Using a Biokinetic Model to Quantify and Optimize Cortisol Measurements for Acute and Chronic Environmental Stress Exposure in Maternal and Child Health

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Maternal stress during pregnancy may have significant health impacts on the developing fetus. To fully understand these impacts, it is necessary to quantify long-term and episodic stress during pregnancy. A wide range of approaches and protocols are available to assess maternal stress and stress biology in pregnancy, including stress-related biomarkers such as cortisol. Cortisol is released in pulses as part of the stress response, therefore instantaneous or short terms measurements in saliva, blood and urine can be unreliable measures of overall stress levels. In isolation of more dynamic measurements, cortisol in hair provides a relatively non-invasive, temporal and cumulative record of stress. Though there is a strong body of research relating psychological stress to elevated hair, blood, saliva and urine cortisol levels, there is little information regarding the relationship between the four compartments. Therefore, we used R (v. 2.14.2) to create a biokinetic model of cortisol levels using parameters obtained from the published literature that relates cortisol concentrations among these compartments. Additionally, we can show overall elevation or depression of cortisol and changes in pulsatile secretion. These values were used to create blood cortisol pulse profiles based on hair cortisol concentrations. This model will ultimately be adapted for pregnant women for potential use in national longitudinal cohort studies such as the National Children's Study to assess and quantify prenatal exposure to stress from maternal hair samples and stress questionnaires.

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Thesis Introduction

While the relationship between stress and disease susceptibility has been studied since at least the 12th century (Rosner 1981), there remains single no comprehensive and objective method for assessing stress across populations. Standard methods of stress assessment include perceived and environmental stress questionnaires and biological measurements of the stress hormone, cortisol, in blood, saliva, urine and hair. The complex biokinetics of cortisol release and distribution lead to a limited utility of a single sample. Here, we create a biokinetic model that relates measures of stress in pregnant women in order to estimate both acute and chronic stress levels. We then test the sensitivity of the model to disease relevant changes in stress levels using posttraumatic stress disorder (PTSD) and generalized anxiety disorder (GAD) as examples. Based on the predictions of our model for cortisol, we discuss the sensitivity and optimization of different stress biomarkers in terms of acute and chronic stress exposure assessment. In order to clearly understand the potential use and development of this model and the optimization of stress assessment techniques, it is first necessary to highlight environmental stress exposure and risks to vulnerable populations, importance of social stress in children's environmental health, the physiological response to stress, disorders of the stress system and current stress assessment techniques and methodologies. This work is part of a formative research project by the National Children's Study (NCS) and may be used to evaluate stress in non-pregnant women in Mexican farmworker communities as well as pregnant women at a national level.

Stress Exposure and Identifying Vulnerable Populations

Stress is one of the most ubiquitous environmental exposures. While generally perceived as having negative health consequences, the stress response is important in maintaining the complex dynamic equilibrium, termed homeostasis, in response to a changing environment(Chrousos 2009). Some stress can be beneficial, stimulating brain activity and providing added energy to complete challenging tasks.

Acute stress, such as a public speaking event or challenging exam causes a short term increase in circulating stress hormones that provide the body with additional energy through increasing the heart rate and increasing blood glucose concentrations (Romero et al. 2007). However, stress becomes toxic when it is chronic, severe or frequent (Cooney 2011). Chronic stress, such as poverty, family stress or job stress lasts weeks to years, while acute stress only lasts minutes to days. Long-term or frequent activation of the stress response can lead to failure to physiologically recover and attenuation or hyper-activation of the stress response (Cooney 2011). Chronic and acute stress lead to very different physiological responses, therefore it is important to consider potential differences in health outcomes. Furthermore, current stress assessment techniques rarely have the ability to measure both chronic and acute stress, leading to either the extrapolation of acute stress levels to more chronic situations or the exclusion of acute stress all together.

The biological response to stress also depends on the individual's perception of stress and physiological conditions of the body(McEwen 1998). The variability in individual perceptions and physical states lead to pronounced differences in responses to stressful stimuli. Lack of social support, financial problems, long work hours, family demands and relationship problems can all contribute to environmental stress exposure and increased stress perception(Cooney 2011; McEwen 1998). While every population is exposed to environmental stress, certain groups may have increased vulnerability due to increased exposure or greater health consequences following exposure. Two subpopulations of special interest to this project are Mexican migrant farmworkers and pregnant women. Mexican American migrant farmworkers are suspected to have a unique social stress exposure profile due to challenges not faced by the general population. Many migrant farmworkers are separated from family, experience a barrier in communication, have higher rates of discrimination and difficult working conditions (Snipes et al. 2007). Similarly, pregnant women are exposed to stressors unique to their life-stage. Many mothers-to-be have concerns about upcoming labor and deliver, parenting skills, changes in

family dynamics and anxiety of potential health problems of their developing baby (Yali and Lobel 1999). Addressing social stress in the context of vulnerable populations is important to understanding the impact of the more broadly termed environment on prenatal development and adverse health outcomes.

Pregnant women may have increased vulnerability to adverse fetal health outcomes following environmental stress exposure. Incidences of preeclampsia and gestational hypertension increase 2-3 times in women reporting anxiety and stress during the first trimester of pregnancy (Kurki et al. 2000; Lansabergis and Hatch 1996). Since preeclampsia is also associated with medically induced preterm deliveries, this can lead to a reduction in fetal growth and development(Goldenberg et al. 2008). Additionally, chronic maternal stress has been shown to increase the risk of delivering a low-birth-weight infant(Borders et al. 2007). Low-birth-weight and preterm infants have increased incidence of immediate and chronic health consequences, making potential risk factors an important subject of public health risks research. Immediate risks to low-birth-weight infants include respiratory, nervous system and immune problems(Hack et al. 1996). Although recent advances in neonatal medicine have increased preterm and low birth weight infant survival rates, there are still concerns over long-term health complications, including adult onset diabetes and hypertension(Barker 1995; Wilson-Costello 2007). Additionally, children of women exposed to extremely stressful events during pregnancy, such as the September, 11th terrorist attacks have altered stress hormone secretion patterns, suggesting an altered *in utero* programming of the stress response(Yehuda et al. 2005). Neurological development can also be affected by prenatal stress exposure. Mothers reporting higher levels of stress during pregnancy had toddlers with greater temperamental and behavioral problems than mothers reporting less stressful pregnancies (Guttelind et al. 2005). This effect remained evident when the children were seven years of age, as those exposed to greater maternal stress *in utero* were more likely to have impaired attention and concentration (Guttelind et al. 2006).

Social Stress in Children's Environmental Health

Recently, social stress has become a new facet to children's environmental health. In 2009 Rosalind Wright wrote an opinion piece highlighting the necessity for inclusion of maternal stress in children's environmental health assessments (Wright, 2009). She commented that both physical environmental toxicants and social environmental stressors perturb key physiological systems at sensitive times during development, such that lifelong effects can be observed. Examples of this include the lifelong effect of fetal alcohol syndrome on the nervous system and the correlation of prenatal stress exposure and lifelong risk for obesity. Further advocating for the inclusion of social stress in children's environmental health are studies documenting prenatal exposure to stress acting as an effect modifier between toxic exposures and adverse health outcomes. This special relationship is thought to exist because both social stress and environmental toxicants can perturb the same physiological systems during development, leading to a magnification of adverse outcomes (Wright 2009). For example, the relationship between air pollution and increased risk of childhood asthma is strengthened with increased prenatal exposure to maternal stress (Sternthall et al. 2011). Maternal stress also is associated with altered cord blood immunoglobulin profiles, suggesting that prenatal stress works within the causal pathway to affect childhood asthma risk. These factors are especially concerning when the overlap of exposure distribution profiles is considered, especially among low-income and minority women and children (Suglia et al. 2010; Wright 2009).

The Physiological Response to Stress:

Environmental stress triggers the release of cortisol through the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Because of this, cortisol is often used as a biomarker for environmental stress exposure in blood, saliva, urine and hair. However, the complex biokinetics and secretion patterns reduce the utility of single cortisol biological samples. In order to better understand the relationship

between biomarkers and their ultimate relationship to stress exposure, it is necessary to develop a detailed understanding cortisol biokinetics. Stress stimulates the paracellular neurons and periventricular nucleus of the hypothalamus to release the neuropeptides, corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) (Romero et al. 2007). These neuropeptides stimulate the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH then stimulates the release of cortisol from the adrenal glands (Romero et al. 2007). Cortisol facilitates the physiological response to stress that manifests through an increase in the heart rate, increase in blood pressure, increase in blood glucose and changes in transcription factors that lead to suppression of the reproductive system, growth and alteration of immune function (Romero et al. 2007). Within 10-20 minutes of stress stimulus, changes in transcription factors are evident. Furthermore, cortisol inhibits the release of ACTH, AVP and CRH through a negative feedback loop within 30-80 minutes of release. Since cortisol is not stored in the body, it mainly exists as a result of short releases in response to environmental stimuli and biological functions (Sapolsky et al. 2000). The short releases are dictated by the quick response of the adrenal glands to ATCH and the negative feedback loop that allows cortisol to inhibit its own synthesis (Keenan and Veldhuis 2003). Additionally, cortisol has a very short, less than an hour, half-life in the blood (Kraan et al. 1997; Perogamvros et al. 2011; Young et al. 2001). The secretion of cortisol follows a strong diurnal pattern; with the highest concentration occurring within 30 minutes of waking followed by a gradual decline of cortisol during the day, such that evening concentrations are usually reduced to approximately 50% of morning cortisol concentrations (Cunningham-Bussel et al. 2009). These multifaceted secretion patterns of cortisol contribute to the complexities associated with its use as a biomarker for stress.

Once released, about 90% of cortisol immediately binds to corticosteroid binding globulin (CBG) and albumin (Dorin et al. 2009). The remaining free cortisol is biologically active and can be transferred to saliva or incorporated in to hair. Bound cortisol, on the other hand, is biologically inactive and is not

transferred to saliva or hair. Cortisol binds to CBG with high affinity, however because CBG is present in the blood at concentrations around 650 nmol/l and total cortisol concentrations can spike to approximately 2000nmol/l, CBG is considered saturable (Dorin et al. 2009; Fernandez-Real et al. 2005). Albumin binds to cortisol with low affinity and is available at concentrations much greater than total cortisol concentrations; therefore it is considered to be non-saturable. At low total cortisol concentrations the majority of cortisol in the blood is bound to CBG, while at higher concentrations a greater amount of cortisol will be free, since CBG is saturated and albumin has a low affinity for cortisol (Dorin et al. 2009). CBG and albumin play a key role in free cortisol concentration regulation and also serve to transport cortisol throughout the body (Gagliardi et al. 2010).

When cortisol is not bound to CBG or albumin it is able to enter cells and bind to the glucocorticoid and mineralocorticoid receptors. The binding influences transcription factors and leads to an increase in blood glucose, alterations in behavior and inhibition of growth and reproduction among other responses (Romero et al. 2007). Many of these responses are governed by the reversible and high affinity (kd=17.5 nmol/l) characteristics of the binding between cortisol and the glucocorticoid receptor (GR) (Mulataro et al. 1997). Cortisol is highly pleotropic and recent estimates have suggested that it affects up to 20% of all expressed human genes and can moderate all homeostatic mechanisms in the body (Chrousos 2009; Chrousos and Kino 2005, 2007). The 11-β hydroxysteroid dehydrogenase (11-βHSD) enzymes in the liver and fat cells convert cortisol to and from the inactive 11-keto form, cortisone (Morineau et al. 1997; Stewart et al. 1995). There are two types of 11-βHSD enzymes. 11-βHSD2 enzymes convert cortisol to cortisone and 11-βHSD1 convert cortisone to cortisol (Stewart et al. 1995), but at a much slower rate. Cortisol and cortisone are also metabolized by irreversible conjugation by the A-ring reductases. In women, A-ring reductases show significant fluctuation in activity based on menstrual cycle phase (Finken et al. 1999). Lastly, about 80% of cortisol and its metabolites are excreted in urine and the remaining 20% are excreted in feces (Kraan et al. 1997).

During pregnancy there are important changes in the HPA axis and cortisol kinetics by trimester. CBG increases drastically during pregnancy, with the third trimester acheiving concentrations almost three times as high as pre-pregnancy levels (Demey-Ponsart et al. 1982). Cortisol self regulates the free blood concentration through a negative feedback loop on cortisol synthesis (Keenan and Veldhuis 2003). Therefore, the increase in CBG caused by pregnancy leads to an increase in total cortisol, but it does not significantly alter the free cortisol concentrations, until the third trimester. The pregnancy induced change in CBG is complex because progesterone also binds to CBG with weak affinity (Demey-Ponsart et al. 1982) and is significantly increased during pregnancy. Therefore, not all the CBG produced is available to bind cortisol. Demey-Ponsart, 1981 provide a detailed quantitative model for assessing CBG availability for cortisol binding during pregnancy. This increase in cortisol may be caused by placental secretion of corticotropin releasing hormone (CRH) that increases exponentially from the 8^{th} week of pregnancy until delivery (Leung, 2001). While the placenta provides a partial barrier to cortisol through its high concentrations of 11-βHSD2, approximately 10-20% of maternal cortisol still reaches the fetus (Beitins et al. 1973; Benediktsson et al. 1997). Because fetal concentrations of cortisol are much lower than maternal concentrations, even a contribution of 10-20% from the mother is still capable of doubling fetal exposure to cortisol (Gitau et al. 1998). At high peaks in cortisol, the 11-βHSD2 enzymes in the placenta may be overwhelmed, allowing more maternal cortisol to pass the placenta. The strong impact of maternal cortisol on fetal concentrations, demonstrates the importance of understanding maternal cortisol kinetics and pulsatile secretion patterns when using cortisol as a biomarker for maternal stress.

Other considerations of the physiology of the stress response include the potential for genetic variation in the HPA axis, cortisol secretion, regulation, binding and excretion. The most significant impacts to cortisol biokinetics resulting from genetic mutations include reduced or elevated 11-β-HSD activity, changes in glucocorticoid receptor affinity and changes in the ultra-short cortisol negative

feedback loop, not yet discussed. Significant variability in the efficiency of the 11-βHSD enzymes exists across individuals, resulting from genetic polymorphisms. Recently, in mice, increased 11-βHSD1 has been associated with obesity and metabolic syndrome development (Masuzaki et al. 2001). In humans, decreased 11-βHSD2 is associated with sodium retention and eventual hypertension (New 1999). Both of these phenotypes increase the amount of cortisol in the blood by either reducing it metabolism to cortisone or increasing the rate that cortisone is converted back to cortisol. Differences in binding affinity to the GR regulate the efficacy of cortisol in facilitating a stress response. A polymorphism in the GR gene that confers relative cortisol resistance is associated with a healthier metabolic profile, lower insulin resistance and cholesterol (van Rossum and Lamberts 2004). Furthermore, once bound to the GR cortisol is subject to the ultra-short negative feedback loop regulated by the *FKBP5* gene. When cortisol binds to the GR, FKBP5 mRNA and protein are produced. FKBP5 is a negative regulator of cortisol (Grad and Picard 2007). Several SNPs in the *FKBP5* gene are associated with differential induction of FKBP5 mRNA by GR activation and therefore causes changes in protein expression (Binder et al. 2008). Binder et al 2008 investigated the impact of four polymorphisms within the *FKBP5* gene on risk of PTSD in a highly traumatized urban cohort. They found that two of the genotypes were associates with increased PTSD risk. These genotypes are also associated with heightened cortisol suppression following dexamethasone (a synthetic glucocorticoid) administration, suggesting that these polymorphisms lead to a strengthened ultra-short negative feedback loop (Binder et al. 2008).

Cortisol in Disease States:

Changes in cortisol secretion and regulation are important in a disease context. Prolonged activation of the stress system is associated with sleep disorders, metabolic disorders, changes in growth and reproduction, immune system suppression and gastrointestinal function (Chrousos 2009). Cortisol secretion stimulates arousal as is evident in the morning cortisol peak observed in healthy individuals

(Cunningham-Bussel et al. 2009). In individuals with chronic fatigue syndrome, or under significant stress the diurnal rhythm is flattened (Adam and Gunna 2000; Jerjes et al. 2006). High cortisol concentrations have also been linked to the development of metabolic syndrome through stimulating insulin resistance, visceral obesity and increasing the circulating levels of low density lipoprotein cholesterols (LDL) and decreasing the circulating concentrations of high density lipoprotein cholesterols (HLD) (van Rossum and Lamberts 2004). In addition, hypertension, obesity and diabetes are all associated with increased cortisol levels (Hammer and Stewart 2006). Since cortisol suppresses the immune system, chronic stress increases susceptibility to disease and conditions of hypocortisolism can lead to an over-active immune system (Chrousos and Kino 2007). Prenatal stress exposure has also been linked to asthmatic events in children (Sternthall et al. 2011). Additionally, cortisol effects behavior and mood (Ellenbogen et al. 2002) . This relationship is particularly interesting considering psychological disorders of the response to stress such as posttraumatic stress disorder (PTSD) and generalized anxiety disorder (GAD). PTSD and GAD will be the focus of the relationship between cortisol kinetics and disease in this analysis.

Posttraumatic Stress Disorder (PTSD) is an anxiety disorder that results from exposure to a traumatic event. Symptoms can be broken into three main categories, re-experiencing, avoidance and numbing coupled with hyper-arousal. Re-experiencing can occur as flashbacks, where the patient believes the event is happening again, repeated upsetting memories of the event, nightmares and adverse reactions to neutral situations that remind him or her of the traumatic event (Vorvick and Merrill 2011). More subtle effects such as avoidance can lead the patient to feel numb and detached from the world around them. The last category, arousal, is generally manifested by difficulty concentrating, increased startle response, hyper-vigilance and disturbances in sleep (Vorvick and Merrill 2011). Many of these symptoms can be severely debilitating and effect the employability and daily functioning of PTSD patients. PTSD also has a high correlation to alcohol abuse and depression and is not always curable, even with treatment. Treatment generally involves support groups or desensitization

therapy, in which patients recall memories and discuss the attached emotions with a healthcare professional (Vorvick and Merrill 2011). Early diagnosis, prompt treatment and a strong social support network can improve the prognosis. Current research is investigating potential cortisol deregulation that may be associated with PTSD etiology.

Since many of the symptoms of PTSD are related to the stress system, cortisol disruption in PTSD has been studied extensively. In individuals with PTSD, hair cortisol levels were found to be much higher than in traumatized controls (Steudte et al. 2011a). This is contrary to published research on PTSD and cortisol levels reporting a reduction in blood and salivary cortisol levels following oral dexamethasone administration (Klaassens et al. 2012). Dexamethasone is a synthetic glucocorticoid that inhibits pulsatile secretion of cortisol. Therefore, blood concentrations measured following dexamethasone administration reflect basal blood cortisol concentrations. Additionally, it is suggested that the diurnal rhythm of cortisol secretion is flattened (Yehuda et al. 2005) and that 24- hour plasma concentrations and urinary excretion levels of cortisol are lower in individuals with PTSD (Yehuda et al. 1990). These findings are inconsistent with Steudte and colleague's recent finding of increased hair cortisol in PTSD individuals. Since the PTSD subjects used in Steudte's recent hair study were still living in a traumatic environment, they hypothesize that recent and current trauma exposures may cause increases in cortisol secretion, while past exposures could lead to a decrease in cortisol. While there is some evidence supporting this argument (Inslicht et al. 2006), it is also possible that this discrepancy is reflective of differences in measurement techniques. Since hair cortisol reflects both pulsatile and basal secretion of cortisol as a result of day-to-day life stressors, it is less affected by the assessment protocol than blood and urinary measurements.

The second stress disorder that will be a focal point of this paper is generalized anxiety disorder (GAD). GAD is characterized by constant worry and anxiety over a variety of activities and events. While

the precise etiology of GAD is unknown, it is believed that genetic factors and stress exposure may play a role. GAD symptoms can vary from mild fatigue and increased worry to debilitating inability to concentrate, restlessness and severe sleep problems (NIH 2009) . Because many of these symptoms are related to stress or the stress response, there has been considerable investigation into the potential for HPA axis dysfunction in GAD.

Steudte and colleagues also measured hair cortisol levels in individuals with generalized anxiety disorder (GAD) (Steudte et al. 2011b). They found that hair cortisol levels were much lower in these individuals than in healthy controls. This is contrary to alternate research demonstrating either no effect (Hoen-Saric 1991; Pomara et al. 2005; van Veen et al. 2008) or a significant increase in salivary cortisol levels in patients with GAD (Mantella et al. 2008). Adding further inconsistency, Phillips 2011 found significantly lower cortisol levels when GAD was comorbid with major depressive disorder, but no effect of GAD alone(Phillips et al. 2011). Steudte addresses these inconsistencies by pointing out the differences in sampling techniques suggesting that since blood and saliva samples only capture instantaneous cortisol concentrations, they may not reflect long-term perturbations in the HPA axis. In this paper, GAD and PTSD are used to show the sensitivity of the biokinetic cortisol model to disruptions in HPA axis functioning as well as to test the ability of single blood and saliva samples to reflect overall changes in HPA axis. Quantifying stress exposure through cortisol can be difficult, since blood and saliva samples are heavily influenced by sampling protocol and urinary cortisol analysis require 24-hour collection of urine. Hair cortisol can reflect long-term changes in cortisol, but may not have the sensitivity to address slight changes in blood cortisol concentrations that may be biologically important. Additionally, since each biomarker reflects a slightly different time-period, assessing long-term overall and acute stress in a single measurement is not possible.

Methods for Assessing Prenatal Stress Exposure:

Maternal stress is generally assessed by questionnaires, surveys or biomarker analyses. Stress questionnaires provide the unique opportunity to rate stress and address stress etiology and are generally low cost, relatively quick and do not place a high burden on the study participant. Two of the most widely used stress questionnaires are Cohen's perceived stress scale (PSS) (Cohen et al. 1983) and the General Well-being Score (GWBS) (Fazio 1977) . While the PSS questionnaire does not attempt to separate environmental and perceived stress, its limited is to 10 questions, easy to administer and has been used across a wide variety of populations. Similarly, the GWBS is relatively short, with 18 questions and has been used at a national level to assess anxiety, depression, positivity and general health. However, neither addresses specific stressors that may be culturally or life stage relevant (Magaña and J.D. 2003).

Previously, Mexican migrant farmworkers and pregnant women were identified as populations that may be particularly susceptible to stress exposure. To assess the specific concerns and stressors of Mexican migrant farmworkers, Snipes and colleagues (2007) developed an environmental stress questionnaire designed to capture the cultural and situational stressors that may be unique to this population. They used community-based focus groups to select items that are subjectively considered a major source of stress. Work stress, personal illness, lack of work, family illness, family stress, lack of money, family in Mexico, desperation, injustice at work, inability to speak English and loss of values in the community were stressors that were repeatedly discussed in the focus groups and were therefore included in the stress questionnaire. While most of these items are common stressors across more general populations, addressing potential language barriers, discrimination, and job specific stressors were important factors in the reliability of the stress questionnaire in the Mexican American farmworker community.

Another population identified to be especially vulnerable to environmental stress is pregnant women. General stress questionnaires, such as the PSS, do not include questions specific to pregnancy or the unique anxieties and stressors that can accompany it. Yali and Lobel (1999) developed the "Prenatal Distress Questionnaire" designed to assess stress caused by pregnancy, upcoming labor, delivery and life changes associated with having a new baby. The questionnaire asks twelve questions about worries over weight gain during pregnancy, physical symptoms, such as nausea and backaches, overall body changes and anxiety over labor and deliver. It also addresses anxiety and stress over handling the new baby, the possibility of having an unhealthy baby and the potential ability for the new baby to disrupt the mother's relationship with the baby's father. Other examples of stress questionnaires include the Social Problems Questionnaire (Corney and Clare 1985), Childhood Trauma Questionnaire (Bernstein et al. 1994), Sarason's Life Experiences Survey (Sarason et al. 1978) and the Stressful Life Events Schedule (Williamson et al. 2003). The variety of stress questionnaires available for both general and specific populations allows researchers to cater the length and specificity of the questionnaire to the goals of their study.

While questionnaires have the ability to determine unique or situational causes of stress, they are not objective measures of stress assessment. Some stress questionnaires focus on perceived or environmental stress, but because it is a self-assessment, it is impossible to completely separate the two. Additionally, it is hypothesized that even the more generalized stress questionnaires, such as the GWBS or the PSS might miss stressors that are important in specific populations, such as migrant farmworkers and pregnant women. If general stress questionnaires do not capture exposure to unique environmental stressors across populations, then their use may be significantly reduced. This is relevant to national longitudinal cohort studies, such as the National Children's Study. Since acute and chronic stress do not elicit the same biological effects, health risks associated with each exposure may be different. Therefore, the ideal stress assessment method addresses both types of stress. Most

questionnaires are designed to capture stress in the past month, providing more of a chronic stress assessment and potentially missing acute stress peaks.

One method for assessing stress that addresses some of the limitations of questionnaires is cortisol biomarker analysis. Cortisol is often used as a prenatal stress biomarker since it is applicable to all populations and hypothesized to play a mechanistic role in some of the adverse fetal outcomes associated with prenatal stress (Field and Diego 2008). However, the complex secretion patterns and biokinetics of cortisol can make interpretation of results from a single blood, saliva or urine biological sample difficult. Changes in blood and saliva cortisol concentrations represent instantaneous stress measurements, while urine and hair reflect cumulative exposures over recent hours and months, respectively. Since acute and chronic stress have different biological effects, understanding the blood cortisol profile provides the best assessment of acute stress. The frequency of acute cortisol pulses in blood is difficult to assess, even through direct measurement. Studies measuring blood cortisol in human subjects every 10 minutes for 24 hours still rely on modeling programs to identify pulses and estimate pulse height, as 10 minute intervals are too wide to capture the shape, amplitude and frequency of the pulses (Keenan and Veldhuis 2003; Keenan 2004). In laboratory settings, environmental stress exposure can lead to a 30-50% increase in total plasma cortisol (Kudielka et al. 2004). While this increase demonstrates the sensitivity of cortisol to stress exposure, it also highlights the susceptibility of blood cortisol samples to the influences of sampling technique and instantaneous stress conditions. The complex characteristics of cortisol release and dominating influence on instantaneous cortisol concentrations decreases the utility of a single blood cortisol sample for assessment in terms daily or chronic stress levels.

Saliva measurements have become a popular proxy for blood cortisol levels. Recent studies have shown peaks in saliva cortisol following acute stress exposure (Kudielka et al. 2004), with an

undetectable lag between blood and saliva peaks (Perogamvros et al. 2011) . Since saliva samples are less invasive than blood samples and less likely to elicit a stress response, they may provide a better method for assessing acute stress. Morning blood cortisol levels peak about 30 minutes after waking (Kunz-Ebrecht et al. 2004) and then slowly declines throughout the day, decreasing by about 50% by bedtime. Since saliva samples can be collected at home, it is possible to capture the cortisol awakening response immediately, without participants needing to stay in a clinic overnight. Though salivary samples have many advantages over blood, a maternal saliva cortisol sample does not reflect the concentration of cortisol available for fetal exposure. Furthermore, multiple saliva samples are needed to provide a daily average stress level and even more samples would be needed to assess chronic stress. While urinary cortisol measurements do not provide instant measures of acute stress or long-term stress averages, they do capture 3-4 hour averages and 24-hour collection protocols can be used to assess daily average stress levels. However, 24-hour urine collection places a relatively high burden on the study subject and has significant potential for noncompliance. Additionally, in order to characterize chronic stress throughout pregnancy, multiple 24-hour urine collections would be needed.

Recently, hair cortisol levels have been used to show chronic stress during long-term unemployment (Dettenborn et al. 2010) and maternal stress (Kirschbaum et al. 2009) as well as long-term changes in cortisol secretion in individuals with posttraumatic stress disorder (PTSD) and generalized anxiety disorder (GAD) (Steudte et al. 2011a; Steudte et al. 2011b). The most likely mechanism through which cortisol is incorporated into hair is by passive diffusion from the blood into the hair follicle (Gow et al. 2010) . Therefore, as hair grows a temporal record of blood cortisol concentrations is created, making hair samples a relatively noninvasive, quick method of assessing cortisol levels for the past nine months, possibly longer (Kirschbaum et al. 2009; Manenschijn et al. 2011). Cortisol levels in hair are not affected by sampling protocol, subjects are not required to adhere to time-specific collection, and a single sample can provide months of cortisol concentration

information. Hair cortisol is the only biomarker that allows for the retrospective assessment of stress levels. Therefore, maternal hair cortisol samples can be used to assess fetal exposure to environmental stress by trimester of development (Kirschbaum et al. 2009) . Since hair cortisol is a relatively new method for assessing chronic stress, uncertainty over washout rates and the effects of cortisol on hair growth rate can cause minor difficulties in data interpretation. While Kirschbaum (2009) and Manenschijn (2009) found no effect of hair dye on cortisol concentration, Suave, 2007 found a significant reduction cortisol hair that had been dyed post collection. However, all studies agreed that further research on the effect of hair dye on cortisol washout rates is needed. Manenschijn collected 18 cm of hair, about 18 months of growth and analyzed for cortisol. No reduction in hair cortisol was found between the 6 consecutive 3 cm segments of hair. These results are consistent with Stalder, 2011 demonstrating that cortisol in hair had high intra-individual stability in the absence of a life changing event(Stalder et al. 2011), making it an ideal tool for assessing long-term and retrospective stressors. Despite these benefits, hair cortisol concentrations do not reflect episodic stress peaks observed in blood.

Thesis Goals:

Since no single method for stress measurement provides a comprehensive tool to assess both the chronic and acute peaks in blood cortisol concentrations that may be critical for prenatal stress assessment, we have developed a biokinetic model for cortisol that relates blood, saliva, urine and hair cortisol concentrations. Using this model, we can relate long-term average cortisol concentrations in hair to pulsatile blood cortisol profiles showing both acute peaks and diurnal variation. This is important in terms of prenatal stress assessment, since blood cortisol concentrations are more closely related to actual fetal exposure than hair concentrations. The main goal of this model is to estimate blood cortisol concentrations over a long period of time from a single hair sample as well as characterize the transfer

of cortisol to saliva and excretion in urine so that all common biomarkers for cortisol assessment can be related.

The second goal of this model is to determine the sensitivity of both the model and various sampling techniques to acute and long-term changes in stress. To do this, published hair cortisol concentrations from individuals with PTSD and GAD were used to create daily blood profiles. Since both hair studies found results contrary to years of blood studies, we tested whether a single blood sample taken from our modeled blood profiles for PSTD and GAD were statistically different from blood profiles modeled after healthy controls. Because a single blood sample is influenced by sampling protocol, can be perturbed by time of day and fails to capture the extreme variation in cortisol secretion through the day, it is possible that a single blood sample will not reflect the differences found in long-term hair records. If this is the case, then hair cortisol would be sensitive to disease-relevant changes in stress response than single blood draws. Though modeling cannot replace measured data, this model serves to assess the sensitivity of various sampling methods and provide a connection between biomarkers of chronic and acute stress.

Chapter One: Using a Biokinetic Model to Quantify and Optimize Cortisol Measurements for Acute and Chronic Environmental Stress Exposure During Pregnancy

Abstract:

BACKGROUND: Environmental stress exposure during pregnancy may have significant health impacts on the developing fetus. To fully understand these impacts, it is necessary to quantify long-term and episodic environmental stress exposure during pregnancy. Cortisol measurements from blood, saliva, urine and hair are commonly used to assess environmental stress exposure. Maternal hair samples provide the unique opportunity to assess environmental stress exposure throughout gestation by collection of a single sample. Though there is a strong body of research relating psychological stress to elevated hair cortisol levels, there is little information regarding the relationship between blood, saliva, urine and hair concentrations.

OBJECTIVES: In this work, we aimed to develop a tool to estimate the episodic peaks of cortisol in blood from hair concentrations. Since hair is a novel biomarker for cortisol, we also aimed to determine the relationship between hair, blood, saliva and urine cortisol concentrations through the development of a biokinetic model.

METHODS: We developed a biokinetic model of cortisol as it moves between blood, saliva, urine and hair. Blood concentrations reflect episodic pulsatile secretions and baseline diurnal variation. Hair concentrations were used to predict blood and saliva concentrations over days and months.

RESULTS: Simulations showed realistic values in all compartments. When hair values were related to pulsatile blood concentrations, we showed that the significant variability in blood leads to a weak relationship between long-term and episodic measurements of stress. Validation of this model was shown through the comparison of our results with published literature.

CONCLUSIONS: This is the first biokinetic cortisol model that incorporates hair. As such, it allows hair concentrations to be used to quantify prenatal environmental stress exposure by trimester of pregnancy. Fetal cortisol exposure is more closely related to the concentration in maternal blood rather than the hair. This model allows for the estimation of maternal blood cortisol concentrations from a hair sample.

Introduction:

Recent exploration into the association between prenatal stress exposure and vulnerability to environmental contamination has brought a new focal point to prenatal environmental health risk assessment (Wright 2009). Since social stress and physical environmental hazards often perturb similar biological systems, the potential for synergism and enhancement of health effects is a growing concern in cumulative risk assessment (Sternthall et al. 2011; Wright 2007). This is especially important considering many exposure distribution profiles for toxicant and stress exposure overlap (Wright 2009). Prenatal exposure to maternal stress is associated with both immediate and long-term health problems. Incidences of preeclampsia and gestational hypertension increase 2-3 times in women reporting anxiety and stress during the first trimester of pregnancy (Kurki et al. 2000; Lansabergis and Hatch 1996). Additionally, chronic maternal stress has been shown to increase the risk of delivering a low-birth-weight infant (Luo 2010). Long-term consequences of low-birth weight include adult onset diabetes and hypertension (Barker 1995; Wilson-Costello 2007). Recently, childhood asthma risk has been associated with both prenatal stress exposure and environmental air pollution (Sternthall 2011), highlighting the importance of maternal stress assessment in children's environmental health research.

In order to incorporate maternal stress assessment in children's environmental health research, it is necessary to develop an assessment technique that is able to address both chronic and episodic peaks in stress throughout pregnancy. Environmental stress exposure stimulates the hypothalamus-

pituitary-adrenal (HPA) axis to release pulses of the hormone cortisol into the bloodstream, providing a biomarker for recent stress exposure. Since questionnaires and surveys must be culturally relevant (Magaña and J.D. 2003; Snipes et al. 2007), focus has turned toward advances in cortisol as a maternal stress biomarker based on its universally applicability and its hypothesized mechanistic role in some of the adverse fetal outcomes associated with maternal stress (Field and Diego 2008). Cortisol is usually measured in blood, urine, saliva and most recently, hair.

Since cortisol is released in a pulsatile manner to the blood, a single blood or saliva sample only reflects instantaneous physiological conditions. It does not reflect stressors in recent days or even hours and has significant intra-individual variability (Hellhammer et al. 2007; Hruschka et al. 2005). Urinary free cortisol concentrations can reflect cortisol levels in recent hours, but also lack the ability to provide a long-term or retrospective look at cortisol secretion patterns. Hair cortisol, in isolation of other methods, can reflect stress exposure for recent months. The difference between the biological response to acute episodic peaks in cortisol and chronic stressors leads to concerns over potential changes in risk, especially for pregnant women, as well as methodological complexities (Hellhammer et al. 2007; McMaster et al. 2011). Improving the characterization of cortisol biokinetics and the relationship between cortisol concentrations in blood, saliva, urine and hair may help reduce these complexities and increase the utility of biomarker samples.

Of particular interest is the relationship between hair and blood cortisol levels. Hair cortisol levels are a novel method of assessing chronic exposure to environmental stress. The most likely mechanism through which cortisol is incorporated into hair is by passive diffusion from the blood into the hair follicle (Gow et al. 2010). Therefore, as hair grows, a temporal record of blood cortisol concentrations is created, making hair samples a relatively noninvasive, quick method of assessing cortisol levels for the past nine months, possibly longer (Kirschbaum et al. 2009; Manenschijn et al.

2011). This allows maternal hair cortisol samples to be used to assess fetal exposure to environmental stress by trimester of development (Kirschbaum et al. 2009). Despite these benefits, hair cortisol concentrations do not reflect episodic stress peaks observed in blood concentrations. Here, we create a model of cortisol biokinetics with the ability to estimate pulsatile concentrations in blood from the baseline concentration determined by hair cortisol analysis. Additionally, the transfer of cortisol between blood, hair, saliva and urine is modeled, increasing the utility of these samples. Determining the blood concentration of cortisol is particularly relevant to assessment of maternal stress exposure because the fetus is exposed to the concentration in the blood. This model relates hair samples to blood cortisol profiles modeled with episodic peaks and diurnal variation.

Methods:

Model Development

We obtained parameters to calculate the rate constants for cortisol transfer between compartments from the published literature. Free cortisol was introduced to the system through a probabilistic function and partial derivatives expressed the transfer between compartments mathematically. We ran the model and generated the graphics using R version 2.14.2. The steps to model development and the biokinetic framework are shown in figure 1.

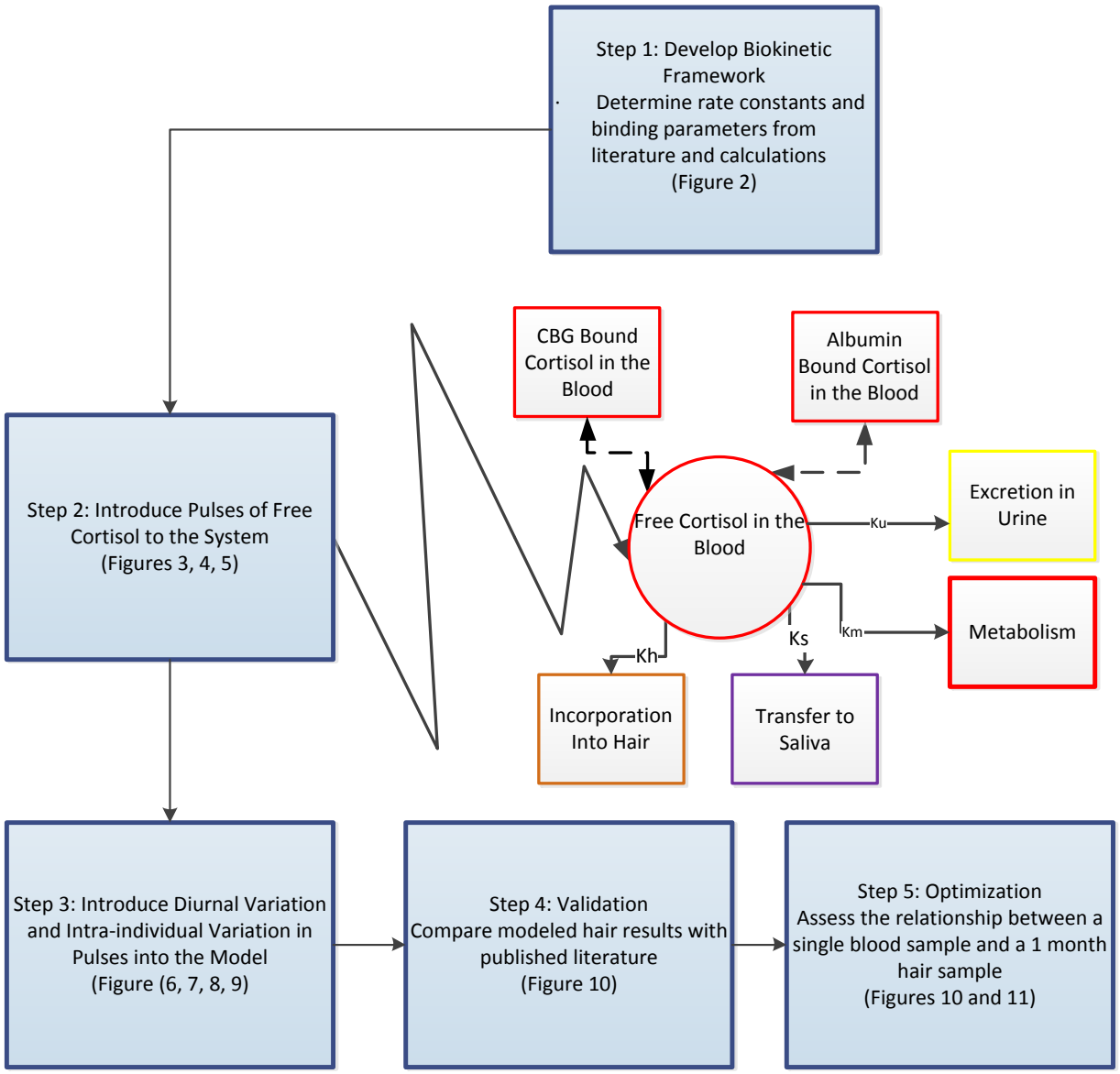

Figure 1*: The steps for model development, validation and optimization are shown along with the biokinetic framework for the model. Step 1, development of the biokinetic framework, is shown by the flowchart in the center with free cortisol in the blood moving into hair, urine and saliva as well as being metabolized and binding with CBG and albumin. Rate constants are represented by the arrows connecting the compartments. Ku is the rate constant for cortisol excretion in urine, Km is the rate constant for cortisol metabolism, Ks is the rate constant for cortisol transfer to saliva and Kh is the rate constant for cortisol incorporation into hair. The biological compartment is represented by the color outlining the box; red is blood, yellow is urine, purple is saliva and brown is hair. Step 2, involves introducing pulses of free cortisol into the framework developed in step 1. The pulses are represented by the spikes in the arrow linking step 2 to the free blood compartment. Step 3, introduction of variation, was the last step in model development. Steps 4 and 5 involved the validation and optimization of the model. Figures found in the results section of each step are referenced in this diagram.*

Step 1: Development of Biokinetic Framework

Binding: Once released into the blood stream, cortisol quickly binds to corticosteroid binding globulin (CBG) and albumin (Stroupe et al. 1978). While both free and bound cortisol contribute to total cortisol concentration in the blood, only free cortisol is bioactive. Bound cortisol cannot be metabolized and is not excreted or incorporated into hair. Therefore, it is imperative that the binding parameters be considered in this model. Since albumin is present in the blood at concentrations around 576 μM and binds to cortisol in a complex cooperative manner with low affinity (equilibrium dissociation constant: 137,800 nM, (Dorin et al. 2009)), it was considered non-saturable within the physiologically normal concentrations of total cortisol (200-1800 nM). CBG, on the other hand, binds cortisol with high affinity (equilibrium dissociation constant: 33nM from Dorin 2009) and is found in the blood at about 625 nM, therefore this interaction is characterized as saturable. Dorin et al 2009 created and validated a model for the binding characteristics of cortisol. Using two equilibrium equations and three conservation of mass equations that were combined to yield a cubic equation for free cortisol, the model calculated free cortisol from total cortisol measurements. Previous methods, such as Coolen's equation did not consider cortisol binding with albumin and therefore these free cortisol estimations were frequently inaccurate (Coolens et al. 1987). We adapted the formula from Dorin et al 2009 to incorporate the binding characteristics mentioned above into our model.

Dorin and colleges (2009) provide the framework for cortisol binding with the following equilibrium equations. F represents free cortisol, C is CBG, FC is CBG bound cortisol, A is albumin and FA is albumin bound cortisol.

Eq. 1.) $FC \Leftrightarrow F+C$ and $FA \Leftrightarrow F+A$

Based on this framework, Dorin and colleges (2009) also provided the following conservation of mass equations. In these examples K_C is the equilibrium dissociation constant for CBG binding with cortisol and K_A is the dissociation constant for albumin binding with cortisol. TotA, TotC and TotF are the total amounts of albumin, CBG and cortisol, respectively.

Eq. 2.) $$\frac{[F][C]}{[FC]} = K_C$$

Eq. 3.) $$\frac{[F][A]}{[FA]} = K_A$$

Eq. 4.) $$C + FC = TotC$$

Eq. 5.) $$A + FA = TotA$$

Solving for FA and FC, TotF is now presented in terms of total CBG, total albumin, equilibrium dissociation constants and free cortisol concentrations.

Eq. 6.) $$TotF = F + F * \frac{TotC}{K_C + F} + F * TotA/(K_A + F)$$

From equation 7, the equation for cubic free cortisol is derived (Eq 8). Since albumin, CBG and Kd constants are relatively stable, it is now possible to solve for free cortisol.

Eq. 7.) $$F^3 + pF^2 + qF + r = 0$$

$$\textit{Where, } p = TotA + TotC - TotF + K_A + K_C,$$

$$q = TotA * K_C + TotC * K_A - TotF * (K_A + K_C) + K_A K_C,$$

$$and \quad r = -K_A K_C * TotF$$

Using the following standard solution for the cubic formula Dorin and colleges (2009) provide a free cortisol calculation given total cortisol measurement and concentrations of CBG, Albumin and Kd for both CBG and Albumin cortisol binding.

Eq. 8.) $$F - cubic = (2\sqrt{-a/3})\cos\left(\frac{\theta}{3}\right) - p/3$$

where, $$a = \frac{3q-p^2}{3},$$

$$b = \frac{2p^3-9pq+27r}{27},$$

and $$\theta = cos^{-1}\left(\frac{-\frac{b}{2}}{\sqrt{\frac{(-a^3}{27)}}}\right)$$

Saliva Transfer: It is believed that cortisol is transferred to saliva by direct diffusion from the blood. Perogavmros and colleagues (2011) compared a time-series concentration plot for free cortisol in the blood with cortisol in the saliva following oral dosing of cortisol. Two important results from Perogavmros influenced this model: 1.) Once the dose was absorbed, there was no significant lag between the spike of free cortisol in the blood and the saliva and 2.) the ratio between free blood and salivary cortisol was fairly constant, remaining around 2.6. Therefore, we assumed that salivary cortisol levels are at instantaneous equilibrium with free cortisol concentrations in the blood.

Fecal Excretion: In this model fecal excretion of cortisol is considered to be directly related to the amount swallowed in saliva. Based on the ratio of free cortisol in the blood to free cortisol in the saliva, it is possible to determine the total amount of cortisol transferred to saliva given an average amount of cortisol in the blood. Assuming that the average person produces and swallows 1.5 liters of saliva daily

(Snyder et al. 1974), the total amount of cortisol swallowed can be calculated and assumed to be the amount of cortisol excreted in feces. Early studies using radiolabeled cortisol showed that about 20% of cortisol is excreted in feces (Kraan et al. 1992). We compared the amount expected to be excreted through transfer to saliva and swallowing with 20% of total cortisol in the blood and found insignificant differences between results and therefore considered this assumption to be reasonable. Based on these assumptions, we calculated the rate constant for fecal excretion of cortisol with the following formula

Eq. 9.)

$$K_f = \frac{F * 2.7}{2.58} * 1.5/(F * 2.7)/24$$

In this formula F is the concentration of free cortisol in the blood, based on the average total cortisol concentration of 1450 nmol/L(Young et al. 2001). This assumes there are 2.7 liters of blood (Snyder et al. 1974) and that the ratio of free cortisol in the blood to cortisol in the saliva is 2.58. This also assumes that the average person produces and swallows 1.5 liters of saliva daily. Finally, we use the 24 hours in one day to convert the rate constant to units of proportion per hour.

Urinary Excretion: Finken 1999 measured urinary free cortisol excretion in women at different phases of menstruation. While most of the cortisol produced is metabolized prior to excretion, a small fraction of unmetabolized cortisol is excreted in urine. The average amount of urinary free cortisol excreted per day was 414 nmols (Finken et al. 1999). Coupling this data with an average amount of total cortisol produced per day (1450 nmol/L Young) and the Dorin model for free cortisol we calculated an average concentration of free cortisol in the plasma and multiplied it by 2.7 L, since this is the average plasma volume for women (Snyder et al. 1974). The equation for the rate constant derivation is shown below.

UFC is urinary free cortisol (414 nmols (Finken et al. 1999)), F is the free cortisol in the blood, 2.7 is the liters of serum in average women, and 24 converts the rate constant to units of portion/hour.

Eq. 10.)

$$K_{u=\mathrm{UFC}/(\mathrm{F}*2.7)/24}$$

Incorporation into Hair: The most likely mechanism for cortisol incorporation into hair is through passive diffusion (Gow et al. 2010). The rate of incorporation into hair was determined based on hair density and growth rate. Assuming all hair growth is derived from biological molecules in the blood stream the density of hair along with the average concentration of cortisol in the blood stream can be used to determine the rate. In order to do this we assumed, a 1 cm/month growth rate, 100,000 strands of hair/head, 0.07 mg/cm/strand, 20 pg/mg average hair concentration and 2.7 L of serum/woman with 60 nmol/l average free cortisol concentration (Linch et al. 2001; Magos and Clarkson 2008; Snyder et al. 1974; Stalder et al. 2011). Such that the rate constant for cortisol incorporation into hair (k_h $hours^{-1}$) is expressed by the product of the concentration of cortisol in the hair (HF nmol/g) and the total amount of hair growth per month (H, grams/month) divided by the product of the amount of free cortisol in the blood (F, nmols) and the number of hours/ month (720 hrs/month).

Eq. 11.) $$K_{h=HF*H/F*720}$$

Metabolism: The three main mechanisms of cortisol clearance are urinary and fecal excretion and metabolism. While excretion through incorporation into hair is an important part of this model, the contribution to overall clearance is relatively small. Because we were able calculate rate constants for urinary and fecal excretion and we have to overall half-life from Young 2001, we used a simple subtraction equation to calculate the rate constant for metabolism.

Eq. 12.) $$Total\ Clearance = K_f + K_m + K_u + K_h$$

In this formula total clearance is calculated from the half-life (49 +/- 42 minutes) reported for healthy women in Young 2001. K_f is the fecal clearance rate constant, K_m is the metabolism rate constant, K_u is the urinary clearance rate constant and K_h is the rate constant for incorporation into hair.

Free Cortisol Excretion and Metabolism:

Eq. 13.)

$$\frac{dF}{dT} = F + F(-K_M - K_U - K_S - K_H)$$

F is the concentration of free cortisol, K_m is the rate constant for the metabolism of free cortisol, K_u is the rate constant for the urinary excretion of free cortisol, K_s is the rate constant for the transfer for cortisol to saliva and K_h is the rate constant for the incorporation of cortisol into hair.

The overall kinetic flowchart of the model is shown in figure 1. In this model, free cortisol binds with and dissociates from CBG and albumin, when unbound, cortisol is eliminated by urinary excretion, transfer into saliva, incorporation in hair and metabolism. Metabolism is the dominant method of excretion, as shown by the rate constants in figure 1.

Step 2: Introduction of Free Cortisol Pulses into the System

Pulse Characteristics: Young et al 2001 studied cortisol pulsatilty in women with and without endogenous depression. Since men and women have different cortisol secretion patterns (Mortola et al. 1987), using the data from the Young 2001 controls allowed the model to be catered toward women. Cortisol pulsatility is highly variable, both among and within individuals. Therefore the number of pulses/day, pulse height, average baseline and half-life were all considered to be distributed based on

the means and standard deviations reported in Young et al 2001 (table 1). Pulses were considered to be instantaneous in order to predict the maximum peak height possible.

Table 1*: Modeled free cortisol pulse characteristics based on parameters from Young, 2001*

Free Cortisol	**Mean +/- Std. Dev.**
Pulses(/day)	38+/-13
Pulse Height (nmol/L)	80+/-44
Baseline Average (nmol)	66+/-50
Half-life (minutes)	49+/-42

Step 3: Introduce Diurnal Variation and within individual Variation to the Model

Diurnal Variation: Cortisol concentrations are lowest at night and gradually rise through the early morning to peak shortly after awakening. After the morning peak, the level slowly declines until night time with the amplitude of approximately 11-13 mg/dl (Young et al. 2001). The natural diurnal rhythms of cortisol were accounted for by using a changing baseline. This allowed for the separation of pulsatile and baseline area under the curve, reported in table 1.

Between and within individual Variation: While Young, 2001 provide standard deviations for pulse height and baseline variation, from their analyses we cannot separate between and within-individual variation. Since this model will eventually be run over multiple days, reflecting hair cortisol accumulation within an individual, it is necessary to address intra and interpersonal variation. Hruschka and colleagues

report the intra-class correlation coefficient for blood cortisol in a US population of 0.37 (Hruschka et al. 2005). We used this as an estimate to distinguish between and within individual variability in our model.

Step 4: Validation with Hair Samples from Published Studies

Given the substantial variability in blood cortisol profiles, a variety of hair concentrations can be modeled. We ran the model 500 times and assessed the hair concentrations, comparing them to the hair concentrations published in Stalder, 2011, Kirschbaum, 2009 and Dettenborn 2010.

Step 5: Optimization of Cortisol Sampling Protocol

In order to promote the optimization of cortisol sampling protocols we examined the connection between a single blood draw and a month of hair growth in terms of cortisol concentrations. We ran 500 simulations of a morning blood peak and corresponding hair samples. We also assessed the variability of cortisol in blood by running the model 500 times and creating hourly distributions of concentrations.

Assumptions:

Based on the methods described above, this model makes the following six assumptions about cortisol kinetics. 1.) Cortisol is released in a pulsatile manner into the blood. This is reflected as an increase in total cortisol which is then partitioned to free, CBG bound and albumin bound. 2.) Cortisol binds with CBG with high affinity (Kd= 33 nM) and albumin with low affinity (Kd= 137,800 nM), but CBG becomes saturated at high cortisol concentrations (Dorin 2009). 3.) Diurnal variation is accounted for by changing the baseline cortisol concentration. 4.) Cortisol found in saliva is swallowed and excreted in feces. 5.) Free cortisol in the blood is at equilibrium with saliva cortisol. 6.) Cortisol is incorporated into hair as it grows through passive diffusion from the blood (Gow, 2010).

Adaptations by pregnancy trimester:

All of the simulations done in this analysis were modeled after non-pregnant women. When this model is used with pregnant women, the CBG concentration will need to be adapted by trimester. CBG concentration changes drastically during pregnancy, achieving concentrations almost three times as high as pre-pregnancy levels by the third trimester. Since we use the equations from Dorin, 2009 to account for cortisol binding, the concentration of CBG in these equations can be changed. The pregnancy-induced change in CBG is complex because progesterone also binds to CBG with weak affinity (Demey-Ponsart et al. 1982) and is significantly increased during pregnancy. Therefore, not all the CBG produced is available to bind cortisol. Demey-Ponsart, 1982 provide a detailed quantitative model for assess CBG available for cortisol binding during pregnancy. We base changes in CBG by trimester on the CBG available to bind cortisol modeled in Demey-Ponsart, 1982. Serum albumin also changes with pregnancy. In the first trimester, serum albumin decreases by approximately 10 % and in the second and third trimester serum albumin can decrease between 20 and 35% (Murphy et al. 2002). These changes can also be entered into the equations from Dorin, 2009.

Results:

The results of our model development and validation are reported as they correspond to the steps addressed in figure 1. Overall we show that our model can create daily profiles of acute peaks in cortisol that relate to long-term measurements of cortisol in hair.

Step 1: Develop Biokinetic Framework

The first step in our model was to determine the kinetic relationship between the compartments shown in figure 1. Since only free cortisol can be metabolized, transferred to saliva and hair and excreted in urine and feces, the first priority was to address the binding properties of cortisol. Figure 2 shows how

total cortisol is distributed by showing the percent bound to CBG, albumin and the percent free. Since CBG is saturable and the equilibrium dissociation constant depends on the concentrations of both cortisol and the binding protein, we show these percentages change, with increasing total cortisol. We show this same concept with amount of cortisol bound to CBG, albumin and free as well.

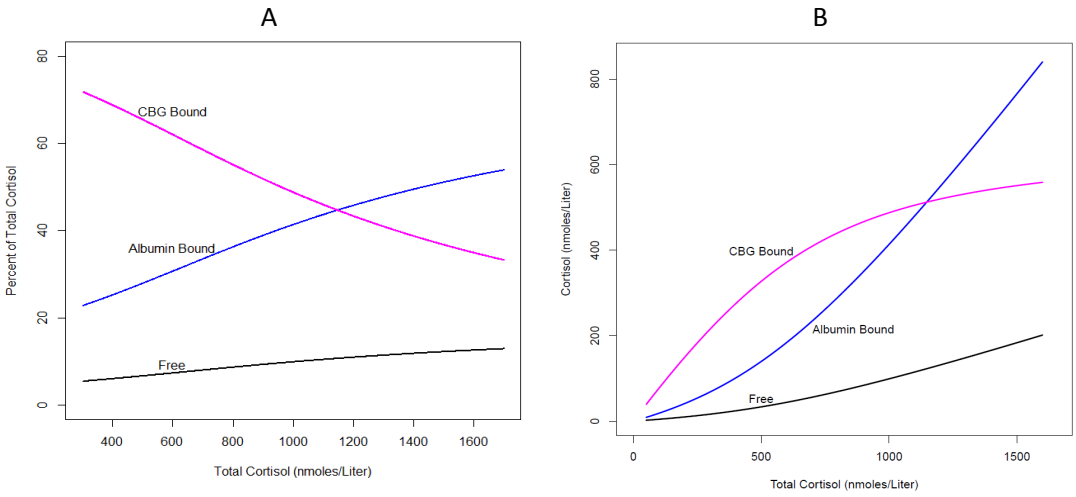

Figure 2*: Free and Bound Cortisol Shown as Percent Total (A) and as Concentration Free and Bound (B) with Increasing Total Cortisol. Modeling of relative concentrations of CBG-bound (pink), albumin-bound (blue), and free cortisol (black) predicted for a normal range for total cortisol concentrations in blood. Blood concentrations of CBG and Albumin are 625 nmol/L and 576 µm/L.*

The rate constants used for transfer of free cortisol to saliva, excretion in urine and metabolism are shown in figure 1. Consistent with urinary analysis, most cortisol is metabolized prior to excretion (Finken et al. 1999).The rate constant for metabolism (Km=16.4 hours $^{-1}$) is orders of magnitude larger than the rate constants for incorporation into hair (Kh=2.07e-5 hours $^{-1}$), transfer to saliva (Ks= 0.024 hours $^{-1}$) and urinary excretion(Ku=0 .627 hours $^{-1}$).

Step 2: Introduce Pulses of Free Cortisol to the System

Before we introduced daily pulses into the model, we assessed how pulses are reflected in free cortisol, bound cortisol and total cortisol. Figure 3 shows a 100 nmol/L pulse in albumin bound, CBG bound and free cortisol. Since CBG is saturable, the pulse height is decreased and the half-life is increased,

compared to free and albumin bound cortisol, which have very similar half-lives. Since albumin is unsaturable, it has the greatest pulse height. Total cortisol is set at 1500 nmols/L. The amount of total cortisol is important because, as shown in figure 4, increasing amounts of total cortisol overwhelm CBG's binding capacity and are therefore related to bigger pulses in free cortisol and shorter half-lives. In figure 5 we show how pulses are reflected in each compartment based on the rate constants discussed above. Metabolites show the greatest change in rate following a 100 nmol/L pulse of free cortisol. Saliva and urine show modest changes and hair shows the smallest change. These rates reflect the rate constants shown in figure 1.

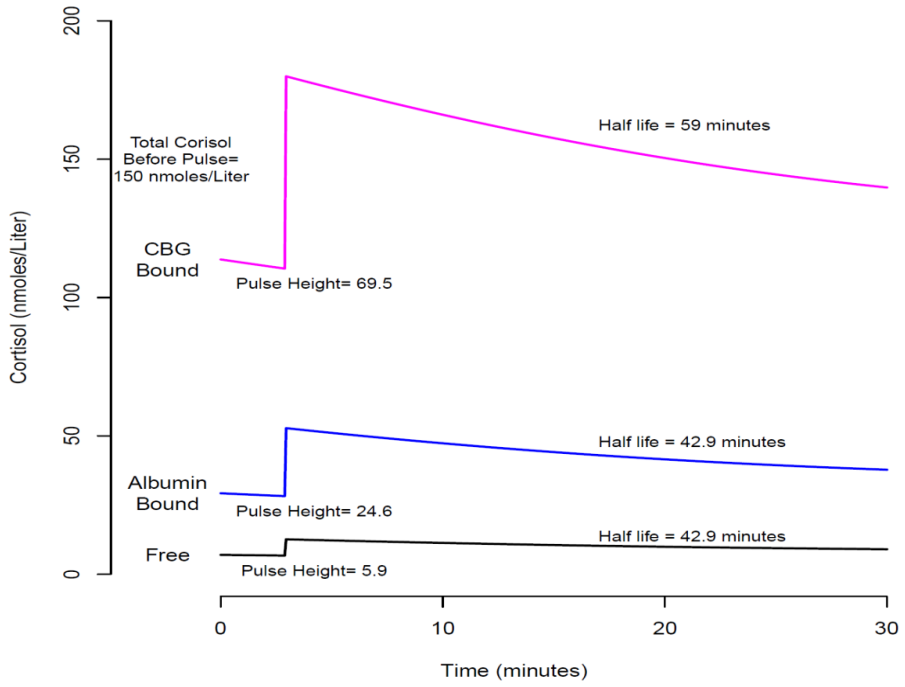

***Figure 3**: Modeling of concentrations of CBG bound (pink), albumin bound (blue) and free cortisol (black) following a physiologically relevant 100 nmol/l pulse in blood already containing a 150 nmol/l total cortisol. The half-lives and pulse heights shown above reflect the differences in binding affinity, saturation and ability for cortisol to be metabolized in each state. CBG can be saturated by cortisol, as reflected by the low pulse height and binds with high affinity, as can be seen by the increase in half-life.*

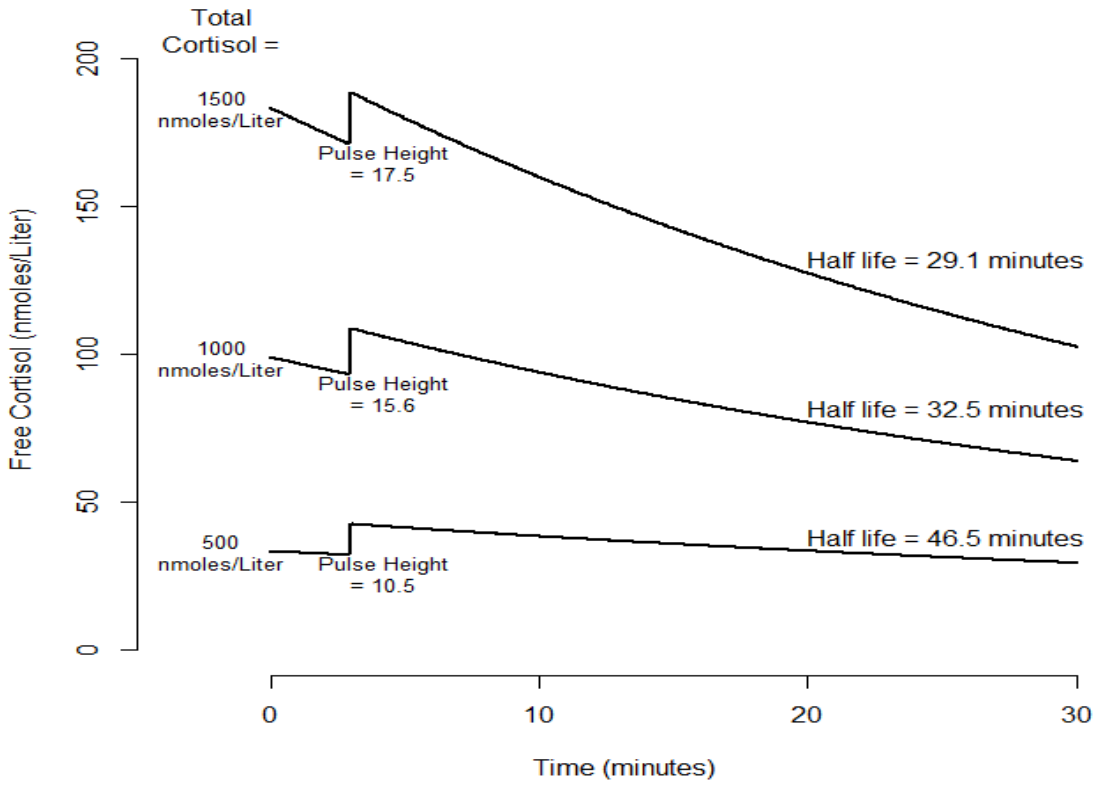

Figure 4*: Modeling of the variation in pulse height and half-life of free cortisol following a 100 nmol/L pulse at different total cortisol concentrations. Normal total cortisol concentrations range between 200 and 2000 nmol/L depending on the time of day and exposure to stress. Because binding to CBG is saturated at high total cortisol concentrations, the free cortisol half-life decreases and the free cortisol pulse height increases with increasing total cortisol.*

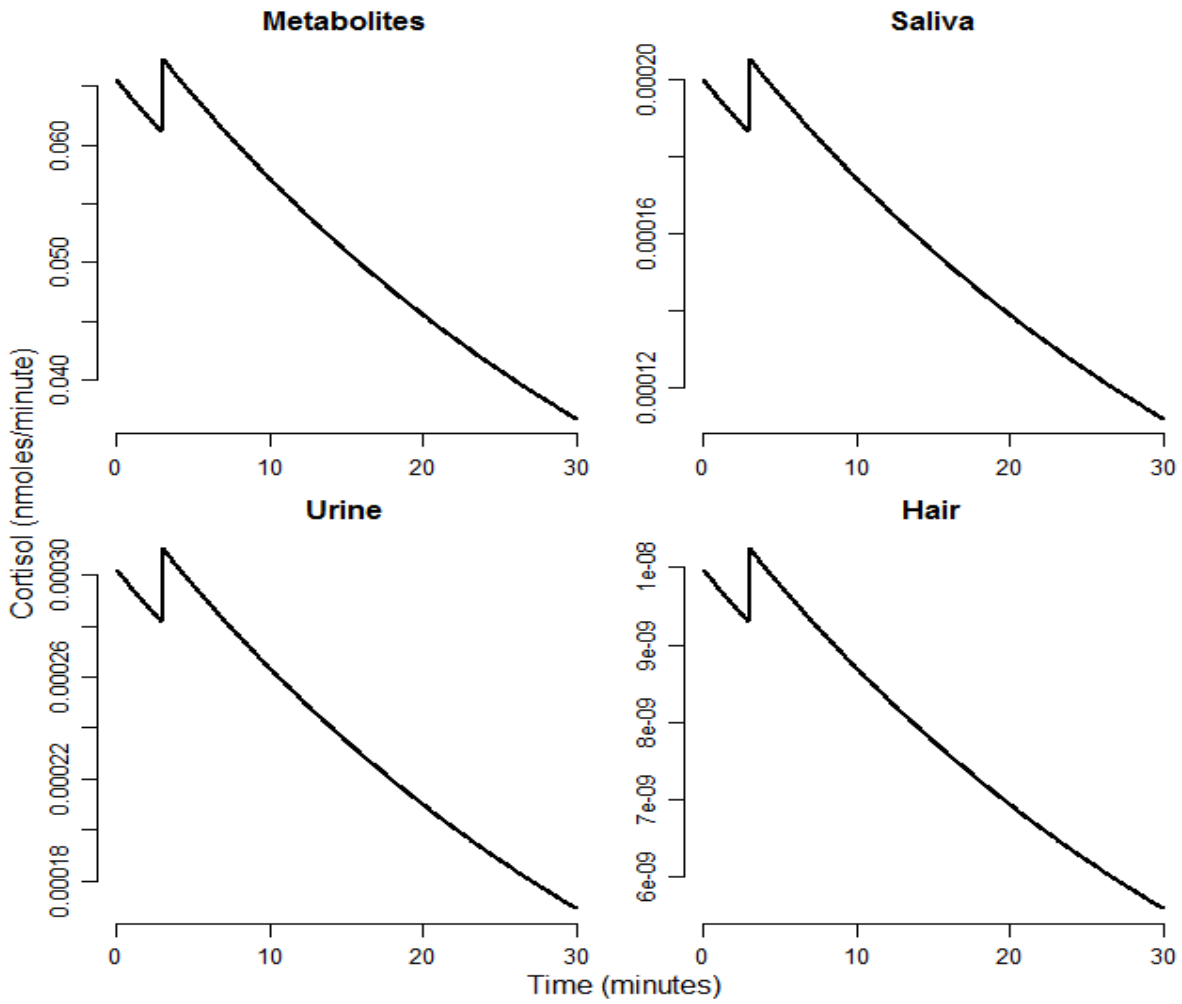

Figure 5: *The change in the rate at which cortisol is metabolized or transferred to saliva, urine and hair following a 100 nmol pulse occurring at 5 minutes. This reflects the changes in half-live shown in figure 4. The y-axis scale changes significantly between compartments, reflecting the 10^6 fold changes in rate constant.*

Step 3: Introduce Diurnal Variation and Intra-individual Variation in Pulses into the Model

Pulses were added to the model based on the pulse parameters shown in table 1. These parameters were extracted from Young, 2001. Pulses were introduced to the free cortisol compartment and then quickly partitioned to bound compartments based on the binding constants. A complete blood cortisol profile is comprised of baseline diurnal variation and pulsatile cortisol secretions. We show the construction of a blood cortisol profile first with just baseline diurnal variation, then just pulsatile secretion and finally the end-result, a complete profile (Figure 6). Based on the between and within

individual variation reported in Hruschka, 2005, we can simulate an entire month of cortisol fluctuation in the blood. Understanding long-term cortisol secretion patterns in the blood is important for assessing the validity of modeled cortisol concentrations in hair. In figure 7, we show an entire month of cortisol concentrations in blood, as they are changed by baseline diurnal variation and pulsatile secretion. The blood cortisol profile shown in figure 7 correlates to a hair concentration of 21 pg/mg. Free cortisol in the blood is incorporated into hair through passive diffusion. The change in the rate of incorporation into hair is based on the concentration of cortisol in blood. Therefore, the pulsatile secretion of cortisol in blood is reflected in hair by changes in the incorporation rate. The change in rate of incorporation into hair and transfer to saliva is shown in figure 8. We also show the accumulation of cortisol in hair after one day (figure 9). The accumulation in hair shown in figure 9 is comparable with a cortisol concentration of about 25.8 pg/mg. This is consistent with the month-long simulations shown in figure 6, which correlates to a 21pg/mg hair concentration.

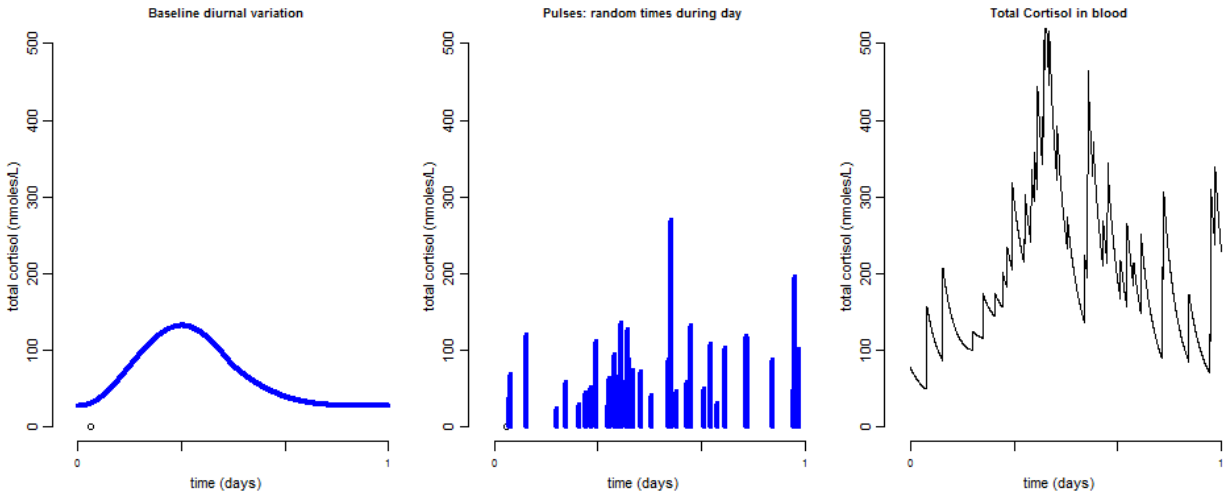

Figure 6: *Construction of a daily blood cortisol profile showing first baseline diurnal variation, then pulsatile cortisol secretion and finally a combination of the two, reflecting a complete blood cortisol profile for one day.*

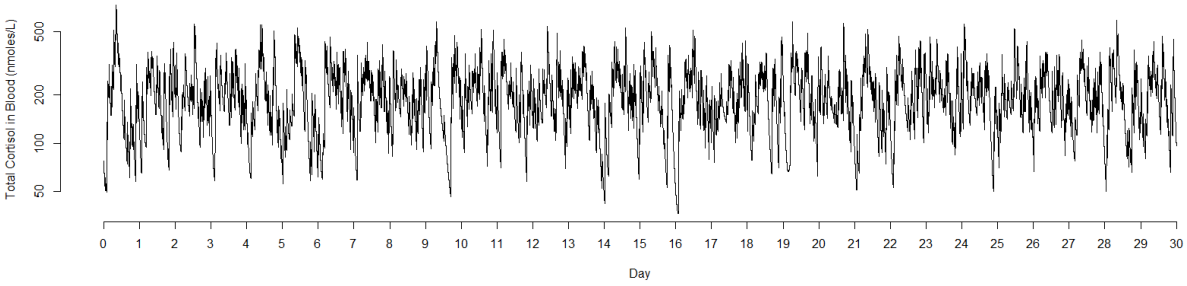

Figure 7: *Baseline diurnal variation and pulsatile secretion of total cortisol in a single individual for 30 days. This blood cortisol profile correlates to a hair concentration of 21 pg/mg.*

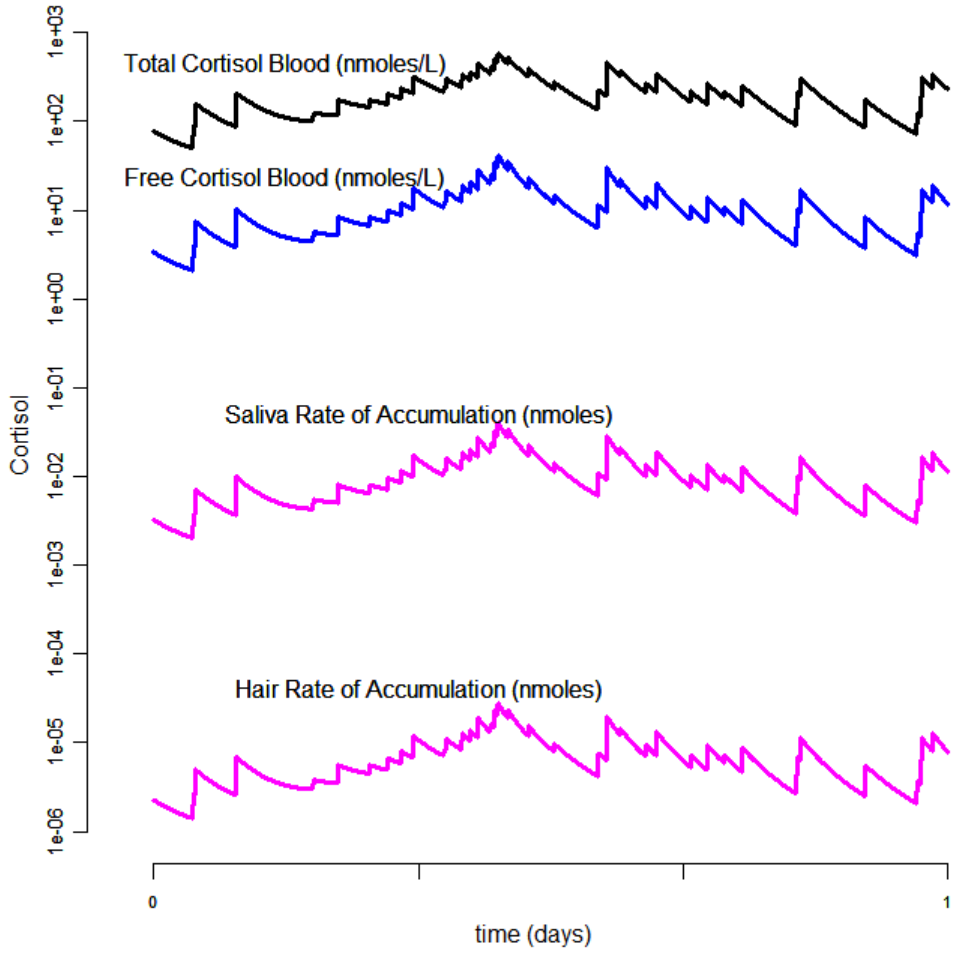

Figure 8*: The variation in total cortisol concentration in blood (black), free cortisol in the blood (blue), the rate of accumulation in saliva (pink) and hair (pink). Saliva and hair are shown in nmols of cortisol accumulation throughout one day.*

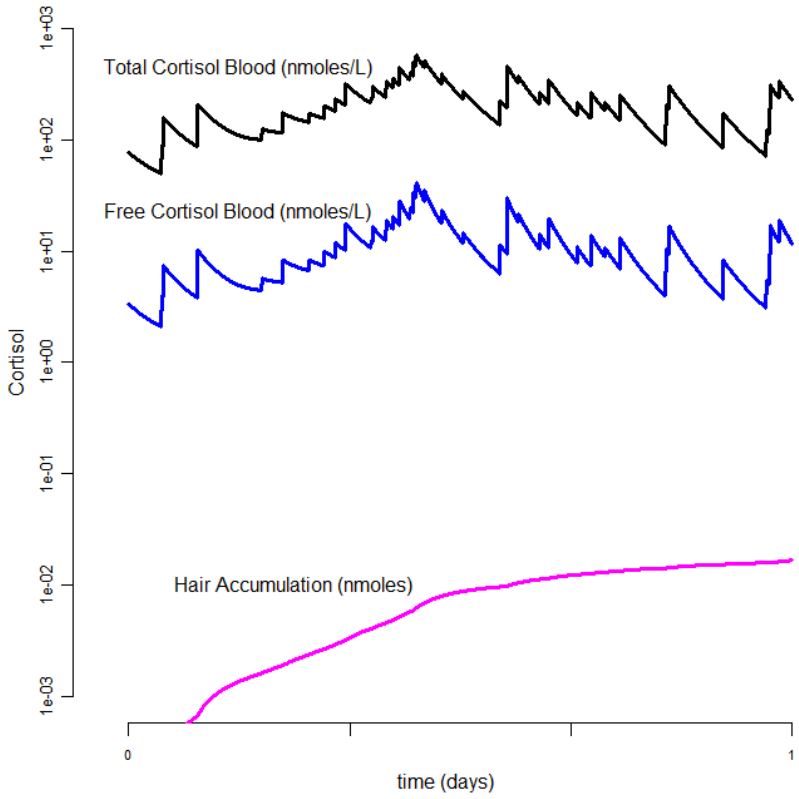

***Figure 9**: The accumulation of cortisol in hair (pink) based on the pulsatile secretion of cortisol in the blood shown in blue and black. The accumulation in hair corresponds to a concentration of 25.8 pg/mg.*

Step 4: Validation: Show cortisol accumulation in hair from longer-term pulsatile cortisol secretion

After running the model 500 times we report a geometric mean hair concentration of 20.7 +/- 1.19. This falls within the range of published data, shown in table 2. The geometric standard deviations show that our modeled data has less variability that published studies.

***Table 2**: Approximate geometric means and standard deviations of three published studies compared to our modeled data show that the modeled hair cortisol concentrations are within the range of published values.*

Study	**Geom. Mean (pg/mg)**	**Geom. SD**	**N**	**Percent Female**
Kirschbaum	19.35	1.53	20	100
Stalder	21.27	1.70	45	66
Dettenborn	14.38	1.49	28	57
Modeled Data	20.7	1.19	500	100

Step 5: Optimization of Cortisol Sampling Protocol

Figure 10 shows the relationship between a single blood sample, taken either in the morning or evening and a hair cortisol concentration for 500 model simulations. The relationship between the two samples is weak, suggesting that a single morning blood sample cannot reflect the chronic stress captured by hair samples. This is also shown by comparing the geometric standard deviations with blood being between 16 -42% more variable than hair samples. We also model the variability in blood (Figure 11), showing that the morning peak has the least variability and therefore may be the most reliable sampling time. This concept is also reflected by the decrease in standard deviation of the morning blood sample, compared to the evening blood sample shown in figure 10. However, even the morning peak had a very weak relationship with hair samples.

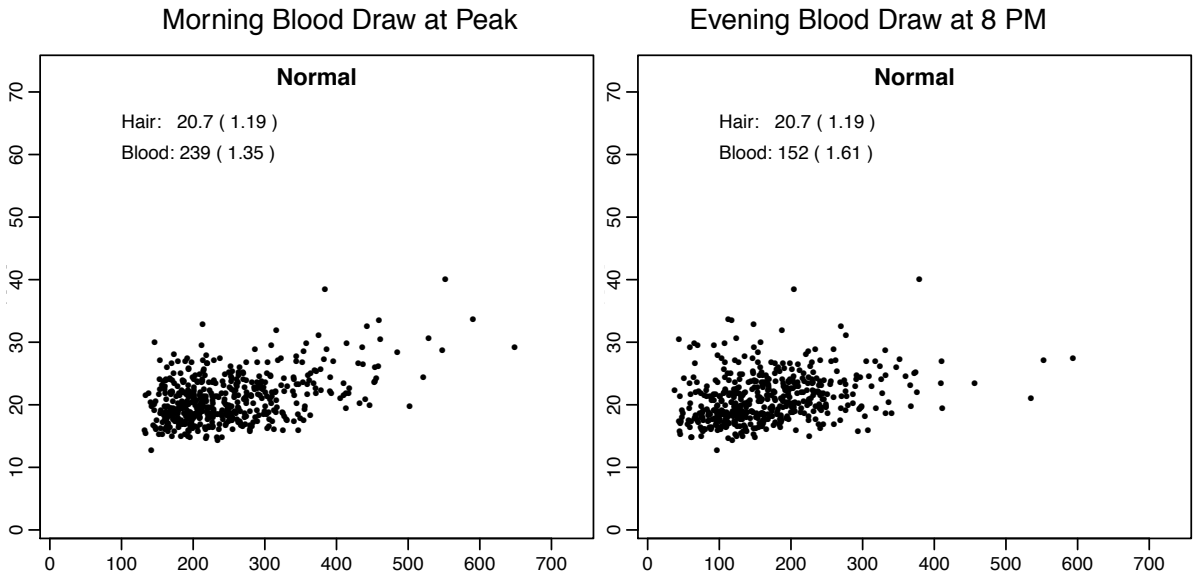

***Figure 10**: The relationship between a single blood cortisol sample taken either at the morning peak (right) or at 8pm (left) and a hair sample following 500 simulations. Geometric mean concentrations are shown for hair and blood as well as the standard deviations in parenthesis.*

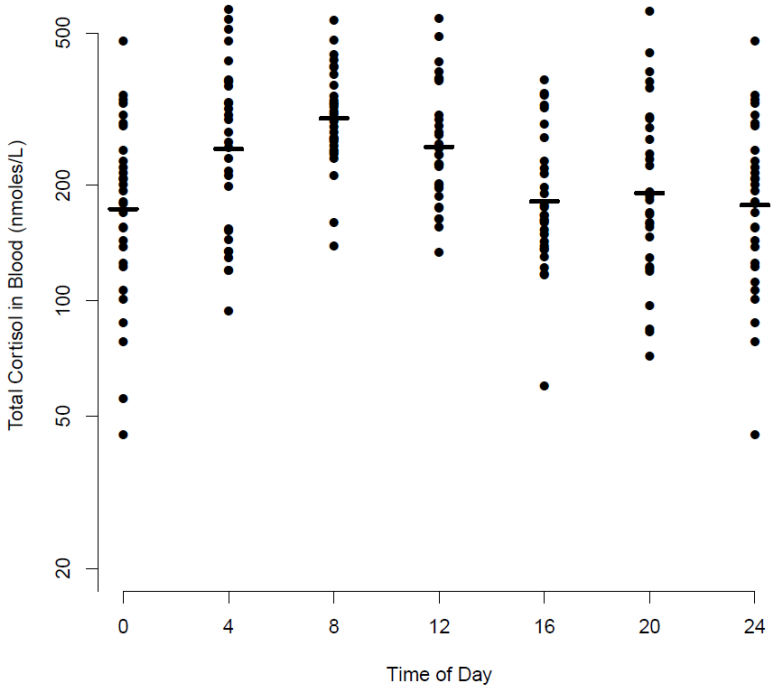

***Figure 11**: Variability in total cortisol concentration (nmols/L) in 500 simulations of 1 day, shown in 24-hour time. The bar indicates the mean concentration.*

Discussion:

We have created a biokinetic model for cortisol transfer between biological compartments with pulsatile parameters for secretion into blood. In this model, cortisol is secreted into the blood in a pulsatile manner and quickly binds to albumin and corticosteroid binding globulin (CBG). Free cortisol is reversibly metabolized by 11-β hydroxysteroid dehydrogenases to inactive cortisone and irreversibly conjugated by A-ring reductases. In this model we used a net reaction rate to express the overall reduction in cortisol from metabolic processes. Only free cortisol is excreted in urine and feces. Again, we assumed cortisol in feces comes from salivary cortisol that is swallowed and excreted. Therefore, in this model free cortisol in the blood is transferred to saliva and excreted as feces. Evidence supporting this assumption is available in Perogavmros 2011 and from the calculations presented in the methods section of this paper showing similar amounts of cortisol excreted under this assumption and using radiolabeled studies (Kraan et al. 1992). Finally, a small amount of free cortisol is incorporated into hair through passive diffusion from the blood.

To date, this is the first biokinetic model of cortisol that includes incorporation into hair. Since hair cortisol is a novel and useful biomarker for chronic stress, determining its relationship to more widely used stress biomarkers contributes to its validation and utility. From modeled blood cortisol profiles we can calculate an estimation of hair cortisol concentrations. When we ran the model 500 times, we found that the average simulated hair concentration correlated well with published studies. However, our model shows about 30% less variability than published studies. Some variability may be introduced in measured samples by the analytical techniques used. Our model does not consider variation in hair cortisol recovery rates, washout rates and other artifacts that may influence cortisol measurements in hair. Other sources of variability that could be added to this model in the future are variability in hair growth rate and variability in CBG and albumin concentrations. Additionally, we relied

on cortisol pulse data from a published study that drew the participant's blood every ten minutes. While this is the best method for capturing cortisol peaks in the blood, study participants were in a controlled environment, so exposure to environmental stressors was limited. Additional environmental stress exposure may be added to this model by using participant specific questionnaire data.

While this model is useful for relating cortisol concentrations across common biomarker compartments, characterizing the relationship between blood and hair cortisol concentrations is particularly relevant to maternal stress research. It has recently been suggested that cortisol may be involved in the mechanism through which maternal stress exerts toxic effects on the fetus (Field and Diego 2008). That being said, it is important that maternal stress studies are able to estimate the concentration of cortisol reaching the fetus. Here, we show how binding parameters influence peaks in the blood and the amount of free cortisol available for transfer to saliva and hair. Since concentrations of CBG change during pregnancy trimester, understanding how this influences free cortisol and hair cortisol concentrations is important for using hair as a biomarker for chronic stress during pregnancy.

Maternal hair samples are ideal for prenatal stress assessment because a single sample allows researchers to quantify nine months of chronic stress exposure(Kirschbaum et al. 2009). However, the fetus is exposed to cortisol in the blood. It is estimated that 40-50% of variation in fetal cortisol is caused by changes in maternal cortisol concentration (Gitau et al. 1998). Since the placenta contains high concentrations of 11-β HSD type 2 that convert cortisol to cortisone, only about 10-20% of maternal blood cortisol crosses the placental barrier. However, fetal cortisol concentrations are typically much lower than maternal blood cortisol; therefore the 10-20% that crosses the placental barrier can cause fetal cortisol concentration to double (Gitau et al. 1998). By using this model, it is possible to estimate fetal exposure to maternal cortisol much more directly.

We also use this model to address sampling protocol optimization. Since many national longitudinal cohort studies are limited in the number of biological samples they can collect, store and analyze, we assessed the relationship between a single blood sample and a hair sample. We found results similar to Sauvé (2007), who report no significant relationship between a single blood sample and a hair sample. We also show that hair samples have less variability than blood samples. This is indicative of the decreased within individual variability found in hair samples, since they reflect long-term accumulation of blood cortisol. A single blood sample, on the other hand, has greater variability because greater amounts of within individual-variability are represented. This analysis demonstrates the benefit of a hair sample, compared to a single blood sample. With the new ability to relate hair concentrations to long-term blood cortisol profiles, there is an even greater benefit to using a one-time hair collection technique, over a single blood sample.

Additionally, cortisol pulses encode an important biological signal that is distinct from constant cortisol concentrations (McMaster et al. 2011). Since hair analysis only shows a general elevation or reduction in cortisol levels, the pulsatile signal may be missed. Using this model it is possible show potential pulse profiles from a hair sample. Future work characterizing pulse profiles and identifying maximum potential peaks might help distinguish between acute and chronic stress health risks. Overall, our biokinetic model provides an excellent framework to aid in the interpretation of cortisol samples from hair, saliva, blood and urine.

Conclusion:

This is the first biokinetic model for cortisol that includes hair concentrations. It has the potential to increase the amount of information obtained from a one-time hair sample, especially in terms of prenatal environmental stress exposure assessment. Not only does this biokinetic model relate hair and blood cortisol concentrations, the nature of hair samples also allows researchers to specify the exposure

assessment by trimester of pregnancy. The development of this tool is part of a formative research project by the NCS.

Chapter Two: Optimizing Cortisol Sampling Protocol for National Longitudinal Cohort Studies by Modeling Cortisol in Stress Disorders

Abstract

BACKGROUND: Hair cortisol has recently been shown to be an effective method for assessing long-term changes in cortisol secretion reflective of chronic environmental stress exposure. Through the use of a biokinetic model hair cortisol concentrations can be related back to the blood. However, the sensitivity of this model to alterations of the HPA axis found in common stress disorders has not been tested, nor has anyone quantified the ability of a single blood sample to reflect slight, but constant changes in the HPA axis that may be indicative of stress disorders.

OBJECTIVES: We use a biokinetic model to create cortisol blood concentration profiles based on hair concentrations from individuals with stress disorders. Using this profile we assess the ability of our biokinetic model to reflect changes in the blood cortisol patterns as well as the ability of a single blood sample from our modeled profile to reflect the long-term changes in cortisol shown in the stress disorder hair samples. We also determine the value of the biokinetic model to national longitudinal cohort studies using a decision model analysis.

METHODS: We ran the biokinetic model using information from two recent publications reporting alterations in cortisol concentrations in hair associated with posttraumatic stress disorder (PTSD) and generalized anxiety disorder (GAD). The modeled blood concentration profiles were used to compare single blood cortisol samples to the reported hair samples. We also assessed the expected value of all common cortisol sampling procedures including, blood, saliva, urine and hair with and without the biokinetic model.

RESULTS: The biokinetic model showed sensitivity to changes in hair concentrations reported in PTSD and GAD patients. A single blood sample from the modeled profile was not able to reflect the long-term changes recorded in hair. We found that the biokinetic model increases the value of hair samples by 17% in national longitudinal cohort studies.

CONCLUSIONS: Many national longitudinal cohort studies are limited in sampling collection; we show that the use of a biokinetic model may reduce the need for multiple collections. The biokinetic model is sensitive to long-term changes in cortisol secretion while a single blood sample is unable to adequately reflect these changes. Therefore we recommend a one-time hair sample as a viable alternative.

Introduction:

Previously, we created a biokinetic model for cortisol transfer between blood and saliva, incorporation into hair and excretion in urine and feces. Cortisol is an end product of the hypothalamus-pituitary-adrenal (HPA) axis that is released in a pulsatile manner in response to environmental stress exposure. Once released into the bloodstream by the adrenal glands, cortisol quickly binds reversibly to albumin and corticosteroid binding globulin (CBG) (Stroupe et al. 1978). While bound, cortisol is inactive. Free cortisol binds to the glucocorticoid receptor to stimulate changes in transcription rates that ultimately lead to the physiological changes commonly associated with acute stress exposure, such as increased heart rate and blood pressure, increased blood glucose, changes in behavior and suppression of the reproduction and immune systems (Romero et al. 2007). Cortisol is metabolized to and from cortisone by 11-β hydroxysteroid dehydrogenases and irreversibly conjugated by A-ring reductases, inactivating cortisol (Finken et al. 1999; Morineau et al. 1997). Finally, cortisol is excreted in urine and feces (Kraan et al. 1992). Our biokinetic model incorporates all these pathways so that blood, saliva and urine cortisol can be estimated from hair concentrations.

Since cortisol is released in a highly pulsatile manner into the blood, a single blood or saliva sample only reflects instantaneous physiological conditions. It does not reflect stressors in recent days or even hours and has significant intra-individual variability (Hellhammer et al. 2007; Hruschka et al. 2005). Urinary free cortisol concentrations can reflect cortisol levels in recent hours, but also lack the ability to provide a long-term or retrospective look at cortisol secretion patterns. Hair cortisol, in isolation of other methods, can reflect stress exposure for recent months. This model relates hair samples to blood cortisol profiles, complete with modeled episodic peaks and diurnal variation. Recently, hair cortisol levels have been used to show chronic stress during long-term unemployment (Dettenborn et al. 2010) and maternal stress (Kirschbaum et al. 2009) as well as alternations in cortisol levels associated with posttraumatic stress disorder (PTSD) and generalized anxiety disorder (GAD) (Steudte et al. 2011a; Steudte et al. 2011b).

In individuals with PTSD, hair cortisol levels have been found to be much higher than in traumatized controls (Steudte et al. 2011a). Most published studies on PTSD report a reduction in blood and salivary cortisol levels, following oral dexamethasone administration (Klaassens et al. 2012). Dexamethasone is a synthetic glucocorticoid that inhibits pulsatile secretion of cortisol. Therefore, blood concentrations measured following dexamethasone administration reflect basal blood cortisol concentrations. Additionally, it is suggested that the diurnal rhythm of cortisol secretion is flattened (Yehuda et al. 2003) and 24- hour plasma concentrations and urinary excretion are lower (Yehuda et al. 1990; Yehuda et al. 1996) in individuals with PTSD.

These findings are inconsistent are with Steudte and colleague's recent finding of increased cortisol in hair of individuals with PTSD(Steudte et al. 2011a). Since the PTSD subjects used in Steudte's recent hair study were still living in a traumatic environment, they hypothesize that recent and current trauma exposures may cause increases in cortisol secretion, while past exposures could lead to an

overall decrease in individuals with PTSD. While there is much evidence supporting this argument (Gola et al. 2012; Inslicht et al. 2006), it is also possible that this discrepancy is reflective of differences in measurement techniques. Since hair cortisol reflects both pulsatile and basal secretion of cortisol as a result of day-to-day life stressors, it is less affected by assessment protocol.

Steudte and colleagues also measured hair cortisol levels in individuals with generalized anxiety disorder (GAD) (Steudte et al. 2011b). They found that hair cortisol levels were much lower in these individuals than in healthy controls. This is contrary to many publication findings demonstrating either no effect (Hoen-Saric 1991; Pomara et al. 2005; van Veen et al. 2008) or a significant increase salivary cortisol levels in patients with GAD (Mantella et al. 2008). Steudte addresses these inconsistencies by pointing out the differences in sampling techniques and suggesting that since blood and saliva samples only capture instantaneous cortisol concentrations, they may not reflect long-term perturbations in the HPA axis.

By using our biokinetic cortisol model, we create 24-hour blood cortisol profiles based on the changes in hair cortisol associated with PTSD and GAD. Blood cortisol profiles show modeled episodic peaks in cortisol as well as diurnal variation. We can also assess the sensitivity of hair cortisol samples to daily changes in cortisol by quantifying the daily change in blood AUC associated with a 10 pg/mg increase or decrease in hair cortisol concentrations. This is a particularly interesting question considering that Steudte found no reduction in the salivary cortisol levels of individuals with GAD whose hair concentrations were significantly lower. Similar, we can determine whether a single blood sample taken from an individual with PTSD or GAD is reflective of the long-term perturbations in the HPA axis shown by the hair samples.

Additionally, we can address differences in sampling protocol by using a decision model to quantify the benefits and drawbacks of each sampling technique. To do this, we compare the expected

value (EV) of a single blood, saliva or urine sample to hair sample with and without the use of our biokinetic model. The EV can be used in a decision model framework to allow researchers to optimize cortisol sampling protocol. Optimization of cortisol sampling protocol is important for the design of national longitudinal cohort studies, like the National Children's Study. While in most of these cases researchers will only be able to collect one sample, the use of this biokinetic model allows researchers to also estimate multiple blood parameters previously unattainable without repeated, more invasive samples. From a one-time hair sample, researchers can use this biokinetic model to estimate blood pulsatility parameters, such as maximum pulsatile secretion from acute stress exposure as well as create daily stress profiles that address longer and repeated acute stress exposure. By using a decision model to calculate the expected value of each sampling protocol it is possible to quantify the favorability of each technique in a single analysis.

The goals of this paper are to test the sensitivities of both our biokinetic cortisol model and hair cortisol levels to abnormalities in long-term cortisol secretion. We synthesize current literature findings to create a blood cortisol profile from hair samples taken from individuals with PTSD and GAD. Then, we determine how much the blood profile changes given the observed change in hair cortisol associated with PTSD and GAD compared to control hair samples, and assess the ability of a single blood sample to reflect this change. Based on the results of this analysis, we address the optimization of cortisol sampling protocol through analyzing the expected value of each sampling technique and the value of information obtained from the biokinetic model with a decision analysis.

Methods:

Biokinetic Model Sensitivity Analysis:

The biokinetic model used in this paper was previously described in great detail. In short, pulses of cortisol are introduced to the free blood compartment and then quickly partitioned to CBG and albumin

bound fractions based on equations from Dorin, 2009. Pulse release and frequency, baseline secretion and diurnal variation parameters were derived from Young, 2001. Unbound cortisol in the blood is free to be metabolized, excreted in urine and saliva and incorporated into hair. The rate constant for transfer to these compartments were derived based on data from (Finken et al. 1999; Kraan et al. 1992; Perogamvros et al. 2011; Young et al. 2001). In this analysis, special attention is given to the relationship between blood and hair cortisol concentrations.

To determine the sensitivity of the model to disease relevant changes in hair cortisol concentrations, we used hair concentrations from patients with GAD and PTSD (Steudte et al. 2011a; Steudte et al. 2011b). Since the hair cortisol concentrations reported in both these papers are inconsistent with years of blood data, we also tested the sensitivity of a single blood draw to a long-term change in hair cortisol concentrations.

Testing the sensitivity of the model to GAD and PTSD hair concentrations:

Steudte reported the average concentration of cortisol of approximately 8 pg/mg in GAD patients and 38 pg/mg in PTSD patients compared to 21 pg/mg in healthy controls (Steudte et al. 2011a; Steudte et al. 2011b). Therefore we adjusted blood input parameters by decreasing the pulsatile and basal secretion of cortisol proportionally until the hair concentration was reduced to the range reported by Steudte for GAD patients. Similarly, we adjusted blood input parameters by increasing the pulsatile and basal secretion of cortisol proportionally until the hair concentration was increased to the range reported by Steudte for PTSD patients. We also adjusted blood cortisol levels by changing just baseline or just pulsatile secretion. Given the controversy in the literature regarding blood cortisol concentrations in both these disease states, we ultimately used the simulation in which basal and pulsatile secretion are changed proportionately. This way changes in pulsatile or basal secretion would not be over-weighted. This allowed us to simulate a month-long blood profile that reflects the change in

hair concentration. From this profile, we assessed the ability of a single blood draw to reflect the significant, long-term change in cortisol shown by the hair samples. This was accomplished by assessing the relationship between a single blood sample and a hair sample in individuals with PTSD and GAD. We also decrease the within-individual variability of blood samples by simulating multiple samples from a single individual in order to determine how many blood samples are needed to show the same between individual variability as a one-time hair sample. All models and statistics were run using R v 2.14.1.

Decision Model Analysis

We assessed the ability of each of the sampling techniques listed in table 1 to address specific concerns relevant to study design and stress assessment goals. The concerns considered in the analysis are the ability to assess instantaneous acute stress, sub-chronic stress (acute stress over a day) and chronic stress as well as participant willingness, reliability, direct relationship to fetal exposure and costs associated with sample collection, analysis and shipping/storage. Each of these concerns will be defined and assigned to a weighting factor based on its importance in study design and optimization of cortisol protocol in the National Children's Study (NCS). While the weighting factors are inherently subjective, examples of the relative importance of each concern was based on published studies as well as the goals and study design of the NCS. Weighting factors were set on a scale of 1 to 5 with 1 being of very little value and 5 being highly valued. The only concerns to be weighted at 5 are associated with reoccurring costs or have the ability to threaten the quality of the data through introduction of biases or unreliability. The ability of each sampling technique to address the concerns discussed above was scored on a scale of 0-1 and multiplied by the weighting factor to provide a weighted average score for each sampling technique. Additional analyses addressing the sensitivity of the results to changes in the weighting factors were conducted to reflect situations in with different goals and study designs.

Weighting Factors

The goals of a longitudinal cohort study rely on the type of stress assessed. For the purposes of this analysis instantaneous acute stress is defined as the current cortisol levels of the study participant, sub-chronic stress is defined as a daily acute stress profile, showing a record of the acute peaks in cortisol concentration over one day and chronic retrospective stress is defined as cortisol levels over previous weeks and months. Based on the definition of toxic stress as frequent, prolonged or severe (Cooney 2011; McEwen 1998), the ability of each technique to assess instantaneous acute stress was assigned to a relatively low weighting factor of 2, since this measurement does not relate to the length, frequency or severity of the stress. Because sub-chronic stress assessment addresses the frequency and severity of stress it scored 3. Chronic retrospective stress measurements reflect all three criteria for toxic stress. Additionally, chronic retrospective stress was given the highest weighting factor because most of the studies relating prenatal stress to adverse fetal outcomes have shown chronic stress to be associated with the greatest risk (Borders et al. 2007). Chronic retrospective stress assessment also has the unique ability to allow researcher to assess previous stress levels and identify changes associated with life-events, such as pregnancy (Kirschbaum et al. 2009). Without the ability to retrospectively assess stress, only prospective cohort stress assessment studies are possible. Therefore retrospective stress assessment opens the door to more types of study design to accommodate unique research goals and questions. These factors all contributed to chronic retrospect stress assessment being weighted at 4 out of 5.

The ability of the sampling technique to provide a reliable assessment of stress, uninfluenced by subject adherence, sampling time, recent food or caffeine consumption, physical activity, common medication and incidental stress exposure associated with the sampling technique was rated highly. Because the fundamental utility of a sample depends upon the reliability, it was given a weighting factor

of 5. Also included in this analysis was the participant willingness of each sampling technique. Placing too high of a burden on study participants can lead to biased recruitment and failure to follow up (Luo 2010). Higher study participant burdens may change the demographics of the recruited cohort. This has been shown in pregnancy cohort studies, such that higher participant burdens lead to greater inclusion of women more invested in their pregnancies, leading to selection bias (Kramer et al. 2009). The loss of study participants, especially when not random can lead to significant biases in the study results (Kristman et al. 2004). The NCS is a 21 year longitudinal cohort study. As such, it already places a relatively high burden on study participants. Therefore, reduction of participant burden through less invasive and less time consuming sampling technique is highly relevant. Since participant willingness is required for unbiased longitudinal cohort studies, it was given a weighting factor of 5.

Of particular interest in maternal stress assessment is the ability of the sampling technique to assess fetal cortisol exposure. Fetal cortisol exposure is related to maternal blood cortisol concentrations since the placental barrier is incomplete (Gitau et al. 1998). Therefore, maternal blood analysis provides the best estimate of fetal cortisol exposure of all common biomarker analysis. Since cortisol has been implicated as a potential mechanism by which maternal stress causes adverse fetal outcomes (Field and Diego 2008), the ability to estimate fetal cortisol exposure is an important aspect of maternal stress assessment. However, unlike reliability or participant willingness, the ability to assess fetal cortisol exposure does not have the potential to bias the study. Furthermore, for some research goals, ranking maternal stress may sufficiently assess fetal cortisol exposure, therefore it was weighted as less important than reliability, willingness to participate and cost and weighted at 3 out of 5.

The financial cost of each sampling technique was also weighted and included in this analysis. The cost of collection, analysis and storage/shipping was considered for each sampling technique. The cost of collection and analysis were both weighted at 4 because these factors are often primary drivers

in determining study size and number of samples obtained. However, in many national longitudinal cohort studies, biological specimens are retained for years following collection. Since cost of storage and shipping is reoccurring it was given a slightly higher weight of 5.

Sampling Technique Scores:

Once weighting factors were determined, each sampling technique was scored on a scale of 0 to 1 based on its ability to address each concern discussed above. The ability of each sampling technique to assess acute, sub-chronic stress and chronic stress, sampling techniques were assess in a dichotomous manner, with one being "yes" and zero being "no". Blood and saliva samples were given 1 in their ability to assess instantaneous acute stress. Saliva cortisol is assumed to be at equilibrium with blood cortisol concentrations, therefore both measurements capture current cortisol concentrations (Perogamvros et al. 2011). This score is also based on studies showing significant changes in saliva and blood cortisol concentrations immediately following an acute stress event (Kudielka et al. 2004). A urine sample was given 0 out of 1 because urinary cortisol reflects blood concentrations in the last few hours, but does not necessarily capture an instantaneous peak in blood cortisol. Hair cortisol is unable to capture acute and instantaneous changes in cortisol (score of 0 out of 1). Though our biokinetic model can predict acute stress peaks, it does not have the ability to reflect the instantaneous stress level at the moment the hair sample was taken therefore it was given a 0.5 out of 1. The model receives 0.5 instead of 0 because daily acute stress profiles can be generated allowing for the determination of maximum peak height. The maximum peak height is important for prenatal risk assessment because high concentrations of maternal cortisol have the ability to overwhelm the placental barrier and significantly increase fetal cortisol levels (Gitau et al. 1998).

The ability of each sampling method to address sub-chronic stress, such as daily acute cortisol peak profiles in blood was also scored. A single blood and saliva sample cannot be used to extrapolate

cortisol secretion over a longer period of time, since the high variability cortisol concentration caused by cortisol pulses is not represented in these analyses (Young et al. 2001). Therefore they both scored 0 out of 1. Urine cortisol analysis reflects blood concentrations over the last 3-4 hours, unless the sample is collected as first morning void. Because we are using sub-chronic to refer to daily stress profiles, a single urine sample only reflect about 16% of the day, therefore urine received a score of 0.16 out of 1. Hair is unable to address daily cortisol profiles in the blood, unless it is used in conjunction with our biokinetic model. The model allows for the extrapolation of long-term average cortisol levels in hair to daily secretion patterns using pulsatile secretion characteristics measured in 24-hour blood concentration profiles. Therefore hair alone received 0, while hair samples and the biokinetic model received 1. While models cannot replace measured data, the only methods for assessing 24-hour acute stress profiles include multiple samples and this analysis only includes a single sample collection.

Chronic retrospective stress is also an important concern in overall stress assessment. A single sample of blood, saliva or urine does not address chronic stress. Hair, on the other hand, can provide a long-term retrospective assessment of cortisol secretion patterns (Dettenborn et al. 2010; Kirschbaum et al. 2009; Manenschijn et al. 2011). Therefore hair and hair in conjunction with the biokinetic model both received a perfect score in their ability to assess chronic stress (1 out of 1).

The next concern we address is participant willingness. We used studies documenting participant willingness to provide biological samples as a metric for the scores of each sampling technique. One study found a 50-60% refusal rate when participants were asked for blood samples (Gjerde et al. 2011; Lacey et al. 2009). Another study found urine samples slightly more acceptable, with a refusal rate of 24% (Fendrich et al. 2004). The acceptability of hair samples has been varied, with a recent study demonstrating a refusal rate of 14-15%, indicating that it may be more favorable than a blood or urine sample (Fendrich et al. 2004). Recent studies in Norway have shown an extremely low

refusal rate for saliva samples of about 3-6% (Gjerde et al. 2012; Gjerde et al. 2011). Based on this information saliva samples scored 1 in terms of participant willingness. Using the formula shown below with the saliva samples refusal rate of 4.5% as the lowest value, blood sampling refusal rate of 55% as the highest value in question scores were calculated. Since in this case, high values indicate lack of favorability, the fraction must be subtracted from one in order to show favorability on a scale of 0-1 with 1 being most favorable.

$$Value\ on\ Scale\ of\ 0-1 = 1 - \frac{Value\ in\ Question - Lowest\ Value}{Highest\ Value - Lowest\ Value}$$

The reliability of a sample was also scored in this analysis. Blood samples can be influenced by anxiety of needle insertion, time of day, recent food consumption and caffeine (Lilliecreutz 2011; Lovallo et al. 1989; Young et al. 2001), leading to artificial peaks in cortisol concentrations. Aside from needle anxiety, saliva samples share many of these same concerns over artificial elevation of cortisol concentrations. Urine is less influenced by sampling protocol than blood, however, time of day and recent food or caffeine consumption can play a significant role in urine cortisol concentrations. Therefore, urine and saliva were given reliability scores of 0.25 while blood scored 0. Hair cortisol is not influenced by sampling protocol, recent food consumption or time of day. However, since the use of hair for long-term cortisol records is still relatively new, some concerns over the effect of hair dye (Kirschbaum et al. 2009; Sauvé et al. 2007)) and the washout rates of cortisol in hair over time (Manenschijn et al. 2011) may lead to a reduced cortisol measurement in older samples compared to more recent growth. Still, there is little concern of the validation of hair cortisol for up to 6 months of growth (Dettenborn et al. 2010). Despite these concerns, hair ranks as the most reliable single sample technique for assessing cortisol, scoring 1.

Of particular interest to maternal stress assessment, is the relationship of the cortisol concentration being measured to fetal exposure concentrations. While blood provides the best

estimate of fetal exposure, our biokinetic model allows for the estimation of blood concentrations from hair measurements. In this case, blood receives a perfect score because it is most closely related to fetal cortisol exposure. Saliva, urine and hair received zeros in this category. However, with the use of our biokinetic model, we are able to estimate blood cortisol concentrations, therefore hair used in conjunction with this model received a 0.75 score. The biokinetic model did not receive a perfect score because modeled data cannot fully replace measurement data.

The last groups of concerns considered in this analysis are the financial costs of sample collection, analysis and storing/shipping associated with each sampling technique. Collection of blood and hair samples requires trained professionals, while urine and saliva can be collected by the study participant at home. The amount of time and training needed to collect a blood sample is slightly greater than the amount required for hair samples. Therefore, the relative cost of collection was scored such that high costs scored zero and low costs scored 1. By these standards blood samples scored 0, hair scored 0.5 and saliva and urine received perfect scores. With the same scale, the cost of analysis was scored. ELISA techniques are relatively inexpensive and can be used for blood, saliva and urine samples. Therefore they all received a 1 in terms of analytical costs. While hair can be analyzed by ELISA, the results generally overestimate cortisol concentrations (Stalder and Kirschbaum 2012). Mass spectrometry is therefore the gold-standard for hair cortisol analysis. Mass spectrometry requires greater personnel time, increasing the cost of hair cortisol analysis. Additionally, a single hair sample can be cut into 3 cm segments, allowing for the retrospective assessment of stress in three-month intervals. Therefore, for every single hair sample collected, multiple analyses may be run, increasing the cost. In this category, hair scored a zero. The last financial concern is the cost of shipping and storage. In many national longitudinal cohort studies, samples are shipped great distances to be analyzed or stored for years before analysis. Blood, saliva and urine must be frozen during shipment and stored in deep freeze. Hair can be shipped in an envelope and stored at room temperature. Based on postal rates, it is

estimated that hair is about five times less expensive to ship than blood, saliva or urine samples. Therefore, hair scored a 1 for storage and shipping costs while blood, saliva and urine received 0.2.

Final Score Calculation:

The weighting factors of the overall importance of each concern were multiplied by the score for each sampling technique. The sums of all the weighted scores were compared as percentages of the sum of all weighting factors. The sum of all the weighting factors was considered the perfect score. In order to achieve that overall score, a single sampling technique would need to score 1 in every category. Therefore, the results are presented as percentage of the perfect overall score.

$$Percentage\ of\ Perfect\ Score\ Calculation$$

$$= \frac{Ia * IaW + Sc * ScW + Ch * ChW + Pw * PwW + R * RW + Fe * FeW + A * Aw * Cl * ClW + St * StW}{IaW + ScW + ChW + PwW + RW + FeW + AW + CLW + StW} * 100$$

Ia is instantaneous acute stress measurement score, IaW is instantaneous acute stress weight, Sc is sub-chronic stress score, ScW is sub-chronic stress weight, Ch is chronic stress score, ChW is chronic stress weight, Pw participant willingness score, PwW is participant willingness weight, R is reliability score, RW is reliability weight, Fe is fetal exposure assessment score, FeW is fetal assessment weight, A is analysis cost score, AW is analysis cost weight, Cl is collection score, ClW is collection score weight and St is storage/shipping score, StW is storage/shipping weight. The score is specific to the sampling technique, while the weight is the same across all sampling techniques. A percentage of perfect score was calculated for each sampling technique.

Decision Model Sensitivity Analysis:

To ensure that no single weighting factor was contributing unfairly to the overall scores of the sampling techniques, we adjusted the weight to reflect a study design that may not value instantaneous acute

stress assessment or direct fetal exposure assessment. This may be the case in longitudinal cohort studies following non-pregnant adults that are interested in the long-term effects of stress exposure.

We also tested the sensitivity of the financial weighting and scoring factors. Since hair analysis may become less expensive as it becomes more common, we set the cost of analysis equal in all cases and report the effect on the overall value of the biokinetic model. In this same analysis, we increase participant willingness for hair, since it is possible that eventually smaller amounts of hair may be used for cortisol assessment.

Results:

Biokinetic Model Sensitivity Analysis

When the blood cortisol concentrations were adjusted by changing just baseline or just pulsatile secretion, results were similar to when both were adjusted proportionally. Since there is little conclusive information available on how blood concentrations change in PTSD and GAD we report the results of the proportional adjustments in baseline and pulsatile secretion. Using the hair sample concentrations reported in Steudte (2011a and b), we show that the decreased hair concentration reported in GAD individuals, approximately 8 pg/mg, leads to a blood profile with less acute peaks and a decreased baseline compared the healthy control blood profile that was based on a hair concentration of 21 pg/mg (Figure 1). In individuals with PTSD, Steudte report an average hair cortisol concentration of approximately 38 pg/mg. Using this value, we show a PTSD blood cortisol profile with increase acute stress peaks, compared to the healthy control (Figure 1). We also show the relationship between a single blood sample (taken either in the morning or at 8pm) to a hair sample within the healthy range, the PTSD range and the GAD range in Figure 3. A single blood draw shows both within and between individual variability since it is reflecting instantaneous blood cortisol concentrations. Because hair reflects accumulation of cortisol from blood over a longer period of time, it tends to be more reflective

of between individual variability. This is evident in these figures because blood concentrations show much more overlap between controls and PTSD as well as between controls and GAD individuals than hair samples. Therefore, at a population level, hair samples differentiate between disease states better than single blood samples. The geometric mean and standard deviations of cortisol concentrations in hair are 20.7+/- 1.19 pg/mg for healthy controls, 7.5 +/- 1.2 pg/mg for GAD patients, 37.9 +/- 1.12 pg/mg for PTSD patients. The geometric mean cortisol concentrations in the morning peak in blood are 239+/- 1.35 nmol/L in healthy controls, 97 +/- 1.36 nmol/L in GAD patients and 479 +/- 1.14 nmol/L in PTSD patients. Evening blood sample geometric means are 152 +/- 1.61 nmol/L for healthy controls, 67 +/- 1.59 nmol/L for GAD patients and 215 +/- 1.41 nmol/L for PTSD patients. From these values we also report the number of blood samples from a single individual needed decrease the within individual variability to the same level as a one-time hair sample (Table 1). Here, we show that multiple blood samples are needed to have the same ability as hair to differentiate between disease states at a populations level. Since morning blood samples have less variability than evening samples, less morning blood samples are needed to reduce the overall variability.

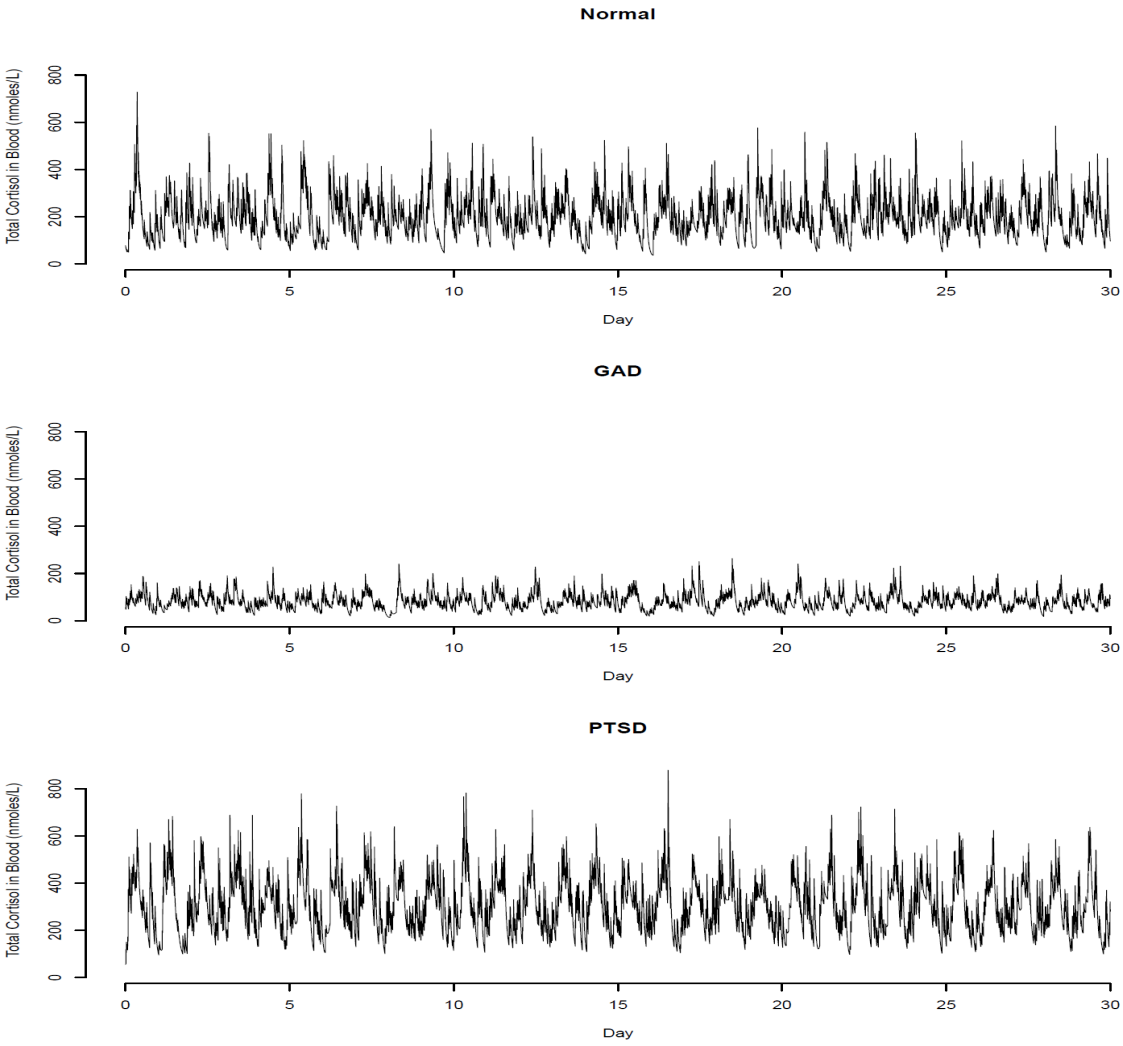

Figure 1: *Based on the hair samples from a healthy control (Top), a GAD patient (Middle) and a PTSD patient (Bottom), we show month long blood cortisol profiles. The GAD patients have much lower acute stress peaks than the controls while PTSD patients have much greater peaks than the controls.*

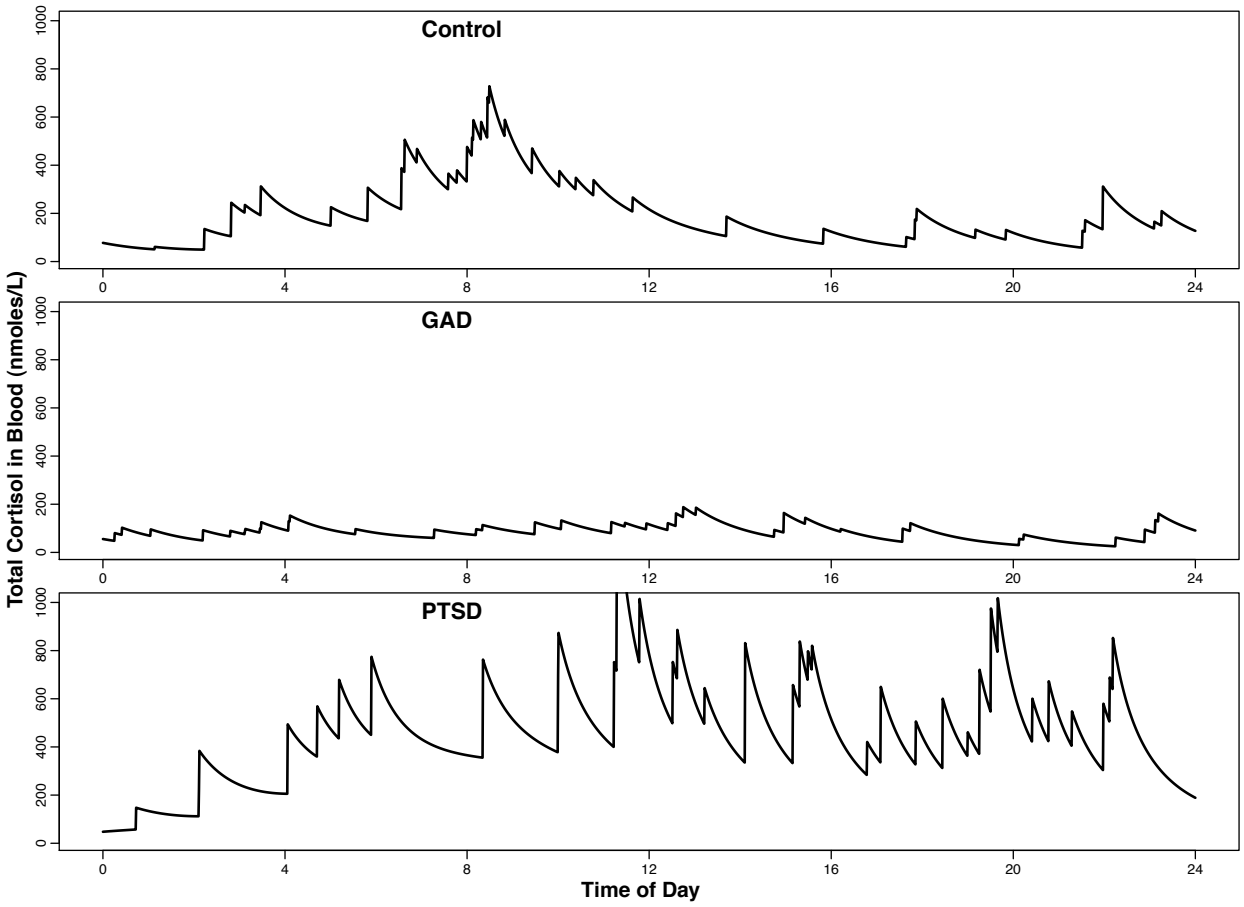

Figure 2: *Daily cortisol blood profile for a healthy control (top), GAD patient (middle) and PTSD patient (bottom). These are potential blood profiles modeled based on hair concentrations of 20.7 pg/mg in healthy controls, 7.5 pg/mg in GAD patients and 37.9 pg/mg in PTSD patients.*

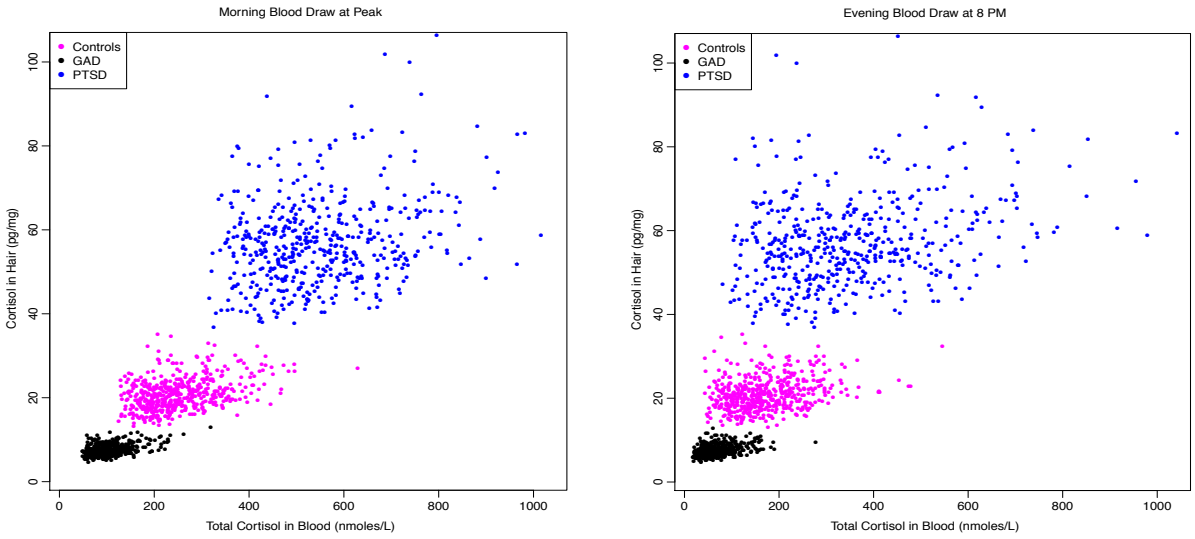

Figure 3: *Scatterplot showing the relationship between cortisol concentrations in hair and blood taken at the morning peak (top) and at 8pm (bottom). Simulations were run 500 times for PTSD (blue), controls (pink) and GAD (black). Blood samples show more variability and do not differentiate between disease states as well as hair samples. Morning samples blood samples show less variability and overlap between populations than evening blood samples, but both show significant overlap between populations, compared to hair.*

Table 1: *Number of blood samples needed to have the same between individual detection of disease relevant changes in cortisol on the population level.*

	Time of Blood Draw			
	Morning Peak 8 AM	Evening 8 PM	Morning 9-10 AM	Daytime 9 AM – 5 PM
Control	3	7	3	5
GAD	4	9	3	5
PTSD	2	7	3	4

Decision Model Analysis

The results of the decision model analysis are shown in table 2. A single blood cortisol sample ranked the lowest, with the overall score of 28.57% of the perfect score. The low score was mostly attributable to the inability to assess chronic stress, lack of reliability of a single sample, and high collection costs, since a trained phlebotomist is required. Additionally, blood samples place a higher burden on the study participant than saliva, urine or hair samples. It is estimated that 7% of the

pregnant population suffers from severe needle phobia, with symptoms ranging from anxiety over blood draws to fainting (Lilliecreutz 2011). Not only can cortisol samples from women with needle phobias be much higher than actual stress levels it is also unlikely that any women in this subset of the population would volunteer for a blood draw. They may, however, be willing to provide a saliva, urine or hair sample.

The next lowest score was urine cortisol analysis. Urine cortisol scored 34.94% of the perfect score. While urine cortisol samples did not score exceptionally low in any single category, the inability of a single urine sample to assess acute or chronic stress significantly decreased the total score. Additionally, a single urine sample can be unreliable, depending on the time of day of collection, caffeine consumption and recent food ingestion. Therefore, urine score low in the reliability category as well, contributing to the relatively low final score. Saliva samples scored 49.29% of the theoretical perfect score, slightly higher than urine, but not as high as hair. While saliva scored well in participant willingness, instantaneous acute stress and many of the financial costs of biomarker analysis, the inability of a single saliva sample to assess chronic stress and unreliability of a single sample lead to a reduction in the overall score.

Hair samples scored the highest of any stand-alone biomarker technique. The score of hair samples is 56.43% of the perfect score. Some of the main contributors for this overall score were the ability of cortisol to assess chronic stress, reliability of hair cortisol samples, relatively low participant burden and low storage and shipping costs. However, this score could be increased, if hair cortisol were able to address acute stress in the blood. Through the use of our biokinetic model, a single hair sample can be used address acute peaks in cortisol concentration in the blood. Not only does this allow a long-term sample to be used for acute stress assessment, it also begins to address fetal cortisol exposure. Since the developing fetus is not exposed to the concentration of cortisol in the hair, relating the hair

cortisol concentration back to concentrations in blood is critical in maternal stress assessment. Using a hair sample in conjunction with our biokinetic model was the highest scoring method of this VOI analysis. This method achieved an overall score of 74.29% of the theoretical perfect score. Therefore, through this analysis we show that our biokinetic model increases the value of information obtained through a hair sample by 17.86%.

Sensitivity Analysis Results:

Previously, weights were assigned to be in line with the values of the National Children's Study. We also tested the decision model using weights that might be more relevant to a longitudinal cohort study not following pregnant women and not interested in instantaneous acute stress assessment. In this example the model scored an additional 6.43% better than hair alone. Blood, saliva and urine cannot provide chronic stress assessment, and therefore scored very low.

We also considered a hypothetical future situation in which hair analysis is less expensive and hair sampling is less invasive. Improvements in analytical technology may reduce the detection limit, allowing smaller samples to be analyzed and reducing the amount of hair needed to be cut from the participant. These improvements may also reduce analytical cost. Therefore the weight of analytical costs was reduced to zero, since all techniques are considered equal and the score of hair sample participant willingness was increased to 1. The biokinetic model scored 17.41% better than hair alone. The results of the sensitivity analysis are shown in table 2.

Table 2: *The simulation type, concerns and sampling techniques used in the expected value calculation for the decision model analysis. The weight assigned to each concern is shown on a scale of 1 to 5 with 5 being the most important. Scores addressing how well each sampling technique meets the concern are shown on a scale of 0-1 with 1 indicating that the sampling technique addresses that concern very well. The weighted average score for each sampling technique is shown as a percentage of the sum of all weights (perfect score). The added value of the biokinetic model is calculating by subtracting the percentage score of hair from the percentage score of hair with the biokinetic model.*

			Sampling Techniques				
Simulation	**Concern**	**Weight**	**Blood**	**Saliva**	**Urine**	**Hair**	**Biokinetic Model and Hair**
NCS	Instantaneous Acute Stress	2.00	1.00	1.00	0.00	0.00	0.50
	Sub-chronic	3.00	0.00	0.00	0.16	0.00	1.00
	Participant Willingness	5.00	0.00	1.00	0.50	0.75	0.75
	Chronic Retrospective Stress	4.00	0.00	0.00	0.00	1.00	1.00
	Reliability	5.00	0.00	0.25	0.25	1.00	1.00
	Direct relationship to fetal exposure	3.00	1.00	0.00	0.00	0.00	0.75
	Low Cost of Collection	4.00	0.00	1.00	1.00	0.50	0.50
	Low Cost of Analysis	4.00	1.00	1.00	0.75	0.00	0.00
	Low Cost of Storage/Shipping	5.00	0.20	0.20	0.20	1.00	1.00
	Weighted Average Score	35.00	10.00	17.25	12.23	19.75	26.00
	Percentage	100.00	28.57	49.29	34.94	56.43	74.29
	Addedd Value of Biokinetic Model						17.86
Low Cost of Hair	Instantaneous Acute Stress	2.00	1.00	1.00	0.00	0.00	0.75
Analysis	Sub-chronic	3.00	0.00	0.00	1.00	0.00	0.75
Low Paricipant Burden	Participant Willingness	5.00	0.00	1.00	0.50	1.00	1.00
	Chronic Stress	4.00	0.00	0.00	0.00	1.00	1.00
	Reliability	5.00	0.00	0.25	0.25	0.80	0.80
	Direct relationship to fetal exposure	3.00	1.00	0.00	0.00	0.00	0.75
	Low Cost of Collection	4.00	0.00	1.00	1.00	0.50	0.50
	Low Cost of Analysis	4.00	1.00	1.00	1.00	1.00	1.00
	Low Cost of Storage/Shipping	5.00	0.20	0.20	0.20	1.00	1.00
	Weighted Average Score	35.00	10.00	17.25	15.75	24.00	30.00
	Percentage	100.00	28.57	49.29	45.00	68.57	85.71
	Addedd Value of Biokinetic Model						17.14
Non-maternal	Instantaneous Acute Stress	0.00	1.00	1.00	0.00	0.00	0.75
No interest in	Sub-chronic	3.00	0.00	0.00	1.00	0.00	0.75
instantaneous acute	Participant Willingness	4.00	0.00	1.00	0.50	0.75	0.75
stress	Chronic Stress	5.00	0.00	0.00	0.00	1.00	1.00
	Retrospective Stress	5.00	0.00	0.00	0.00	1.00	1.00
	Reliability	5.00	0.00	0.25	0.25	0.80	0.80
	Direct relationship to fetal exposure	0.00	1.00	0.00	0.00	0.00	0.75
	Low Cost of Collection	0.00	0.00	1.00	1.00	0.50	0.50
	Low Cost of Analysis	0.00	1.00	1.00	0.75	0.00	0.00
	Low Cost of Storage/Shipping	0.00	0.20	0.20	0.20	1.00	1.00
	Weighted Average Score	22.00	0.00	5.25	6.25	17.00	19.25
	Percentage	100.00	0.00	15.00	17.86	48.57	55.00
	Addedd Value of Biokinetic Model						6.43

Discussion:

The biokinetic model demonstrated the sensitivity for detecting slight, but long-term changes in cortisol secretion in blood by showing changes in monthly blood cortisol profiles based on 10 pg/mg changes in hair cortisol concentration associated with PTSD and GAD. Hair cortisol levels have been shown to be increased by approximately 7 pg/mg with long-term unemployment (Dettenborn et al. 2010) and 30 mg/pg during the third trimester of pregnancy (Kirschbaum et al. 2009). The ability of our model to shown changes in the blood cortisol profile related to increases in hair cortisol is important to its utility as a tool for detecting elevated stress during pregnancy.

We also demonstrated that a single blood sample does not show the same long-term changes recorded in hair. The model was used to provide single blood sample estimates based on the daily cortisol profiles related to the reduction and elevation of hair cortisol recorded in individuals with PTSD and GAD. Since both PTSD and GAD hair samples showed cortisol levels contradicting years of blood and saliva studies, we show that it is possible for a single blood sample to contrast a hair sample. This is primarily because hair samples record long-term averages, while blood, saliva and urine are affected by instantaneous pulses of cortisol and reflect greater within than between-individual variability. In our results we report the geometric mean hair concentration for a healthy individual to be 20.7 pg/mg with a standard deviation of 1.19, in blood the geometric mean cortisol concentration is 239 nmol/L with a standard deviation of 1.35. In all cases the standard deviation in blood is much greater than the standard deviation in hair. Because hair reflects long-term accumulation of cortisol it is more reflective of between individual variability than within individual variability. Therefore, hair may be the better method for showing long-term changes in HPA axis functioning and differentiating between disease states at a population level. This finding is important because it shows that small changes in daily

cortisol secretion may be represented in hair, but may not be distinguishable in single blood samples because of the high within individual variability of pulsatile cortisol secretion.

The variability of cortisol secretion in blood makes instantaneous measures of cortisol concentration difficult to interpret. While Steudte 2011a report elevated hair cortisol concentrations in PTSD patients compared to controls, most blood cortisol studies have found lower or insignificant differences while a few have even found increased blood cortisol. In our sensitivity analysis, we show that a multiple blood samples may be needed to reflect longer-term changes in cortisol secretion, especially if samples are not collected in the morning. Some of the difficulties of single blood, saliva and urine samples are demonstrated in a meta-analysis of 37 cortisol studies measuring blood, saliva and urine in PTSD and non-PTSD individuals. The combined data found no significant changes in cortisol secretion associated with PTSD (Meewisse et al. 2007). However, the authors also report a significant reduction in cortisol associated with PTSD in afternoon samples, but not in morning samples and also when only serum or plasma studies were considered, there was a significant relationship. In the 17 studies that compared PTSD patients to traumatized controls, no differences in cortisol concentrations were found (Meewisse et al. 2007). The differences in findings associated with sample type, sampling timing and trauma exposure show the difficulty of using a single cortisol sample to reflect changes in overall secretion patterns. More invasive sampling, such as multiple blood draws in a 24-hour time period can also lead to unclear results. Yehuda, 1996 examined 24-hour cortisol secretion patterns in individuals with PTSD and healthy controls. There was a significant reduction in blood cortisol levels of PTSD individuals. However, the differences were found between 7 and 9pm (Yehuda et al. 1996). Since most clinics do not collect participant blood in the late evening, they may miss significant changes in cortisol. This particular study required participants to be hospitalized for 24-hours with blood draws every 10 minutes. Not only can hospitalization interfere with exposure to environmental stressors, but also, the costs associated with 24-hour hospitalization significantly reduce the number of study

participants that can be evaluated, which can lead to lack of statistical power for analysis. While the findings of elevated hair cortisol in individuals with PTSD have not yet been replicated, our analysis shows that hair samples may provide a more reliable measure of cortisol secretion overtime than a single blood, saliva or urine sample.

Similarly, the hair cortisol concentrations from individuals with GAD contradicted many blood cortisol studies. The hair samples showed a significant decrease in cortisol, while previous work with blood showed no change, or an increase. Interestingly, Steudte and colleagues measured saliva at three time periods in the individuals diagnosed with GAD and healthy controls. While the hair results showed significant differences, no changes in salivary cortisol were observed. This finding supports the results of our model, showing that a single blood or saliva sample cannot always predict long-term changes in cortisol concentration. Both of these results are consistent with Suave and colleagues finding of no significant correlation of cortisol concentrations between a two month hair sample and a single serum sample ($r = 0.064$)(Sauvé et al. 2007). Our findings help to better characterize the abilities of different sampling techniques to assess instantaneous and chronic stress.

Our biokinetic model may be used in future studies attempting to better characterize cortisol changes in PTSD and GAD. The blood profiles produced from the stress disorder hair samples have different areas under the curve, indicating the presence of an alteration in cortisol secretion. This allows researcher to model the effects of flattening of the diurnal rhythm and increased pulsatile secretion on long-term cortisol accumulation in hair. Since both PTSD and GAD are surrounded by a wealth of literature documenting changes in cortisol, it is possible to begin to piece together conflicting studies. For example, the diurnal rhythm may be flattened in PTSD (Yehuda et al. 2003), which could lead to a reduction of overall cortisol production. However, if the cortisol response to stressors is increased (Gola et al. 2012), then it is possible for total cortisol production to be increased. In order to document the

cumulative effects of changes in both the diurnal rhythm and pulsatile secretion, multiple samples would need to be taken throughout a subjects normal day. Hospitalization with 24-hour blood draws would not reflect the increased pulsatility because the patient may not be exposed to stressors while being monitored. Therefore, hair cortisol in conjunction with out biokinetic model may provide some insight into potential causes for seemingly contradictory findings.

Since this biokinetic model was developed as part of a formative research project by the NCS, we also conducted a decision model analysis to quantify the added value of the information obtained by using this biokinetic model for maternal stress assessment. In most national longitudinal cohort studies sample collection is limited, therefore we quantified the expected value of information from a single blood, saliva and urine sample as well as a hair sample with and without the biokinetic model. The biokinetic model increased the overall score of using hair as a sampling technique by 17%, mostly because a hair sample is now able to provide some information on acute stress exposure and is related to blood concentrations. The relationship of hair cortisol to blood concentrations is especially important in the NCS because the developing fetus is exposed to a fraction of the concentration of cortisol in maternal blood. Single blood, saliva and urine samples have the potential to be unreliable measures of longer-term stress, as shown by the sensitivity analysis discussed above. Therefore, these sampling techniques are not recommended as stand-alone methods.

Validation of this model was done through comparisons to published literature; however future work measuring saliva, blood, urine and hair cortisol in the same individual would significantly improve our ability to validate this model. Overall, our biokinetic model provides an excellent framework to aid in the interpretation of cortisol samples from hair, saliva, blood and urine and increase the utility of hair samples in isolation of other methods. This analysis has important implication in future study design. In order for researchers to design the most cost-effective and meaningful study, the benefits and

drawbacks of using a single blood, saliva or urine sample compared to using a hair sample, with and without the biokinetic model for maternal stress assessment must be quantified. Our biokinetic model increases the value of a hair sample by 17%, through its ability to address acute and chronic stress and relate hair concentrations to more well-known and biochemically understood biomarker, such as blood. The ability to address acute stress peaks through the use of a relatively non-invasive hair sample greatly improves the ability of national longitudinal cohort studies to assess maternal stress.

Conclusion:

Here we show that our biokinetic model is sensitive to disease relevant changes in hair cortisol. We also show that a single blood sample cannot reflect slight, but long-term changes in cortisol secretion patterns that a one-time hair sample can. These factors contribute to the added of this biokinetic model. We use a decision model to show that hair, in conjunction with our biokinetic model is the optimal sampling protocol for national longitudinal cohort studies following pregnant women.

Thesis Conclusion:

In this biokinetic model, cortisol is secreted into the blood in a pulsatile manner and quickly binds to albumin and corticosteroid binding globulin (CBG). Free cortisol is reversibly metabolized by 11-β hydroxysteroid dehydrogenases to inactive cortisone and irreversibly conjugated by A-ring reductases. We used a net reaction rate to express the overall reduction in cortisol from metabolic processes in this model. Only free cortisol is excreted in urine and feces. We assumed cortisol in feces comes from salivary cortisol that is swallowed and excreted. Therefore, in this model free cortisol in the blood is transferred to saliva and excreted as feces. Finally, a small amount of free cortisol is incorporated into hair through passive diffusion in the blood.

To date, this is the first biokinetic model of cortisol that includes incorporation into hair. Since hair cortisol is a novel and useful biomarker for chronic stress, determining its relationship to more widely used stress biomarkers contributes to its validation and utility. Here, we show that hair cortisol can be used to develop blood pulse profile estimation. Additionally, we show how binding parameters influence peaks in the blood and the amount of free cortisol available for transfer to saliva and hair. Since concentrations of CBG change during pregnancy trimester, understanding how this influences free cortisol and hair cortisol concentrations is important for using hair as a biomarker for chronic stress during pregnancy.

While this model is useful for relating cortisol concentrations between any common biomarker compartments, characterizing the relationship between blood and hair cortisol concentrations is particularly relevant to maternal stress risks research. It has recently been suggested that cortisol may be involved in the mechanism through which maternal stress exerts toxic effects on the fetus (Field and Diego 2008), making it important that maternal stress assessment methods characterize the amount of cortisol reaching the fetus. Maternal hair samples are ideal for prenatal stress assessment because a single sample allows researchers to quantify nine months of chronic stress exposure. However, the fetus is not exposed to the concentration of cortisol in maternal hair, but rather the blood. It is estimated that 40-50% of variation in fetal cortisol is caused by changes in maternal blood cortisol concentration. Since the placenta contains high concentrations of 11-β HSD type 2 that convert cortisol to cortisone, only about 10-20% of maternal blood cortisol crosses the placental barrier. Fetal cortisol concentrations are typically much lower than maternal blood cortisol; therefore the 10-20% that crosses the placental barrier can cause fetal cortisol concentration to double (Gitau et al. 1998). By using this model, it is possible to estimate fetal exposure to maternal cortisol much more directly.

Additionally, cortisol pulses encode an important biological signal that is distinct from constant cortisol concentrations (McMaster et al. 2011; Young et al. 2004). Since hair analysis only shows a general elevation or reduction in cortisol levels, the biologically important signal may be missed. By using this model, we can create daily blood cortisol pulse profiles that relate acute secretion events to a long-term chronic stress measurement, such as a hair concentration. Because the variability and peaks in blood cortisol concentrations encode a unique biological signal, short-term, high concentration exposures may have different risk than long-term slightly elevated concentrations. By using this model, it is possible identify potential pulse heights associated with the area under the curve.

Furthermore, we tested the sensitivity of our biokinetic model, as well as blood and saliva samples to a long-term change in overall hair cortisol concentrations. The biokinetic model demonstrated the sensitivity for detecting slight, but long-terms changes in cortisol secretion in blood by predicting changes in blood cortisol profiles based on 10 pg/mg changes in hair cortisol concentration associated with PTSD and GAD. Hair cortisol levels have been shown to be increased by approximately 7 pg/mg with long-term unemployment (Dettenborn et al. 2010) and increased by about 30 mg/pg during the third trimester of pregnancy (Kirschbaum et al. 2009). The ability of our model to show increases in cortisol concentrations in blood profiles related to increases in hair cortisol is important to its utility as a tool for detecting elevated stress during pregnancy.

We also demonstrated that a single blood sample may not reflect these slight, but long-term changes recorded in hair because of the increased variability. The model was used to provide single blood sample estimates based on the daily cortisol profiles related to the reduction and elevation of hair cortisol recorded in individuals with PTSD and GAD. Since both PTSD and GAD hair samples showed cortisol levels contradicting years of blood and saliva studies, we show that it is possible for a single blood or saliva sample to contrast a hair sample. This is primarily because hair samples record long-term

averages, while blood, saliva and urine are affected by instantaneous pulses of cortisol. This finding is based on the premise than blood has greater within-individual variability than hair, since it reflects an instantaneous measurement. Hair reflects long-term cortisol accumulation and is therefore more indicative of between-individual variability. We also report that multiple blood samples were needed to have the same ability as a one-time hair sample to differentiate between disease states as a population level. This finding is important because it shows that small changes in daily cortisol secretion may be represented in hair, but may not be distinguishable in single blood samples because of the high variability of pulsatile cortisol secretion.

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long-term cortisol accumulation in hair. Since both PTSD and GAD are surrounded by a wealth of literature documenting changes in cortisol, it is possible to begin to piece together conflicting studies. For example, the diurnal rhythm may be flattened in PTSD (Yehuda et al. 2003), which could lead to a reduction of overall cortisol production. However, if the cortisol response to stressors is increased (Gola et al. 2012), then it is possible for total cortisol production to be increased. In order to document the cumulative effects of changes in both the diurnal rhythm and pulsatile secretion, multiple samples would need to be taken throughout a subjects normal day. Hospitalization with 24-hour blood draws would not reflect the increased pulsatility because the patient may not be exposed to stressors while being monitored. Therefore, hair cortisol in conjunction with out biokinetic model may provide some insight into potential causes for seemingly contradictory findings.

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