medical records rather than the historically assumed 1 cm/month. HCC correlated positively with age ($p<0.0001$), puberty status ($p<0.001$), and BMI ($p<0.01$) in males and females 7-17 years old. Nitrogen evaporation resulted in greater cortisol recovery than air evaporation ($p=0.0003$), and hair digestion using NaOH resulted in more rapid extraction of cortisol. These results provide incremental improvements to previous methods and assumptions for HCC analysis and elucidate normal HCC changes in children and adolescents.

Keywords

Cortisol, Hair Analysis, Therapy Monitoring, Paediatric, Stress

Summary for Lay Audience

Cortisol, the main glucocorticoid hormone in humans, is part of the normal stress response and has historically been assessed in serum, saliva, and urine. Long-term assessment from these acute measures requires repeated collection over time which is both invasive and costly. Cortisol in scalp hair has been growing in popularity as a less invasive, longer-term correlate of cortisol exposure. We retrospectively evaluated whether cortisol levels in hair correlated to reported symptomatology in the medical records of Cushing's or Addison's patients over months to years. Historical hair cortisol timelines generated by segmental hair analysis of these patients reflected reported changes in clinical states. In another study, we collected hair samples from a healthy cohort of 250 children and adolescents to assess hair cortisol changes with age, puberty status, BMI and sex. Hair cortisol increased with age, puberty status and BMI, but did not differ between sexes or correlate with age-adjusted BMI. In an effort to develop a high-throughput protocol for hair cortisol analysis we tried two methods of hair digestion. We assessed sodium hydroxide or enzymatic hair digestion to decrease extraction time and improve method reproducibility compared to the standard method which requires mincing with scissors and a 16-hour methanol extraction. We were unable to assay cortisol from the enzyme digests but were able to recover cortisol using sodium hydroxide digestion. When comparing two evaporation methods, we found that nitrogen evaporation provides a better environment for hair cortisol recovery.

Co-Authorship Statement

Chapter 2 was written in collaboration with Dr. Michael Greff, Dr. Awatif Abuzgaiaa, Abdelbaset Elzagallaai, PhD, Dr. Michael Rieder, and Dr. Stan Van Uum. They were involved with the writing and editing of the manuscript prior to submission to the journal.

Chapter 3 was written in collaboration with Abdelbaset Elzagallaai, Dr. Michael Rieder, and Dr. Stan Van Uum. They were involved with the writing and editing of the manuscript.

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List of Abbreviations

α-MSH α-Melanocyte Stimulating Hormone
11β-HSD1 11β-Hydroxysteroid Dehydrogenase Type 1
11β-HSD2 11β-Hydroxysteroid Dehydrogenase Type 2
3βHSD2 3β-Hydroxysteroid Dehydrogenase Type 2
ACTH Adrenocorticotropic Hormone
AI Adrenal Insufficiency
ANOVA Analysis of Variance
BMI Body Mass Index
BSA Bovine Serum Albumin
CAD Coronary Artery Disease
CAH Congenital Adrenal Hyperplasia
CCS Cyclical Cushing's Syndrome
CD Cushing's Disease
CRH Corticotropin-Releasing Hormone
CS Cushing's Syndrome
DHEA-S Dehydroepiandrosterone Sulfate
DM Diabetes Mellitus
DST Dexamethasone Suppression Test
DTT Dithioerythritol
ELISA Enzyme-Linked Immunosorbent Assay
GAD Generalized Anxiety Disorder
GR Glucocorticoid Receptor
HDL High-Density Lipoprotein
HPA Hypothalamic-Pituitary-Adrenal
HPG Hypothalamic-Pituitary-Gonadal
IMM Inner Mitochondrial Membrane
LC-MS Liquid Chromatography-Mass Spectroscopy
LDL Low-Density Lipoprotein
LIA Luminescence Assay
LNSC Late-Night Salivary Cortisol
MC1R Skin Melanocortin 1 Receptor
MR Mineralocorticoid Receptor
OMM Outer Mitochondrial Membrane
P450 Side-Chain Cleavage Enzyme
PBS Phosphate Buffered Saline
POMC Pro-opiomelanocortin
PTSD Post-Traumatic Stress Disorder
RIA Radioimmunoassay
SD Standard Deviation
SRB1 Scavenger Receptor B1
StAR Steroidogenic Acute Regulatory Protein
UFC Urinary Free Cortisol
ULN Upper Limit of Normal

Chapter 1

1 Introduction

The discovery of the corticosteroids was one of the seminal events in understanding the biology of the endocrine system and physiological responses to stress. Although corticosteroid biology has been studied for some time, many questions remain, notably as to the impact of stress on corticosteroids and how altering stressors may alter corticosteroid production. The use of corticosteroid concentrations in hair as biomarkers of stress has now been established for a number of conditions, but questions remain as to fundamental issues such as the ontogeny of corticosteroid deposition in hair.

1.1 Cortisol

1.1.1 Regulation and Major Physiological Effects

Cortisol is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (**Fig. 1**). The function of this system is to facilitate adequate response to various physiological stressors and restore systemic homeostasis in order to protect the body from overshooting the stress response[1]. In an acute stress paradigm, cortisol ensures sufficient energy levels and blunts inflammation to overcome the stressor[1]. It does this by increasing blood glucose via the mobilization of energy stores and the inhibition of additional storage, causes vasoconstriction and diversion of blood to muscles, and modulates immune cells and inflammatory gene expression[1]. Chronically elevated cortisol, however, results in impaired glucose and fat metabolism, inhibits bone formation, and negatively impacts health resulting in poor outcomes[2–4]. These symptoms are typical of Cushing's, a disease resulting from too much cortisol[5]. In response to stress, hypothalamic paraventricular cells secrete corticotropin-releasing hormone (CRH) into the hypophyseal portal system. Anterior pituitary corticotrophs respond to CRH by releasing adrenocorticotropic hormone (ACTH) into systemic circulation. This hormone is derived from the cleavage of pro-opiomelanocortin (POMC), which also produces α-melanocyte stimulating hormone (α-MSH) [6,7].

Cortisol is produced in the adrenal glands, which are found sitting above each kidney. The adrenal glands are composed of an outer cortex and an inner medulla. Within the cortex, *zona fasciculata* cells are stimulated by ACTH to produce and release cortisol. Cortisol is regulated in a typical negative feedback manner whereby cortisol travels back to both the hypothalamus and pituitary, subsequently inhibiting secretion of CRH and ACTH, respectively (**Fig. 1**). One symptom of adrenal insufficiency, a disease of too little cortisol, is darkening of the skin[8,9] which is caused by a lack of negative feedback and continued cleavage of POMC to generate ACTH and α-MSH. The α-MSH stimulates melanocytes in the skin. In healthy individuals, systemic cortisol secretion follows a circadian secretion pattern with lowest levels found late at night and peak levels found early in the morning[10,11].

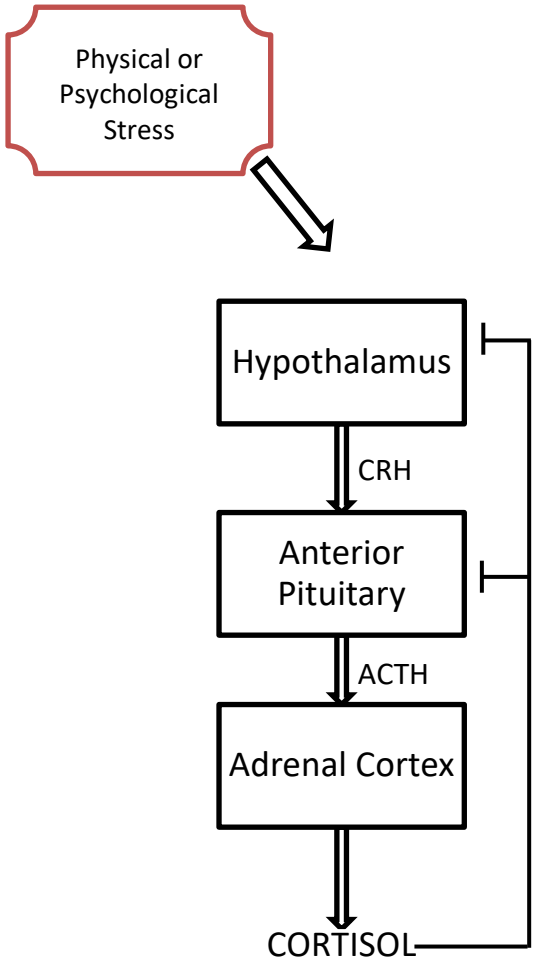

Figure 1: Activation and Negative feedback of the Hypothalamic-Pituitary-Adrenal (HPA) Axis. ACTH, Adrenocorticotropic Hormone; CRH,

1.1.2 Cortisol Biosynthesis

Beginning with cholesterol, steroid hormone synthesis, or steroidogenesis, results in the production of numerous steroid hormones. Cholesterol can be sourced from either *de novo* synthesis in the endoplasmic reticulum or from circulating cholesterol[12,13]. Cells with steroidogenic capacity uptake low-density lipoproteins (LDL) via receptor-mediated endocytosis after binding the LDL receptor, or high-density lipoprotein (HDL) upon binding of scavenger receptor B1 (SRB1)[13–15]. Enzymes within the endosome cleave cholesterol esters from these lipoproteins resulting in the export of cholesterol into the cytoplasmic compartment[12,13].

Cytoplasmic cholesterol is trafficked towards the outer mitochondrial membrane (OMM) (**Fig. 2)** or stored in lipid droplets associated with the outer OMM [14]. During maximal steroidogenesis, steroidogenic acute regulatory protein (StAR) facilitates cholesterol influx from the OMM to the inner mitochondrial membrane (IMM)[13,14,16]. Cholesterol is converted to pregnenolone by side-chain cleavage enzyme, P450scc, found associated with the IMM (**Fig. 2)**[13,14]. In the adrenal *zona fasciculata*, P450c17 converts pregnenolone to 17OH-pregenenolone. Pregnenolone and 17OH-pregnenolone are metabolized by 3β-hydroxysteroid dehydrogenase type 2 (3βHSD2) to progesterone and 17OH-progesterone, respectively. Progesterone can be metabolized to aldosterone via a multi-step pathway or, alternatively, to 17OH-progesterone by P450c17. The 17OH-progesterone pool from either of these two reactions is then hydroxylated by P450c21 to 11-Deoxycortisol, and finally by P450c11β to complete the biosynthesis of cortisol (**Fig. 2)**. The majority of cortisol synthesis occurs in the adrenal gland, with several other tissues including the liver, CNS, adipose and skin[17,18] having the capacity for extra-adrenal cortisol steroidogenesis. Cortisol that is released into circulation can interact with the glucocorticoid receptor (GR) that is found ubiquitously throughout the body.

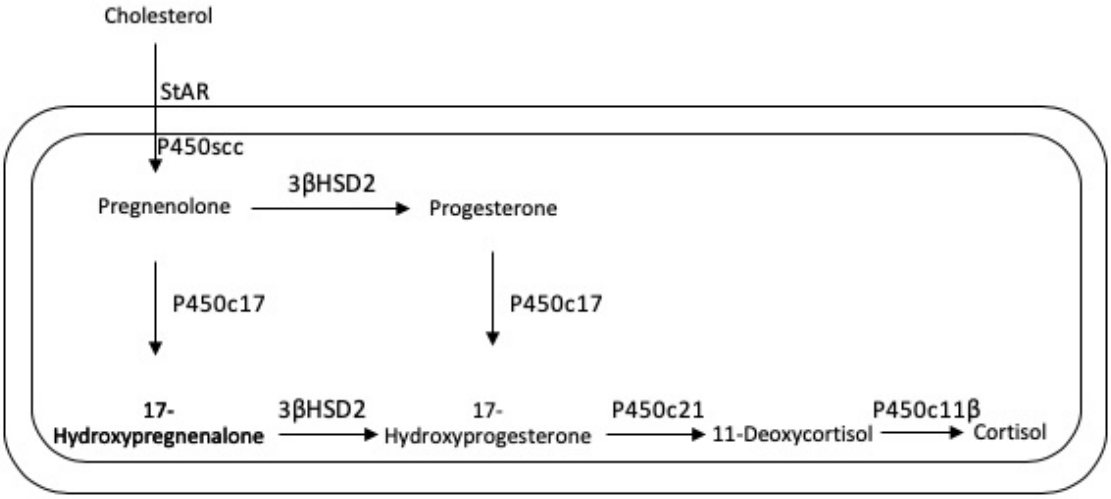

Figure 2: Mitochondrial Intracellular Cholesterol Trafficking and Cortisol Steroidogenesis of a Cortisol Producing Cell. StAR, Steroidogenic Acute Regulatory Protein. Adapted from [14,15]

1.1.3 Cortisol Transport, Metabolism, and Excretion

Most cortisol (90-95%) is transported in the blood bound to corticosteroid-binding globulin, while the remainder travels as free cortisol[19,20]. Free cortisol diffuses through the cell membrane and binds to the cytoplasmic GR. The cortisol-GR complex is translocated into the nucleus where it binds to hormone response elements to regulate target gene transcription[21,22]. Interestingly, cortisol binds the mineralocorticoid receptor (MR) with such high affinity, that low cortisol levels will maximally activate the MR while minimally activating the GR[23,24]. As a protective mechanism to minimize off-target activation of the MR, mineralocorticoid-responsive tissues such as the kidneys express 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which oxidizes cortisol to inactive cortisone[14,18,25,26]. Muted expression of 11β-HSD2 leads to apparent mineralocorticoid excess, a syndrome that mimics excess mineralocorticoids leading to symptoms such as hypertension and hypokalemia[26]. On the other hand, 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) increases intracellular cortisol levels by reducing cortisone to cortisol[18,26,27]. Activation by 11β-HSD1 is important in adipocyte differentiation and expression is elevated in obese individuals[18].

The half-life of cortisol is about 60 – 80 minutes[11,26]. Metabolism of cortisol prior to excretion primarily occurs in the liver and kidneys, with a small fraction being excreted in the urine without prior metabolism[26,27]. Cortisol and cortisone are reduced in a multistep pathway to produce reduced metabolites that can be glucuronidated or sulfated, and eventually excreted[26].

1.2 Changes in the HPA axis

Physiological changes in HPA reactivity are a consequence of signals that inform the body of the time of day[28,29] or season[30,31], the current developmental stage of life[32–34], or other environmental signals[35] that require the so-called sympathetic ("fight-or-flight") system. The appropriate HPA response is both situational- and time-dependent, involving complex regulatory biology with intricate control to avoid negative outcomes from too much or too little cortisol production. This dynamic system can become dysregulated by psychological experiences or pathophysiological changes to developmental signals, or the development of lesions within the HPA axis.

1.2.1 Pathological changes in HPA function

Aberrations in how the body responds to any of these signals may arise from the way in which an individual's brain perceives environmental stressors with memories of past experiences or the nature of current circumstances[1,36]. These types of stress-induced changes, at least in part, might be responsible for propagating symptoms of PTSD, depression and anxiety[37,38]. Though mostly beyond the scope of the current thesis, mental health is briefly discussed in chapter 2. Cellular changes at any level of the HPA axis might also be responsible for aberrant stress response signaling; the two most common forms of HPA dysregulation are Adrenal Insufficiency (AI) and Cushing's Syndrome (CS)[5,8,39].

1.2.1.1 Hypocortisolemia

Adrenal insufficiency is a potentially life-threatening condition wherein the adrenal glands fail to produce enough cortisol[8,40,41]. The precise subclass of AI can be

determined based upon where the abnormality occurs within HPA axis. Primary AI results from a loss of function at the level of the adrenal glands; secondary AI occurs at the level of the pituitary gland; and tertiary AI originates at the level of the hypothalamus (**Fig. 3)**[42].

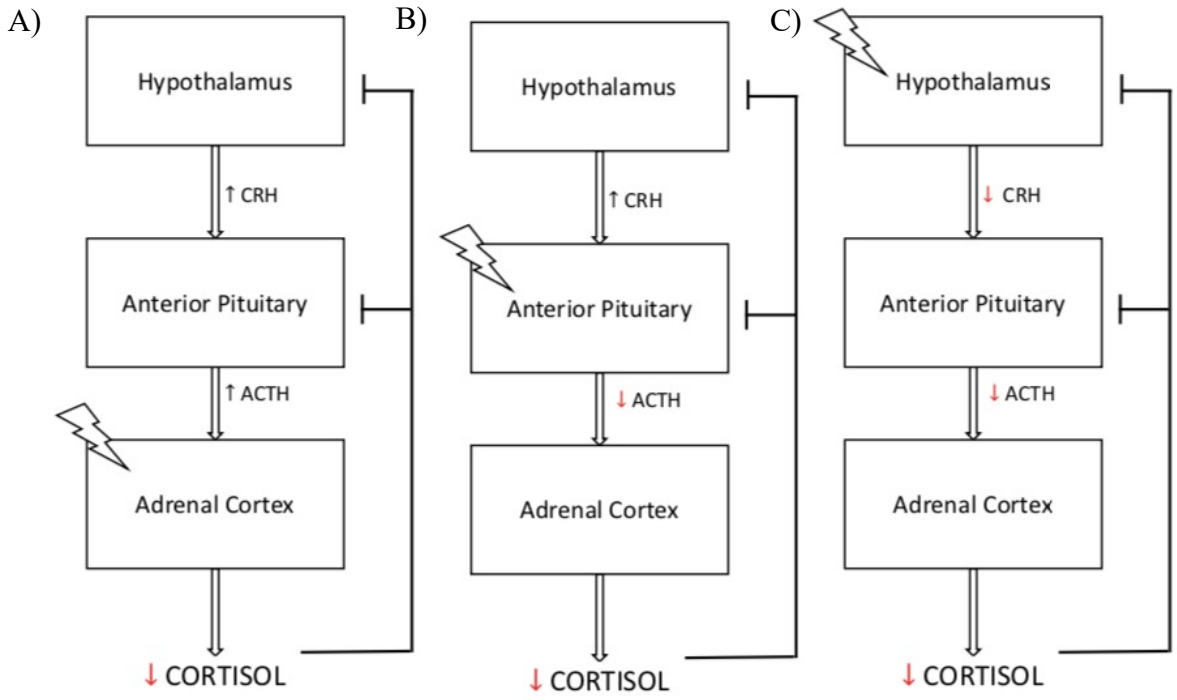

Figure 3: Classification of AI. A) Primary; B) Secondary and C) Tertiary AI. Adapted from [42]

Addison's disease is the most common form of primary AI and is caused by autoantibody-induced destruction of the cortisol-producing cells in the adrenal glands[8,43]. Addison's disease has a prevalence of about 1 in 10,000[8] and occurs most frequently in women above the age of 30[42]. Autoantibodies are found against several steroidogenic enzymes within the adrenal glands; anti-21-hydroxylase antibodies being the most common[44]. Congenital adrenal hyperplasia (CAH), an autosomal recessive condition, is another form of primary AI, where genetic mutations in one or more of the cortisol biosynthetic enzymes result in the inability to produce adrenal hormones[45,46]. Notably, mutations in the CYP21A2, HSD3B2, CYP11B1, CYP17A1 or STAR genes

can have profound effects on an individual's ability to produce adrenal hormones. Reduced activity of the 21-hydroxylase (P450c21) enzyme resulting from mutations in CYP21A1 account for 95% of all CAH cases[40,46]. Combined, the prevalence of CAH is about 1 in 15,000[46].

Primary AI might also arise from medications that inhibit steroidogenesis or infections within the adrenal glands[42,43]. Drug-induced AI can result from direct inhibition of steroidogenesis, and the induction or inhibition of cortisol metabolizing enzymes[43]. The antifungal ketoconazole inhibits various steroidogenic enzymes and is commonly used to treat hypercortisolism[39,42,43]. Barbiturates, phenytoin, and rifampin induce P450-enzymes resulting in lower systemic of exposure to cortisol[47,48]. Infectious adrenalitis is the most common form of primary AI in developing nations resulting from tuberculous, HIV-related cytomegaloviral, or syphilis infections[42].

Secondary and tertiary AI can occur due to hypothalamic or pituitary tumors, or alternatively from surgery and radiotherapy used to eliminate these tumors[42]. Administration of high-dose exogenous glucocorticoids is the most common cause of tertiary AI[41,43]. Long-term administration of exogenous glucocorticoids suppresses the HPA axis by inhibiting the release of CRH and ACTH from the hypothalamus and the anterior pituitary gland, respectively[41].

Diagnosis of AI is frequently delayed because many of the symptoms are non-specific[41]. Initial symptoms typically include fatigue, loss of appetite, nausea, weight loss, and muscle or joint pain[40,42,49]. More specific signs of AI involve skin hyperpigmentation from enhanced activation of skin melanocortin 1 receptors (MC1R) by high ACTH levels, salt cravings, and orthostatic hypotension due to mineralocorticoid deficiency[9,43]. In the case of primary AI, symptoms become apparent when 90% of adrenocortical tissue has already been lost[42]. Low cortisol levels are a clear indicator of AI, but typically hyponatremia and hyperkalemia are identified before more specific cortisol investigations are warranted.

Primary treatment revolves around glucocorticoid replacement therapy, typically in the form of hydrocortisone administered at doses sufficient to mimic the overall cortisol

exposure that might be expected in a healthy individual[43,50]. Most of the hydrocortisone is given in the morning to replicate the circadian secretion of cortisol with increased doses suggested during minor illness to facilitate appropriate physiological response to the stress[43]. Mineralocorticoid replacement with fludrocortisone is indicated to help control body weight and blood pressure[40,51]. Therapeutic drug monitoring is important in these patients to prevent side effects from excess glucocorticoid exposure.

1.2.1.2 Hypercortisolemia

Inappropriately high exposure to glucocorticoids result in many signs and symptoms that are typical of CS[52]n. Like AI, the classification of CS is dependent upon the primary cause of hypercortisolism (**Fig. 4**).

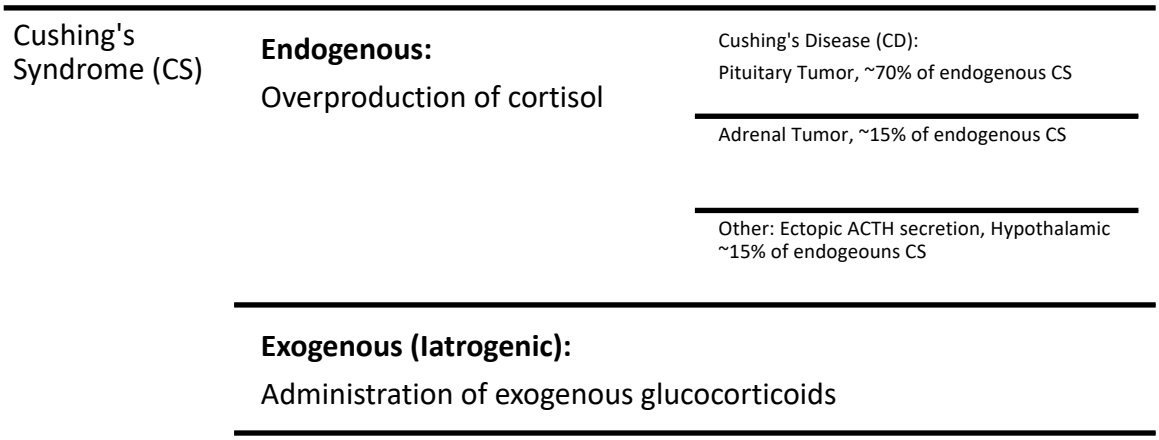

Figure 4: Classification of Cushing's Syndrome (CS).

The most common cause of CS is exogenous administration of glucocorticoids, known as iatrogenic CS[52]. Glucocorticoids are prescribed for many common inflammatory conditions including skin conditions like psoriasis[53], as well as life-threatening indications such as immunosuppression after organ transplantation[54] or in severe autoimmune diseases like systemic lupus erythematosus, lupus nephritis[55], or ANCA-associated vasculitis[56]. Dosage reduction of exogenous steroids is indicated in less severe indications. Glucocorticoids still remain important in the standard of care in many life-threatening indications because their benefits outweigh the associated side effects.

Endogenous CS occurs less frequently, with the highest estimates indicating a prevalence of approximately 10,000 cases in the Unites States[57]. ACTH-dependent CS is the most common form of endogenous CS[39,52]. Cushing's Disease (CD) is a subset of ACTH-dependent CS caused by ACTH secreting micro- or macroadenoma in the pituitary that do not adequately respond to negative feedback signals from circulating cortisol[47,52,58]. Adrenal neoplasms and hyperplasia of steroid-producing cells account for the next most common form of endogenous CS. This is known as ACTH-independent CS since the adrenal tissue continues to produce cortisol in the absence of signaling from the pituitary; ACTH levels are low-to-normal in these patients[47]. Ectopic ACTH-secreting tumors, such as those found in non-small cell lung cancer, secrete ACTH and result in excess cortisol production by the adrenal gland[59].

Patients with overt CS have a characteristic Cushingoid phenotype; on the other hand, it is often difficult to diagnose patients with mild-to-moderate CS because of the overlapping signs and symptoms with other conditions[47,52]. Signs that best discriminate CS include easy bruising, facial plethora, proximal muscle weakness, striae (reddish purple stretch marks), dorsocervical and supraclavicular fat, facial fullness, and obesity[39,52]. Women commonly have menstrual abnormalities including amenorrhea, grow facial hair (hirsutism), are balding, and have acne[47,52]. Many of the clinical findings overlap with obesity and metabolic disorders and include hypertension, cardiovascular disorders, hypokalemia, polycystic ovarian syndrome, and diabetes mellitus[40]. Symptoms include depression, fatigue, irritability, and decreased concentration, libido and memory[39,52]. CS in children is most apparent by weight gain, with decreasing growth velocity, short stature, and delayed or pseudo precocious puberty[39,60].

The primary goal in these patients is to eliminate the signs and symptoms of CS and treat comorbidities by normalizing cortisol levels or blocking its action at the receptor level[39,61]. Surgical resection of the pituitary adenoma or adrenalectomy is indicated as first line therapy in CS and often restores normal HPA control. However, in about 20-30% of patients, surgery might not be possible due to the location of the tumor, risks associated with repeated surgery or other underlying conditions[39,61]. In this group of

patients, the goal is to minimize the effects of excess cortisol. Second-line therapy in eligible patients is radiotherapy to target the lesioned tissue, or medicinal intervention to lower cortisol levels or inhibit GR binding in target tissues[39]. Although not approved in many countries for the CS, the cortisol synthesis inhibitor ketoconazole is often used to lower cortisol production in these patients but is associated with a risk of hepatotoxicity[52,61,62]. Ketoconazole inhibits CYP17A1, CYP11A1, CYP11B1, and CYP11B2[61]. Mifepristone is a GR antagonist that is approved to reduce blood glucose in these patients, but side effects are frequent because systemic cortisol levels remain elevated[39,58,61]. Mifepristone has off-target affinity for the progesterone receptor leading to endometrial thickening in women and is also used for medical abortions[39,58]. Other therapies include somatostatin analogues[58,63] and dopamine receptor agonists[39,58].

1.2.2 HPA changes during adolescence

Significant research has gone into the development of the HPA axis during the peri- and post-natal period, much of which is influenced by the mother's surroundings and exposure to stress. The ontological changes in HPA function during this period are extremely important as they set the trajectory for the individuals' life, but further discussion is beyond the scope of this thesis. After the neonatal period, the next major period of change occurs around puberty[64].

The maturation of the hypothalamic-pituitary-gonadal (HPG) system is an important milestone that allows an organism to reproduce. The hormones produced during this process: estrogens, testosterone and dehydroepiandrosterone sulfate (DHEA-S), interact with the HPA axis at many levels[7,64]. Steroidogenic enzymes compete for the basic cholesterol precursors, but also interact via known and unknown mechanisms at the level of the hypothalamus, pituitary, and adrenal glands[7]. Most literature on the topic derives from animal models with a paucity of data in humans. Some studies have shown that young males have high levels of cortisol which decreases before rising again at puberty to reach the levels seen in adults[65–67]. Sexually dimorphic differences in cortisol vary based upon the time of day samples were taken, the matrix assayed, and the

developmental stage of the individuals being studied. Increased basal HPA activity with age and puberty are fairly consistent, especially in females[32,68].

1.3 Measurement of Cortisol

Diagnostic evaluation of cortisol has classically used sampling of the serum, saliva, or urine. The preferred assessment depends upon the clinical suspicion of high or low cortisol and the subset (i.e. primary, secondary or tertiary) of the disease in question. Specific tests are useful for diagnosing CS, while others are more helpful for AI; cortisol is frequently assessed in combination with ACTH. Circadian secretion of cortisol is an important aspect of cortisol assessments, since the time of day significantly impacts the anticipated reference range.

1.3.1 Cortisol Assessments for AI Diagnosis

Exploring the suspicion of AI can be pursued by measurement of a morning serum cortisol concentration. Healthy individuals have high morning cortisol concentrations of about 150 to 550 nmol/L (10 - 20 mcg/dL)[69,70]. An individual with morning cortisol below 100 nmol/L (~3 mcg/dL) is likely to have AI[69]. If morning cortisol is low, this would predict even lower levels later in the day, or a disruption in the normal circadian rhythm. ACTH levels might help with a more conclusive diagnosis. If cortisol is low and ACTH levels are very high, then primary AI is likely (**Fig. 3)**. If both cortisol and ACTH are low, then secondary or tertiary AI should be considered (**Fig. 3)**. Follow-up tests are indicated after an abnormal morning cortisol measurement.

The ACTH stimulation test uses synthetic ACTH in order to investigate the likelihood of primary AI. In the morning, healthy individuals respond to an ACTH test by producing more cortisol than later in the day, whereas the amount of cortisol produced by AI patients does not differ throughout the day. Normally serum cortisol rises to approximately 500 - 550 nmol/L (18 - 20 mcg/dL) within 30 minutes of a high-dose (250 mcg) IV ACTH stimulation test[71]. If ACTH challenge results in normal response, it is probable that the individual does not have primary AI.

Corticotrophin releasing hormone stimulation tests can be helpful when trying to differentiate between secondary and tertiary AI. Synthetic CRH (ACTHREL; 1 mcg/kg or 100 mcg total IV) is injected and blood samples are drawn at intervals for up to 2 hours to assess ACTH levels. In healthy individuals, ACTH and cortisol concentrations increase by 400% and 250% within the first hour, respectively[72]. A robust and prolonged ACTH secretion profile is seen in tertiary AI as a result of the novel signal to release ACTH[73]. On the other hand, there is little ACTH response in patients with secondary AI[73].

Additional tests such as the metyrapone test, and the insulin-induced hypoglycemia test might also be used by specialists to determine the severity of AI. The metyrapone test assesses partial ACTH deficiency by inhibiting 11β-hydroxylase, the final biosynthetic step in cortisol synthesis leading to lower cortisol and increased CRH and ACTH[74]. Low blood glucose is induced in the insulin-induced hypoglycemia test to test if the individual has a normal cortisol response at intervals following insulin administration[74]. In patients with sufficient evidence of primary AI, assessment of serum antibodies against the steroidogenic enzymes P450scc, 17α-hydroxylase and 21-hydroxylase might elucidate the cause of their AI[9].

1.3.2 Cortisol Assessments for CS Diagnosis

Guidelines recommend assessing cortisol in patients with signs and symptoms of CS. Generally, no single test can be used alone to accurately diagnose CS[75]. Initial testing can include urinary free cortisol (UFC; at least two tests), late-night salivary cortisol (two measurements), 1-mg overnight dexamethasone suppression test (DST), and the longer low-dose DST (2 mg/d for 48 h)[47,75].

Urinary cortisol collected over 24 hours has been a mainstay of CS diagnosis for many years[75,76]. UFC represents the integral of cortisol exposure for an entire day by quantifying free cortisol (i.e. active, not bound to plasma proteins) that is excreted by the kidney[76]. Assessing urinary free cortisol alone results in relatively high false negative rates[75]. In most cases, the false negative rate is driven by individuals with mild or moderate CS and not overt CS. Specific normal values differ between labs, but generally,

the UFC upper limit of normal (ULN) is approximately 138 nmol/24 hours (50 μg/24 hr)[77]. Limitations of UFC include the requirement of collecting urine for 24 hours, the exclusion of individuals with kidney problems, alteration by hydration status, low sensitivity, and inability to capture changes in the circadian rhythm[78].

Measuring late-night salivary cortisol (LNSC) is an important tool used to distinguish between individuals with elevated cortisol due to overlapping conditions or CS[28]. Obese and depressed individuals have preserved circadian cortisol secretion, but those with CS do not[75]. Salivary cortisol is preferred by many patients because it is non-invasive and can be collected at home.

The 1 mg DST involves the administration of 1 mg of dexamethasone between 11 PM and midnight followed by the measurement of serum cortisol at 8 AM the next morning[47]. In healthy individuals the administration of a supraphysiological glucocorticoids suppresses the output of CRH, ACTH, and cortisol. Suppressed morning serum cortisol with a cut-off below 1.8 mcg/dL (50 nmol/L) provides 100% sensitivity and 91% specificity for discerning CS[79]. The two-day, low-dose DST requires dexamethasone to be dosed every six hours for two days, with a total dose of 4 mg (2mg/day). Normal response to this prolonged dosing schedule is to have decreased UFC, serum cortisol, and ACTH[75].

For patients with abnormal initial results, follow-up tests such as the 8 mg high-dose dexamethasone test can be used to discern a diagnosis of Cushing's disease versus non-pituitary causes of CS such as adrenal or ectopic ACTH tumors[75]. There is also a subset of CS patients with a cyclical disease, which means that they undergo periods of excess cortisol production followed by normal cortisol secretion[80,81]. Cyclical CS (CCS) is difficult to diagnose using acute plasma or saliva samples because the patient might be experiencing a period of normal cortisol secretion. Experienced endocrinologists might implement more advanced testing in their diagnosis.

1.3.3 Cortisol in Hair

Over the past decade, there has been growing interest into the utility of assessing human scalp hair as a matrix for quantifying exposure to cortisol. Hair cortisol represents a much larger time window than the assessments discussed above, with segments of hair representing a month or more in historical cortisol exposure.

1.3.3.1 Hair Physiology

Mammalian hair functions as a protective layer from the cold and ultraviolet radiation, thermoregulator, sensory organ that provides tactile information, and as an outward display involved in social and sexual communication[82]. Hair is found almost ubiquitously on the body expect for parts of the external genitalia, sole of the foot, palm of the hand, and the buccal surface of the lips[82]. Terminal hair is the thick and pigmented, androgen-dependent hair found in the pubic region, axilla, scalp, beard, and chest[82,83]. Vellus hair is the thin, lightly colored hair that protrudes from the skin prior to puberty and is more noticeable on regions of the body not typically covered in darker hair (i.e. forehead, eyelids, or the ear lobe)[82,83].

Hair is a derivative of the epidermis that is principally composed of fully keratinized epithelial cells[82]. The visible hair shaft protrudes externally from the skin, growing outwards from a hidden follicle[82–84]. The hair shaft is composed of a thin outer cuticle, a larger cortex, and a smaller central medulla[82–84]. Microfibrils, composed of densely packed filament proteins, comprise approximately 65-95% of the matrix, with the remainder including 1-9% lipids, 3-5% water, and <1% trace elements[84,85]. Alpha-keratin and keratin-associated proteins are the major protein constituents of the matrix and have abundant cysteine residues that form crosslinks to maintain structural rigidity and hydrophobicity of the hair structure[82,85]. Hair color is determined by the amount of melanin pigment in hair[83,85,86]. Melanin, a blackish-brown pigment found in the hair, skin, and eyes, is produced by melanocytes[82].

The hair follicle is an important structure responsible for maintaining and replenishing hair. It is found near the junction of the epidermis and dermis and is principally

composed of the inner and outer root sheath and the dermal papilla[82,85]. Structures closely associated with the follicle include sweat and sebaceous glands, the arrector pili muscle, and the stem cell bulge that controls the rate of hair growth and re-growth (**Fig. 5**)[82,83]. The dermal papilla is highly vascularized by arterioles that originate in the subcutaneous fat[82]. Nutrients are provided by the vasculature allow for rapid keratinocyte proliferation and hair growth. Endogenous and exogenous compounds within systemic circulation also enter the follicular microenvironment through this vasculature[82,83,85,86].

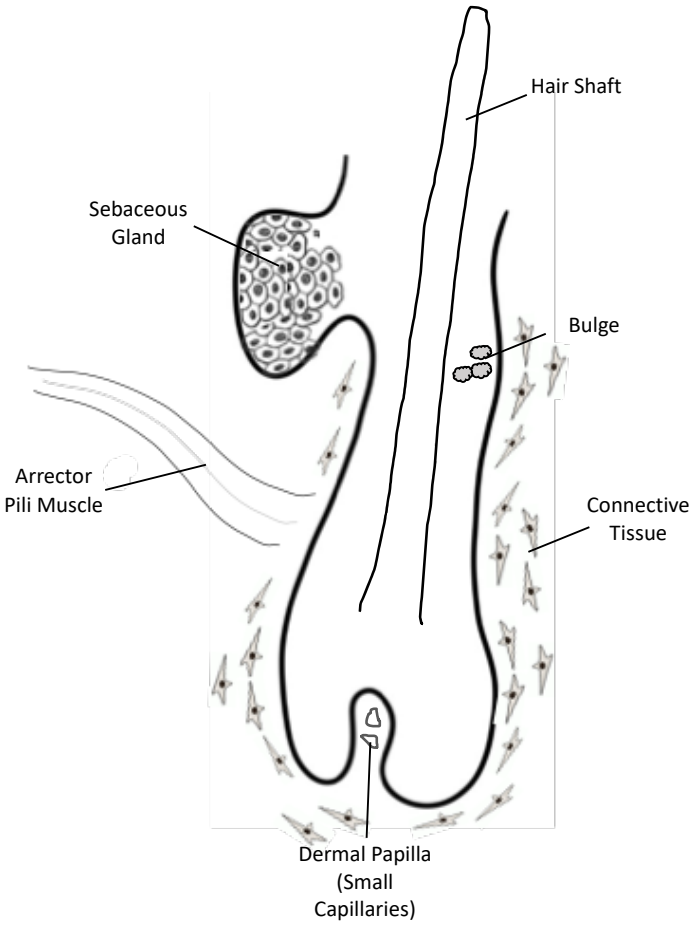

Figure 5: The hair follicle and its associated structures. Adapted from [69]

Hair follicles grow in a cyclical pattern that is divided into three phases: 1) Growth or Anagen phase; 2) Transitional or Catagen phase; and 3) Resting or Telogen phase[82,83,87,88]. Genetic and environmental factors determine the length of each of these phases. Anagen is the active growth phase[82,83] whereby bulge progenitor cells proliferate into the skin, differentiate into the various parts of the follicle, and drive proliferation and differentiation of keratinocytes[89]. The length of this phase determines how long an individual's hair might grow and can last between 2 and 7 years[82–84]. Cell proliferation rapidly decreases in the catagen phase followed by regression of the follicle for several weeks. Follicular resting occurs during telogen phase and upon completion the hair will fall out. Approximately 10-15% of hairs are in telogen at any moment[82,83]. This cycle repeats for an individual's lifetime unless interrupted by age, androgen, or disorder-related hair loss[17,90].

1.3.3.2 Integration and stability of compounds in hair

Compounds, such as cortisol, can be incorporated into hair from the blood, sweat, sebum, and from external environmental contamination[85,86,91]. Passive diffusion of compounds from the vasculature into dividing cells of the follicle is the principal mechanism thought to account for the incorporation of compounds into hair[83,92]. By this mechanism, the concentration of a compound that is added to a growing hair is proportional to the plasma concentration at that time[83,92]. In a similar paradigm, diffusion of compounds into the follicular microenvironment might precede excretion into sweat and sebum that coats the growing hair[83].

The physiochemical properties of a compound and the rate of metabolism determine the extent to which it is incorporated into hair. Generally, small, lipophilic molecules have the greatest capacity to diffuse into the lipophilic structure of hair[85,86,93]. Cortisol's low molecular weight (362 Daltons) and lipophilic structure permits passive diffusion through the cell membrane to bind the GR[21]. Similarly, these properties allow cortisol to readily diffuse into hair as demonstrated by the quantification of tritium-labelled cortisol in the hair of non-human primates after intravenous injection[94]. Lastly, as documented in hair analysis for drugs of abuse such as cocaine, passive exposure in the

environment might account for an additional deposition into hair[83,85]; hydrocortisone preparations used for scalp psoriasis might contribute to hair cortisol, for example.

1.3.3.3 Hair Cortisol

Serum and salivary cortisol are valuable for capturing aberrations in cortisol reactivity at specific points in time such as assessing changes in circadian secretion or response to stimulation or suppression tests. Salivary cortisol has the benefit of being less invasive and representative of free cortisol. Both measures, however, are less suitable for assessing questions associated with adrenal steroid production and exposure over time. Repeated collection might overcome this shortcoming, but this becomes expensive, time-consuming, and highly invasive in the case of serum cortisol[92].

Twenty-four-hour UFC extends the window of analysis to one day by capturing free cortisol that is excreted by the kidney[76]. This medium-term analysis window is useful for determining the overall exposure, or area under the curve for 24 hours (AUC_{24hr}) but does not capture circadian secretion patterns. Urinary cortisol is not reliable in populations with kidney problems, differs by hydration status, and might not reflect tissue cortisol since the kidney is a major site of metabolism and clearance. Collection of urine over a day or longer is cumbersome such that assessing longer-term exposure with this method is suboptimal.

The act of drawing blood for serum samples must also be considered when assaying cortisol because doctor visits result in elevated blood pressure, transient increase in serum cortisol, and psychological stress[92,95]. This effect is particularly pronounced in children and adolescents who have an innate fear of needles with the result being inaccurate measures of basal cortisol levels[95]. Additionally, the infrastructure required for these specimens includes needles, vials, urine containers, transportation, qualified individuals to draw blood, and refrigerators or freezers for storage[92]. Access to appropriate infrastructure might be limited in some regions of the world, or following disasters, making it difficult to investigate population-based questions or explore HPA changes in times of unrest[96,97].

Hair cortisol may be used to address some of these shortcomings. Hair is easy to collect, is stable at room temperature, represents a time window of a month or longer, is non-invasive, and can be collected over a larger period of time[92,98]. A small hair sample with approximately 100-150 hairs can be collected using a pair of scissors. Hair is collected from the posterior vertex of the scalp because of the smallest interindividual variability in growth rate and cortisol concentration[99]. After sampling, hair can be stored in a dry, dark area for many weeks at room temperature. Uniquely, hair cortisol captures retrospective cortisol data over months by integrating the sum of cortisol exposure including production and the metabolism of cortisol[81,98]. Hair cortisol might also be more representative of tissue cortisol exposure than a plasma sample because the cortisol in hair navigated through the small follicular capillary beds and diffused into the hair microenvironment. Hair cortisol concentration correlates with repeated saliva assessments, indicating coherence with more standard measures[100]. Importantly, as reviewed in Chapter 2, hair cortisol has been used to capture changes in cortisol exposure in diabetes, cardiovascular disorder, CS and AI, psychological disorders (depression, PTSD, and anxiety), as well as the effect of natural disasters and trauma.

1.3.3.3.1 Knowledge Gaps in the Biology of Hair Cortisol

Empirical, well-designed studies to address methodology, reliability, and under-studied populations are needed to validate hair cortisol as a useful biomarker of HPA function. Methodological differences between labs has led to substantial variability in absolute hair cortisol values stemming from the particular methods being employed[101]. Cortisol extraction methods include pulverizing or cutting hair into small pieces followed extraction in an organic solvent for up to 24 hours and then cortisol quantification by immunoassay or liquid chromatography–mass spectrometry[102]. Extraction temperature, choice of organic solvent, procedure used to mince the hair, number of extractions, and method used to quantify cortisol are all variables that affect the absolute cortisol measurement. Methodological improvements borrowed from hair toxicology such as enzymatic degradation or hydrolysis of hair might reduce variability and increase the reliability of hair cortisol as a result of complete extraction[84]. Inexplicably, these

methods have not been explored in published analysis of hair cortisol and represent a potentially large step towards standardization in the field of hair cortisol research.

Arguably, one of the most important applications of hair cortisol is for the more rapid diagnosis of difficult to diagnose diseases including CCS. Patients with CCS present with periods of normal cortisol secretion making it difficult to capture elevated cortisol with single saliva or serum measurements[59,81,103]. Patients with suspected CCS might benefit from having their hair cortisol assessed because it allows the physician a window into historical cortisol levels. Without the use of hair cortisol, physicians have to rely on repeated sampling procedures for weeks to months to reach a conclusive diagnosis. Several studies have shown the utility in this paradigm using 1 cm/month hair growth rate to construct retrospective hair cortisol timelines[81,98]. Hair growth of 1 cm per month is the average cited in the literature[104], but there is substantial hair growth rate variability that is driven largely by ethnicity, nutritional status and seasonality[87,104,105]. Generating unique hair timelines is important because it is impossible to know an individual's hair growth rate *a priori*. No prior studies have considered variability in hair growth rates when constructing hair cortisol timelines for individual patients.

Hair cortisol has historically been assessed in adult patients with an emphasis on studying the underlying etiology or response to disease[98]. Fewer studies have assessed the utility of hair cortisol in younger populations, healthy populations, and in individuals from various ethnic backgrounds. Hair cortisol studies in children have typically looked at early life trauma or psychosocial stressors, indicating elevations in those exposed to stressors compared to a small number of controls[106]. There is currently less known about normal changes in hair cortisol as children age through to adulthood. Some small studies have looked at changes in hair cortisol with age but many of these have reported conflicting results[65,107]. Hair cortisol might elucidate the total exposure to cortisol on a background of HPG axis-interactions and provide context to the differences that have been observed in studies measuring cortisol in the serum or saliva. There have been no studies to date that have measured hair cortisol in a large cohort of healthy children and adolescents in order to assess the relationship between puberty and hair cortisol exposure.

1.4 Thesis Overview and Hypotheses

1.4.1 Chapter 2: Objective

In Chapter 2, we review methodological aspects of hair cortisol measurement, including some of the current limitations and differences amongst labs conducting hair cortisol analysis, and describe current knowledge on the clinical utility of measuring cortisol in hair.

1.4.2 Chapter 3: Objective & Hypothesis

1. To explore the utility of relating hair cortisol concentrations to past cortisol-related symptomatology in CS and AI patients by constructing several timelines of cortisol exposure.

2. To determine to what extent the commonly assumed hair growth rate of 1 cm per month results in retrospective hair cortisol-based timelines adequately reflect individual patient histories.

In chapter 3, we hypothesized that hair cortisol timelines could be generated to accurately reflect CS and AI patient medical histories, and that the clinical timelines could be improved upon by generating timelines that vary from the assumed 1 cm/month growth rate.

1.4.3 Chapter 4: Objective & Hypothesis

1. To investigate the relationship between hair cortisol and age, puberty, sex and BMI in healthy children and adolescents.

In chapter 4, we hypothesized that hair cortisol could be measured in children and adolescents in order to establish reference ranges as they relate to normal development. Specifically, that hair cortisol would correlate with age and puberty status, not differ between males and female, and would increase with BMI.

1.4.4 Chapter 5: Objective & Hypothesis

1. To compare hair cortisol recovery from the extraction solvent by utilizing room air or nitrogen gas evaporation.

2. To investigate if novel hair cortisol extraction methods, including enzymatic digestion and basic digestion, could be used to improve reproducibility and decrease variability in hair cortisol analysis in a pilot study.

In chapter 5, we hypothesized that cortisol recovery for hair would not differ when using nitrogen gas or room air for evaporation and that enzymatic or basic digestion of hair would enhance cortisol recovery compared to the standard mincing method.

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Chapter 2

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

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

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2.1 Introduction

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

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

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

2.2 Methodology and technical aspects of analysis

2.2.1 Incorporation of cortisol into hair

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

2.2.2 Hair Growth

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

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

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

2.2.3 Sampling, storage, and segmentation

Sauve et al. found that the relative variability in hair cortisol, as determined by the coefficient of variation (CV), was high (30.5%) when hair was sampled from different regions of the head compared to samples taken from the posterior vertex (CV, 15.3%) [13]. Standardization between laboratories may be accomplished by collecting hair from the posterior vertex of the head, even when exact hair sampling procedures vary slightly between laboratories (**Fig. 6**).

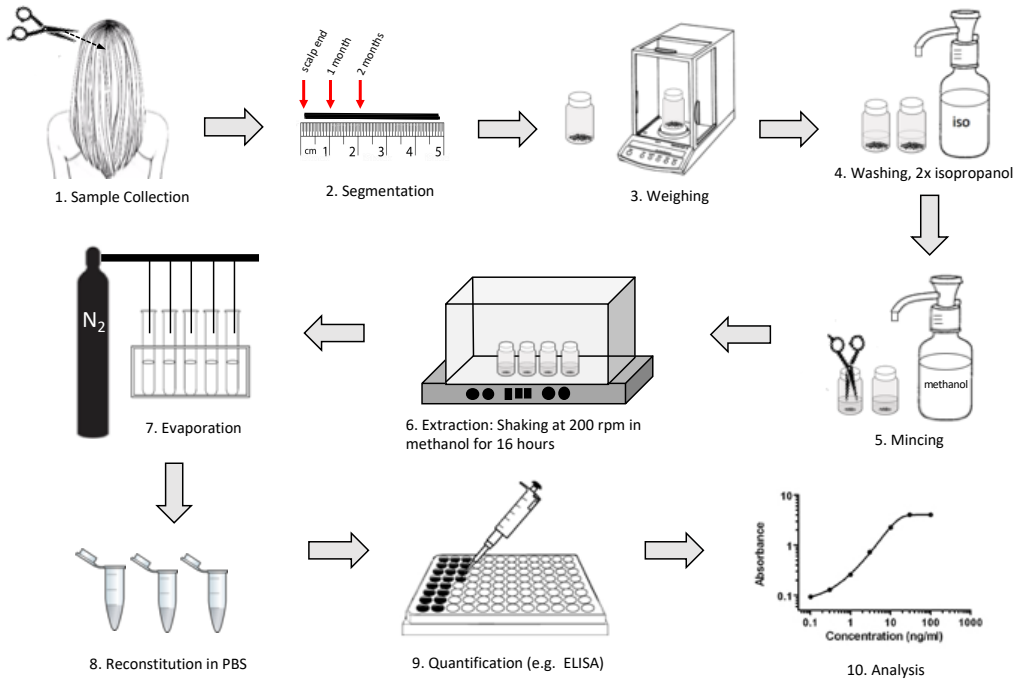

Figure 6: Standard method for the quantification of hair cortisol concentrations.

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

meaning that samples should be stored away from UV exposure to allow analysis of samples in the distant future [20].

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

2.2.4 Weighing and washing

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

2.2.5 Mincing versus grinding

Efficient cortisol extraction requires a large surface area for the interaction of the hair medulla and the extraction solvent. The hair extraction surface has been increased by manually mincing the hair, blending the hair, and grinding or pulverizing the hair by means of milling. Particle size reduction has classically been accomplished by mincing the hair into ‘small pieces’ using surgical scissors. Mincing of the sample with scissors is far from being standardized as the size of the minced pieces is determined by how

meticulous the technician is in the cutting process. This may potentially lead to variations in the amounts of cortisol extracted. However, intra-laboratory validation of hair cortisol measurement has been shown to have high correlation between results when using the mincing method (r^2: 0.9692) [21]. Alternatively, blenders or mills with zirconium oxide beads and liquid nitrogen cooling (cryomilling) can produce hair particles of ~5 μm in size.

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

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

2.2.6 Extraction, evaporation and reconstitution

Cortisol is extracted from minced or ground hair using organic solvents (e.g., methanol, acetone). Typically, cortisol is extracted from 10 mg of hair by adding 1 to 2 ml of methanol to the glass vial in which the hair was washed and weighed. Shaking of the sample on a rocker overnight in methanol at ambient temperature or at 52 °C is the most common method for extracting cortisol from hair. However, data has suggested that a single methanol extraction method may only yield 40–60% with an average of 46% of absolute HCC [22]. A 4-step method that employs alternating methanol and acetone for 15 h at 52 °C and 5 min at room temperature, respectively, repeated twice, has yielded an

estimated 98–100% of hair cortisol [22]. Although the effect of extraction efficiency on the clinical utility of the technique is not certain, it can be argued that following a consistent protocol still results in useful comparisons between subjects and controls.

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

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

2.2.7 Measurement of cortisol, ELISA versus LC-MS/MS

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

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

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

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

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

Quantitative hair analysis is known to be more difficult than other matrices due to the solid, heterogeneous solid composition of hair, and insufficient amounts of reference material [24]. In order to ensure quality control in hair analysis, further work should be done to formulate standard practices with regards to sampling, preparation, extraction,

reconstitution, and calibration. Calibration and validation between laboratories can be accomplished using the same quality control spiked hair samples. Methods to facilitate heterogeneity and more reproducible results between laboratories should be explored as recommended by the Society of Hair Testing [25].

2.2.8 Comparison of hair as a matrix to traditional matrices and methods

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

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

Traditionally serum, saliva, and urine have been used for the measurement of cortisol to assist with clinical diagnosis. **Table 1** compares the traditional forms of cortisol measurement with that of hair. Use of these acute and short-term cortisol biomarkers are currently the standard for screening of endocrinological disorders [5]. Hair cortisol levels

appear to be fairly stable over time. Stalder et al. demonstrated strong intra-individual stability of HCC in two clinical studies where hair was first sampled after 1-year, and secondly at 2-month intervals [35]. The results from these time points help to demonstrate the utility of this technique in clinical applications and the estimation of cortisol secretion over long periods of time [35].

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

Table 1: Comparison of sample types for cortisol measurement.

2.2.9 Limitations of hair cortisol analysis

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

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

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

2.3 Applications of hair cortisol in animals

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

2.4 Clinical applications of hair cortisol measurement

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

2.4.1 Cushing syndrome

Cushing Syndrome (CS) is characterized by signs and symptoms of exposure to excess glucocorticoids for an extended period of time. For patients with clinical suspicion of CS, the first step is to exclude exogenous causes, usually iatrogenic due to treatment with

dexamethasone, prednisone, or other glucocorticoids [59]. The next step is to evaluate if there is indeed endogenous cortisol overproduction. In clinical practice, three tests are currently used for diagnosing CS, these are urine free cortisol (UFC; at least two measurements), late-night salivary cortisol (two measurements), and the 1mg overnight dexamethasone suppression test (DST) [59–62]. Typically, two concordantly positive tests from two methods are required to diagnose CS. Our group was the first to demonstrate elevated hair cortisol in CS as compared to healthy controls [63]. Since then, several groups have analyzed the performance of the measurement of cortisol in the most proximal hair segment. Using the upper limit of normal for healthy non-obese individuals as cut-off, two HCC cut-off values have been suggested for the diagnosis of CS: Firstly an upper limit of 75.9 ng/g with 86% sensitivity and specificity of 98% (lean individuals) to 93% (individuals with abdominal obesity) [64] (**Fig. 7**), and secondly, a lower cut-off of 31.1 ng/g with 93% sensitivity and 90% specificity [5]. Hodes et al. found higher hair cortisol values in 36 CS patients than in 6 controls, with mean hair cortisol of 266 ± 738.4 and 38.9 ± 25.3 pmol/g in each group, respectively (P = .003) [65]. Overall, these results appear to be comparable to commonly used tests.

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

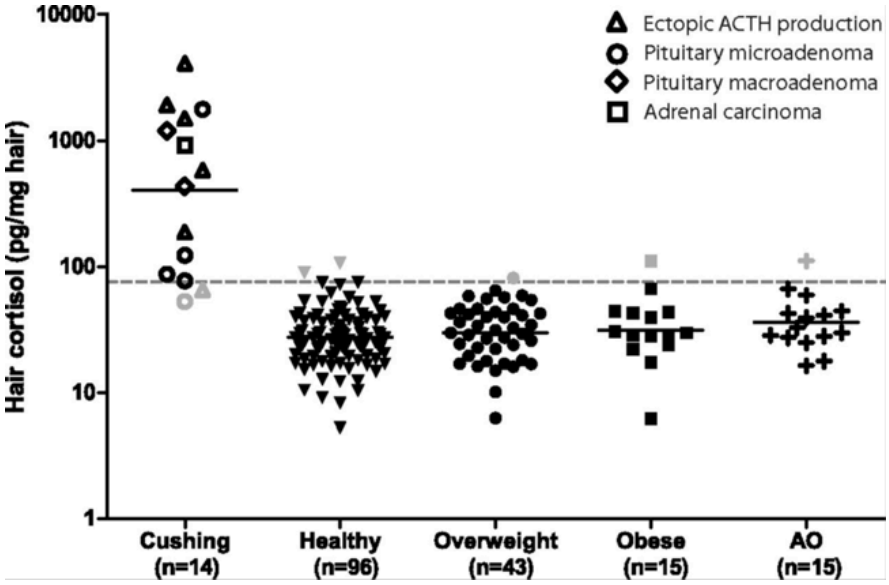

Figure 7: Hair cortisol levels in patients with CS and healthy overweight and obese controls and individuals with abdominal obesity. The dotted line represents the upper limit of normal hair cortisol levels (75.9 pg/mg hair). The gray symbols represent the individuals with abdominal obesity. The dotted line represents the upper limit of normal hair cortisol levels (75.9 pg/mg hair). The gray symbols represent the individuals with cortisol levels below the upper limit of normal (in case of confirmed CS) or above the upper limit of normal (no CS). The nature of the CS is indicated by the different symbols used for the patients. From [64].

The assessment of cortisol production over time may be particularly useful for evaluation of cyclical CS (CCS). Cyclical CS is a rare condition characterized by recurrent episodes of hypercortisolemia interspersed by episodes of normal or decreased cortisol production [66]. The observed cyclicity can be either regular or irregular in nature, ranging from days to years, making a diagnosis of CCS especially difficult [64]. HCC is able map this fluctuation in cortisol levels over time. A diagnosis of true cyclicity of CS requires evidence of at least 3 peaks and 2 troughs in cortisol levels over time [66]. **Fig. 8** demonstrates the cyclical nature of a patient measured in our lab, with a subsequent diagnosis of CCS. Depending on the nature of a patient's condition it may take months to observe these findings with typical laboratory assessments. In fact, a recent study has shown that CS patients have normal cortisol secretion for large periods of time, with one patient having normal late-night salivary cortisol levels in 33/34 samples [67]. HCC has

the potential to eliminate weeks to months of daily measurements, allowing for earlier diagnosis and treatment.

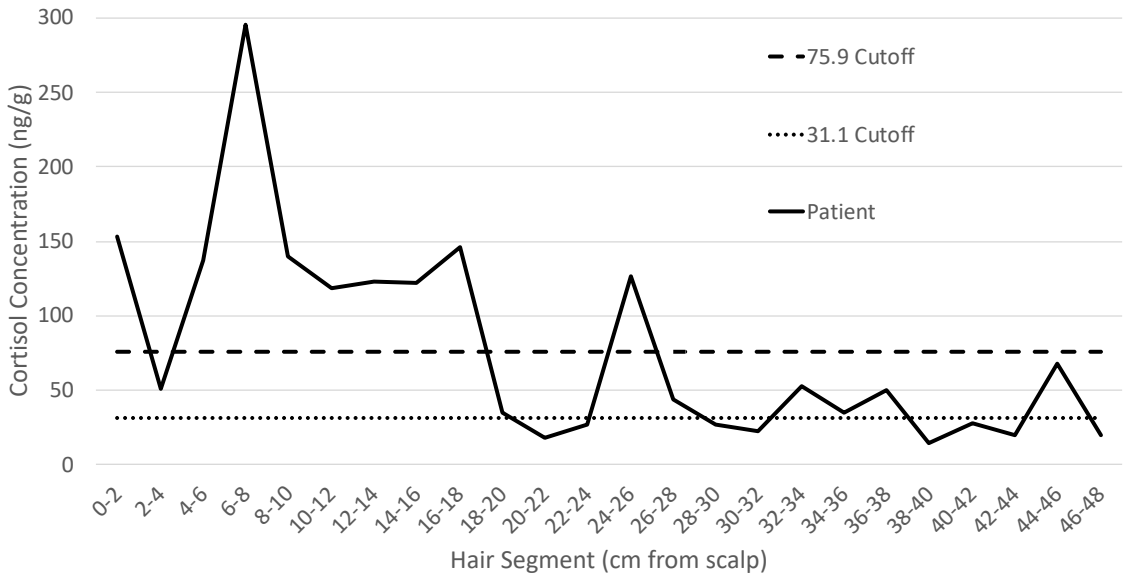

Figure 8: Hair cortisol analysis of a patient with cyclical Cushing's syndrome with overlapping proposed CS diagnosis cut-off values.

2.4.2 Adrenal Insufficiency

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

For patients with sufficiently long hair, segmental analysis of cortisol production over time provides a unique record of the natural development of auto-immune adrenal insufficiency [70]. **Fig. 9** shows the development of AI over a period of more than two years, with the patient gradually progressing from mild chronic AI symptoms toward adrenal crisis, necessitating admission to intensive care. Based on this pattern, hair cortisol measurement may also help in diagnosing AI in patients who have already been started on glucocorticoid treatment, which jeopardizes the ability of saliva and serum cortisol measurements

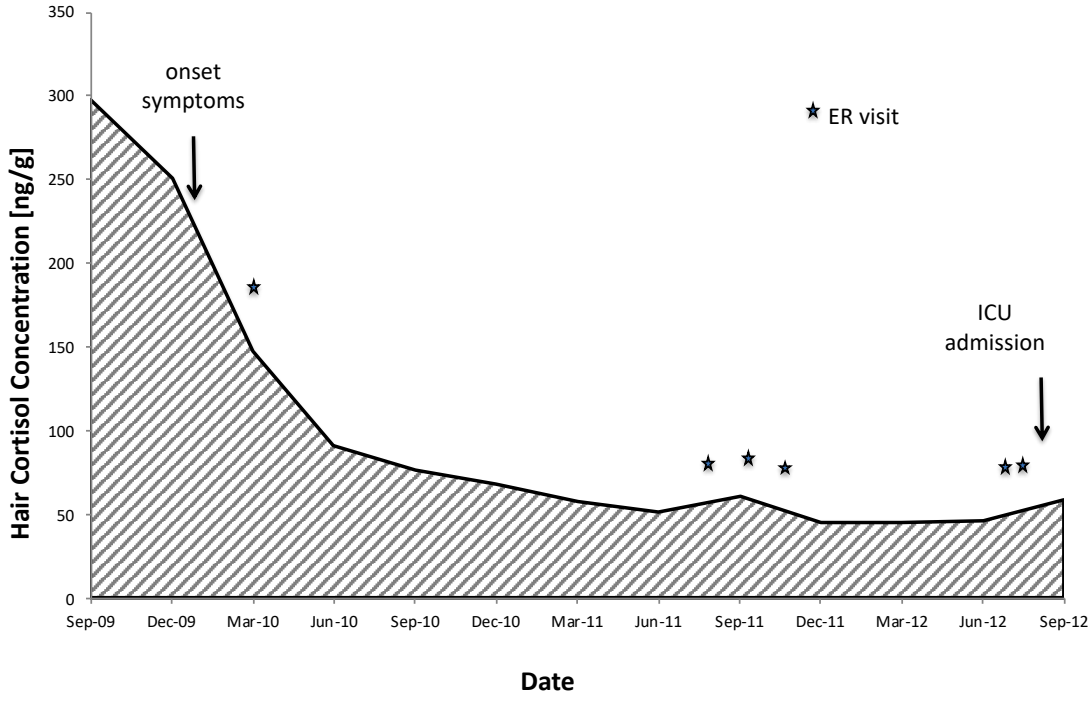

Figure 9: Hair cortisol concentration in a patient with adrenal insufficiency over time.

2.4.3 Hair cortisol and cardiovascular disease

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

2.4.4 Hair cortisol analysis as a biomarker of chronic and acute stress

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

Hair cortisol analysis is now at the forefront of research addressing the effects of both acute and chronic stress. Endogenous cortisol levels are influenced by a broad range of

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

2.4.5 Mental health

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

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

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

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

2.5 Post-mortem applications

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

2.6 Discussion and future directions

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

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

Future research should test the accuracy of ELISA techniques compared with the use of mass spectrometry. Immunoassays provide a relatively easy and inexpensive option for analysis, however this is an indirect measurement based on binding of the assay, whereas MS directly measures the molecule of interest. Prior research comparing the use of ELISA and MS has highlighted the contrasting differences in both sensitivity and specificity. Techniques need to be developed to better detect hair cortisol using MS to more easily allow for the detection of hair cortisol without the cross reactivity associated with ELISA.

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

Additional research should address the time from the formation of the hair shaft in the follicle to the eruption of the hair shaft from the skin, which relates individual hair

growth rates. There is conflicting research on whether this process ranges from a few days up to 2 weeks [94,95]. What kind of clinical effect this may have is yet to be determined.

2.7 Conclusion

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

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Chapter 3

3 Retrospective hair cortisol analysis in the clinical setting

This chapter explores utility of hair cortisol in capturing long-term cortisol exposure in patients with Cushing's Disease and Addison's Disease by retrospectively depicting cortisol levels over several months or even years. The aim of this study was to determine to what extent the commonly assumed hair growth rate of 1 cm/month results in retrospective hair cortisol-based timelines adequately reflect individual patient history-based fluctuations in cortisol exposure that result from disease states and medical interventions. Postulated hair cortisol timelines reflect the clinical status of patients with cortisol-associated symptomatology. The growth rate timeline for the 4 patients were 1, 0.75, 0.75 and 1 cm/month. Historical hair timelines generated by segmental hair analysis of patients with cortisol-related symptoms reflect reported changes in clinical states. Variations in hair growth rate may need to be used to determine the best historical record of cortisol secretion. Clinicians should keep this in mind when using hair cortisol as an s additional tool to diagnosed conditions with abnormal cortisol secretion.

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Retrospective Hair Cortisol Analysis in the Clinical Setting

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3.1 Introduction

Secretion of cortisol, the predominant glucocorticoid in humans, is dynamically regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Various physiological and psychological stressors can initiate the secretion of corticotrophin releasing hormone (CRH) from the hypothalamus. This induces the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) that travels to the adrenal cortex to release cortisol. Under physiological conditions cortisol is released in a circadian pattern with the highest

systemic concentration occurring upon waking[1]. Cortisol rises following stressful experiences and increases blood-glucose levels via proteolysis, lipolysis, and gluconeogenesis to match the increased energy demands and restore systemic homeostasis[2]. It is also an important modulator of immunity and electrolyte balance[3]. Due to its ubiquitous activity, chronic exposure resulting from prolonged stress, or one of many pathologies, can have negative consequences[2].

Cushing Syndrome (CS) is the result of prolonged exposure to high levels of cortisol or other glucocorticoids. Symptoms of CS include hypertension, weight gain, osteoporosis, diabetes mellitus, depression and mood changes, easy bruising, fat redistribution and purple striae[4–7]. Endogenous CS is due to elevated cortisol secretion from the adrenal glands, and is caused by an adrenal adenoma or by excess production of ACTH, either from a pituitary adenoma or from an ectopic tumor[5,8]. Cyclical Cushing Syndrome (CCS), once believed to be a rare condition, is characterized by cycles of variable steroidogenesis with episodes of hyper secretion of cortisol interspersed by periods of lower cortisol production [7]. The frequency of these cycles can vary, ranging from days to months[6].

Adrenal insufficiency (AI) is characterized by low secretion of cortisol and most commonly results from autoimmune destruction of the adrenal cortex. It may also be caused by cancer, infections, medications, and genetic factors that influence steroidogenesis or adrenal response to ACTH[9], or by insufficient pituitary ACTH secretion. In the case of primary AI, sometimes referred to as Addison's disease, the symptoms of AI begin to appear when about 90% of the adrenal cortex has been destroyed. They may include weight loss, hyperpigmentation, low blood pressure, abdominal pain, weakness, anorexia, nausea, vomiting, and salt craving[9].

In clinical practice, suspicion for cortisol overproduction is assessed by 24-h urinary free cortisol excretion, late night salivary cortisol, and the 1-mg overnight dexamethasone suppression test[6]. Typically, two of these tests need to be abnormal to diagnose Cushing's[10]. Salivary and serum cortisol are considered to be acute measures as they are reflective of cortisol concentrations at a single point-in-time[3,8]. Patients with CCS are known to frequently have salivary or serum levels in the normal range [4,9,11], making

salivary and serum cortisol measurements suboptimal for accurately capturing cortisol abnormalities in these individuals. Ambiguity in diagnosing variable cortisol production can also be caused by other factors such as obesity and diabetes, use of exogenous glucocorticoids, and states such as renal failure, adrenal masses, and pregnancy, meaning that careful selection of screening tests and repeated testing is usually required [12].

Over the last decade, human hair has emerged as a novel biological matrix for the retrospective analysis of long-term cortisol exposure[3,8,13]. Because scalp hair grows at an average of about 1 cm every month[14], retrospective timelines of cortisol exposure can be generated by cutting hair into segments to detect the fluctuation of cortisol over time which can then be compared to reports of clinical symptoms [4]. Hair samples are easily collected from the posterior vertex region of a patient's scalp, can be stored at room temperature[3], and the window of detection is generally only limited by the length of the hair sample. Measuring multiple hair segments provides an insight into the past variation in systemic cortisol exposure[3,5]. Based on this unique feature, hair cortisol concentration (HCC) also has the potential to aid in a more rapid diagnosis of CCS as it allows retrospective analysis over months to years[5,7]. Analysis of blood, saliva, or urine, in contrast, would need to be done prospectively over such a time period, and would require frequent sampling in order to detect cortisol variability[4,6]. Further, segmentation of hair to detect the fluctuations of cortisol over time can be compared to the chronology of the patient's clinical symptomatology to paint a picture of historical changes in cortisol exposure that can assist clinicians with diagnosis[5]. These timelines, however, may vary depending on an individual patient's hair growth rate and may vary with factors such as diet, age, stress, disease states, and seasonality[14]. Hair growth variation in adults aged 18-35 was found to be large between ethnicities, with individuals of recent African descent having the slowest, and those of recent Asian descent having the fastest hair growth rates[15]. Any hair growth rate that differs from the 1 cm/month assumption becomes more compounded as one travels more distal along a hair sample, representing periods further back in time.

The aim of this study is to explore the use of hair cortisol in relation to past cortisol-related symptomatology by constructing several timelines of cortisol exposure.

Specifically, we aimed to apply various hair growth rates in an attempt to optimize retrospective timelines of cortisol exposure to adequately reflect individual patient medical histories.

3.2 Methods

3.2.1 Patients

We collected data from patients who attended our tertiary Endocrinology referral clinic at St. Joseph's Hospital, London, Ontario, Canada between September 2015 and May 2018, and who had a hair sample collected as part of their clinician's diagnostic assessment. The study was approved by the Western Research Ethics Board, with a waiver of informed consent due to the retrospective design of the study. Approval for this study can be found in **Appendix B.** All information collected from the patient charts was considered germane to their condition, and included factors such as demographics, prior cortisol assessment results, medications, surgeries and reported symptomatology. We carefully extracted very detailed information on the time course of patients' symptoms.

3.2.2 Hair Collection & Analysis

The primary physician cut a lock of approximately 150 strands of hair as close to the scalp as possible from the posterior vertex of the scalp. The scalp end of each hair sample was clearly labelled and taped to a piece of paper with scotch tape. Hair samples were then placed in a sealed envelope labelled with the collection date and sent to the Drug Safety Laboratory at Robarts Research Institution (London, Canada) for analysis. Hair samples were segmented into 1- or 2-cm sections in order to generate a retrospective timeline reflective of monthly or bi-monthly hair cortisol exposure. The hair was then prepared as previously described[3]. In brief, the hair was weighed and washed twice with 3mL of isopropyl alcohol, then allowed to airdry for a minimum of 5 hours. Samples were minced into 1 mm pieces in 2mL of methanol using surgical scissors and left to extract on an incubator shaker for 16 hours at 50 °C. The next day, the methanol extraction solvent was transferred into glass test tubes and evaporated under a stream of nitrogen and heat. Subsequently, the sample residues were dissolved in PBS and the cortisol was quantified using a commercially available ELISA Kit for salivary cortisol (Alpco Diagnostics®,

Windham, NH) as per the manufacturer's directions with the reagents provided. Cross-reactivity of other steroids with the kit's antibodies was reported as follows: Prednisolone (13.6%), Corticosterone (7.6%), Deoxycorticosterone (7.2%), Progesterone (7.2%), Cortisone (6.2%), Deoxycortisol (5.6%), Prednisone (5.6%), Dexamethasone (1.6%). Intraassay variation was below 5% and the interassay variation below 8%, as reported by the supplier.

3.2.3 Hair Cortisol Timelines

Retrospective timelines were created based upon the length of hair samples provided. For each patient we created three unique timelines to assess for variation in individual hair growth rates. Each patient's hair timeline is presented with segmental HCC based on three postulated growth rates; a growth rate of 1 cm/month, a slower hair growth rate of 0.75 cm/month, and a more rapid hair growth rate of 1.25 cm/month. These rates were chosen to include 3 SD (0.06 cm/ month)[14] above and below the average hair growth rate of 1 cm per month found in the literature[14]. Each month was considered 30 days for any calculations. Reported symptoms and interventions were then overlaid on top of the graph. Exact dates were used to produce the graphs, however the dates were removed from publication to protect the identity of patients. The best-fitting retrospective timeline was determined by 4 independent reviewers who were given the 3 timelines for each patient in addition to the clinical description. The hair growth rate that best matched the particular patient's history and was chosen for publication required majority (3/4) agreement.

3.3 Results

Hair cortisol levels were determined in 4 female patients with symptoms of cortisol-related pathology. We included three patients with Cushing syndrome and one patient with AI. We provide great detail on the history of each individual patient to allow best correlation with hair cortisol results.

3.3.1 Detailed Individual Clinical Courses and Hair Cortisol Timelines

Patient A, a 25-year-old woman with CS, was referred to our clinic in August 2016. Her symptoms started around October 2014, with the inability to cope with stress being the most prominent. Oral contraceptives were prescribed for acne control in April 2015 but were discontinued shortly after because there was no improvement in the patient's symptoms. In addition to worsening facial and back acne, and hirsutism on the neck and face, she reported a 11.5 kg weight gain over the 3 months beginning around May. In the period around December 2015, she visited her family physician and reported proximal myopathy making climbing stairs difficult, amenorrhea, unexplained bruising, new striae on her axilla and hips, blurred vision, anxiety requiring the use of clonazepam, and polyuria. She had not previously had diabetes mellitus or hypertension. In February 2016, laboratory testing found an elevated plasma cortisol of 1,165 (upper limit of normal [ULN] 720) nmol/L, and ACTH of 26.8 (ULN14) pmol/L. A urinary cortisol measurement was severely elevated at 3474 (ULN 275) nmol/24hr in the first week of March 2016. The MRI scan of the sella showed a cystic pituitary lesion with a maximum diameter of 1.3 cm in mid-July 2016. She was referred to our clinic and physical examination in mid-August 2016 was remarkable for prominent Cushingoid features including moon face, cystic acne, facial plethora, dorso-cervical and supraclavicular fat depositions, purple striae on her thighs and axilla, widespread bruising and proximal myopathy. Her blood pressure was 131/81 mmHg and she weighed 53.9 kg. Spine X-rays demonstrated micro fractures consistent with osteoporosis, without a previous history of fractures. Petrosal vein sampling, undertaken in mid-September 2016 because of the cystic nature of the pituitary mass, confirmed the pituitary as the primary source of the hypersecretion of ACTH. She underwent endoscopic pituitary macroadenoma resection in mid-November 2016. Subsequently, she was prescribed hydrocortisone at a dose of 30 mg q.am, tapering to 10 mg over several months. From January to March 2017, her Cushingoid symptoms improved, she lost 7 kg, her menses restarted, and her lab values normalized (AM cortisol 188 nmol/L, ACTH 4.6 pmol/L).

The hair cortisol timeline of patient A is presented in **Fig. 10.** Her reported symptoms correlate best with hair timelines based upon a growth rate of 1 cm/month (**Fig. 10B**), but not with the other growth rates (**Fig. 10 A, C**). An irregular peak in her hair cortisol timeline appears at about 22 to 27 cm from the scalp (**Fig. 10B**). Using 1 cm/month, this region lines up with the weight gain that was reported in May 2015. Initial and worsening Cushingoid features were reported throughout 2015 and 2016; this is the period of time with elevated hair cortisol values in the range of 600 ng/g (**Fig. 10B**), and this graph shows a decrease in hair cortisol following successful adenoma resection. **Fig. 10A**, based on a growth rate of 0.75 cm/ month, shows elevated cortisol prior to any symptoms, and a decrease in hair cortisol before, and not after surgery. **Fig. 10C**, reflecting a growth rate of 1.25 cm/month, depicts symptoms appearing prior to any increase in hair cortisol, while **Fig. 10A** shows elevated cortisol prior to any symptoms. Initial and worsening Cushingoid features were reported throughout 2015 and 2016; this is the period of time with highly elevated hair cortisol values in the range of 600 ng/g (**Fig. 10B**). It would be expected that hair cortisol would drop off following resection, and this is what is seen the best in the 1 cm/month graph. Hair cortisol drops significantly after resection in the chosen graph but does not appear to match **Fig. 10A** or **Fig. 10C**.

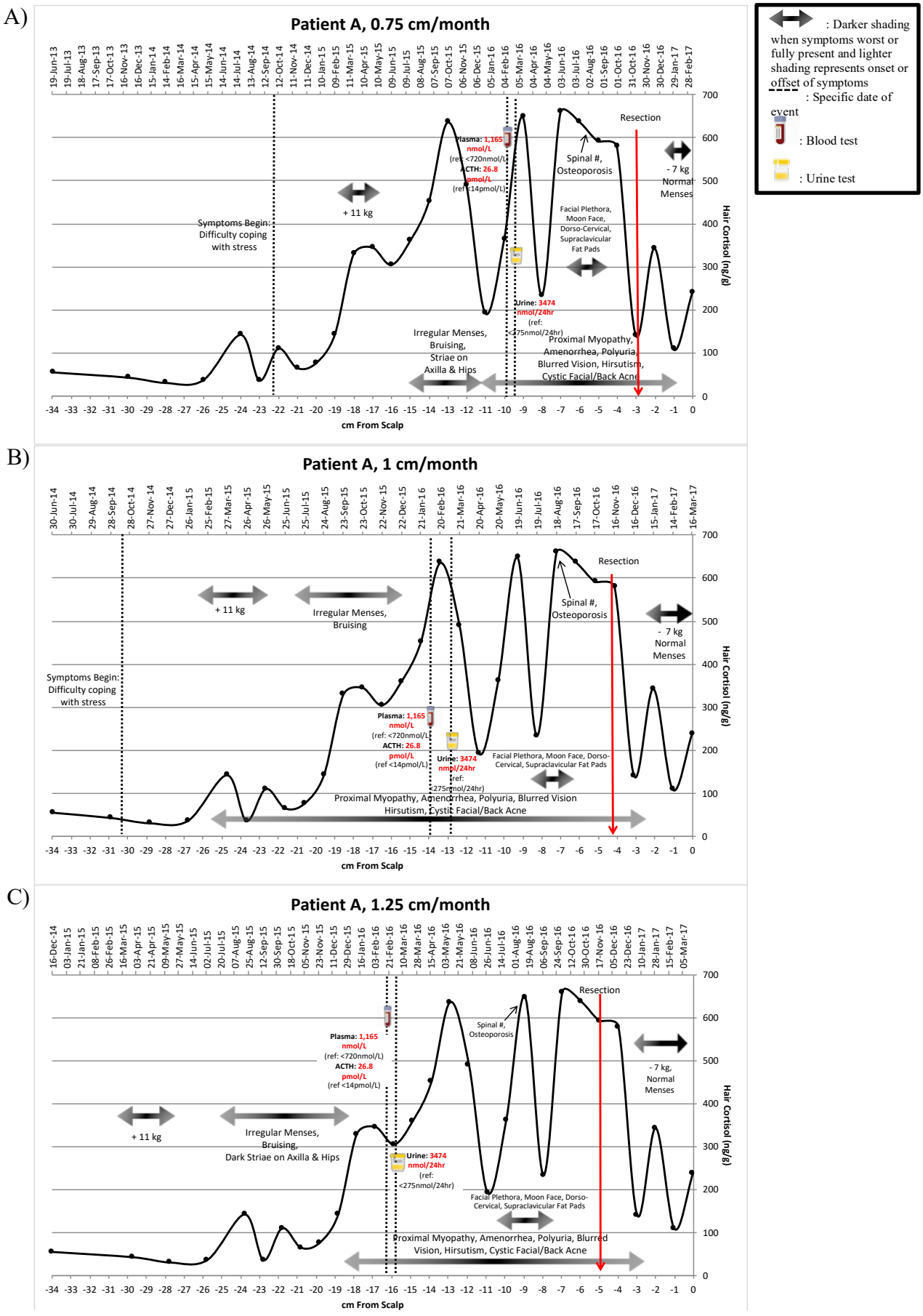

Figure 10: Patient A Hair Cortisol Timelines utilizing A) 0.75, B) 1, or C) 1.25 cm/month growth rate.

Patient B, a 34-year-old woman, was diagnosed with premature ovarian insufficiency and infertility in June 2015. At the first visit to our clinic in June 2016, she did not report any symptoms of AI, her sitting blood pressure was 110/76 mmHg and her weight was 72.6 kg with a BMI of 22.6 kg/m^2. in September 2017 she was referred back to our clinic because of new noticeable hyperpigmentation of the skin. In early October 2017 she reported a significant decrease in appetite, loss of approximately 5 kg in 3 weeks, nausea and worsening of her skin hyperpigmentation. At this time, her standing BP was 78/62 mmHg and her weight 70.2 kg, with unmeasurable AM cortisol of <3 (reference range 130-540) nmol/L and an ACTH of 325 (ULN 14) pmol/L. She was found to be positive for adrenal antibodies confirming a diagnosis of autoimmune AI.

Fig. 11 shows her hair cortisol timelines. The graphs suggest that for this patient a growth rate of 0.75 cm per month (**Fig. 11A**) provided the best retrospective time for her clinical symptoms. The timeline constructed using 0.75 cm/month provided the best representation for this patient's clinical course because she was not demonstrating any AI symptoms at her visit in June 2016 which appeared to occur around the time of a peak in hair cortisol at 12-13 cm from the scalp (**Fig. 11A**). The other growth rates would put this visit at times of decreased hair cortisol troughs, suggesting decreased cortisol secretion.

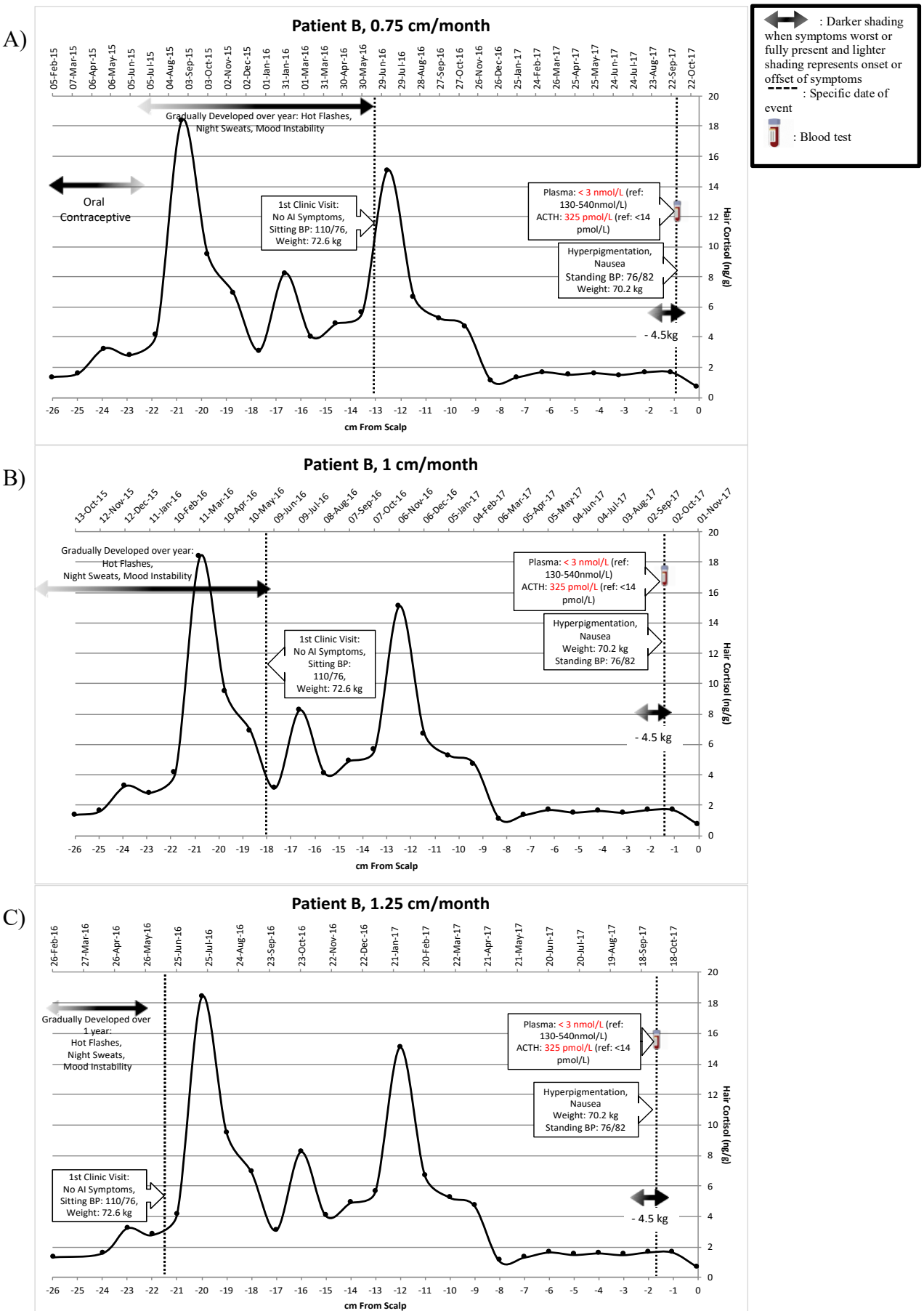

Figure 11: Patient B Hair Cortisol Timelines utilizing A) 0.75, B) 1, or C) 1.25 cm/month growth rate.

Patient C is a 26-year-old woman was first seen at our clinic at the end of June 2016 when she was 24 weeks pregnant. She described symptoms suggestive of undiagnosed hypercortisolism during her previous pregnancy about 2 years earlier. Her symptoms recurred during the current pregnancy, and included weight gain, worsening diabetes mellitus and hypertension prompting investigation into the cause of these symptoms. Physical examination revealed Cushingoid features including a round flush face, hirsutism on the neck, cheeks and lips, prominent striae on her forearms and axilla, thin skin and dorso-cervical and supraclavicular fat pads. Biochemical investigations showed elevated late-night saliva cortisol and increased urinary cortisol of 1141 (ULN < 275) nmol/24hr with an undetectable ACTH of < 0.3 (1.6 - 14) pmol/L. MRI scan of the abdomen showed a left adrenal mass with a maximum diameter of 4 cm. She was started on metyrapone at 25 weeks' gestation to lower her cortisol and clinically responded well. The baby was delivered by caesarean section at the beginning of September 2016 (35 weeks' gestation). Post-delivery she was switched to ketoconazole. Her Cushingoid features had improved by the final week of September 2016, and 30 days after the delivery of her baby, her Cushing symptoms had significantly improved. In the middle of November 2016, she underwent a left adrenalectomy. Post-operatively she was started on hydrocortisone 120 mg daily for 2 days, and then 60 mg for 2 days which was tapered to a maintenance dose of 40 mg and tapered towards discontinuation in February 2017. In the third week of January 2017 she appeared to have improved facial filling and no new facial hair. Subsequently, there was a rise in ACTH with sequential measurements over the following weeks and months being 1.0, 1.4, 3.7, 9.1 pmol/L and AM cortisol production increasing from < 28 to 62 nmol/L indicating return of endogenous pituitary-mediated cortisol production. This time period corresponds with weight loss of approximately 35 kg, restored menses, improvement in external Cushingoid features and resolution of her diabetes and hypertension. Her cortisol and ATCH were subsequently found to be normal by repeated measurements in June and August 2017.

Her hair cortisol timeline in **Fig. 12** shows a continual increase in HCC throughout the 2nd and 3rd trimester, peaking around the time of her child's birth and then dropping sharply from 16 ng/g to approximately 4 ng/g post-delivery with the addition of 200 mg of Ketoconazole t.i.d. The timing of these peaks and troughs suggest that the most

appropriate timeline for this patient is based upon a hair growth rate of 0.75 cm/month. Between October and her surgery in November, the proposed hair timeline indicates increasing cortisol levels which abruptly plunged following her surgery corresponding to the trough in her HCC in January 2017 and her AM cortisol levels (**Fig. 12**). Subsequently, we see a rise in her HCC levels corresponding to an increase in her ACTH and AM cortisol production which indicates the return of endogenous pituitary feedback cortisol production.

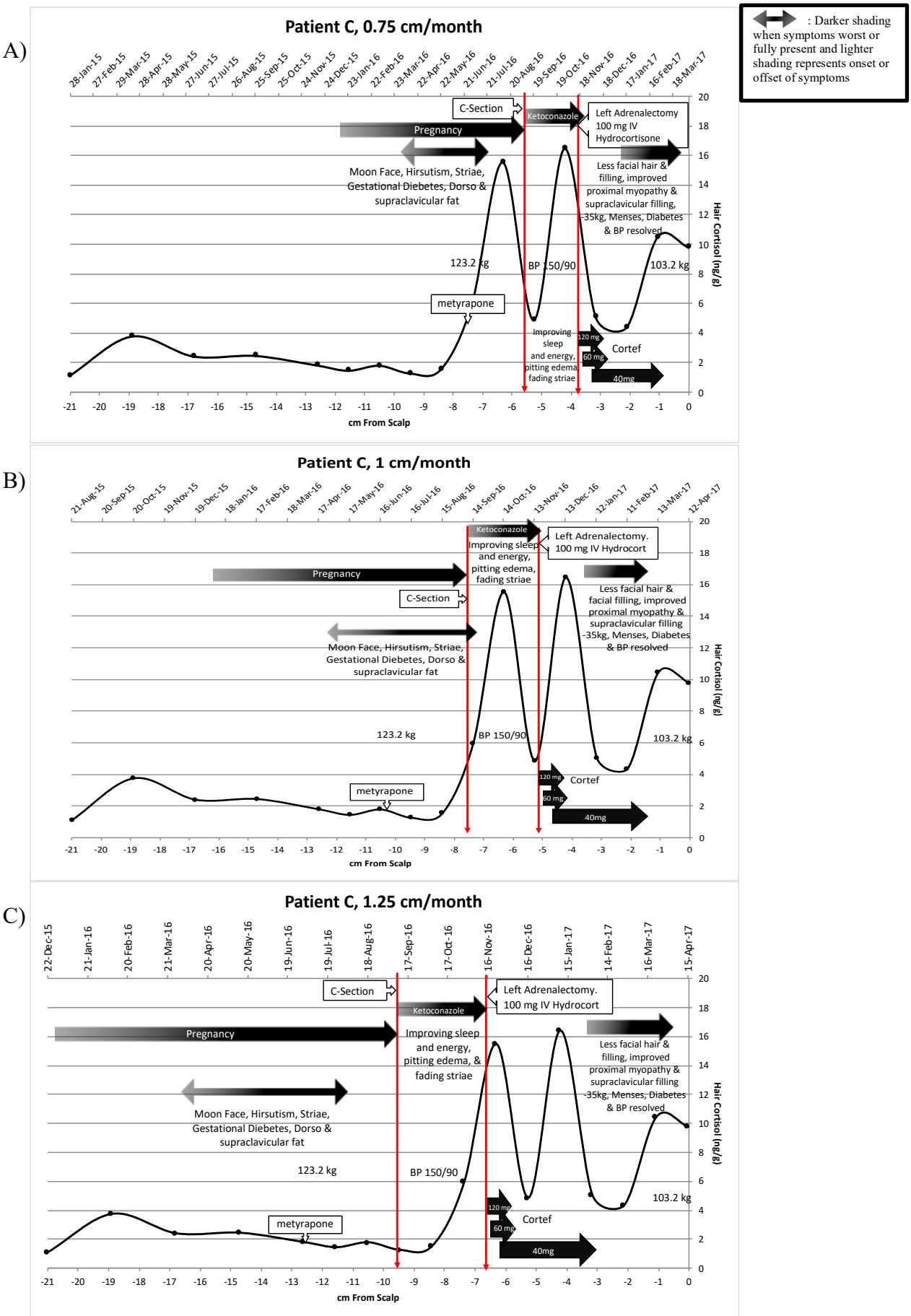

Figure 12: Patient C Hair Cortisol Timelines utilizing A) 0.75, B) 1, or C) 1.25 cm/month growth rate.

Patient D is a 25-year-old woman who gave birth to a healthy baby in September 2016. She was seen in the outpatient clinic at the end of January 2017 because of a full face and abnormal weight gain. Biochemical testing demonstrated excess 24-hr urinary cortisol of 691 (ULN 275) nmol/24hr. A 8 mg dexamethasone suppression test was abnormal with a cortisol of 572 (reference ≤ 50) nmol/L and a spontaneous ACTH value of 29.6 (ULN < 14) pmol/L indicating ACTH-dependent Cushing syndrome. Her AM cortisol results were 752 and 1115 (Ref range 135-527) nmol/L and ACTH of 25.7 and 49.7 (ULN < 14) pmol/L in late February and early March 2017, respectively. MRI scan of the sella demonstrated a 3 mm pituitary adenoma and inferior petrosal vein sampling confirmed the pituitary as the source of the excess ACTH secretion. At the end of March 2017, shad had been started on ketoconazole 200 mg t.i.d. leading to an improvement in strength, facial swelling and hirsutism, loss of 5 kg and her first reported menses since before pregnancy occurred in the middle of April 2017. In June 2017 she underwent successful resection of her pituitary adenoma She was discharged on hydrocortisone was 30 mg q.a.m. & 10 mg q.p.m. followed by a dose decrease to 20 mg q.a.m., 15 mg q.a.m, and finally 10 mg q.a.m. Upon examination in August 2017, she had reduced facial swelling, substantial weight loss, improved mood and less proximal myopathy. Her overall Cushing symptoms continued to gradually improve. In May 2018, she reported having regular periods and feeling excellent with a weight loss of 18 kg and demonstrated no residual Cushingoid features.

The hair cortisol timelines for various postulated growth rates are presented in **Fig. 13**. The hair growth rate of 1cm/ month was thought to best match with the detailed course of her symptoms (**Fig. 13B**). Reduction in hair cortisol is seen following birth of her child in September 2016. Her symptomatic period overlaps with highly elevated hair cortisol of over 1000 ng/g. Ketoconazole therapy induces a clear decrease in hair cortisol. The timing of the other two graphs do not show a reduction in hair cortisol corresponding to the start of ketoconazole (**Fig. 13A, C**). The 1 cm/month timeline shows a sharp decline in hair cortisol following resection, while **Fig. 13A** shows decreasing cortisol before resection. Improvements in symptoms appear to correlate well with continually decreasing hair cortisol until hair cortisol reaches about 100 ng/g and the patient is asymptomatic (**Fig. 13B**).

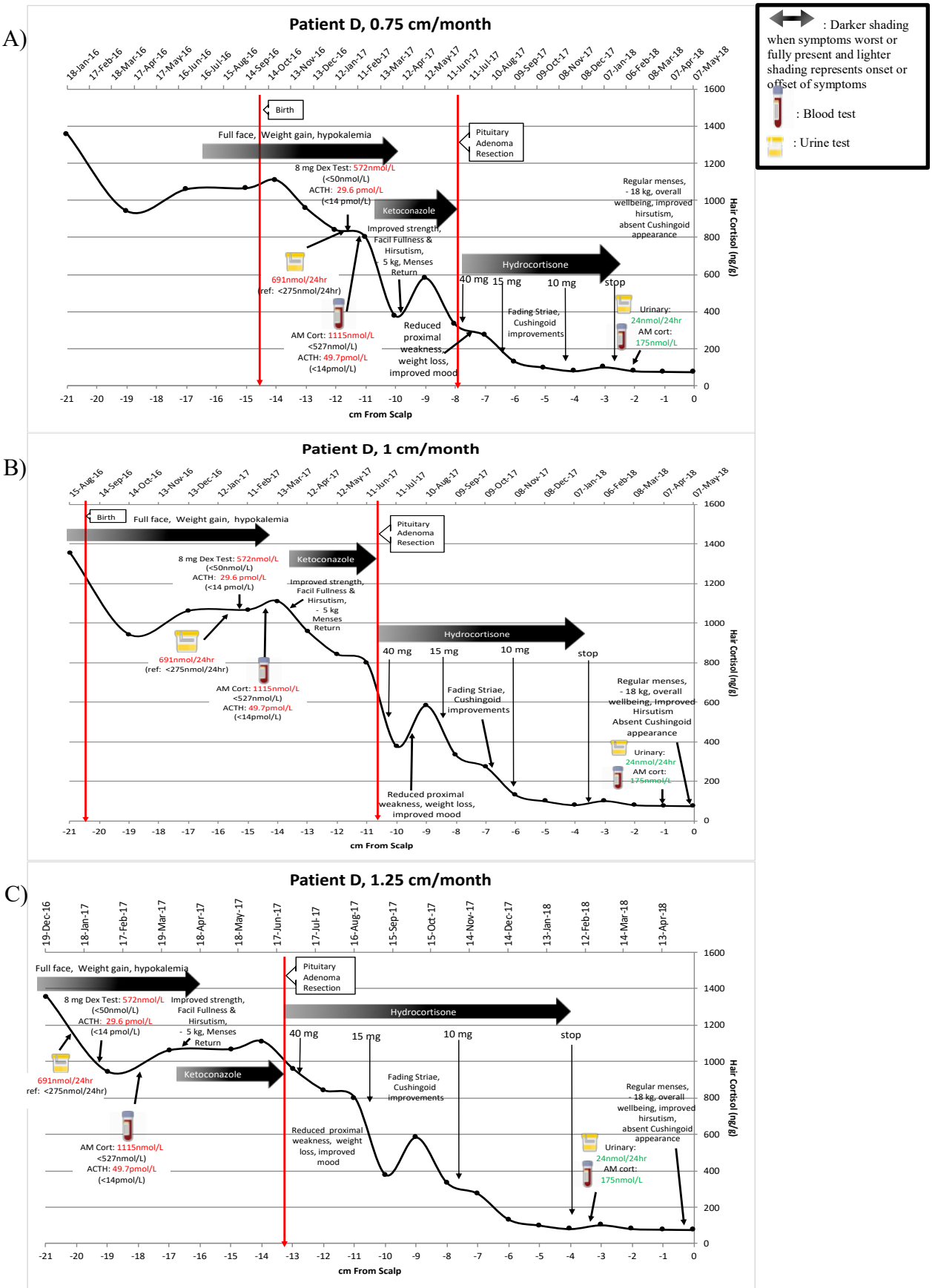

Figure 13: Patient D Hair Cortisol Timelines utilizing A) 0.75, B) 1, or C) 1.25 cm/month growth rate.

3.4 Discussion

The current study investigated retrospective hair cortisol levels over several months to years in three patients with CS and one patient with AI and determined the hair growth rates best fitting with the individualized time course of the clinical symptoms. Our findings suggest that the commonly presumed hair growth rate of 1 cm per month did not always best match with the known clinical time course in each patient. This suggests there is a need to utilize various hypothetical hair growth rates when attempting to construct individual timelines representing historical cortisol secretion in each unique patient.

Prior studies have accepted 1 cm per month hair growth rate as the basis for their timelines[7,16,17]. It has always been known that there is variation in growth rates dependent upon seasonality, ethnicity, age and other factors[14]. Yet it has become evident that there is more variability between individuals than previously thought. For example, individuals from recent African descent have been shown to have slower hair growth on average of 0.75 cm/ month, and women of Asian ancestry can have hair growth rates approaching 1.5 cm/month with an average of 1.25 cm/ month[18]. This variability becomes more problematic with longer hair samples as any variation from 1 cm/month compounds over time. Because it is not possible to know an individual's hair growth rate *a priori* with absolute certainty, we created three timelines for each individual based upon hypothesized hair growth rates of 0.75, 1, and 1.25 cm/month. This technique was by another group when trying to quantify Cyclosporine A (CsA) in liver transplant patients[19]. By utilizing this method, they were able to detect CsA in hair segments corresponding to times of intake, whereas hair segments without immunosuppressant therapy were negative. In the present study, the most suitable timelines we determined were 0.75 cm/month for patients B and C, while for the remaining patients a timeline generated based on a growth rate of 1 cm/month appears to provide the best fit. This method gives clinicians the ability to more accurately represent the timeline in a given patient.

Previous work has reported an upper limit of hair cortisol in healthy individuals to be 75.9 pg/mg hair, with a sensitivity for distinguishing between healthy individuals and

those with CS of 86% [20]. A recent study in Spanish adults indicates an average hair cortisol of 127.91 pg/mg[21]. Three of the patients presented in this study had confirmed diagnosis of CS. Patient A had a peak hair cortisol value of 700 ng/g, patient C (who was pregnant when she was diagnosed with adrenal Cushing) had a peaked at 17 ng/g, and patient D peaked at 1400 ng/g hair. Both patient A and patient D did report cessation of CS symptoms corresponding to hair cortisol approaching 100 ng/g hair and were considered in remission around the same time. Future research should assess how disease and physiological changes such as pregnancy act as covariates that effect cortisol exposure.

Patient B was diagnosed with adrenal insufficiency. Her course suggests that autoimmune AI may present in a cyclical fashion rather than the more commonly presumed linear fashion. Waxing and waning of clinical manifestations is well documented in rheumatic autoimmune diseases including systemic lupus erythematosus[22], rheumatoid arthritis[22], psoriasis[23], and other autoimmune diseases such as Graves' disease[24,25] and Hashimoto's thyroiditis[25]. The honeymoon phase in type 1 Diabetes Mellitus, another a relapsing–remitting condition, is proposed to be another example of cyclic tug-of-war between immune regulation and inflammation[26]. These cycles of worsening and cessation of symptoms are thought to be induced by environmental factors and stress. Thus, in this patient's hair timeline the peaks and troughs may be attributed to the ebb and flow of autoimmune insult on the cortisol producing cells within the adrenal cortex. Future studies need to evaluate additional autoimmune AI patients to determine if this is a common phenomenon.

Patient C had CS, yet her hair cortisol values similar to Patient B who was diagnosed with autoimmune AI. Interestingly, patient C, who had the lowest hair cortisol levels, was the only one with ACTH-independent CS, indicating that there may be a difference in the availability of cortisol to be deposited in the hair of these patients or that the hair follicle may be responsible for part of the cortisol incorporated in hair[27]. She was also pregnant. Cortisol is known to increase in the second and third trimester of pregnancy in response to an increased production of corticosteroid binding globulin[28]. During pregnancy, plasma cortisol has been shown to be 2- to 3-fold greater in pregnant women

compared to controls[29,30] and 5-fold greater at delivery than during the first trimester[31], followed by a sharp drop-off postpartum[28]. This increase in cortisol during the third trimester has been demonstrated in HCC as well[16,21]. Reports of CS and pregnancy are sparse, with approximately 200 cases in the literature[32]. Two pregnant patients (C & D) with CS are presented here. To our knowledge this is the first report of hair cortisol being used to track cortisol exposure in a pregnant patient with concomitant CS. The hair cortisol timeline of Patient C appears to capture a 5-fold increase in in hair cortisol from pre-conception to birth in addition to a return to these elevated hair cortisol levels associated with her CS state. Subsequent reduction of hair cortisol appears to occur around the time that her adrenal mass was removed. Patient D did not have long enough hair to detail hair cortisol exposure prior to the birth of her child, yet there was a decrease in hair cortisol around the time of birth.

Although there was large variation in the absolute values of hair cortisol between patients, the best representative timelines matched reported worsening or improvement of symptoms, as well as the effect of surgery and initiation of medications affecting cortisol secretion. Further, as hydrocortisone is the pharmacological form of cortisol, treatment with hydrocortisone will be reflected in hair cortisol measurement. It is known that hair cortisol is not highly correlated with serum, saliva, or urinary cortisol values as hair cortisol represents a much larger time period of exposure and is less prone to capturing acute fluctuations in systemic cortisol. Retrospective hair timelines assume that hair growth rate is fairly stable, and not affected by (variation in) the underlying disease or external factors such as seasonality. Elevated cortisol is known to reduce turnover of the hair follicle[33], yet very high hair cortisol in patient A did not seem to effect the rate of hair growth in this patient. True hair growth rates may be different from the postulated values, limiting the utility of hair cortisol as a technique in isolation from additional laboratory diagnostics. In the future, clinicians may be able to use a test-retest method by collecting a hair sample and then measuring the hair growth in that spot over a given period of time in between clinic visits to get a better idea of true hair growth. In addition, the retrospective nature of this study is inherently problematic since patient reports of symptoms are subjective and biased, and the only information available is that which is recorded in patient medical records. Future study design should take into consideration

the inherent variability of growth rates and subjective recall by examining hair cortisol and clinical symptoms in a prospective manner.

In conclusion, retrospective hair cortisol timelines reflect historical fluctuations in cortisol exposure as a result of disease states, medical interventions, and variable steroidogenesis. By analyzing three different hair growth rates and selecting the best match, we were able to improve the relation between retrospective cortisol levels and the patient history. Considering variable hair growth rates provides clinicians a modification to this powerful diagnostic tool to more accurately represent an individual patient's hair timeline. We propose that hair cortisol be implemented in clinical practice for assisting in earlier recognition of patients suffering from pathological states with cortisol variability.

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Chapter 4

4 Hair cortisol in a sample of children and adolescents: age, BMI, puberty, and sex

This study investigates the relationship between levels of cortisol in hair and sex, pubertal development, age, and BMI in healthy children and adolescents. Hair cortisol levels were examined in 250 normally developing subjects from 7 to 17 years of age. Subjects provided a hair sample at a hospital emergency department visit. Pubertal stage was assessed with a self-questionnaire that included Tanner stage sketches. Subjects were grouped according to their general status of pubertal development (early-puberty: Tanner stage I & II; mid- to post-puberty: Tanner stage III - VI). Hair cortisol increased with age, pubertal development and BMI but there were no sex differences. Changes in hypothalamic–pituitary–adrenal (HPA) axis function that occur during puberty may have implications for immediate and long-term adolescent health.

4.1 Introduction

The hypothalamic-pituitary-adrenal (HPA) axis mediates the secretion of corticosteroids, including cortisol, from the adrenal cortex. This axis is responsible for controlling the body's response to deviations from homeostasis, making the secretion of cortisol essential for activation of the normal stress response[1]. Cortisol is vital for energy metabolism, electrolyte balance[2], modulating the immune system, and interacting with the central nervous system to effect cognition and memory[3]. Perturbations in normal cortisol secretion, whether hypo- or hyper-secretion, can lead to poor outcomes[4–6]. In particular, changes in cortisol secretion during development can have long-lasting implications for cognition, psychopathology, and physical and emotional development in children and adolescents. Other than perinatal development, the period of time in development marked by the most drastic physiological changes is puberty, with a number of these changes being steroid-dependent [7]. This period of time during adolescence is critical for undergoing striking changes in physical and emotional growth that can last with the individual for many years[8].

Sex differences in the reactivity of the HPA axis and the resulting stress response largely arise during puberty[8,9]. There is a complex interplay between the HPA axis and the hypothalamic-pituitary–gonadal (HPG) system. Glucocorticoids and the sex hormones estrogen and testosterone, products of the HPG axis, interact at the receptor level as well as the hypothalamic and pituitary levels[9]. The gold standard for assessing puberty is physician assessment using the Tanner scale, which defines pubertal maturation into 5 stages (I-V) based upon external sexual characteristics including breast, genitals and pubic hair.

Cortisol is routinely measured in clinical practice in serum, saliva and 24-h urine[1,10]. Salivary and serum cortisol are considered to be acute measures as they are reflective of cortisol concentrations at a single point-in-time[1,10,11]. These assays require repeated samples, limiting their utility for determining long-term cortisol exposure[11,12]. Similarly, urinary cortisol collected from 24-hr urine samples is labor intensive and is not feasible in renal patients. These standard sampling techniques are suboptimal for a young population who may be fearful of needles, or clinics in general[13]. A number of studies have assessed cortisol levels during puberty with varying conclusions[14–16]. Differences between males and females have generally varied based upon the matrix assayed, the time of day samples were taken, and the stage of development[9,16].Yet increases in basal HPA activity with age and puberty are consistent, especially in females[16].

Measuring hormones in human hair provides a practical and non-invasive alternative to the collection of blood samples, and eliminates the variability that is captured from circadian fluctuations in salivary, serum, or urinary assessments[10]. The non-invasive nature of hair sampling may be particularly important in paediatric populations, where serum cortisol may be elevated as a result of the stress associated with venipuncture sampling procedures[17]. Most hair cortisol studies have looked at adults[12,18,19] or young children[13,20–22]. Fewer studies have measured hair cortisol in adolescents[23–26]. These studies have generally found an increase in hair cortisol with age, with 3 assessing puberty status[19,25,27].

This study investigates the relationship between cortisol in hair and age, puberty, sex and BMI in children and adolescents. The goal of this work is to add to the literature by determining normal changes in hair cortisol values in paediatric and adolescent individuals. Hair cortisol may prove to be important for capturing long-term cortisol exposure in children and adolescents, a population in which recurrent venipuncture is problematic because of this population's innate fear of needles. In the future this information can be used as a comparison for assessing perturbations in cortisol exposure that may be caused by disease states, or modern social stressors such as technology and social media that may be impacting the health of this population.

4.2 Methods

4.2.1 Sample Selection

The recruitment process involved screening all patients attending the Paediatric Emergency Department, Children's Hospital of Southwestern Ontario, London, Ontario, Canada. Electronic medical records were reviewed for complete medical history including recent hospital visits, medical diagnoses, and list of medications being used. All potential participants and their substitute decision makers (if under the age of 14) were asked about any past and current physical or emotional problems including recent stressors, sickness, as well as any diagnosed mental or physical issues, and any medications being taken. Exclusion criteria included use of glucocorticoids, both systemic and topical, or medication known to alter glucocorticoid metabolism, a history of serious health problems, diseases, or psychiatric illnesses, abnormal stress (e.g. moves, school, parental divorce), vaccinations within the last 2 months, severe trauma in the last 2 months, and infection (e.g. cold, flu) within the last 2 months. Once deemed eligible, participants were given an information letter and request for consent. A total of 250 (130 boys, 120 girls) seven to 17-year-olds were included in the study. All subjects and a substitute decision maker provided written informed consent. This study was approved by Western University Research Ethics Board. The approval for this study can be found in **Appendix C.**

4.2.2 Measures

4.2.2.1 Pubertal stage

Morphological pubertal stage was assessed using validated schematic drawings of secondary sex characteristics associated with the five Tanner stages of pubertal development [28]. The self-questionnaire (**Fig. 14**) was validated for assessing sexual maturation in children by comparing the questionnaire to physician assessment and blood hormone levels[28]. At the study visit, the objective of the questionnaire was explained to the parent and subject was then shown a sample of the figures. They were given the option of completing the questionnaire individually or together. It was explained that when choosing the image that looked most like them, to not include the practice of pubic hair shaving. Similar ratings have been widely used and demonstrated good reliability. Subjects were then classified as Tanner stage I–V; males were staged based upon genitals and pubic hair, and females were staged based upon breast and pubic hair.

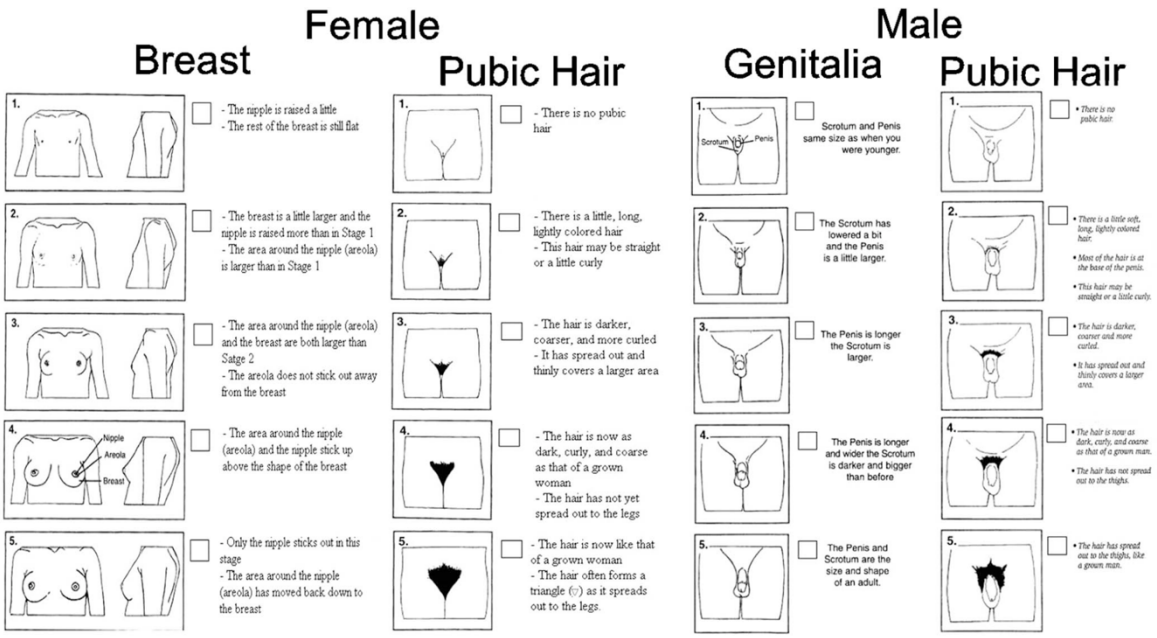

Figure 14: Tanner Self-Questionnaire.

4.2.2.2 Body Mass Index (BMI)

Height and weight were measured at the subjects' hospital visit. Height was measured in centimeters (cm) using a standard stadiometer, and weight was measured in kilograms (kg) using a digital scale. The same height meter and weighing scales were used throughout the study. Body mass index (BMI), a standard index of a person's weight in relation to height, was determined for each subject by dividing the weight (kg) by the square of the height (m^2).

4.2.2.3 Hair Sampling

Subjects provided a hair sample about the thickness of a pencil eraser (~100 hairs) which was collected from the posterior vertex of the scalp as close to the scalp as possible. The sample was then taped to a hair collection form and secured by placing it in an envelope labelled with the patient's study ID. Samples were transported to the laboratory and stored at room temperature until analysis.

4.2.2.4 Cortisol Measurement

Hair samples were prepared and cortisol was analyzed using a modified commercial salivary cortisol competitive ELISA (Alpco Diagnostics®, Windham, NH) as previously described[29]. Briefly, the 2 cm of hair proximal to the scalp was weighed to 10 mg, then washed twice with isopropyl alcohol. The hair was minced using surgical scissors in methanol and the cortisol was extracted for 16 hours on an incubator shaker with heat. The supernatant was evaporated under nitrogenous conditions, leaving a cortisol residue that was reconstituted in PBS. Cortisol was quantified according to the manufacturer's instructions using a micro-well plate reader at a wavelength of 450 nm. Cross-reactivity of other steroids with the kit's antibodies was reported as follows: Prednisolone (13.6%), Corticosterone (7.6%), Deoxycorticosterone (7.2%), Progesterone (7.2%), Cortisone (6.2%), Deoxycortisol (5.6%), Prednisone (5.6%), Dexamethasone (1.6%). Intraassay variation was below 5% and the interassay variation below 10%. Assay results were converted to ng of cortisol per g of dry hair (ng/g).

4.2.3 Statistical Analysis

Statistical analyses were performed using SAS University (SAS Studio 3.8, SAS 9.4M6) and R (version 3.5.3, 2019-03-11). Hair cortisol was $\log_{10}$-transformed to achieve normality and equalize variance. Variance was assessed by Levene's test for homogeneity. Continuous data was analyzed by analysis of variance (ANOVA) followed by Tukey post-hoc for significant results. Standard deviation (SD) scores (z-scores) for BMI were calculated based on the WHO growth reference 5-19 2007 using the WHO 2007 R macro package[30]. Differences between groups were assessed using Student's t-tests. Relationships between hair cortisol and age, and hair cortisol and BMI were analyzed by linear regression.

4.3 Results

4.3.1 Characteristics of Participants

Subjects ranged from 7 to 17 years old. At total of 120 females (48%) and 130 males (52%) were included. The general characteristics of study participants are found in **Table 2**. There were no significant sex differences in mean age, BMI or Tanner stage.

	Pooled Sample (N=250)	**Male (n=130)**	**Female (n=120)**	
Age (years ± SD)	11.98 ± 0.19	11.76 ± 0.28	12.2 ± .27	p = 0.25
BMI (kg/m^2 ± SD)	20.65 ± 4.83	20.72 ± 5.26	20.57 ± 4.34	p = 0.80
BMI z-score ± SD	0.61 ± 1.44	0.71 ± 1.53	0.50 ± 1.34	p = 0.25
Tanner Stage ± SD				
Breast/ Genitals	2.72 ± 1.30	2.82 ± 1.27	2.63 ± 1.32	p = 0.25
Pubic hair	2.53 ± 1.45	2.49 ± 1.55	2.58 ± 1.33	p = 0.10

Table 2: Characteristics of Study Participants

The majority of participants (78%) were of Caucasian background, followed by Hispanic or Latino (5.2%), Asian (4.4%), and Middle Eastern (3.6%). The ethnicities of all participants can be found in **Table 3.**

Ethnicity	Frequency	Percent
Caucasian	195	78.00
Hispanic or Latino	13	5.20
Asian	11	4.40
Middle Eastern	9	3.60
Unknown	6	2.40
Aboriginal	4	1.60
African American	4	1.60
African American/Caucasian	2	0.80
Asian/Caucasian	2	0.80
Caucasian/Latino	2	0.80
Aboriginal/Hispanic	1	0.40
Middle Eastern/Caucasian	1	0.40

Table 3: Ethnicity of Study Participants

The reason for each participant's hospital emergency visit, coded according to the Medical Dictionary for Regulatory Activities patient friendly terms (MedDRA, v22.0), are provided in **Table 4.** If the reason was listed only once, it was included in the 'Other' category. The full listing is available in **Appendix E**.

Reason	Frequency	Percent
Abdominal discomfort	26	10.40
Ankle injury	17	6.80
Head injury	15	6.00
Laceration	15	6.00
Wrist injury	15	6.00
Finger injury	13	5.20
Headache	10	4.00
Elbow injury	8	3.20
Wrist fracture	8	3.20
Arm injury	7	2.80
Shoulder injury	7	2.80
Clavicle fracture	6	2.40
Knee injury	6	2.40
Leg injury	6	2.40
Sore throat	6	2.40
Syncope	6	2.40
Chest pain	5	2.00
Cough	5	2.00
Hand injury	5	2.00
Urinary tract infection	5	2.00
Fever	4	1.60
Neck pain	4	1.60
Nose injury	4	1.60
Vomiting	4	1.60
Anaphylactic reaction	2	0.80
Constipation	2	0.80
Earache	2	0.80
Skin rash	2	0.80
Toe injury	2	0.80
Other	32	12.8
Unknown	1	0.40

Table 4: Reason for Hospital Visit (abbreviated)

Status of pubertal development (Tanner stage I-V) was assessed for all participants. Based upon pubic hair development, 50.8% of participants were in pre- to early-puberty (Tanner stage I & II) and 49.2% were in the later stages of puberty (III-V, **Table 5**). Based upon breast or genital development, 44.8% were in pre to early puberty and 55.2% were in stages III-V (**Table 5**). There was good agreement with both measures.

Tanner Stage	Pubic Hair by Sex			Genital/Breast by Sex		
	F	M	Total	F	M	Total
1	39 15.60 41.05 32.50	56 22.40 58.95 43.08	95 38.00	34 13.60 60.71 28.33	22 8.80 39.29 16.92	56 22.40
2	17 6.80 53.13 14.17	15 6.00 46.88 11.54	32 12.80	20 8.00 35.71 16.67	36 14.40 64.29 27.69	56 22.40
3	28 11.20 59.57 23.33	19 7.60 40.43 14.62	47 18.80	35 14.00 51.47 29.17	33 13.20 48.53 25.38	68 27.20
4	28 11.20 59.57 23.33	19 7.60 40.43 14.62	47 18.80	19 7.60 46.34 15.83	22 8.80 53.66 16.92	41 16.40
5	8 3.20 27.59 6.67	21 8.40 72.41 16.15	29 11.60	12 4.80 41.38 10.00	17 6.80 58.62 13.08	29 11.60
Total	120 48.00	130 52.00	250 100.0	120 48.00	130 52.00	250 100.00

Frequency
Percent
Row Pct
Col Pct

Table 5: Puberty Status by Sex

Participants were grouped based on standardized BMI scores according to the WHO 2007 definitions: Obesity: > + 2SD (equivalent to BMI 30 kg/m^2 at 19 years), Overweight: > + 1SD (equivalent to BMI 25 kg/m^2 at 19 years), > -2 SD Normal < + 1 SD and, Underweight < - 2SD[30]. The frequency for reported BMI subgroups are found in **Table 6.**

Growth	Frequency	Percent
Normal	143	57.20
Overweight	63	25.20
Obese	37	14.80
Underweight	7	2.80

Table 6: BMI Growth Groupings Based on WHO 2007 z-score Cut-Off.

4.3.2 Hair Cortisol

Hair cortisol concentrations were 250.56 ± 314.07 ng/g (mean ± SD), ranging from 4.47-2703.64 ng/g. After log-transformation, the mean (± SD) hair cortisol was 2.17 (±0.65) ng/g. Sex did not influence hair cortisol ($p = 0.29$).

4.3.2.1 Hair Cortisol in Relation to Age

Regression analysis showed a positive linear relationship between hair cortisol concentration and age ($\beta = 0.29$, $p<.0001$, **Fig. 15**). This relationship remained in both the male ($\beta = 0.32$, $p < 0.0005$) and female ($\beta = 0.25$, $p < 0.01$) subgroups.

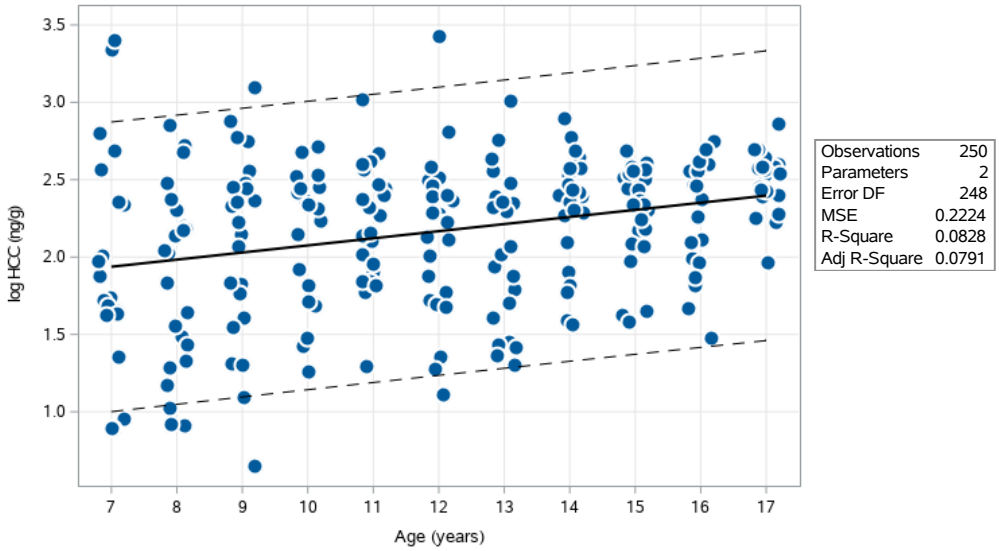

Figure 15: The relationship between log-transformed Hair Cortisol (ng/g) and Age (years). Dotted lines represent limits of 95% prediction intervals based on linear regression; ($\beta = 0.29$, $p<.0001$).

4.3.2.2 Hair Cortisol in Relation Pubertal Status

There was a significant difference between hair cortisol concentration in early and late puberty groups as determined by 2-way ANOVA ($F_{(3,246)} = 4.29$, $p = 0.006$) for genital or breast development. A Tukey post-hoc test revealed that hair cortisol was significantly lower ($p < 0.001$) in the early puberty group (Tanner stage I & II, 2.05 ± 0.54 ng/g) compared to the late puberty group (Tanner stage III-V, 2.26 ± 0.42 ng/g) as assessed by genital or breast Tanner stage (**Fig. 16a)**. There was no main sex or interaction effect.

There was a significant difference between hair cortisol concentration in early and late puberty groups as determined by 2-way ANOVA ($F_{(3,246)} = 5.00$, $p = 0.002$) for pubic hair development. A Tukey post-hoc test revealed that hair cortisol was significantly lower ($p < 0.001$) in the early puberty group (2.06 ± 0.55 ng/g) compared to the late puberty group (2.28 ± 0.38 ng/g) as assessed by pubic hair Tanner stage (**Fig. 16b).** There was no main sex or interaction effect. There was a positive correlation between both measures of puberty and hair cortisol (not shown, $p< 0.001$).

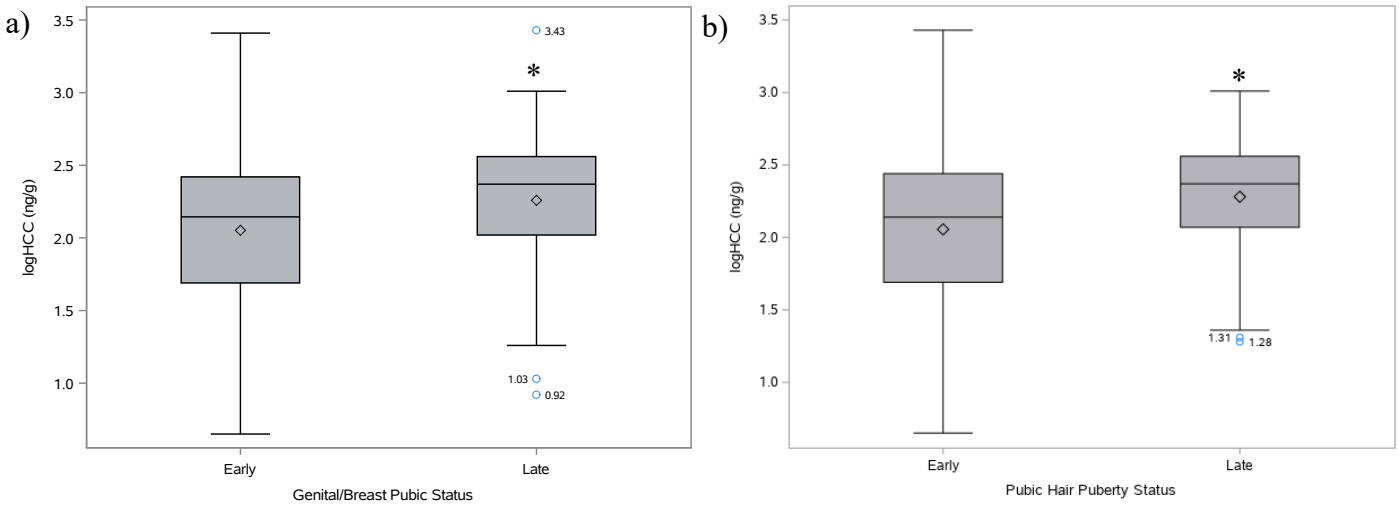

Figure 16: Boxplots comparing mean hair cortisol by early or late stage of puberty assessed by **a)** breast or genital Tanner stage and **b)** Pubic Hair Tanner stage. * $p < 0.001$, Tukey.

4.3.2.3 Hair Cortisol in Relation BMI

Regression analysis showed a positive linear relationship between hair cortisol concentration and BMI ($\beta = 0.16$, $p = 0.01$, **Fig. 17**). This correlation remained in male ($\beta = 0.20$, $p < 0.05$), but not the female ($p = 0.20$) subgroup. Hair cortisol did not regress with BMI adjusted for age ($\beta = 0.038$, $p=0.55$).

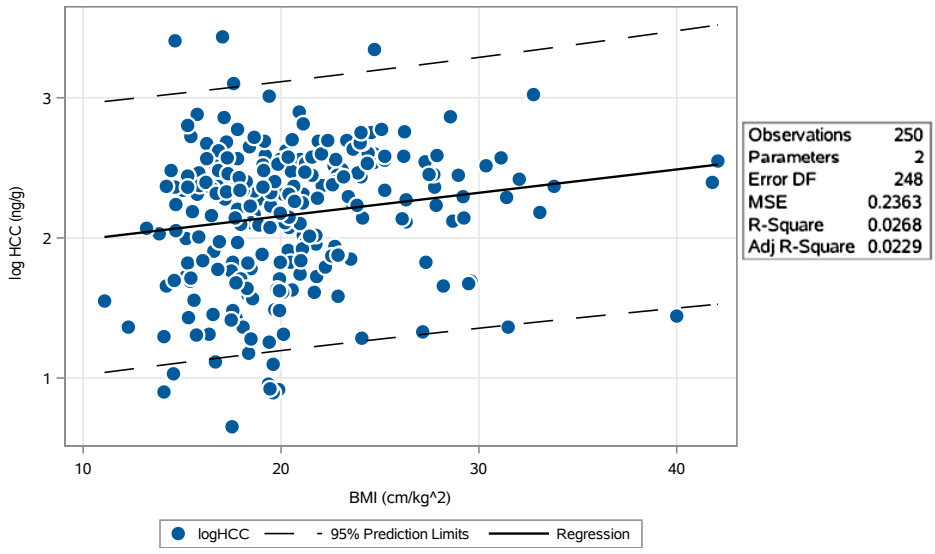

Figure 17: The association between log-transformed Hair Cortisol (ng/g) and BMI (cm/kg^2); Dotted lines represent limits of 95% prediction intervals; ($\beta = 0.16$, $p=0.01$).

There was no significant difference between thin, normal, overweight, or obese groups as determined by one-way ANOVA ($F_{(3,246)} = 1.92$, $p = 0.13$) for BMI cut-offs (**Fig. 18**).

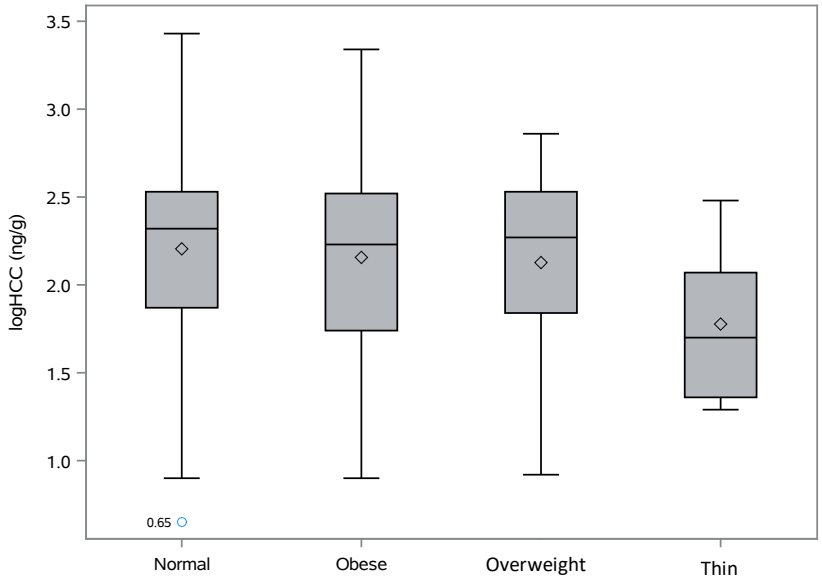

Figure 18: Boxplot for BMI subgroups according to WHO 2007; p = 0.13

4.4 Discussion

This study assessed hair cortisol as a function of age, sex, BMI and puberty status in a large sample of healthy children and adolescents. Each participant was screened to exclude those with known diagnoses, recent stressors or any medication use. Hair cortisol increased positively with age. A number of studies found no significant association between hair cortisol and age, though they had small sample sizes, and limited age ranges[31]. The positive correlation is in agreement with a previous study of 128 children aged 4-14[25] and a study of 245 3-16 year-olds[27], both of which assessed hair cortisol over a wider age range of children and adolescents compared to the studies that found no relationship. On the other hand, two studies found that hair cortisol decreased with age in children below 9 years of age[19,22]. This may indicate a quadratic relationship between hair cortisol and age, with high levels at very young ages declining within the first 7-8 years of life, followed by increasing cortisol levels into adulthood[19]. In a subset analysis of the 7 to 9-year-olds in our cohort, however, the positive relationship remained. Variability in hair cortisol decreased with age in the current study. Decreasing variance from infancy has been previously reported in hair cortisol [22] as well as serum cortisol measurements up until the age of 17, when variance begins to increase once again[32].

Hair cortisol did not differ between male and female participants. This finding is similar to many studies which found no significant difference between sexes[19,20,22,25,33,34]. While other studies found higher cortisol in males than in females[35,36]. These studies, however, were in younger children and thus it may be true that boys have higher cortisol levels during early childhood. It was hypothesized that differences would arise during puberty because adolescence is a sensitive period in the development of both the HPA and HPG axes[8,9]. Similar to White et al.[27], puberty status was associated with increased hair cortisol, with those in later stages of puberty having higher cortisol than those in early puberty. Noppe et al. [25] showed no difference from pre to post-puberty, though that study had only 26 children (20%) in later stages of puberty thus limiting the ability to draw conclusions on the effect of puberty.

Body mass index ranged from very thin to obese according to the WHO 2007 reference data[30]. There was a positive relationship between hair cortisol and BMI which did not remain when calculating BMI-for-age z-scores. A positive relationship with BMI in children has previously been shown[35,37]. This positive correlation remained in previous studies assessing standard deviation scores for BMI[27,38–40], while, similar to our study, two other groups did not find a correlation between BMI z-score and hair cortisol[25,41]. Obesity in children has been associated with increased hair cortisol[38,42], though in the current study, hair cortisol did not differ between obese, overweight, normal and underweight groups. None of these studies used the WHO reference data for comparison. Waist circumference, as a measure of body composition, may provide a more consistent relationship with hair cortisol[31,37].

A number of limitations of the present study must be discussed. Recruitment from the emergency department at Children's Hospital of Western Ontario was chosen because it services a large area of Southwestern Ontario with a diverse multicultural background. However, the results indicate that the vast majority of participants were Caucasian limiting the generalizability of our results to other ethnicities. Hair cortisol concentrations in the present investigation are quite high compared to previous studies, but similar to studies using the same methods and even the same ELISA kit[22,43–45]. Immunoassay measurement of cortisol overestimates cortisol[43,46], and most studies are moving

towards the use of liquid chromatography mass spectrometry to increase the specificity of measurements[46–48]. Hair cortisol is decreased with UV exposure[49]. The majority of samples were taken during the winter months meaning that most participants would not have been exposed to UV as a result of cold weather as well as low winter UV index in Ontario. This could be one contributory factor to the high cortisol levels. Recruitment in the emergency setting also leads to sampling bias for active children who are prone to physical injury. Intensive aerobic exercise activates the HPA axis leading to increased hair cortisol levels[50].

This study was well powered with a wide age range, including approximately equal numbers of individuals in pre- and post-puberty. Our sample was large enough to have about 20 individuals for each year of age, with approximately equal distribution between males and females. The wide geographical sampling area, drawing from London, Ontario and many of the surrounding regions in Southwestern Ontario indicates a potentially representative sample of the general healthy population within our target age range. Because recruitment occurred in the hospital, thorough screening by electronic medical record and questioning at the time of recruitment was utilized to exclude those exposed to recent stressors, and those with medication use or any diagnosed medical conditions.

This study showed an increase in hair cortisol with age, BMI and puberty status in a large population of relatively healthy children and adolescents. Hair cortisol has the potential to assess cortisol exposure in a non-invasive manner. This has important implications for assessing cortisol exposure during an important and stressful period of human development. Hair cortisol may be a useful tool for future research to delve into the cornucopia of stressors facing adolescents today, including the way technology is impacting physical and mental health. Future studies need to address the use of immunoassays for cortisol measurement because of the tendency to overestimate cortisol levels in hair.

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Chapter 5

5 Hair Cortisol Methods Update

5.1 Introduction

The keratinized structure of hair and the relatively predictable growth rate [1] allows for the retrospective determination of exposure to endogenous and exogenous compounds. Hair as a matrix, is composed of 65-95% protein, 1-9% lipids, 3-5% water, and <1% trace elements [2]. The surface of hair is hydrophobic, and the color is determined the melanin pigment[2]. Compounds in the blood diffuse from capillaries within the dermal papilla of the hair follicle into dividing cells and are subsequently deposited in the hair shaft as it grows[3,4]. The use of hair to measure exposure to exogenous compounds, especially in the case of drugs of abuse, has been used for many years[2,5]. Notably, the development of hair assays for exposure to cocaine[3,6–9], amphetamines[7,10], opiates[7,8,11], and cannabinoids[6,8] in human hair.

Analysis of exogenous corticosteroids in hair [12] sparked the first interest in measuring the endogenous corticosteroid, cortisol[13]. Since this time, hair cortisol analysis has rapidly gained in popularity, with the number of newly published manuscripts increasing every month. Studies have looked at hair cortisol values in healthy controls[14,15] and a number of diseased populations from those with Cushing's disease[16,17], cardiovascular disease[18,19] and mental health[20–22].

The standard methods for hair cortisol analysis involves weighing hair samples, followed by washing the samples with isopropanol to remove cortisol that may have been deposited on the outside of the hair from sweat, sebaceous secretions or from handling[23]. Following this washing step, samples are allowed to air dry before the cortisol is extracted. A prerequisite to maximal cortisol extraction is increasing the surface area of the hair's keratin structure. To date, this has been achieved by using ball mills to grind or pulverize the hair, blending the hair, or most commonly by mincing the hair with surgical scissors[23]. Pulverizing the hair with ball mills suffers from the high expense of the machinery as well as high sample loss[24] and carryover between

samples[23]. Other groups have used blender-like ball mills which are less costly[25], but still suffer from the potential for sample loss during the transfer of the fine powdered dust from the blender to the tube which the sample will be extracted in. Mincing hair with surgical scissors to pieces smaller than 1 – 2 mm has been the most common method for increasing hair surface area[23]. The benefits of this method are that the mincing can be done directly in the methanol without the need for transfer into another vial, it is highly cost efficient, and there is no chance for carryover. This method, however, is time consuming and may lead to variability depending on the skill or care taken by the technician. Cortisol is then usually extracted in methanol or acetone at room temperature or in heat from 15 – 24 hours[23].

Following the extraction of cortisol, the supernatant (i.e. solvent and cortisol) is transferred to a clean test tube and evaporated under a steady stream of nitrogen while being heated until dry[23]. The use of nitrogen way hypothesized to prevent the oxidation and degradation of cortisol, however it is known that drying cortisol extracts for LC-MS/MS does not cause signal loss[23]. One study found no significant difference between the nitrogen versus room air extraction on hair cortisol concentration[25]. Finally, the dried samples are typically analyzed by ELISA or LC-MS/MS[23].

Hair color, determined by melanin content, may also effect hair cortisol content[26,27]. The effect of hair pigmentation on hair cortisol may arise during the incorporation phase, or alternatively may be an artefact of the stronger binding of cortisol to the melanin in hair. Some groups have assumed that cortisol incorporation is independent of melanin, though it is possible that cortisol can bind melanin via weak interactions[26]. Solubilization of hair has the possibility of freeing all compounds in the hair from the pigment and keratin, thus eliminating one source of variability. This may be particularly important for hair cortisol as it has been estimated that a single methanol extraction only yields about 46.1% of absolute cortisol from hair[24]. The time required for digestion and extraction is also considerably less[2,6].

Reduction in potential sample loss, variability, and extraction time, may be achieved by alternative methods for hair particle size reduction. Enzymatic, basic, and acidic

hydrolysis of hair has been used in the determination of drugs of abuse and other compounds in hair[6,9,28]. Hydrochloric acid, sulfuric acid, nitric acid, perchloric acid, and sodium hydroxide solutions have all been employed to dissolve hair samples[2]. The concentrations of acid or base used in these methods range from 0.1M to pure acid or base, as reviewed elsewhere[2]. Yet for some reason, these methods have not been developed for extracting cortisol from hair.

Faster, more reliable and efficient extraction of hair cortisol is desirable for the development high throughput analysis to reduce technician time and cost and increase confidence in analytical results. The aim of the current study is to compare various extraction and evaporation methods to optimize hair cortisol analysis for future use.

5.2 Methods

A sample of hair was taken from participants as part of a larger study assessing hair cortisol levels in healthy children and adolescents. Participants were recruited from the Paediatric Emergency Department, Children's Hospital of Southwestern Ontario, London, Ontario, Canada. This study was approved by the Western Research Ethics Board. All participants gave assent and, a substitute decision maker provided written informed consent if under the age of 14, and those 14 – 17 provided informed consent. Participants were screened for current or past emotional or psychiatric problems, any abnormal medical diagnoses or conditions, medication use, recent abnormal stressors, vaccination, or infections. Screened subjects were excluded for any past medical history of smoking, psychiatric illnesses, cardiovascular diseases, autoimmune diseases or medication use.

5.2.1 Nitrogen vs. Air Evaporation

A 20 mg sample of hair was cut from the posterior vertex of the head as close to the scalp as possible. The hair was washed twice with a 3 mL of isopropanol and allowed to fully dry in air. For this part of the study, the typical mincing method using surgical scissors was used to increase the surface area of the hair. Four (4) mL of methanol was added to the hair which was then minced into small pieces less than 1 mm in size. The vials were then placed on an incubator shaker at 52 °C for 16 hours. The supernatant was then split

into two separate test tubes, each with 2 of the 4 mL of supernatant. One of these test tubes was dried with nitrogen and the other with air, both at a temperature of 50°C until completely dry. The entire process is highlighted in **Fig. 19.**

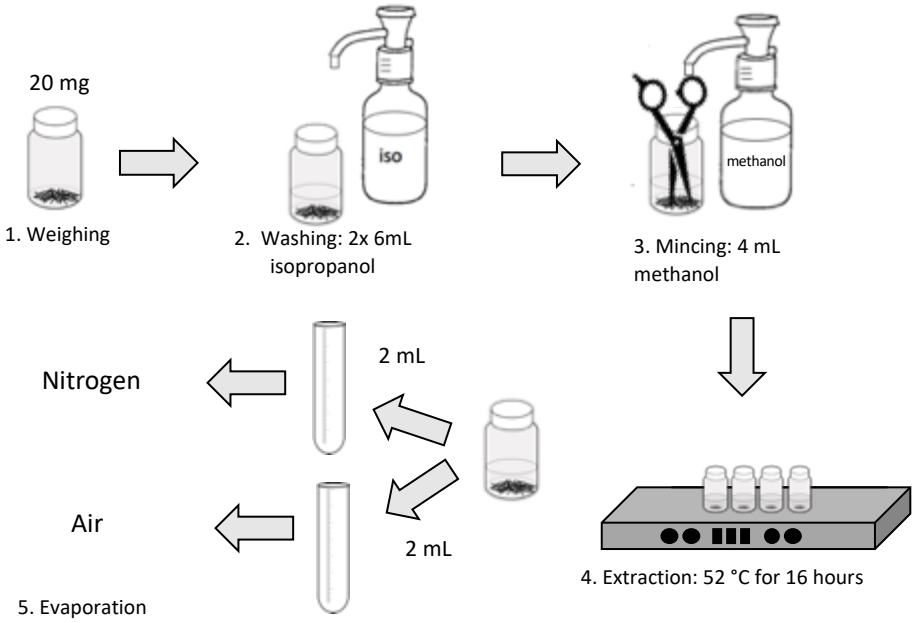

Figure 19: Protocol for nitrogen versus air evaporation.

5.2.2 Enzymatic Digestion

For enzymatic digestion, 10 mg of Proteinase K enzyme (Sigma, P8044), 100 mg of 1,4-Dithiothreitol (DTT, Cleland's reagent; Roche 10708984001) and 1 mL of Tris HCl buffer (pH 8.0) was added to 10 mg of hair. The digestion was undertaken for 60 min at 37.5°C on a shaker to ensure continuous mixing. The hair digest was centrifuged for 10 min at 2380g and the hair pellet was discarded. Two (2) mL of dichloromethane was added, the mixture was vortexed for 10 seconds, and the organic layer was transferred into a fresh glass tube. This was repeated once more. The dichloromethane was evaporated under a gentle stream of nitrogen gas at 50°C.

5.2.3 Sodium Hydroxide Digestion

5.2.3.1 Spike-Recovery and Dilution

For basic digestion, spike-recovery and dilution was assessed (**Fig. 20)**. Hydrocortisone (Sigma, H4001) was diluted in PBS at pH 8.0 by serial dilution. In order to assess linearity of dilution, 3 concentrations (1, 10, 100 ng/mL) of hydrocortisone were added to individual test tubes (**Fig**. **20**). To minimize the volume of PBS added to the assay, 25µL of each hydrocortisone solution was added to the test tubes to achieve a final concentration of 1, 10, and 100 ng/mL in 250µL of PBS for the ELISA (**Table 7)**.

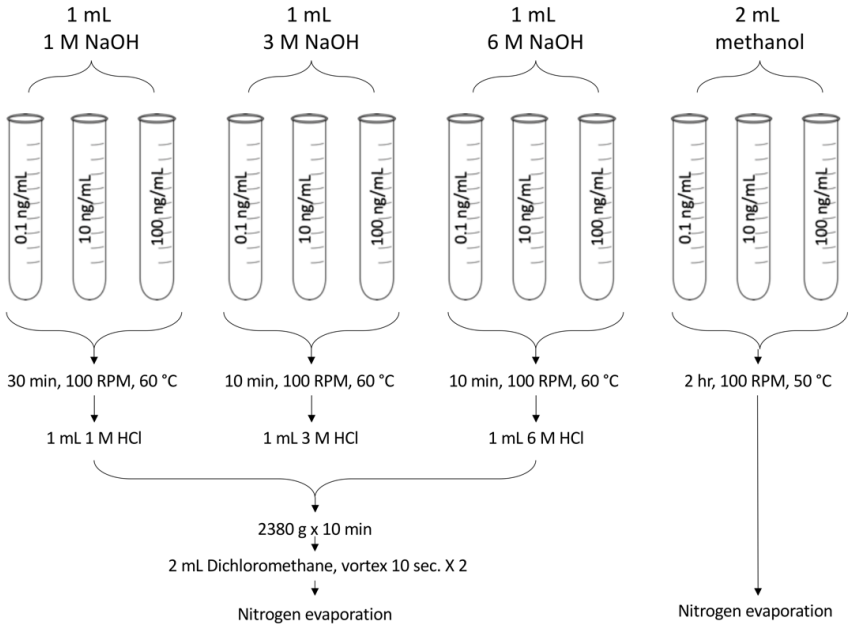

Figure 20: Cortisol spike-recovery dilution assay protocol. Comparing 3 concentrations of NaOH to methanol extraction, each with 3 spiked concentrations of cortisol.

Dilution (ng/mL)	Volume (µL)	Expected Recovered Mass (ng)	Expected Final Concentration in 250µL of PBS (ng/mL)
10	25	0.25	1
100	25	2.5	10
1000	25	25	100

Table 7: Cortisol solutions spiked into samples for recovery and expected recovery in 250µL of PBS. Solutions made by serial dilution of hydrocortisone.

Three sodium hydroxide (NaOH) and 3 hydrochloric acid (HCl) solutions of 1M, 3M and 6 M concentration were made using NaOH pellets or 12M stock HCl in deionized water, respectively. One (1) mL of each of the 3 NaOH solutions was added to each test tube (**Fig. 20**) and incubated at 60°C and 100 RPM for either 10 min (3M & 6M) or 30 min (1M, **Fig. 20**). The length of incubation time was determined by digesting 5 hair samples each at 1M, 3M, or 6M, until visually disintegrated.

To neutralize the NaOH, 1 mL of 1M, 3M, or 6M HCl was added to each sample, and then centrifuged for 10 min at 2380g. The hair and salt pellet was discarded. To the supernatant, 2 mL of dichloromethane was added and vortexed for 10 seconds, and the organic layer was transferred to a fresh test tube. This was repeated twice. The dichloromethane was then evaporated under a steady stream of nitrogen and the residue was frozen until analysis. The NaOH extracts were compared to the standard methanol method (**Fig. 20**).

Percent recovery was calculated as the ELISA result from each extract divided by the ELISA result for the standard dilutions plated directly multiplied by 100. Relative percent recovery, defined as the recovery compared to the standard methanol method, was calculated as the ELISA result from each extract divided by the ELISA result for the methanol extraction multiplied by 100.

5.2.3.2 Recovery from Hair

To assess the hypothesis that NaOH digestion of hair frees a larger percentage of absolute cortisol in hair compared to methanolic extraction, NaOH digestion was undertaken on 27 hair samples. Each 10 mg sample received 1 mL of 6 M NaOH as described above. The results of this digestion were then compared to the standard methanolic extraction of hair cortisol. Relative recovery was calculated by dividing the ELISA result from the NaOH digestion by the ELISA result from the standard method and multiplying by 100.

5.2.4 Reconstitution and Quantification of Cortisol

Dried residue from the above-mentioned evaporations was reconstituted with 250 µL PBS at pH 8.0 and vortexed for 10 seconds. Cortisol concentration was measured using a modified salivary cortisol ELISA (Alpco Diagnostics®, Windham, NH) as per the manufacturer's instructions. Samples were plated in duplicate and the average of the two was taken as the result. Any result outside of the standard curve was re-run and diluted if required. Hair cortisol was log transformed to achieve normality for statistical tests. Intra- and inter-assay CV was 7% and 9%.

5.3 Results

Log hair cortisol concentrations after evaporation with nitrogen gas versus air were 2.41 and 2.37 ng/g, respectively (p=0.0003, **Fig. 21)**

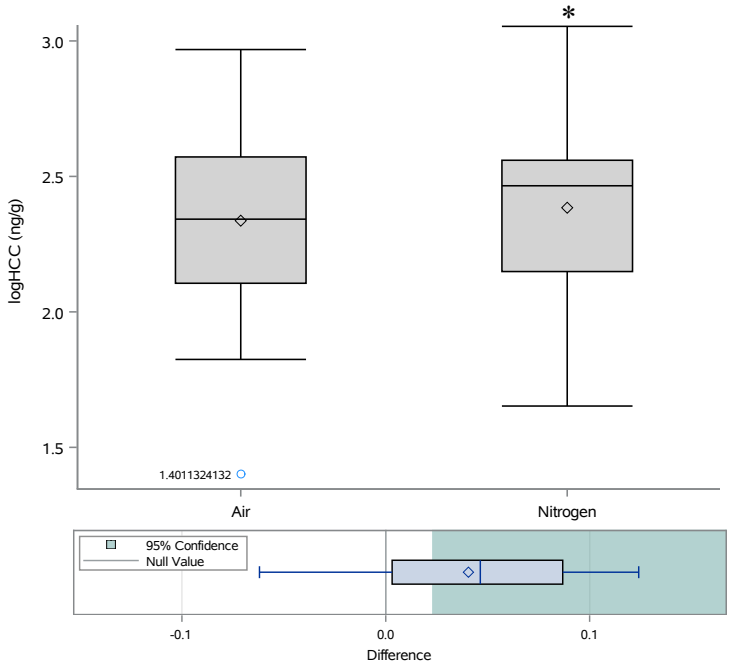

Figure 21: Comparison of distribution of log hair cortisol (ng/g) for Nitrogen vs Air evaporation with 95% upper CI for the difference in paired samples (N=28). Difference represents log(hair cortisol)nitrogen – log(hair cortisol)air; * p = 0.0003, upper one-tailed t-test.

Cortisol measurement was not possible from the proteinase K hair digests because DTT blocked ELISA readout. To confirm this, DTT was added to a hydrocortisone standard and measured via ELISA.

Standard curves were generated to assess the linearity of dilution and recovery of cortisol from standard dilutions of 1, 10, and 100 ng/mL (**Fig. 22).**

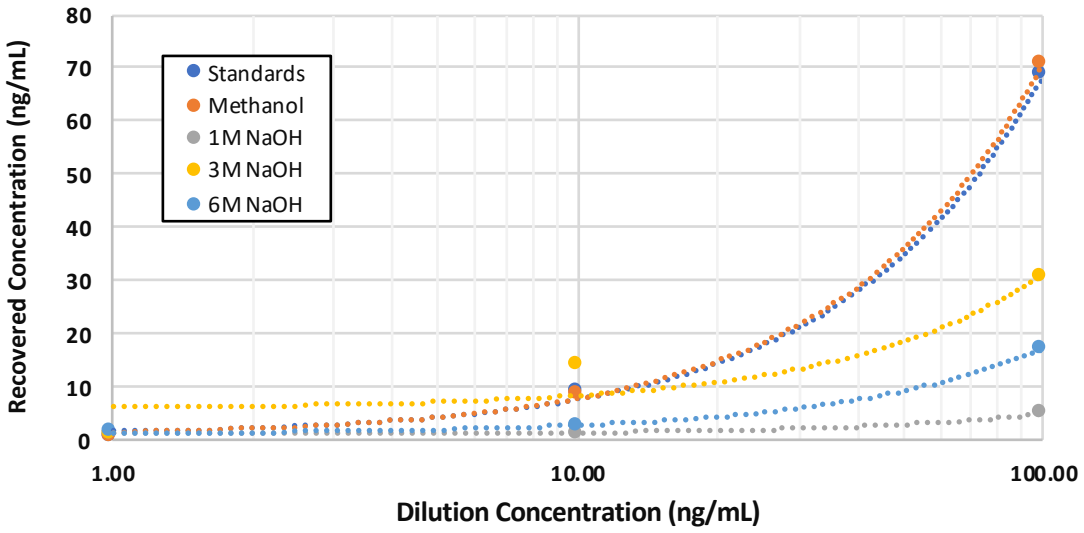

Figure 22: Standard curves generated from 1, 10, 100 ng/mL dilutions and corresponding recovery curves from methanol and NaOH extractions.

Percent recovery and relative percent recovery are presented in **Table 8.**

Standard Dilution (ng/mL)		Methanol	1M NaOH	3M NaOH	6M NaOH
% Recovery	1	123.5	211.6	149.6	257.7
	10	98.4	15.5	159.3	29.7
	100	103.4	7.4	44.7	25.4
% Relative Recovery	1		171.4	121.2	208.7
	10		15.8	162.0	30.2
	100		7.1	43.2	24.5

Table 8: Recovery of hydrocortisone standards from methanolic and NaOH extractions. Recovery represents recovered cortisol in ng/mL divided by standard dilutions, and relative percent recovery equals recovered cortisol from NaOH extraction divided by recovered cortisol from methanol extraction.

The mean ± SD log hair cortisol was lower in the NaOH digestion group (1.91 ± 0.28 ng/g) compared to the standard methanol extraction group (2.21 ± 0.29 ng/g, $p < 0.0001$). The average cortisol recovery from the NaOH group compared to the standard extraction was 63.3%.

5.4 Discussion

Nitrogen gas is commonly used in the evaporation of solvents because of its inert properties. Though nitrogen evaporation in cortisol analysis is not consistent across protocols. For example, in non-hair cortisol LC-MS protocols, air drying is sometimes employed without loss of signal. In the present study, nitrogen dried samples had higher cortisol measurements compared to those dried with air. Curiously, room air is composed of about 78% nitrogen yet there was still a difference between the two evaporation conditions. This is in contrast to a previous study which did not find a difference between the two evaporation conditions[25]. That study had a small sample size (12 pairs) while we ran more than double the samples. To remove any sources of variability, we conducted a single extraction on a sample of 20 mg and then equally separated the extract into two vials before evaporating under either nitrogen or room air. It is therefore suggested to continue using nitrogen for drying hair cortisol samples.

A prerequisite to assaying cortisol in a solid matrix is to free the analyte of interest out of the sample and into solution. This may be achieved by disintegrating the hair before extraction or extracting the compound directly from solid hair. The typical method of mincing or pulverizing the hair may lead to sample loss and incomplete extraction, is laborious and time consuming, and can the equipment required for grinding can be expensive. We explored the possibility of digesting hair with the use of proteinase K to digest the hair, and also using a basic milieu to break down the hair structure.

Proteinase K, a serine protease, rapidly digests hair with the addition of a thiol compound such as DTT which acts as a disulfide reduction agent. The addition of DTT increases enzyme activity 10-fold[29], leading to complete hair digestion within an hour[6]. However, even after centrifugation of the hair extracts, the presence of DTT prevented

ELISA readout. This was confirmed by adding DTT to a hydrocortisone sample and running an ELISA. Testing of alternative agents, further purification within the protocol, or the quantification of cortisol with LC/MS which does not rely on assay binding, could lead to positive results in the future.

In a pilot study, we tested three concentrations of sodium hydroxide in a cortisol spike-recovery assay compared to the standard methanol extraction method. The methanol extract had very similar ELISA results compared to the standard dilutions plated directly. Whereas the NaOH extracts had lower recoveries with the 3M NaOH having the highest recovery, followed by 6M and 1M. There are two possible causes for the signal loss: 1) The basic strong basic environment leads to cortisol breakdown, or 2) complete recovery requires additional volume of dichloromethane to allow for maximal cortisol dissolution from the extract. Unexpectedly, the 1M NaOH extraction resulted in the lowest recovery. This may be due to the longer 30-minute digestion required to dissolve the hair at this concentration compared the 10 minutes required for 3M or 6M NaOH.

Previous studies have shown a single methanolic extraction yields approximately 46% of absolute cortisol from hair[24]. This suggests a method for completely disintegrating hair could, in theory, release all of the cortisol in hair and result in a better method for hair cortisol quantification. We found an average recovery of 63.3% in hair samples digested by 6M NaOH compared to the standard methanol extraction method. Based on the spike-recovery assay, we would have expected between 24-30% recovery. This may suggest that the NaOH digestion released a larger fraction of total cortisol from the hair, and the decreased cortisol measured from the NaOH method may be a result of the basic environment. In order to validate and optimize this method, lower concentrations of NaOH should be used to reduce the potential for cortisol degradation.

The use of hair cortisol as a measure of retrospective cortisol exposure is currently limited by inter-laboratory variability, cost, and laborious extraction protocols. Development of automated digestion methods with the use of an enzyme or basic digestion has the potential to reduce variability as well as the length of time required for analysis. The current results suggest that nitrogen evaporation is required for optimal hair

cortisol measurement. The NaOH digestion and extraction method discussed may hold promise as an inexpensive and rapid hair cortisol method. Further studies need to be done to optimize this protocol before it can be wide-spread application for high-throughput hair cortisol analysis.

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Chapter 6

6 General Discussion

This thesis aimed to explore hair cortisol as a biomarker of cortisol exposure by addressing previous gaps in hair cortisol literature: 1) the failure of current hair growth rate assumptions to apply to individual patients, 2) a lack of available normative data in children and adolescents, and 3) inefficiencies in the standard methods used for hair cortisol extraction and recovery. This chapter presents an overview of the current findings in the context of the available hair cortisol literature. Additionally, relevant implications of these findings, and strengths and limitations of this research are examined. Finally, recommendations for future research are discussed.

6.1 Summary of Findings

The main contribution of this thesis to the existing literature is the incremental improvement to previous methods and assumptions for hair cortisol analysis, and the determination of normal changes in hair cortisol in a large sample of children and adolescents.

In chapter 3 it was hypothesized that hair cortisol timelines could be generated to accurately reflect CS and AI patient medical histories, and that the clinical timelines could be improved upon by generating timelines with different hair growth rates than the commonly assumed rate of 1 cm/month. This hypothesis was supported in two of the four patients examined. Prior rationale for using 1cm/month is based on the average hair growth rate in the literature. Using this number is baseless for individualized timelines because each patient's hair growth may substantially differ from 1 cm/month. The most accurate hair cortisol timeline for 50% (2/4) of the patients in our sample was generated using a slower growth rate of 0.75 cm/month, while the remaining two patients were sufficiently represented by a 1 cm/month growth rate.

In Chapter 4 it was hypothesized that hair cortisol reference values could be generated as they relate to normal adolescent development. Specifically, it was hypothesized that hair cortisol would correlate with age and puberty status, not differ between males and

females, and would increase with BMI. This study found that hair cortisol correlated positively with age and puberty status confirming the hypothesis relating to age and puberty. Hair cortisol did not differ between males and females in this study suggesting no sexual dimorphism in hair cortisol values. Hair cortisol increased with BMI, but this relationship did not remain when correcting for age-adjusted BMI.

In chapter 5, we explored the hypothesis that nitrogen gas evaporation would not lead to greater cortisol recovery from hair than room air evaporation. A second hypothesis was that enzymatic or basic digestion of hair would enhance cortisol recovery compared to the standard mincing method. Evaporation of methanolic hair cortisol extracts resulted in greater cortisol recovery than evaporation using room air resulting in rejection of the first hypothesis. This was surprising considering that air is comprised of approximately 78% nitrogen and a small published study found no difference between the two methods[1]. Enzymatic degradation of hair was a time-efficient method to reduce hair particle size, but cortisol quantification was not possible from these samples. This might be due to the ELISA binding inhibition from the DTT reagent used to enhance the digestion rate. Basic digestion also efficiently reduced hair particle size. Cortisol recovery was lower from the basic extracts compared to those from the standard methanolic extracts indicating that the second hypothesis should be rejected.

6.2 Implication of Findings

In the context of clinical applications, a review of applicable literature indicates increasing utilization of hair cortisol analysis. These publications have shown that retrospective timelines can be generated from patient hair samples that correlate with patient medical records in CS and AI, however, individualization of hair growth rates is absent in these studies[2,3]. Retrospective timelines can be viewed as an important adjunct to current cortisol diagnostics which can only be prospectively collected and represent short windows of analysis.

Individualized hair cortisol timelines have important implications for clinicians. In the context of cyclical CS, for example, a clinician might produce an individualized hair

timeline that best correlates to a patient's previously reported symptoms. If the symptoms align with the peaks or troughs in hair cortisol, then the clinician can be more confident in a potential diagnosis of cyclical CS. Otherwise, the clinician would have to collect many blood or urine samples to prospectively determine if there are variable cycles of steroidogenesis, leading to delayed diagnosis while the patient remains untreated, leading to worsening symptoms and comorbities. Naturally, a positive corollary between symptoms and hair cortisol would require a follow-up confirmatory test, as with all other available CS diagnostic tests. The flexibility of being able to use multiple hair growth rates might allow hair cortisol analysis to be implemented in a broader patient population since the 1 cm per month might not fit every patient.

There are scarce prior publications that have assessed hair cortisol in healthy, young, or ethnic populations. The major contribution of chapter 4 in this thesis, was to show that hair cortisol increases with age and puberty status in a large population of healthy young individuals. Within the context of young populations, prior hair cortisol studies have addressed hair cortisol changes with a focus on psychosocial issues[4]. The studies that have focused on healthy children and adolescents have been small and included a narrow age range when compared to the current thesis. Studies in healthy children using serum or salivary cortisol have resulted in mixed results, likely because of the variable sampling times, and the possibility that sampling itself might alter HPA secretion[5–7]. The results from our large cohort of healthy children might be used for future studies as a comparator, with the knowledge that findings of increased cortisol in hair might be due to maturation rather than some other factor being studied. Several groups have attempted to link hair cortisol trajectories with events such as natural disasters or humanitarian crises[8]. In this particular case, hair can be useful because of scarce availability of infrastructure. Knowledge that hair cortisol increases with age might be useful as a comparison in longitudinal studies where hair cortisol trajectories are found to have a negative slope, become flattened, or have a higher rate of increase than presented in the present discussion.

Lastly, the totality of hair cortisol research has relied on nitrogenous evaporation of solvent-based hair cortisol extracts. Agreement between labs utilizing hair cortisol has

been weak on an absolute basis, and studies using multiple extractions or alternative solvents have yielded different results[9]. Nitrogen has been used in the evaporation process because it is believed that oxygen in room air would result in oxidization and lower cortisol measurements. We showed that nitrogen did result in higher cortisol measurements than extracts that were dried with room air. These results indicate that nitrogen should be used to achieve the greatest recovery of hair cortisol.

In chapter 5, we conducted a small methodological pilot study with the goal of increasing the rate and completeness of hair digestion using proteinase K or basic sodium hydroxide solutions. Proteinase K rapidly digested hair samples but we were unable to measure cortisol in these samples. Various concentrations of sodium hydroxide resulted in rapid hair digestion and recovery of cortisol. Although highly variable, this might be a promising method for future experimentation. Optimization of this protocol would be a great contribution to the field of hair cortisol research. Digestion of hair would be highly beneficial because it would standardize the extraction procedure, reduce the required laboratory technician labor, and be more cost-efficient than other available methods.

Previously implemented methods for reducing hair sample particle size to improve extraction efficiency have included mincing with surgical scissors, ball mills or blenders. Mincing with scissors is laborious for the technician and is associated with inherent variability stemming from quality control, typically based on visual inspection of pieces hair. This method has most frequently been used, but it is time-consuming and inadequate for a standardized lab procedure. Ball mills have been associated with degradation of cortisol and carryover between samples[10]. In hair analysis of drugs of abuse and in toxicological analysis, hair size is commonly reduced via digestion with enzymes or acidic and basic solutions[11]. Application of these methods would significantly reduce the time required for analysis, allowing for high-throughput analysis, reduce variability from technician mincing, and dissociate all compounds from hair regardless of melanin content.

6.3 Strengths and Limitations

This research has a number of strengths and limitations that must be considered. Chapter 3 was a unique study because it is the first to assess hair cortisol in pregnant women with concomitant CS. Clinical timelines were highly detailed because of the access to in-depth medical histories from the treating physician, including many laboratory analyses to corroborate our findings. This study was the first of its kinds to try to implement a method that captures unique patient hair growth rates.

In spite of the strengths of this study, recognition of the limitations of this study is key for future applications. As with all retrospective studies, data is limited by the information that recorded patient medical charts. For the proposed adjunct diagnostic purposes, hair was seemingly sufficient to capture many of the reported symptomatic periods and medical interventions. Retrospective hair timelines assume that hair growth rate is stable, which is likely not the case because several factors influence growth rates. This study assessed hair samples that retrospectively represent many months, but we did not account for washout effects that might have significantly reduced cortisol values more distal to the patients' scalps. Additionally, there was large interpatient hair cortisol variability which we cannot account for. Finally, the nature of this study design represents a *post hoc* analysis based upon subjectively matching symptoms to hair cortisol measurements; we tried to objectify the selection of the correct timeline by utilizing an independent review committee.

Chapter 4 represents the largest study of hair cortisol ever conducted in a healthy sample of children and adolescents. This has typically been a group that has not been studied. We included a wide age range to capture pre-, peri- and post-puberty which facilitated the assessment of hair cortisol changes with puberty. Participants were heavily screened by reviewing electronic medical records in addition to in-person questionnaires to select for those without concomitant illnesses or medication use that might influence cortisol levels.

Participants were recruited from the emergency room, which typically selects for unwell children. The emergency population can be divided into chronically unwell patients, and

acutely sick patients; the latter was targeted for recruitment. Acutely sick patients consisted of patients with the common cold or influenza, for example, or musculoskeletal injuries. A large proportion of the participants that were enrolled had musculoskeletal injuries, possibly representing a selection bias for highly active children. It is known that strenuous exercise increases hair cortisol levels. Psychosocial factors such as family dynamics and socio-economic status reflect changes in children's cortisol levels, however these were not assessed. The target population was healthy children meaning that the number of participants with abnormal BMI was small, reducing the confidence in findings associated with BMI. Measuring other hormones in hair associated with pubertal changes, such as testosterone and DHEA-S, would have been informative to confirm puberty status. This was discussed prior to the completion of the trial; however, such analysis would have required a larger hair sample and not been feasible because of the concern from participants of potential bald spots from large samples. Finally, the majority of participants in this study were Caucasian so it is unclear if these results are applicable to children of other ethnicities.

Chapter 5 represents the early stages of a potentially large contribution to the field of hair cortisol research, for many reasons, as previously discussed. The comparison between nitrogenous and air evaporation included a larger number of samples than was analyzed in a previous study that compared the two methods. Dividing the methanol extracts into two parts from a single extraction likely decreased variability compared to the prior study that did two separate extractions for comparison. Both the enzymatic and basic digestions methods were highly effective in reducing hair particle size for extraction and would be expected to completely liberate trapped hair cortisol, compared to mincing, and regardless of melanin content.

This was a pilot study to assess the futility of utilizing hair digestion to recover cortisol. Unfortunately, it was not possible to measure the cortisol from the enzymatically digested samples using ELISA. Basic hair digests were highly variable for hair cortisol and were unexpectedly lower than methanolic extraction suggesting that the strong basic solution might have caused cortisol degradation.

One of the major limitations throughout this thesis was the use of ELISA to quantify hair cortisol. Prior studies have shown that the correlation between ELISA and LCMS is high, but the absolute values differ substantially between the two methods. Typically, values from ELISA are greater than LCMS, possibly due to a lack of specificity to cortisol.

6.4 Future Directions

Future studies aiming to utilize hair cortisol timelines in clinical patients should be prospectively conducted. For example, hair samples could be collected periodically while concomitantly recording patient symptoms from a patient diary. In such as study, there would be increased confidence that a segment of hair is representative of the symptomatic period of interest. Alternatively, hair growth rates could be determined for an individual patient prior to study initiation instead of generating multiple timelines.

Additional studies in children might aim to include a larger proportion of children from different ethnic backgrounds. Many biomarker levels vary with genetic background meaning that cortisol exposure might differ from largely Caucasian population studied here. Importantly, future studies might include stress or socioeconomic questionnaires because of the known effect of these types of stressors on children. As such, some of the variability seen in this study might be explained by background stressors.

The hair digestions methods presented in this thesis represent a proof-of-concept of the possibility of recovering cortisol from hair that has been digested. As this was a small pilot trial, future studies will have to optimize and validate these methods. Experimenting with different concentrations of sodium hydroxide, digestion times, or alternative digestion solvents might improve cortisol recovery. Future research might also apply more sophisticated quantification methods such as LCMS for the determination of absolute cortisol values as a way to reduce variability from non-specific binding associated with immunoassays.

6.5 Conclusion

This research was the first of its kind to address unique hair growth rates for the production of clinical hair cortisol timelines as a potential add-on diagnostic tool for clinicians. The major finding was that hair timelines generated from different growth rates best match individual patients. In the largest study of its kind, hair cortisol was found to increase with age and puberty and not differ between sexes in healthy children and adolescents. Finally, novel methods for improved hair cortisol extraction were investigated in an important proof-of-concept of a more rapid, reliable, and high-throughput method of hair cortisol measurement.

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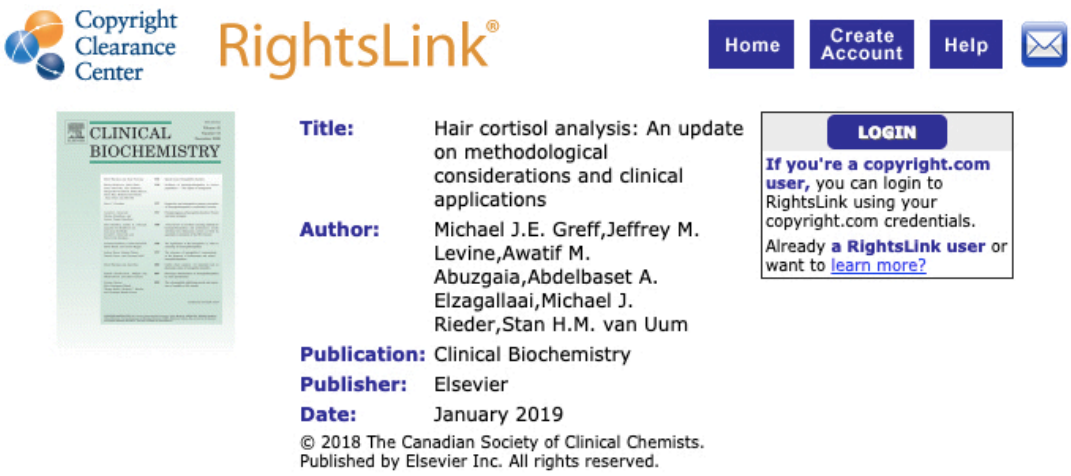
Appendices



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Appendix A: Copyright for Chapter 2

Western Research

Date: 6 April 2018

To: Dr. Stanislaus Van Uum

Project ID: 111330

Study Title: Measurement of Hair Cortisol for the Diagnosis of Cushing's Disease: A Retrospective Chart Review

Application Type: HSREB Initial Application

Review Type: Delegated

Meeting Date / Full Board Reporting Date: 17/Apr/2018

Date Approval Issued: 06/Apr/2018

REB Approval Expiry Date: 06/Apr/2019

Dear Dr. Stanislaus Van Uum

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
DATA COLLECTION FORM_restrospective chart Review_Version 2.0_13Jan2018	Other Data Collection Instruments	13/Jan/2018	2.0

Documents Acknowledged:

Document Name	Document Type
Cushing's Retrospective Chart Review References	References

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB , except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

[redacted] Ethics Officer (ext. 85990) on behalf of Dr. Joseph Gilbert, HSREB Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Appendix B: Western Research Ethics Board Approval for Retrospective Study

Western Research

Date: 20 March 2018

To: Dr. Michael Rieder

Project ID: 110823

Study Title: Reference Ranges of Hair Cortisol in Healthy Children and Adolescents

Application Type: HSREB Initial Application

Review Type: Full Board

Full Board Reporting Date: 20/Feb/2018 13:00

Date Approval Issued: 20/Mar/2018 09:31

REB Approval Expiry Date: 20/Mar/2019

Dear Dr. Michael Rieder

The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above mentioned study as described in the WREM application form, as of the HSREB Initial Approval Date noted above. This research study is to be conducted by the investigator noted above. All other required institutional approvals must also be obtained prior to the conduct of the study.

Documents Approved:

Document Name	Document Type	Document Date	Document Version
Assent Letter_Version 3.0 21Feb2018	Assent Form	21/Feb/2018	3.0
Data Collection Form_PaediatricRef_V2.0_06Jan2018	Other Data Collection Instruments	06/Jan/2018	2.0
Hair Sample Collection Form_PH Study_V 2.0 06Jan2018	Other Data Collection Instruments	06/Jan/2018	2.0
ICF_Version 3.0 21Feb2018	Written Consent/Assent	21/Feb/2018	3.0
Tanner Self Questionnaire_V 2.0_06Jan2018	Paper Survey	06/Jan/2018	2.0

No deviations from, or changes to, the protocol or WREM application should be initiated without prior written approval of an appropriate amendment from Western HSREB , except when necessary to eliminate immediate hazard(s) to study participants or when the change(s) involves only administrative or logistical aspects of the trial.

REB members involved in the research project do not participate in the review, discussion or decision.

The Western University HSREB operates in compliance with, and is constituted in accordance with, the requirements of the TriCouncil Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Sincerely,

[redacted] Ethics Officer on behalf of Dr. Marcelo Kremenchutzky, HSREB Vice-Chair

Note: This correspondence includes an electronic signature (validation and approval via an online system that is compliant with all regulations).

Appendix C: Western Research Ethics Board Approval for Paediatric Hair Cortisol Study

Reason	Frequency	Percent
Abdominal discomfort	26	10.40
Ankle injury	17	6.80
Head injury	15	6.00
Laceration	15	6.00
Wrist injury	15	6.00
Finger injury	13	5.20
Headache	10	4.00
Elbow injury	8	3.20
Wrist fracture	8	3.20
Arm injury	7	2.80
Shoulder injury	7	2.80
Clavicle fracture	6	2.40
Knee injury	6	2.40
Leg injury	6	2.40
Sore throat	6	2.40
Syncope	6	2.40
Chest pain	5	2.00
Cough	5	2.00
Hand injury	5	2.00
Urinary tract infection	5	2.00
Fever	4	1.60
Neck pain	4	1.60
Nose injury	4	1.60
Vomiting	4	1.60
Anaphylactic reaction	2	0.80
Constipation	2	0.80
Ear ache	2	0.80
Skin rash	2	0.80
Toe injury	2	0.80
Abscess on thigh	1	0.40
Allergic rash	1	0.40
Allergic reaction	1	0.40
Back injury	1	0.40
Back pain	1	0.40
Blisters	1	0.40
Bloodwork	1	0.40
Breast mass	1	0.40
Breathlessness	1	0.40
Common cold	1	0.40
Confusion	1	0.40
Ear injury	1	0.40
Ear piercing infection	1	0.40
Eye infection	1	0.40
Eye injury	1	0.40
Face injury	1	0.40
Feeling unwell	1	0.40
Fever/Vomiting	1	0.40
Foot injury	1	0.40
Frostbite	1	0.40
Hand fracture	1	0.40
Head Pressure	1	0.40
Headache/vomiting	1	0.40
Insect bite	1	0.40
Leg pain	1	0.40
Migraine	1	0.40
Numbness in face	1	0.40
Overdose	1	0.40
Rib pain	1	0.40
Skin infection	1	0.40
Thumb injury	1	0.40
Toothache	1	0.40
Unknown	1	0.40

Appendix D: Full Listing of Reasons for Hospital Visit

Curriculum Vitae

Name: Jeffrey Levine

Post-secondary Education and Degrees:

Western University
London, Ontario, Canada
M.Sc. (Candidate**)** Physiology & Pharmacology
2017 - present

Western University
London, Ontario, Canada
BMSc Hon
2012-2017

Honors and Awards:

Dean's Honor List
2012 - 2017

Western Scholarship of Excellence
2012

Related Work Experience

PHYS 2139, Human Physiology
Western University
2018-2019

PHYS 4120b, Stem Cell Physiology and Regenerative Medicine
Western University
2018

Publications:

M.J.E. Greff, J.M. Levine, A.M. Abuzgaia, A.A. Elzagallaai, et al., Hair cortisol analysis: An update on methodological considerations and clinical applications, Clin. Biochem. 63 (2019) 1–9.