Measurement of Hair Cortisol Concentration in Pregnant Women and Children with Asthma to Assess Its Potential as a Biomarker of the Hypothalamic-Pituitary-Adrenal Axis

by

Laura Smy

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Pharmaceutical Sciences
University of Toronto

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Abstract

The recommended long-term treatment for asthma is inhaled corticosteroid (ICS) therapy, but research has shown that ICS use is associated with adrenal suppression. Cortisol is important physiologically and involved in fetal maturation and epigenetic programming. Hair cortisol has been used as a biomarker of the long-term effects on the hypothalamic-pituitary-adrenal (HPA) axis for a variety of psychiatric and medical conditions. This research hypothesizes that hair cortisol may be able to detect lower cortisol concentrations in pregnant women and children on ICS therapy and, therefore, have potential as a useful biomarker for possible adrenal suppression due to ICS use.

This retrospective cohort study collected hair samples from children and pregnant women with asthma, treated and not treated with ICS, and those without asthma. Hair samples were segmented, based on an average growth of 1 cm/month, and analyzed using a validated ELISA method. A children's pilot study analyzed the hair prior to and during ICS therapy for the same children. The proximal 3 cm hair segment was analyzed for the large cohort of children. Hair segments for preconception, trimesters 1-3, and postpartum were analyzed for the pregnant women. The results were compared within and among the groups, as appropriate.
The children's pilot study showed hair cortisol concentrations were twofold lower during ICS therapy (median 89.8 ng/g vs. 198.2 ng/g, p=0.0015, n=18). For pregnant women (n=118), asthma was a significant factor associated with lower hair cortisol. Women with asthma and consistent ICS use had significantly lower third trimester hair cortisol (47%, p=0.029) compared to Controls. The comparison of median hair cortisol concentrations among the three groups in the large cohort of children (n=460) was not significant. However, 5.3% of children on ICS therapy had hair cortisol below 2 ng/g compared to none in the control groups, which may indicate children with or at risk of adrenal suppression. The significant factors associated with hair cortisol in children were age, female sex, intranasal corticosteroid use, and BMI.

Hair cortisol analysis successfully detected significantly lower hair cortisol concentrations suggesting it is a useful biomarker of the HPA axis in pregnant women and children. The correlation of hair cortisol concentrations with a diagnosis of adrenal suppression or insufficiency and the potential impact of decreased maternal cortisol in women with asthma on perinatal outcomes remain to be determined.
Acknowledgments

It would be possible to describe absolutely everything scientifically, but it would make no sense. It would be without meaning, as if you described a Beethoven symphony as a variation of wave pressure.

– Albert Einstein –

I have completed my degree with the collective efforts of: my graduate advisory committee, Drs. Carolyn Cummins, Michael Thompson, and Bhushan Kapur; the staff of CPNDS and the DSEN collaborating clinics that aided with study participant recruitment and data collection; the undergraduate volunteers and summer students who helped with data collection and/or sample analysis; and the whole department of Clinical Pharmacology and Toxicology and Motherisk at the Hospital for Sick Children.

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To my fellow graduate students, where do I begin? Without you, I would not know how to be a graduate student. Your wisdom and experience, encompassing everything from the administrative side of graduate work, astute scientific observations, and technology savvy to what's hot with the young crowd have allowed me to metamorphose from a Medical Laboratory Technologist trying to find my way to a graduate student who has had fun amongst our challenges.

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To my family, I am so happy that I make you proud.
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<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADR</td>
<td>adverse drug reaction</td>
</tr>
<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>area-under-the-curve</td>
</tr>
<tr>
<td>β</td>
<td>beta, regression coefficient</td>
</tr>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>cm/mo</td>
<td>centimetre(s) per month</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DSEN</td>
<td>Drug Safety and Effectiveness Network</td>
</tr>
<tr>
<td>DSEN-SEARCH</td>
<td>Drug Safety and Effectiveness Network for active Surveillance and Evaluation of Adverse Reactions in Canadian Healthcare</td>
</tr>
<tr>
<td>GAD</td>
<td>generalized anxiety disorder</td>
</tr>
<tr>
<td>HFA</td>
<td>hydrofluoroalkane</td>
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<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
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<tr>
<td>ICS</td>
<td>inhaled corticosteroids</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>IgE</td>
<td>immunoglobulin-E</td>
</tr>
<tr>
<td>IQR</td>
<td>interquartile range</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LC-MS</td>
<td>liquid chromatography-mass spectrometry</td>
</tr>
<tr>
<td>ln</td>
<td>natural logarithm</td>
</tr>
<tr>
<td>LR</td>
<td>likelihood ratio</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>mo</td>
<td>month</td>
</tr>
<tr>
<td>OLS</td>
<td>ordinary least squares</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>OR&lt;sub&gt;adj&lt;/sub&gt;</td>
<td>adjusted odds ratio</td>
</tr>
<tr>
<td>p</td>
<td>p-value</td>
</tr>
<tr>
<td>p&lt;sub&gt;adj&lt;/sub&gt;</td>
<td>adjusted p-value</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>preconception</td>
</tr>
<tr>
<td>PP</td>
<td>postpartum</td>
</tr>
<tr>
<td>PSS</td>
<td>Perceived Stress Scale</td>
</tr>
<tr>
<td>PTSD</td>
<td>post-traumatic stress disorder</td>
</tr>
<tr>
<td>r</td>
<td>Pearson product-moment correlation coefficient</td>
</tr>
<tr>
<td>R&lt;sup&gt;2&lt;/sup&gt;</td>
<td>coefficient of determination</td>
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ρ  Spearman's rank correlation rho

ROC  receiver operating characteristic

rpm  revolutions per minute

RR  relative risk

$RR_{adj}$  adjusted relative risk

SD  standard deviation

$T_{1/2}$  elimination half-life

T1  first trimester

T2  second trimester

T3  third trimester

$T_{Max}$  Time to maximum plasma concentration

µg  micrograms

µL  microlitre

$V_d$  volume of distribution
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Chapter 1
Introduction

1.1 Statement of the Problem

Asthma is a common chronic respiratory condition that affects 2.4-million Canadians, with 1.8-million of those having active asthma requiring treatment with medication in the last 12 months (Public Health Agency of Canada, 2015; Statistics Canada, 2015). The recommended therapy for long-term control in patients aged six years and older is the use of inhaled corticosteroids (ICS) (Global Initiative for Asthma, 2015), of which there are five available in Canada: fluticasone propionate, budesonide, beclomethasone diopropionate, mometasone furoate, and ciclesonide. However, these medications are used "off-label" in children younger than that recommended by the manufacturer, sometimes in high doses (Thomas et al., 2006), and an average of 30% of pregnant women discontinue taking their medication in the first or second trimester likely due to the concerns about their safety during pregnancy from the patients and possibly their physicians (Enriquez et al., 2006; Schatz et al., 2005).

The effects of long-term ICS use in children are a concern and have not been fully ascertained. There is evidence that children who use budesonide for four to six years will be 1.2 cm shorter in adulthood than a placebo control group (Kelly et al., 2012), but it is not known if this extends to other ICS. Of greater concern is the potential for children to experience adrenal suppression when using ICS. Adrenal suppression, characterized by decreased production of cortisol rather than inadequate production of cortisol as for adrenal insufficiency, can occur through activation of the negative feedback associated with cortisol by the ICS mimicking cortisol in the body and binding to glucocorticoid receptors. Research shows that 65% of children who use ICS experience whole or partial suppression of the hypothalamic-pituitary-adrenal (HPA) axis (Zollner et al., 2012). This suppression could manifest in adrenal crisis should the child abruptly discontinue their ICS or experience a stressful event, such as trauma or infection, and not have the adrenal capacity to respond by releasing the required amount of cortisol (Lipworth, 1999). Children that experienced adrenal crisis more often used fluticasone propionate than any other ICS (Todd et al., 2002), but the potential for adrenal suppression is likely present with any ICS, especially when used in high enough doses.
For pregnant women with asthma who require ICS therapy for proper disease control, discontinuing their ICS early in pregnancy may result not only in poor asthma control, but also in poor pregnancy outcomes. Uncontrolled asthma or exacerbations early in pregnancy are associated with an increased risk of small for gestational age or low birth weight neonates, and preterm birth (Bakhireva et al., 2008; Murphy et al., 2011). Treatment with ICS significantly reduces the risk of acute asthma attacks requiring hospitalization in pregnant women (Stenius-Aarniala et al., 1996). However, it is unknown if pregnant women with asthma treated with ICS have a risk for adrenal suppression similar to children on ICS therapy. Cortisol plays an important role in pregnancy, with a surge in the third trimester participating in fetal maturation and epigenetic programming of numerous biological systems, the most well-known being the respiratory system (Liggins, 1994; Moisiadis & Matthews, 2014b).

For both children and pregnant women, finding an easy and effective way to detect adrenal suppression prior to the occurrence of a crisis or poor perinatal outcome would be beneficial. The matrices currently used for cortisol measurements are saliva, serum, or 24-hour urine. Over the last fifteen years, the measurement of cortisol in head hair has emerged as a viable alternative. This thesis research will assess whether hair shows lower cortisol concentrations in children and pregnant women on ICS therapy and, therefore, has potential as a useful biomarker for possible adrenal suppression due to ICS use. Ultimately, the ability to easily detect adrenal suppression using hair analysis may improve patient healthcare and reduce healthcare costs by reducing medical visits due to previously undetected adrenal suppression.
1.2 Study objectives

Objective 1: To measure hair cortisol concentrations in pregnant women with and without asthma as a potential biomarker of the HPA axis.

Objective 2: To assess any dose or type effects of ICS therapy on hair cortisol concentrations in pregnant women with asthma.

Objective 3: To measure hair cortisol concentrations in children with and without asthma as a potential biomarker of the HPA axis.

Objective 4: To assess any dose or type effects of ICS therapy on hair cortisol concentrations in children with asthma.

1.3 Study hypotheses

Hypothesis 1: Pregnant women using ICS will exhibit lower hair cortisol concentrations than those with asthma not using ICS and those without asthma.

Hypothesis 2: Children using ICS will exhibit lower hair cortisol concentrations than those with asthma not using ICS and those without asthma.
Chapter 2
Literature Review

2.1 What is a biomarker?

The term *biomarker*, from *biological marker*, is used quite prevalently in the research literature, but a specific definition has not been consistently applied. This ambiguity has led to the formation of the National Institutes of Health Biomarkers Definitions Working Group. Historically, biomarkers include a wide range of physiological or chemical indicators that may be measured from the body or using its blood and tissues, such as blood pressure, hemoglobin, or blood glucose, to assess biological parameters. Biomarkers such as these fall under the definition of "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working Group, 2001). Biomarkers defined as such may further be diagnostic of disease presence or stage, prognostic of disease progression, or used for predicting or monitoring therapeutic interventions (Biomarkers Definitions Working Group, 2001).

Because biomarkers may indicate responses to therapeutic interventions, clinical pharmacology and toxicology research has evolved to use biomarkers to assess the efficacy and/or toxicity of pharmaceuticals in drug development and evaluation (Figure 1). In some instances, biomarkers have been found to be good surrogates for clinical endpoints, which are defined as "a characteristic or variable that reflects how a patient feels, functions, or survives" (Biomarkers Definitions Working Group, 2001). As a result, some biomarkers are called surrogate endpoints, and while all surrogate endpoints are biomarkers, not all biomarkers are surrogate endpoints.

One form of validation of a biomarker is the assessment of assay performance characteristics such as sensitivity, specificity, and reproducibility. However, "evaluation" is the term considered most appropriate for the assessment of a biomarker as a surrogate endpoint because when surrogate endpoints are considered "validated" it implies generalizability to other diseases or interventions that would also affect that surrogate endpoint (Biomarkers Definitions Working Group, 2001). The surrogate endpoint must be proven to accurately, and reliably,
predict either clinical harm or benefit (Strimbu & Tavel, 2010). Major benefits of using biomarkers as surrogate endpoints are that they may reduce the duration and cost of clinical trials, as well as reduce potential harm to study participants by indicating if an intervention should be stopped (Strimbu & Tavel, 2010).

**Figure 1.** Biomarker Definitions Working Group schematic of the relationship between biomarkers for efficacy, toxicity, and as surrogate endpoints in clinical research. (Reprinted from *Clinical Pharmacology and Therapeutics*, 69, Biomarkers Definitions Working Group, Biomarkers and surrogate endpoints: preferred definitions and conceptual framework, 89-95, Copyright (2001), with permission from John Wiley and Sons.)

Hair cortisol concentration, in the context of this thesis, is not being evaluated as a surrogate endpoint but rather assessed as a potential biomarker of the hypothalamic-pituitary-adrenal axis for future use as a biomarker of toxicity with regard to the suppressive effects of ICS on the hypothalamic-pituitary-adrenal axis (Figure 1). As such, it would be considered a "response biomarker" that can be more specifically classified either as a "safety biomarker to monitor adverse effects on biology" or a "pharmacodynamics biomarker as an indicator of intended drug activity" (Amur et al., 2015).
2.2 Asthma

2.2.1 Brief overview of asthma

Asthma is a chronic inflammatory lung condition affecting between 235-334 million people worldwide that is frequently under-diagnosed and undertreated (World Health Organization, 2011; The Global Asthma Network, 2014). In Canadian children aged 0 to 11 years, the prevalence of asthma increased significantly from 11% in 1994-95 to 13% in 2000-01, but this increase was predominantly for those with mild symptoms (Garner et al., 2008). Further, for this age group, asthma is more common in boys than girls (16.1% vs. 10.5%, in 2000/01). However, the prevalence of asthma dropped significantly by 2008-09 (for children aged 2 to 7 years) (Thomas, 2010). Asthma remained more common in boys than girls in 2008-09 (11.4% vs. 7.9%). The gender difference shifts by the late teens with more females being affected than males for all ages thereafter (Statistics Canada, 2010).

The prevalence of asthma in pregnant women, as in children, appears to be on the rise. It was reported to be as low as 0.43% in the Canadian population for the years 1989-1996 (Wen et al., 2001). Alexander et al. (1998) assessed pregnant women in Nova Scotia and reported the rate increased from 4.8% in 1991 to 6.9% in 1993. In the United States in 2001, the prevalence was reported to be 8.4% (Kwon et al., 2003), but then was reported as 6.5% for the period of 1995 to 2003 (Enriquez et al., 2007). Significantly, for approximately one-third of women, asthma worsens during pregnancy, whereas it improves for one-third and remains unchanged for the remaining third (Clifton et al., 2004).

Asthma symptoms vary in severity and are exacerbated by a variety of inflammatory stimuli including allergic triggers, such as mould, dust mites, pollen, or animal dander; or non-allergic triggers, such as aspirin, cold, dry air, viral respiratory infections, or air pollution (Asthma Society of Canada, 2013). Maternal smoking, smoking by a parent in the household, and exposure to respiratory viruses in the early years are risk factors increasing the number of children diagnosed with or found to have asthma-like symptoms, such as wheezing and whistling in the lungs (Garner & Kohen, 2008; Wahn, 2013). For children in non-smoking homes, the presences of pets may be a reason for an increase in asthma-like symptoms (Garner & Kohen, 2008). Asthma is typically characterized by mucous overproduction, cellular infiltration, structural remodelling, and constriction of the bronchioles resulting in shortness of breath,
wheezing, coughing, and chest tightness (Asthma Society of Canada, 2013; Lemanske & Busse, 2010). The molecular mechanism underlying the development of asthma varies between children and adults and will be presented in the next sections.

### 2.2.2 Overview of the molecular mechanism of asthma in adults

Exposure to allergens or viral infections triggers the network of molecular processes that result in constriction of the airways, mucus overproduction, and airway hyperreactivity. In the allergic response, the antigen is captured by the antigen presenting cell, which is a mast cell covered in immunoglobulin-E (IgE) molecules, and presented to the T-helper cell. This triggers a cell-mediated immune response. Additionally, the IgE on the mast cell surface form complexes, causing degranulation with release of histamine and other inflammatory agents (Stanfield et al., 2008). Histamine is a potent mediator of bronchoconstriction by causing smooth muscle contraction in the airways (Triggiani et al., 2013; Stanfield et al., 2008).

In the chronic phase of asthma (Figure 2), the cell-mediated immune response mainly involves the T-helper cell type 2 (Th2) (Hiemstra et al., 2015). The Th2 cell releases cytokines, such as interleukin (IL)-3, -4, -5, and -13, that recruit mast cells, eosinophils, and fibroblasts, and causes further production of IgE (Triggiani et al., 2013; Lloyd et al., 2009). Wardlaw et al. (1988) determined there are significantly higher numbers of eosinophils, mast cells, and epithelial cells in the bronchioles of individuals with airway hyperreactivity compared to those without airway hyperreactivity, and that airway hyperreactivity occurs when eosinophils are activated (not just present), though this does not explain all hyperreactivity. They proposed that airway smooth muscle susceptibility is also a contributor. The activated eosinophils release major basic protein, which is damaging to airway epithelia (Wardlaw et al., 1988), as well as histamine and other agents from their granules. The epithelial damage results in increased permeability, altered ion transport, impaired anti-oxidant activity through reactive oxygen or nitrogen species, increased goblet cell activity that releases mucus, and impaired innate response to viruses (Hiemstra et al., 2015). Further, macrophages release tumour necrosis factor-α, which, like Th2 cells, causes histamine release from mast cells and neutrophils, eosinophil recruitment (Berry et al., 2007), and release of IL-4 and -13 that stimulates the production of more IgE (Triggiani et al., 2013).
Figure 2. Molecular mechanisms contributing to asthma during acute, chronic, and remodelling phases of the disease. (Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology (Gern & Busse, 2002), copyright (2002).)

With continued chronic inflammation, airways undergo remodelling (Figure 2), which leads to persistently reduced lung function and increased hyperreactivity (Triggiani et al., 2013). The airway epithelia contribute to the chronic inflammation, as they are also a source of inflammatory mediators and growth factors (Lemanske & Busse, 2010). Remodelling is characterized by epithelial damage, smooth muscle hyperplasia and hypertrophy, mucus gland hyperplasia, reticular basement membrane thickening, and fibroblast activation with collagen deposition (Triggiani et al., 2013; Gern & Busse, 2002; Lemanske & Busse, 2010).

It has not been fully determined whether chronic asthma is a result of ineffective anti-inflammatory or wound healing responses, but IL-10 and transforming growth factor-β, from regulatory T-cells are important in controlling the immune response (Lloyd & Hawrylowicz, 2009). Individuals with asthma have lower levels of IL-10 mRNA and protein production, and those with a polymorphism in the IL10 promoter, resulting in downregulation of IL-10, tend to have more severe asthma (Lloyd & Hawrylowicz, 2009).
2.2.3 Overview of asthma molecular mechanism in children

Diagnosis of asthma in children younger than 6-years-old is difficult due to the variety of differential diagnoses possible for wheezing in young children, belief that asthma does not occur in young children, or the inability to perform lung function tests before the age of 6 years (Ducharme et al., 2014; National Heart Lung and Blood Institute, 2007). Still, it has been determined that elements of inflammation and remodelling similar to adults occur in childhood asthma pathophysiology, but remodelling may or may not include reticular basement membrane thickening (Lemanske & Busse, 2010; Papadopoulos, 2013). The cell-mediated immune response is activated in children, but may not always be due to eosinophils (Papadopoulos, 2013). In a comparison of healthy children and children with a wheeze, 1- to 2-years-old, those with a wheeze had an increase in cellular infiltration in their bronchioles. Similar to adults, there was an increase in epithelial cells; but unlike adults, there was also an increase in lymphocytes and neutrophils (Krawiec et al., 2001). Remodelling is seen in preschool-aged children, the same as in older children and adults, but this does not occur in infants (Papadopoulos, 2013). Reeves et al. (2015) found that children aged 10- to 11-years-old, similar to adults, also experienced deposition of collagen type I, and this was due to an increase in transforming growth factor-β, which elicited fibroblast transition to myofibroblasts.

2.2.4 Effects of asthma on pregnancy and perinatal outcomes

Asthma in pregnancy has been associated with a number of poor perinatal outcomes. A Norwegian birth registry study comparing the outcomes of pregnancy in 1967-68 for women with bronchial asthma (information regarding asthma severity and treatment, if any, was unavailable) versus healthy pregnant women found that there was a significant increase in the incidence of hyperemesis gravidarum, vaginal hemorrhage, toxemia of pregnancy, prematurity (delivery < 37 weeks of gestation), and low birth weight (birth weight < 2500 g) (Bahna & Bjerkedal, 1972). Canadian and American database studies have found asthma was significantly associated with preterm labour, hypertensive disorders, gestational diabetes, antepartum hemorrhage, membrane-related disorders (i.e., premature rupture of membranes and infection of amniotic cavity), cesarean section, low birth weight, small for gestational age (<10th percentile for gestational age and sex), and postpartum hemorrhage (Wen et al., 2001; Enriquez et al., 2007). Increasing the power of the available evidence, the meta-analysis by Murphy et al. (2011)
found a significant risk for low birth weight (relative risk (RR) = 1.46, CI$_{95\%}$ 1.22-1.75), small for gestational age (RR = 1.22, CI$_{95\%}$ 1.14-1.31), preterm delivery (RR = 1.41, CI$_{95\%}$ 1.23-1.62), and preeclampsia (RR = 1.54, CI$_{95\%}$ 1.32-1.81), although there was significant heterogeneity among the studies for all of these analyses. With respect to malformations, one study found an increased risk of malformations involving the nervous system (excluding spina bifida), respiratory system, and digestive system (Blais et al., 2010). But, in a subsequent study, the authors determined their cohort may have been biased due to over-representation of women who were "publicly insured with social welfare" (Blais et al., 2015). Finally, a 2013 meta-analysis also found a weak association between asthma and congenital malformations (inclusive of major and minor) (RR = 1.11, CI$_{95\%}$ 1.02-1.21) and oral cleft (RR = 1.30, CI$_{95\%}$ 1.01-1.68) (Murphy et al., 2013). However, when only major malformations were assessed, asthma was not associated with an increased risk (Blais et al., 2008; Murphy et al., 2013; Tata et al., 2008).

Uncontrolled asthma in pregnancy is associated with some of the same poor perinatal outcomes as asthma, although they may become more severe. For example, a case report described the development of cerebral palsy and diplegia in a 4-month old baby that experienced hypoxic necrosis of the periventricular area of the brain (Sugai et al., 2006). In their meta-analysis, Murphy et al. (2011) showed an increased risk of preterm delivery (RR = 1.50, CI$_{95\%}$ 1.28–1.75) in ten studies of women without active management of their asthma, which was not present in the analysis of five studies with active asthma management (RR = 1.07, CI$_{95\%}$ 0.91-1.26). Included in the Murphy et al. meta-analysis, but worth mentioning specifically, is the study by Bakhireva et al. (2008), in which pregnant women who had fair to poor asthma control (specifically in first trimester) or who were hospitalized for their asthma, had a significantly higher incidence of preterm delivery compared to those with adequate asthma control or those not hospitalized (11.4% vs. 6.3% and 16.4% vs. 7.6%, respectively, p = 0.02 for both comparisons).

With regards to asthma exacerbations during pregnancy, Namazy et al. (2013) examined the effects of exacerbations on perinatal outcomes and found a significant increase in the risk of low birth weight (RR = 3.02, CI$_{95\%}$ 1.87-4.89), but no increased risk of preterm delivery or small for gestational age. In a recent study, while there were no significant findings for preterm labour, preeclampsia, placenta previa, or gestational diabetes in women who experienced exacerbations during pregnancy, there was a significant increase in the rate of cesarean section (27.1% vs.
18.9%, p < 0.001) (Kim et al., 2015). The comparison group used for both of those studies was pregnant women with asthma who did not experience an exacerbation. Additionally, women who experienced asthma exacerbations in first trimester may have a slightly increased risk of having a baby with any congenital malformation (major and minor) (adjusted odds ratio (OR_{adj}) = 1.48, CI_{95%} 1.04-2.09), but not a major malformation (OR_{adj} = 1.32, CI_{95%} 0.86-2.04) (Blais & Forget, 2008). However, as previously mentioned, the authors acknowledge that their cohort was biased by over-representation of women with a lower socioeconomic status (Blais et al., 2015).

There is valid concern for fetal hypoxia in pregnant women with asthma. Babies born to women with asthma were found to be hypoxic significantly more often than those born to healthy pregnant women (1.6% vs. 0.7%) (Bahna & Bjerkedal, 1972). Women with asthma, generally, were found to have cesarean sections ~1.5% of the time due to fetal distress or uterine hypoxia (Kim et al., 2015). Fetal or neonatal hypoxia can cause neonatal death or cerebral palsy, as seen in the case study. There is an increased risk of neonatal death for women with asthma as found by Bahna & Bjerkedal (1972) (18.5/1000 vs. 8.0/1000) and the meta-analysis by Murphy et al. (2013) (RR = 1.49, CI_{95%} 1.11-2.00, I^2 = 0%).

The mother in the case report mentioned above had decided to discontinue her medication for fear of harming her baby. Research shows that 23% to 36% of pregnant women discontinue their ICS therapy in first or second trimester (Enriquez et al., 2006; Schatz & Liebman, 2005). A Korean database study of health insurance claims found a significant decrease (~50%) in prescriptions of ICS during pregnancy and for the two years afterwards compared to the year prior to pregnancy, except when the women experienced acute asthma exacerbations (Kim et al., 2015). ICS prescription rates for women who experienced acute exacerbations were more than double that for women who did not experience acute exacerbations (54.9% vs. 22.1%, p < 0.001). From this evidence, it appears that proper asthma control using the appropriate therapy throughout pregnancy, including first trimester, should be the goal to prevent exacerbations or uncontrolled asthma that may lead to poor perinatal outcomes.

2.2.5 Uncontrolled asthma in children

Asthma is associated with an increased risk of serious morbidity, and rarely mortality (Johnston & Sears, 2006; The Global Asthma Network, 2014), that may be even further
increased if the disease is left uncontrolled or untreated. Research indicates that, for children, the number of previous acute care visits (to the emergency room, urgent care centre, or an inpatient admission) in the last year increases the odds of a future acute care visit in an increasing manner. One previous visit had an OR$_{adj}$ of 3.60 (CI$_{95\%}$ 3.14-4.12) for future visits, but five previous visits had an OR$_{adj}$ of 58.71 (CI$_{95\%}$ 24.34-141.61) (Hanson et al., 2015). Some of the main reasons for uncontrolled asthma in children include inaccurate assessment, nonadherence to therapy, unresponsiveness to therapy, poor technique, and lack of a written asthma action plan (Maykut et al., 2010). The majority (80%) of asthma exacerbations in children are brought on by respiratory viral infections. Treatment with ICS for the prevention of exacerbations during respiratory viral infections is controversial, but the level of asthma control prior to exposure to respiratory viral infections and other asthma triggers likely plays a role in the outcome (Johnston & Sears, 2006). In their review, O’Byrne et al. (2013) discuss how children with uncontrolled asthma have reduced physical activity, have a higher BMI and percentage body fat, are reported to have a learning disability twice as often, experience depression and anxiety more frequently, and are prone to lung infections more often compared to children with good asthma control. Unfortunately, the research supporting these findings is not without limitations or controversies, such as whether obesity precedes asthma or the other way around. Nevertheless, evidence suggests that proper asthma control could reduce the sequelae of this common chronic condition, which would be beneficial for the patients, families, and healthcare system.

2.3 Treatment of Asthma

2.3.1 Guidelines for the treatment of asthma with inhaled corticosteroids

There is a recommended step therapy for asthma management with a goal of achieving proper asthma control (Figure 3). Step therapy encompasses increasing medications in an ordered fashion to gain control of asthma symptoms, but also to decrease them, when possible (though not during pregnancy), while still maintaining control (Global Initiative for Asthma, 2015). One of the main components of asthma management is to remove or avoid potential triggers (National Heart Lung and Blood Institute, 2007). The initial step involving medication is the use of short-acting $\beta_2$-adrenergic receptor agonists, such as salbutamol, which are known as "reliever" medications because of their fast bronchodilating action. The recommended therapy for long-term asthma control is the use of ICS for children 0- to 4-years-old who meet certain
criteria, and for all patients aged 5-years and older with persistent asthma, including pregnant women (National Heart Lung and Blood Institute, 2007; National Asthma Education and Prevention Program Asthma and Pregnancy Working Group, 2005). Budesonide is the preferred ICS for use during pregnancy due to the evidence supporting its safety (National Asthma Education and Prevention Program Asthma and Pregnancy Working Group, 2005), but if budesonide is not effective in controlling a woman's asthma during pregnancy, use of another ICS that controls her asthma adequately is preferred. ICS use has a host of clinical effects aside from anti-inflammatory effects, such as reducing exacerbations, improving asthma control and quality of life for the patient, and reducing emergency visits, hospitalization, and death due to asthma (National Heart Lung and Blood Institute, 2007).

When treating asthma, it is important to consider the patient's age due to the lack of evidence available for the use of different ICS in younger age groups and because the course of the disease may change over time. In children younger than 5-years-old, the disease may often go untreated due to misdiagnosis given the large number of differential diagnoses available, such as foreign body aspiration, lower respiratory infections, or heart disease, and the inability to properly conduct diagnostic tests in this age group (Ducharme et al., 2014; National Heart Lung and Blood Institute, 2007). Generally, clinical trials have not determined the safety of ICS in children under 4- to 6-years-old with the exception of the fluticasone metered dose inhaler, which can be used in children as young as 12 months (Table 1) (Takeda Canada Inc., 2012; Valeant Canada LP, 2013; GlaxoSmithKline, 2014; Merck Canada Inc., 2015; AstraZeneca Canada Inc., 2015).
The latest ICS dosage categories for children and adults (inclusive of pregnant women) are shown in Table 1. For children, low-dose ICS are recommended at first and, with each subsequent step of the management continuum, the ICS dose increases with eventual addition of add-on therapy, such as a long-acting β2-adrenergic receptor agonist (National Heart Lung and Blood Institute, 2007). The use of high doses of ICS (equivalent to >400 μg/d of beclomethasone-hydrofluoroalkane (HFA), *i.e.* beclomethasone-HFA equivalent) in children is not recommended unless absolutely necessary and has very little to no adequate supporting evidence (Ducharme et al., 2015; Lougheed et al., 2012; National Heart Lung and Blood Institute, 2007).
Table 1. Inhaled corticosteroid dosage categories for children and adults.

<table>
<thead>
<tr>
<th>Corticosteroid</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beclomethasone dipropionate HFA</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤ 200</td>
<td>201-400</td>
<td>&gt; 400</td>
<td>≤ 250</td>
<td>251-500</td>
<td>&gt; 500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Budesonide</td>
<td>≤ 400</td>
<td>401-800</td>
<td>&gt; 800</td>
<td>≤ 400</td>
<td>401-800</td>
<td>&gt; 800</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciclesonide</td>
<td>100</td>
<td>200</td>
<td></td>
<td>≤ 200</td>
<td>201-400</td>
<td>&gt; 400</td>
<td>≤ 200</td>
<td>201-400</td>
<td>&gt; 400</td>
</tr>
<tr>
<td>Fluticasone propionate</td>
<td>50-125</td>
<td>200-250</td>
<td></td>
<td>≤ 200</td>
<td>201-400</td>
<td>&gt; 400</td>
<td>≤ 250</td>
<td>251-500</td>
<td>&gt; 500</td>
</tr>
<tr>
<td>Mometasone Furoate</td>
<td>≤ 100</td>
<td>≤ 100</td>
<td></td>
<td>200</td>
<td>≥ 400-800</td>
<td>&gt; 800</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Ducharme et al. (2015), Lougheed et al. (2012), and Thomas et al. (2006). Shaded areas indicate these dosages are not approved by Health Canada or the manufacturer for that age group with the exception of beclomethasone dipropionate HFA, which is approved for use in children ≥ 5-years-old, and mometasone furoate, which is approved for children ≥ 4-years-old for the dry powder inhaler (Merck Canada Inc., 2015).

2.3.2 Corticosteroid pharmacodynamics and molecular mechanism

The term corticosteroid encompasses both the endogenous steroid hormones found in our bodies (e.g., cortisol), and a class of drugs, such as the glucocorticoids discovered in the 1940s (Coutinho & Chapman, 2011). They have been invaluable in treating a variety of conditions of varying severity from leukemia to skin lesions due to their immunosuppressive effects. Inhalation for the treatment of asthma is generally considered safe and it is thought that inhaled corticosteroids are not absorbed into the systemic circulation, but this appears to be an incorrect assumption as some of the systemic side effects seen with high dose or long-term use of systemic corticosteroids have been documented (Lipworth, 1999). For some people, corticosteroid ADRs may outweigh their beneficial actions. Common adverse effects include gastrointestinal bleeding, ulceration or perforation, delayed puberty, short stature in children, skin thinning, immune suppression, osteoporosis or osteonecrosis, muscle atrophy, diabetes, glaucoma or cataracts, or even adrenal suppression or insufficiency, which is the focus of this thesis (Lowenberg et al., 2008; Rhen & Cidlowski, 2005; Stahn et al., 2008).
The molecular effects of corticosteroids are numerous and complex and can be broken down into two main categories: genomic and non-genomic (Figure 4). Corticosteroid genomic mechanisms require time to take effect, ranging from minutes to hours or days (Borski, 2000; Liu et al., 2005). Corticosteroids will activate gene expression of anti-inflammatory proteins and repress pro-inflammatory gene expression, referred to as transactivation and transrepression, respectively (Rhen & Cidlowski, 2005). The lipophilic nature of corticosteroids allows them to cross cellular plasma membranes to interact with the cytosolic glucocorticoid receptor (GR). By binding to the cytosolic GR and subsequent translocation of the complex to the nucleus, corticosteroids activate the production of proteins such as annexin-1, mitogen-activated protein kinases (MAPK) phosphatase I, and an inhibitor of nuclear factor-κB (IκB) (Lowenberg et al., 2008; Marwick et al., 2010; Rhen & Cidlowski, 2005). Genomic transactivation is theorized to be responsible for most of the adverse effects of corticosteroids (Lowenberg et al., 2008; Marwick et al., 2010). Corticosteroids carry out transrepression also by binding to the cytosolic GR that consequently interferes with the binding of nuclear factors important in inflammation, such as nuclear factor-κB and activator protein 1 (Lowenberg et al., 2008; Marwick et al., 2010; Rhen & Cidlowski, 2005).

In contrast, rapid actions of corticosteroids were noticed as early as 1942 by Hans Seyle and were described by Grosman and Jensen in 1984 (Grosman et al., 1984; Losel et al., 2003). These rapid actions were later determined to be non-genomic and have been studied quite extensively in neural cells (Liu et al., 2005; Zhou et al., 2008), but their mechanisms are still poorly understood (Haller et al., 2008). Key criteria defining a non-genomic mechanism include a short time frame of action (from seconds to minutes), and that the outcome is not susceptible to inhibitors of transcription, protein synthesis, or receptor blockade (Haller et al., 2008). It is postulated that the therapeutic, non-genomic effects occur from the use of high dose corticosteroids, when cytosolic GRs are saturated, and are mediated through membrane-bound GRs or by physicochemical interactions with cell membranes (Liu et al., 2005). Two examples of non-genomic effects of corticosteroids are the decrease in phosphorylation of MAPK p38 impairing T-cell signaling pathways (Spies et al., 2010), and the inhibition of degranulation in mast cells (Zhou et al., 2008), both actions that are beneficial for the treatment of asthma.
2.3.3 Inhaled corticosteroid pharmacology and systemic exposure

There are five ICS licensed for use in Canada: fluticasone propionate, beclomethasone dipropionate, mometasone furoate, ciclesonide, and budesonide. The structures of each ICS are shown in Figure 5 along with the structure for cortisol. There are four main parts of the steroid molecule, shared by cortisol and each ICS, that are responsible for the corticosteroids properties (Shaw, 2002). The pharmacokinetic characteristics of each ICS are listed in Table 2. Generally, the ICS are not adequately studied for use in pregnant women or children less than 4- to 6-years-old.
Figure 5. Chemical structures of the five available ICS in Canada and cortisol for comparison. The substitutions highlighted in yellow on cortisol are the components that are responsible for the corticosteroid properties. (Structures prepared using ChemDraw Professional, v.15.0)
### Table 2. Overview of the pharmacological characteristics of each ICS available in Canada.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Beclomethasone dipropionate/Beclomethasone-17-monopropionate</th>
<th>Budesonide</th>
<th>Fluticasone propionate</th>
<th>Ciclesonide/21-desmethylpropionylciclesonide</th>
<th>Mometasone furoate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approved Age for Use (years)</td>
<td>≥5</td>
<td>≥3 months (nebulizer) ≥6 (dry powder inhaler)</td>
<td>≥4 (Diskus®) ≥1 (puffer)</td>
<td>≥6</td>
<td>≥4</td>
</tr>
<tr>
<td>Studied in Pregnant Women?</td>
<td>not adequately studied</td>
<td>no risk of malformations</td>
<td>not adequately studied</td>
<td>not adequately studied</td>
<td>not adequately studied</td>
</tr>
<tr>
<td>Usual Dosage</td>
<td>100 – 800 µg/day</td>
<td>200 – 1600 µg OD</td>
<td>50 – 1000 µg BID</td>
<td>100 – 800 µg/day</td>
<td>200 – 800 µg OD</td>
</tr>
<tr>
<td>Systemic Bioavailability (%)</td>
<td>1 – 4 (inhaled) / ~62 (inhaled + oral)</td>
<td>49 (inhaled dry powder) 39 (inhaled aerosol) 11 (oral)</td>
<td>100 (inhaled aerosol) 18 (inhaled dry powder) &lt; 1 (oral)</td>
<td>&lt; 1 (oral), 22 (inhaled / &lt; 1 (oral), 63 (inhaled) 10 (inhaled, with asthma) 16 (inhaled, healthy) &lt; 1 (oral)</td>
<td></td>
</tr>
<tr>
<td>Protein Binding (%)</td>
<td>87% / 98.4%</td>
<td>85-90%</td>
<td>90-91%</td>
<td>99% / 98%</td>
<td>98.99%</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>0.5 / 2.7</td>
<td>2.8 (adult) 1.9 (child)</td>
<td>8</td>
<td>&lt;1 / 5.2 – 6.7</td>
<td>4.5</td>
</tr>
<tr>
<td>Time to Max. Plasma Conc. (h)</td>
<td>0.3</td>
<td>0.5</td>
<td>N/A</td>
<td>~1</td>
<td>1 – 2.5</td>
</tr>
<tr>
<td>Vd</td>
<td>20 L (by IV) / 424 L (by IV)</td>
<td>3 L/kg</td>
<td>4.2 L/kg (by IV) 282 L (with asthma)</td>
<td>2.9 L/kg / 12.1 L/kg</td>
<td>152 L (by IV)</td>
</tr>
<tr>
<td>Clearance (systemic, L/h)</td>
<td>150 / 120</td>
<td>81</td>
<td>6.6</td>
<td>152 / 228</td>
<td>N/A</td>
</tr>
<tr>
<td>GR Binding Affinity*</td>
<td>0.4 / 13.5</td>
<td>9.4</td>
<td>18.0</td>
<td>0.12 / 12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Polar Surface Area (Å²)</td>
<td>106.97</td>
<td>93.06</td>
<td>80.67</td>
<td>99.13</td>
<td>93.81</td>
</tr>
<tr>
<td>LogD at pH 7.4</td>
<td>4.07</td>
<td>3.20</td>
<td>3.73</td>
<td>6.13</td>
<td>3.59</td>
</tr>
<tr>
<td>Comments</td>
<td>• approved since 1972 • highly lipophilic • rapid extra-hepatic metabolism by esterases to active metabolite beclomethasone-17-monopropionate (17-BMP), and inactive beclomethasone-21-monopropionate (21-BMP) and beclomethasone</td>
<td>• approved since 1990 • potent glucocorticoid activity • moderately lipophilic, • ~90% biotransformation on first pass metabolism, mainly by CYP3A4 • the metabolites have &lt;1% activity of budesonide • only ICS considered a Category B drug for use in pregnancy</td>
<td>• approved since 1998 • highly potent and lipophilic • rapidly and prolonged absorption through the lungs • extensive first-pass metabolism by CYP3A4 to inactive 17-(beta)-carboxylic acid metabolite • excreted ~100% in the feces</td>
<td>• highly lipophilic • approved since 2006-2007 • prodrug converted by esterases in the lungs to C21 des-methylpropionylciclesonide, a potent glucocorticoid metabolite • metabolite is metabolized in the liver by CYP3A4 (mainly) and CYP2D6</td>
<td>• highly lipophilic, • biphasic elimination • extensively metabolized by CYP3A4 in the liver or to multiple metabolites that are not all characterized • excreted mainly in the feces (~92%)</td>
</tr>
</tbody>
</table>

Although inhalation of ICS is considered topical application and it is assumed there is minimal systemic absorption (National Heart Lung and Blood Institute, 2007), many pharmacologic factors may influence whether the ICS enter the systemic circulation. The ICS are very lipophilic molecules as shown by their high logD values at pH 7.4 (logD is equivalent to logP at a specified pH) (Table 2), which allows them to easily pass through cell plasma membranes in the lungs to achieve their topical action. Their lipophilicity also results in large volumes of distribution (V_d) (Table 2). Although many of the ICS undergo extensive first-pass metabolism, when entering the body through the lungs, they will be in the systemic circulation for a time before being metabolized by the liver enzymes (predominantly cytochrome P450 3A4 (CYP3A4)), unlike orally administered drugs that pass through the portal circulation and will be metabolized immediately after gut absorption but before entering the systemic circulation. Further, drug interactions or inter-individual variations in the enzyme kinetics of CYP3A4 and esterases (beclomethasone dipropionate and ciclesonide are prodrugs converted to their active metabolite by esterases) will result in increased or decreased susceptibility to the ICS (National Heart Lung and Blood Institute, 2007). Theoretically, once in the systemic circulation, with their large V_d, increased affinity for the GR, and variable metabolism, the ICS would be able to affect other cells and systems in the body. This accessibility to the systemic circulation is a likely explanation for the ADRs seen with ICS use, such as adrenal suppression.

2.3.4 Inhaled corticosteroid use in pregnant women

The content in this section has been previously published in Smy, L., Chan, A. C., Bozzo, P., & Koren, G. (2014). Is it safe to use inhaled corticosteroids in pregnancy? Can Fam Physician, 60(9), 809-12, e433. Minor changes have been made for use in this thesis. (Used with permission from Canadian Family Physician.)

Currently, budesonide is the ICS most studied during pregnancy. Studies from the Swedish birth and health registries reported no significant increase in congenital malformations in more than 10 000 prenatally-exposed infants (Kallen et al., 2007). As well, no adverse outcomes were found in terms of gestational age, birth weight, birth length, or stillbirths (Norjávaara et al., 2003). No significant associations have been found between the use of other ICSs during pregnancy and adverse perinatal outcomes (Rahimi et al., 2006; Rocklin, 2011). Meta-analyses did not find associations between use of ICSs, as a group, and increased risk of
major malformations (OR 0.96, CI\textsubscript{95%} 0.51 to 1.83, \(n = 847\)) or any congenital malformations (OR 0.96, CI\textsubscript{95%} 0.89 to 1.04, \(n = 17\ 220\) exposed to ICSs), when compared with outcomes for women with asthma not using ICSs (Murphy et al., 2013; Rahimi et al., 2006). Further, active management of asthma was found to decrease the odds of preterm delivery (Murphy et al., 2011).

A large Canadian cohort study \((n = 4\ 392)\), included in the meta-analysis discussed above, confirmed the safety of using low to moderate doses (0 to 1000 \(\mu\)g/d beclomethasone-chlorofluorocarbon equivalent) of ICSs in the first trimester (Blais et al., 2009). Of interest, they also included a cohort of women taking high-dose ICSs (>1000 \(\mu\)g/d, \(n = 154\)), and although they reported no increased risk of major malformations, they did report a small but statistically significant higher risk of having a baby with congenital malformations (major and minor) compared with those who used \(\leq 1000\ \mu\)g/d (RR\textsubscript{adj} 1.63, CI\textsubscript{95%} 1.02 to 2.60) (Blais et al., 2009). Women with moderate to severe asthma would likely be prescribed high-dose ICSs and experience more frequent exacerbations, which can confound the effects found with the high-dose ICSs. As a result, it is difficult to distinguish the effects of asthma from those of ICS use. In addition, as discussed in section 2.2.4, many of the same pregnancy and perinatal outcomes are observed in women with asthma regardless of their ICS use.

One study followed children born to women with asthma treated with ICS up to a median age of 6.1 years (range 3.6 to 8.9 years, \(n = 1\ 231\)) (Tegethoff et al., 2012). Budesonide, and all ICSs used, were associated with an increased risk of endocrine and metabolic disorders; however, there was no association with 14 other disease categories (Tegethoff et al., 2012). The study did not report the details of the disorders or account for asthma severity, oral corticosteroid use, or low birth weight, which might confound these results (George et al., 2012; Lim et al., 2011). The use of ICSs was still supported by the authors, and they emphasized the need for continued research (Tegethoff et al., 2012).

2.3.5 Inhaled corticosteroid use in children

Some of the factors that lead Italian primary care physicians to prescribe ICS for children from 1- to 5-years-old include frequent wheezing, emergency department visits for wheezing, attending a day care or kindergarten, and a personal history of allergic disease (Montella et al., 2013). Approximately, 45% of Canadian children with asthma used inhalers in 2000-01, which
was not significantly different from 1994-95 (Garner & Kohen et al., 2008). Interestingly, the rates of asthma exacerbations decreased significantly from 1994-95 to 2000-01 (51% vs. 39%) (Garner & Kohen et al., 2008), which does not appear to be explained by ICS use. It may be due to awareness and reducing exposure to triggers. For most ICS, their use in patients under 4- to 6-years-old is off-label, since appropriate clinical studies in younger children have not been performed. Yet, Thomas et al. (2006) found that 5.6% of children less than 5-years-old and 10% of children 5- to 11-years-old were on doses >400 µg/d, which is considered high dose therapy for those age groups (Table 1, p. 16). One ICS in particular, fluticasone propionate, was prescribed in higher doses, but it is one of the most potent ICS having an affinity for the glucocorticoid receptor 18-times greater than dexamethasone (Table 2, p. 20). Not surprisingly, the use of higher ICS doses can lead to increased ADRs in children. The ADR of interest for this thesis, adrenal suppression, and adrenal insufficiency and crisis, have been frequently discussed in the literature as occurring in children on high-dose and normal therapeutic dose ICS, with fluticasone propionate frequently implicated (Ahmet et al., 2011; Christensson et al., 2008; Crowley, 2003; Kelly, 2003; Lipworth, 1999; Smith et al., 2012; Todd et al., 2002; Zollner, 2007a & 2012). There is great concern that under-diagnosed adrenal suppression may have severe repercussions should the child undergo significant stress from surgery, illness, or accident (Ahmet et al., 2011; Smith et al., 2012). Moreover, it is unknown what the effects of a lifetime of treatment with ICS will be on health, especially if adrenal suppression should occur but not be properly detected.

2.4 Cortisol and adrenal suppression

2.4.1 The hypothalamic-pituitary-adrenal axis and production of cortisol

Cortisol is released in a diurnal fashion with the highest concentration in the morning and lowest at night. In the systemic circulation, cortisol is ~90% bound to corticosteroid-binding globulin and ~10% free, active cortisol (Bancos et al., 2015), which is important in many biological activities. It is involved in control of inflammatory processes; blood vessel response to vasoconstriction or dilation; fat, carbohydrate and protein metabolism; and a variety of functions with the skeletal, nervous, and urinary systems (Stanfield et al., 2008). When the body experiences a perceived stress, the response is initiated in the locus caeruleus/norepinephrine autonomic nervous system (Nicolaides et al., 2015). The signal is transmitted to the
hypothalamus, which releases corticotropin-releasing hormone (CRH) stimulating the anterior pituitary to release adrenocorticotropic hormone (ACTH). One target organ for ACTH is the zona fasiculata of the adrenal cortex that predominantly produces and releases cortisol. Altogether, this signal cascade is one aspect of the HPA axis.

Synthesis of cortisol is one pathway of steroidogenesis and occurs through a multi-step enzymatic conversion of cholesterol (Figure 6). Additionally, the interconversion of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase types I and II is also shown in Figure 6. Importantly, 11β-hydroxysteroid dehydrogenase types I and II and CRH are present in the placenta and control the amount of cortisol a fetus is exposed to during pregnancy (discussed in more detail in section 2.4.3)

**Figure 6.** Cortisol synthesis pathway. Enzyme names in orange indicate they are located in the mitochondria. Enzyme names in blue indicate they are located in the endoplasmic reticulum. Reactions with the same enzyme are indicated with a grey bar across the reaction arrows and the enzyme listed inside. *This enzyme is found in the endoplasmic reticulum and mitochondria.

CYP11A1 – P450scc or cholesterol side-chain cleavage enzyme; CYP17A1 – P450c17 or 17α-hydroxylase; CYP21A1 – P450c21 or 21α-hydroxylase; CYP11B1 – P450c11β or 11β-hydroxylase

(Adapted from Bremer et al. (2014), prepared using ChemDraw Professional v.15.0)
2.4.2 Adrenal suppression in relation to inhaled corticosteroid use

Adrenal suppression and insufficiency are conditions characterized by decreased or inadequate production of cortisol by the adrenal gland (Ahmet et al., 2011). There are a variety of causes of adrenal insufficiency, which are classified as primary, secondary, or tertiary. Primary adrenal insufficiency results from conditions affecting the adrenal gland directly, for example Addison's disease (an autoimmune disease) or adrenoleukodystrophy, a genetic metabolic disorder that results in destruction of the adrenal glands (Chrousos et al., 2009). Primary causes will not only suppress the release of glucocorticoids, such as cortisol, but likely that of mineralocorticoids and androgens too. Secondary adrenal insufficiency is a result of decreased release of ACTH due to pituitary or hypothalamic disease (Crowley et al., 2014). Tertiary adrenal insufficiency occurs due to impaired hypothalamic function with decreased release of its hormones, such as CRH (Charmandari et al., 2014). Secondary and tertiary adrenal insufficiency is often caused by excess endogenous or exogenous glucocorticoids (Charmandari et al., 2014; Crowley et al., 2014). The term adrenal suppression is typically used to describe the HPA axis suppression associated with exogenous glucocorticoid use that may lead to adrenal insufficiency (Ahmet et al., 2011). Cortisol release is regulated by negative feedback. Due to the structural similarity of ICS to cortisol (Figure 5), ICS that enter the systemic circulation can mimic cortisol and potentially disrupt cortisol release from the adrenal cortex. The body interprets the ICS as cortisol and downregulates the release of ACTH from the pituitary, as well as release of CRH from the hypothalamus (Lipworth et al., 2005). Reduced cortisol release manifests in a variety of symptoms including, but not limited to, chronic fatigue, muscle weakness, weight loss, irritability or depression, hypoglycemia, and, for children, poor weight gain and linear growth (Ahmet et al., 2011; National Institute of Diabetes and Digestive and Kidney Diseases, 2012; Liu et al., 2013). It has been expressed in the literature that adrenal suppression is an under-diagnosed ADR of ICS treatment (Ahmet et al., 2011). While many studies have found that children experience adrenal effects due to ICS use more often than adults, such adverse reactions are also documented for adults (Ahmet et al., 2011; Broersen et al., 2015; Crowley, 2003; for a good meta-analysis and review see Lipworth, 1999; Molimard et al., 2008; Todd et al., 2002; Wlodarczyk et al., 2008). There is a paucity of research investigating adrenal suppression in pregnancy. The prevalence of adrenal insufficiency in pregnancy is considered rare (Mastrogiannis et al., 1994). But, even though researchers have published
pregnancy reference ranges for serum cortisol (Abbassi-Ghanavati et al., 2009), adrenal suppression or insufficiency may go undetected because laboratories have not determined cortisol reference ranges specific for pregnancy based on their testing method and instead apply the reference range for the general population when reporting results.

2.4.3 Diagnosis of adrenal insufficiency

Adrenal insufficiency is diagnosed by the clinical presentation as well as laboratory tests. The easiest laboratory test to perform is the first morning serum cortisol taken at 0800. This test has a specificity close to 100% and sensitivity of ~60% when a low cut-off value of 85-112 nmol/L is used and is considered diagnostic of adrenal insufficiency (Ahmet et al., 2011, Bancos et al., 2015; Charmandari et al., 2014). When the concentration is 100-500 nmol/L, adrenal insufficiency cannot be ruled out (Charmandari et al., 2014), possibly indicating adrenal suppression. Adrenal suppression due to exogenous corticosteroids is ruled out if the concentration is > 500 nmol/L (Charmandari et al., 2014). In children, Zollner (2007b) recommends an intra-individual comparison of serum cortisol concentrations due to the marked inter-individual variability as a result of the pulsatile nature of cortisol secretions. Additionally, plasma ACTH concentrations may be measured. In tertiary adrenal insufficiency, ACTH will be low or normal (Bancos et al., 2015). Salivary cortisol concentrations have also been reported as a screening test but have not been fully validated as a diagnostic test (Charmandari et al., 2014). A concentration < 5 nmol/L suggests a high probability of adrenal insufficiency and > 16 nmol/L excludes adrenal insufficiency (Charmandari et al., 2014). Research studies have measured 24-h urinary free cortisol as an indicator of adrenal suppression, but it is not considered useful for diagnosing adrenal insufficiency since there is pronounced inter-individual variability (Bancos et al., 2015; Charmandari et al., 2014). Neither saliva nor 24-h urinary cortisol concentrations have been well-studied in children with adrenal suppression (Ahmet et al., 2011).

Confirmatory tests for adrenal insufficiency may be performed for individuals with indeterminate morning serum cortisol concentrations. The confirmatory tests measure whether there is an inadequate production of cortisol in response to a stimulus. The "gold standard" confirmatory test is the insulin tolerance test, but its use is declining because it is cumbersome, expensive, and potentially unsafe in certain patient populations, e.g., children (Bancos et al, 2015). The metyrapone test tests the HPA axis negative feedback control by inhibiting 11β-
hydroxylase, which converts 11-deoxycortisol to cortisol (Figure 6). Plasma 11-deoxycortisol concentrations, and possibly ACTH concentrations, are measured and will rise if the hypothalamus and pituitary are functioning properly (Zollner, 2007b). The metyrapone test is also declining in use because of decreased availability of metyrapone and the potential to cause adrenal crisis (Shulman et al, 2007). Furthermore, it is not recommended for pregnant women (Lindsay & Nieman, 2005). The most frequently performed confirmatory test currently is the ACTH stimulation test (also known as the cosyntropin or short synacthen test). The test is performed by administering either 250 µg (standard dose) or 1 µg (low dose) of corticotropin intravenously or intramuscularly with measurement of serum cortisol concentrations at 0, 30, and 60 minutes. A cortisol concentration > 500-550 nmol/L after stimulation, depending on the cortisol assay used, indicates a normal response (Bancos et al., 2015). While there is an ongoing debate as to which corticotropin dose is best (Bancos et al, 2015; Zollner, 2007b), the low dose test has been found to be more sensitive at detecting mild adrenal suppression in children with asthma taking moderate doses of ICS (Kannisto et al., 2000), and has been recommended by some authors for HPA axis assessment in children (Ahmet et al., 2011). The standard- and low-dose ACTH stimulation tests have also been used to diagnose adrenal insufficiency in pregnancy, but the data are limited. The proposed cut-offs indicating a normal response are higher owing to the heightened adrenal response to ACTH during pregnancy (Bancos et al., 2015; Lindsay & Nieman, 2005; Suri et al., 2006). Bancos et al. suggest cut-offs of 700 nmol/L, 800 nmol/L, and 900 nmol/L for first, second, and third trimester, respectively.

2.4.4 Role of cortisol in pregnancy

During the course of normal pregnancy, in early pregnancy, the fetus is protected from maternal cortisol by placental 11β-hydroxysteroid dehydrogenase type II (and a lesser degree by type I), which converts cortisol to less active cortisone (Bremer & Miller et al., 2014; Moisiadis & Matthews, 2014b). Later in pregnancy, from mid-second trimester onward, the fetus begins producing cortisol and is able to release cortisol in response to stress, and maternal serum cortisol concentrations rise 2- to 3-fold higher than a non-pregnant state due to increased levels of corticosteroid-binding globulin and because the placenta is stimulated to release its own CRH and ACTH, which further stimulates the maternal HPA axis to release cortisol (Kamoun et al., 2014). The excess maternal serum cortisol can mimic a hypercortisolic state, such as Cushing's syndrome, and because the placenta produces less 11β-hydroxysteroid dehydrogenase type II
enzyme later in pregnancy, cortisol may saturate the enzyme allowing excess cortisol to potentially cross the placenta (Kivlighan et al., 2008; Moisiadis & Matthews, 2014b). Even if the majority of maternal cortisol is converted to cortisone when crossing the placenta, maternal cortisol has been found to account for approximately 40% of cortisol in the fetus (Gitau et al., 1998). Additionally, the uterus increases cortisone to cortisol conversion near term (Murphy, 1977). Taken together, these results potentially indicate a role for maternal cortisol in fetal maturation, rather than solely relying on cortisol produced by the fetus.

The role of cortisol in fetal maturation has been known for many decades. Cortisol has been associated with preparing the fetus by:

a) maturing lung cellular structure and stimulating production of surfactant,
b) increasing fetal T₃ levels,
c) promoting in utero liver glycogen storage,
d) inducing gastrointestinal enzymes and maturation of cellular morphology,
e) preparing the adrenal medulla to release the required catecholamines during labour and delivery, and the zona fasciculata to respond to ACTH,
f) enhancing kidney glomerular filtration rate and sodium reabsorption, and
g) switching erythropoiesis from the liver to the bone marrow (Liggins, 1994).

The majority of research concerning glucocorticoids in pregnancy has investigated the effects of increased exposure to either endogenous or exogenous forms (Moisiadis & Matthews, 2014b). Exposure to increased levels of glucocorticoids can inhibit organ system functions, whereas lower doses promote maturation of those systems (Liggins, 1994). It would be expected that fetal exposure to suboptimal levels of endogenous and maternal cortisol may have an adverse effect, but this theory has not been well studied.

Although they mainly focus on the effects of increased endogenous or exogenous glucocorticoids, Moisiadis & Matthews (2014b) recognize that improper timing or dysregulation of exposure to the required glucocorticoids for fetal maturation can alter fetal programming and lead to future disease, which may be different depending on the sex of the fetus. Furthermore, over the last few years, it has been realized that the surge in cortisol in late pregnancy is involved in epigenetic processes, which are ultimately part of programming the fetal cardiovascular, neurologic, endocrine, and metabolic systems (Moisiadis & Matthews, 2014b). If the required
surge is diminished, it stands to reason that there could be alterations in the fetal programming of those systems that might result in disease. Therefore, a method to determine and monitor maternal cortisol levels, such as hair cortisol analysis, may be of significant value in the evaluation of pregnancy and perinatal outcomes.

2.5 Hair cortisol

2.5.1 Brief overview of hair structure, composition, and cortisol incorporation

A strand of hair forms in a hair follicle located in the dermis and extends to the skin surface after passing through the epidermis (Robbins, 2012). Because of the follicle's depth, approximately 10 to 14 days of hair growth resides below the surface of the skin (Kintz (Ed.), 2007). Hair has three stages anagen, catagen, and telogen (see Table 3 for details) (Robbins, 2012). The structure of hair is complicated; but, put simply, hair is composed of an outer cuticle, inner cortex (which contains the majority of fiber mass), and a central medulla if the hair is thick (Figure 7) (Robbins, 2012). Hair consists of approximately 65-95% proteins, 1-9% lipids (both structural and free lipids), 0.1-5% pigments such as melanin, trace elements, and up to 32% water by weight (Kintz (Ed.), 2007; Robbins, 2012). Although the exact mechanism of incorporation is still unknown, the multi-compartment model likely best describes the incorporation of endogenous compounds via the bloodstream, and secretions of the sebaceous, apocrine, and eccrine glands (Figure 7) (Boumba et al., 2006; Harkey, 1993; Kintz (Ed.), 2007). This means that some of the compound is internally bound in the hair cortex and medulla and some may be incorporated externally via the cuticle.

Table 3. Details of hair growth stages (Robbins, 2002 & 2012).

<table>
<thead>
<tr>
<th>Hair Stage</th>
<th>Action</th>
<th>% Hair in Stage</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anagen</td>
<td>Active growing with significant metabolic activity</td>
<td>80-90</td>
<td>2 to 6 years</td>
</tr>
<tr>
<td>Catagen</td>
<td>Transition stage with decreasing metabolic activity</td>
<td>1-2</td>
<td>A few weeks</td>
</tr>
<tr>
<td>Telogen</td>
<td>Resting stage with total cessation of growth and eventual shedding</td>
<td>10-20</td>
<td>4 to 8 weeks</td>
</tr>
</tbody>
</table>
Figure 7. Diagram of hair structure and proposed multicompartment model for incorporation of cortisol into hair by the A) systemic circulation, B) sebaceous glands, and C) sweat glands. (Reprinted from Psychoneuroendocrinology, 37(5), Russell E, Koren G, Rieder M, and Van Uum S. Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions, 589-601, Copyright (2012), with permission from Elsevier.)

Although the exact interaction between cortisol and hair is unknown, cortisol has some physicochemical properties that would allow for binding. Cortisol is a small (molecular weight 362.5 g/mol), lipophilic (logD at pH 7.4 is 1.66) molecule, which would potentially allow for easy incorporation into hair matrix as it is formed and interaction with hair lipids. Additionally, cortisol has the ability to form hydrogen bonds with five H-bond acceptors and three donors per molecule (all values from ChemSpider, 2015).

2.5.2 Hair cortisol concentrations compared to that of saliva, blood, and 24h-urine

Currently, the samples used for cortisol determination are saliva, serum, or 24-hour urine collection. Saliva and serum samples provide a cortisol level for that particular moment when the sample was collected. A 24-hour urine collection is effective at assessing cortisol levels over a longer period, but is cumbersome if used to assess cortisol levels for any longer time frame. Hair is an easy, non-invasive sample to collect by simply cutting hair close to the scalp with scissors.
from a specific location (discussed in Chapter 3). With an average growth rate of 1 centimetre per month (cm/mo) (Kintz (Ed.), 2007), hair is a unique sample for obtaining and monitoring cortisol levels retrospectively.

An ACTH stimulation test repeated on days 0, 7, and 14 in Holstein cows showed a significant increase in hair cortisol in the hair samples collected on day 14 ($p < 0.05$). No increase was seen in Holstein cows injected with saline or control Holstein cows not given an injection (del Rosario González-de-la-Vara et al., 2011). The hair cortisol concentration of the stimulated cows remained significantly increased until day 28, but by day 44 was the same as the comparison groups. The increase in endogenous cortisol for each ACTH administration was confirmed by serum cortisol determination of samples collected prior to ACTH administration to those collected 60 and 90 minutes after ACTH administration. This research demonstrated that the cortisol incorporated into mammalian hair reflects endogenous levels of cortisol.

Studies in humans have compared hair cortisol results to other sample matrices. Serum measurements of cortisol consist of the total cortisol concentration inclusive of the free, active cortisol and the inactive cortisol bound to corticosteroid-binding globulin (Russell et al., 2012). Thus, the measurement of cortisol concentrations in serum will be affected by changing concentrations of corticosteroid-binding globulin, e.g., in pregnancy, and, therefore, not accurately represent the free active cortisol. In contrast, saliva and hair measure the free, active cortisol only (Russell et al., 2012). When compared to a single serum or saliva cortisol concentration, it is not surprising that a correlation was not found with hair cortisol (Sauvé et al., 2007; Vanaelst et al., 2012). Serum and saliva samples are affected by the diurnal nature of cortisol release, whereas hair appears to represent the average hair cortisol over time. Unless these samples are collected multiple times over a long period and the results integrated, they cannot be directly compared. Research comparing hair cortisol to four saliva samples collected on each of two days (Spearman's rank correlation rho ($\rho$) = 0.398, $p = 0.024$) and six saliva samples on each of three days in a seven day period (Pearson $r = 0.41$, $p = 0.03$) suggests that hair cortisol is more representative of longer periods (van Holland et al., 2012; Vanaelst et al., 2012). Similarly, hair cortisol in a one-month segment was compared to the average cortisol of morning saliva samples collected once a week for three weeks of the corresponding period and showed a significant correlation ($r = 0.383$, $p < 0.05$) (Xie et al., 2011). Finally, the strongest support of this theory is from a recent study comparing the summed 30-day saliva cortisol area-
under-the-(concentration-time) curve (AUC) with the proximal 1-cm hair segment that reported
the highest significant correlation to date ($r = 0.61$, $p = 0.01$) (Short et al., 2016). In pregnancy,
because of the dynamic nature of cortisol secretion, the results of two rounds of saliva sampling
within a trimester about five weeks apart (consisting of three days with three samples per day)
correlated with hair cortisol concentrations for that trimester better than one round of saliva
sampling during a trimester (e.g., two rounds of sampling in third trimester $R^2 = 0.57$, $p = 0.01$
vs. one round of sampling in first trimester $R^2 = 0.29$, $p = 0.22$) (D’Anna-Hernandez et al.,
2011). When compared to a single 24-hour urine sample, hair cortisol was significantly
correlated ($r = 0.33$, $p = 0.041$) (Sauvé et al., 2007), which is similar to the correlation between
AUC$_{0-22h}$ serum cortisol and 24-hour urinary cortisol corrected for creatinine excretion ($r = 0.44$,
$p = 0.0001$) (Nelson et al., 2002). However, the comparison between hair and urine did not
remain significant when the corresponding 1-cm of hair was compared to one-month of
integrated 24-hour urine samples collected weekly in each of four weeks ($r = 0.30$, $p = 0.28$)
(Short et al., 2016). The lower correlation values of 24-hour urine samples compared to multiple
saliva samples may be a reflection of the shorter period captured by a 24-hour urine collection
compared to the saliva sampling protocols of the previous research, or it may be due to
challenges of proper collection and specimen preparation when analyzing a 24-hour urine sample
(Nelson et al., 2002). Nonetheless, taken together, the results of these studies suggest that hair
cortisol is reflective of a longer period and likely represents the average cortisol over the period
associated with the hair segment length, whether that is 1, 2, or 3 centimetres that represents the
past 1, 2, or 3 months.

To connect hair cortisol concentrations further, albeit indirectly, to endogenous cortisol
concentrations, the research comparing serum or plasma and saliva cortisol concentrations may
be effectual. Numerous studies have reported a significant correlation between matched samples
for measurement of total serum/plasma and salivary cortisol concentrations in a variety of
situations: random-timed samples ($r = 0.62$, $p < 0.0001$), morning samples ($r = 0.54-0.74$),
samples collected during an ACTH stimulation test ($r = 0.86$), pre- ($r = 0.52$, $p = 0.005$) and post-
resistance exercise ($r = 0.62$, $p = 0.001$), after oral ($r = 0.83$, $p < 0.0001$) or intravenous ($r = 0.94$,
$p < 0.0001$) hydrocortisone administration, and in patients with Cushing's syndrome ($r = 0.74$)
(Cadore et al., 2008; Hananda et al., 1985; Jung et al., 2014; Kahn et al., 1988; Lac et al., 1993;
Luthold et al., 1985; Restituto et al., 2008) The correlations tend to be strong when comparing
saliva cortisol to free unbound serum/plasma cortisol with values of 0.92 (\( p < 0.001, n = 15 \)) and 0.893 (\( p < 0.001, n = 150 \)) (Meulenberg et al., 1987; Umeda et al., 1981). A strong comparison was also reported for matched saliva and plasma cortisol concentrations in 36 full-term neonates (\( r = 0.83 \)) (Francis et al., 1987). It should be noted that in some of these studies there were individuals that did not fit the established correlation and were considered outliers or whose correlations between serum and saliva was not significant or, for a couple of individuals, had an unexpected negative correlation (e.g. Jung et al., 2014; Kahn et al., 1988). These anomalies may be due to limitations in testing methods or due to inter-individual variability in endocrine system function, for example, variability in cortisol or corticosteroid-binding globulin production.

Most relevant to the work in this thesis, a few studies have produced interesting comparisons between serum and saliva cortisol. First, Vinning et al. (1983) found that the very strong correlation between salivary and free unbound serum cortisol (\( r = 0.97, n = 93 \) total) was reliable regardless of oral contraceptive usage (\( n = 9 \)), sex (M:F = 59:13), or being in T3 of pregnancy (\( n = 12 \)) indicating these concentrations are also highly correlated in pregnant women in T3. Second, adults with mild to moderate asthma were tested to determine the utility of different methods (24-hour urine, 8 a.m. saliva, or AUC\(_{0-22h}\) serum) for the determination of adrenal suppression due to ICS use (Nelson et al., 2002). The morning and AUC\(_{0-22h}\) serum cortisol concentrations were reported to correlate significantly with the morning salivary cortisol concentration, as well as with the 24-hour urine (reported above), and a 50% reduction in salivary cortisol was reported to be 63% sensitive and 81% specific for detecting a 50% reduction in the serum AUC\(_{0-22h}\) (Nelson et al., 2002). Finally, studies that included patients with Addison's disease found that the total serum and saliva cortisol concentrations correlated well (\( r = 0.73, p = 0.011 \)) although they were lower than normal expected values, did not respond to ACTH stimulation, increased after hydrocortisone therapy, and decreased after dexamethasone administration, thus showing that even in these patients the serum and saliva cortisol concentrations responded in the same manner and as expected to different stimuli (Hiramatsu, 1981; Kahn et al., 1984; Restituto et al., 2008; Vinning et al., 1983).

In summary, significant associations have been reported between saliva and serum/plasma cortisol concentrations, especially when measuring free unbound cortisol. These associations were determined in a variety of study participants including neonates, adults, pregnant women in T3, and patients with Addison's disease. Further, hair cortisol has been
significantly associated with saliva AUC. Taken together, the data is consistent with the theory that hair cortisol reflects the serum/plasma cortisol concentration profile over time.

2.5.3 Current use of hair cortisol in research

Initial work studying hair cortisol was performed by Koren et al. (2002) examining the feasibility of measuring cortisol in the hair of the hyrax, a small, herbivorous mammal. This analysis was further used to correlate the hair cortisol levels of the more dominant “singing” hyraxes, which also experience more stress, from their less dominant counterparts (Koren et al., 2008). A more recent pilot study by Bechshøft et al. (2011) ascertained it was also possible to measure cortisol in polar bear hair and concluded that hair analysis may be a useful tool to study long-term stress in these animals. Many other newer studies have evaluated hair cortisol as a measure of stress in animals, but they will not be discussed in this thesis.

Continuing from animal studies, research teams have been using hair cortisol levels as a tool to study stress in humans since early 2004 (for an excellent review see Russell et al., 2012). Of note was the positive correlation between hair cortisol results and the occurrence of acute myocardial infarction (AMI) in men, thus linking chronic stress, indicated by the hair cortisol biomarker, as a potential risk factor for AMI (OR = 17.4, CI95% 2.15– 140.5; p = 0.007) (Pereg et al., 2011). Manenschijn et al. (2013) also found increased risk of cardiovascular disease, inclusive of congestive heart disease, stroke, and peripheral artery disease, adjusted for age, sex, smoking status, alcohol consumption, and physical activity with each increasing quartile of hair cortisol concentrations (ORadj = 1.9, 2.0, 2.7, for second, third, and fourth quartiles, respectively), but only the fourth quartile was significant (p = 0.01).

Although there is disagreement in the findings, hair cortisol is gaining momentum as a tool for use in research into neuropsychiatric disorders such as severe depression, post-traumatic stress disorder (PTSD), or generalized anxiety disorder (GAD) (Luo et al., 2012; Stalder et al., 2012; Steudte et al., 2011; Steudte et al., 2013). Two studies comparing people with PTSD to traumatized or non-traumatized controls both showed that the group with PTSD had lower hair cortisol than the traumatized group and it was lower than expected, but they differed in their findings when comparing the PTSD group to the non-traumatized group (Luo et al., 2012; Steudte et al., 2013). This difference may be due to the variation in time when the study was performed after the event (months vs. years) or the presence of psychiatric comorbidities.
(Steudte et al., 2013), or it may relate to the different sorts of trauma experienced. Regardless, as the authors commented, their results suggest a different HPA axis response to trauma with and without PTSD and proposed that the higher cortisol levels of the non-PTSD group may have been a protective measure against developing PTSD (Luo et al., 2012). As a last example, Steudte et al. (2011) found lower hair cortisol results for those with GAD compared to controls, whereas previously basal cortisol levels were believed to be unchanged for these patients. The authors concluded that hair was more representative of normal HPA axis function and not prone to acute changes upon sample collection, and thus may indicate that GAD is associated with hypocortisolism.

Hair cortisol has also been used to evaluate the effects of illicit drug use and alcohol dependence, but with mixed results. Interestingly, recent heavy use of 3,4-methylenedioxymethamphetamine (MDMA) or 'Ecstasy' (which causes cortisol release), defined as use on more than five occasions in the last five months, was significantly associated with an increase in hair cortisol concentrations, although there was a larger variance for this group compared to the comparison groups (Parrott et al., 2014). The authors acknowledged that the increased concentrations might also be due to individual differences in levels of physical exertion, since this drug is commonly ingested at dance clubs (see information on the effects of physical activity in the next section – Overview of factors affecting hair cortisol concentrations). Additionally, using ultra fast liquid chromatography coupled with ion trap time-of-flight mass spectrometry, cortisol was identified as one of the potential biomarkers in hair for heroin use in a metabonomic study (Xie et al., 2015). Alternatively, Wells et al. (2014) did not find any difference in hair cortisol results based on illicit drug use, but did find significantly increased hair cortisol in people with a higher hazardous drinking score \( (\beta_{\text{adj}} = 0.129, p = 0.022) \). Alcoholics in current alcohol withdrawal also had significantly higher hair cortisol concentrations than controls or alcoholics that have been abstinent for 14 weeks (Stalder et al., 2010). However, Wosu et al. (2015) and Stalder et al. (2013) did not find any effects from increased alcohol consumption.

With more agreement than some of the other areas of research and, therefore, potentially of greater benefit, hair cortisol has been evaluated for monitoring treatment, diagnosing endocrine disease, or evaluating the effects of disease. Studies have effectively showed that hair cortisol levels correlate well with hydrocortisone therapy of adult and child patients with adrenal insufficiency and that some individuals may be overmedicated (Gow et al., 2011; Noppe et al.,
These results are supported by a pharmacokinetic study of hydrocortisone therapy in adult adrenal insufficiency patients where it was demonstrated that ~ 50% of patients are overmedicated and ~20% are under-medicated, but the authors concluded "that it is impossible to treat in a perfect way all patients with primary or secondary adrenal insufficiency with the dosing and formulations currently available" (Simon et al., 2010). Additionally, studies involving patients with Cushing’s syndrome confirm hair cortisol levels are reflective of endogenous cortisol levels by effectively reflecting the characteristic, increased cortisol levels (Manenschijn et al., 2012a; Thomson et al., 2010). Manenschijn et al. (2012a) reported that hair cortisol is 86% sensitive and 98% specific for detecting cyclic Cushing’s syndrome. To date, hair cortisol has been used to detect adrenal suppression or insufficiency in only two studies. In a study evaluating hair cortisol as a measure of long-term cortisol concentrations, the hair cortisol analysis of one participant diagnosed with Addison's disease that was treated with hydrocortisone successfully showed the lower cortisol concentrations prior to treatment and subsequent increase after treatment (Manenschijn et al., 2011a). In another study, hair cortisol concentrations for children with and without asthma (n = 10 for each group) were compared and revealed that children with asthma (ICS use was not discussed) had cortisol concentrations ~50% lower than control children (Kamps et al., 2014). Although we do not currently know the threshold for hair cortisol concentrations to represent clinically significant adrenal suppression or insufficiency, one study has determined normal ranges for children 4- to 14-years-old using one available enzyme immunoassay (Noppe et al., 2014b), although the ranges would not necessarily be generalizable to other immunoassay methods. The research in this thesis will build upon the evidence currently available for the use of hair cortisol as a biomarker by assessing its ability to detect lower hair cortisol concentrations in pregnant women and children on ICS therapy, which may be suggestive of adrenal suppression.

2.5.4 Overview of factors affecting hair cortisol concentrations

This overview of factors is not meant be an exhaustive list, but rather a presentation of some of the better-known, and some new, variables that are theorized to be or have experimentally been associated with altered hair cortisol concentrations (Table 4). Many factors are associated with mixed findings except for pregnancy, which is always associated with an increase in hair cortisol over the course of pregnancy (Table 4). For a more comprehensive analysis, Wosu et al. (2013) have written an excellent review on the subject.
Of importance, and surprisingly, many researchers have not found a significant association with perceived stress (Boesch et al., 2015; Dettenborn et al., 2010; Dowlati et al., 2010; Gerber et al., 2013; Skoluda et al., 2012; Saleem et al., 2013; Stalder et al., 2010). However, more recent research has found an association of increased hair cortisol with higher perceived stress until the highest level of perceived stress, which was associated with lower hair cortisol (Wells et al., 2014). A similar relationship was found by Karlén et al. (2011) who reported a weak inverse relationship between perceived stress and hair cortisol (r = -0.061, p = 0.025), but it did not remain significant when included in a multiple regression model along with perceived health and serious life events.

Another area of concern, but with conflicting evidence, is the effect of physical activity. Gerber et al. (2013) reported vigorous physical activity, but not moderate physical activity, was associated with increased hair cortisol ($\beta_{adj} = 0.33, p < 0.05, R^2 = 0.126$ vs. $\beta_{adj} = -0.05, p > 0.05, R^2 = 0.023$). Skoluda et al. (2012) also found the same in 304 endurance athletes, consisting of long-distance runners, triathletes and cyclists. Extreme training significantly increased hair cortisol concentrations but not moderate exercise. This may be due to the neuroendocrinological stress response of the exercise itself or from cortisol incorporation into hair through sweat (Gerber et al., 2013). However, Grass et al. (2015) found that there were no acute changes in hair cortisol due to sweat; their study did not find that physiologic stresses that induced sweating, such as treadmill running and being in a sauna, were associated with increased hair cortisol concentrations. Additionally, two other studies did not find an effect from regular physical activity (Stalder et al., 2013; Wosu et al., 2015). Noppe et al. (2014b) also found no effect of frequent sweating in 128 children. Their research did not rule out that, over time with repeated sweating, cortisol from sweat could be incorporated into hair. The in vitro work by Russell et al. (2014a) showed that hair soaked in a cortisol phosphate buffer solution mimicking sweat absorbs cortisol and that this cortisol cannot be removed by the usual wash with isopropanol. Two possible explanations for the discrepancy between in vivo and in vitro studies are that there may be other substances in real sweat, such as lactic acid, urea or amino acids (Harkey, 1993), that disrupts the incorporation of cortisol into hair, or letting the cortisol treated hair air dry for 12 h, as Russell et al. did, allowed cortisol to bind to the hair more than it would from sweat exposure in vivo.
The effect of sex on hair cortisol also has had mixed results with many groups finding no effect (Table 4), two of which were in children (Karlén et al., 2013; Noppe et al., 2014b). Frequently, male sex is associated with higher hair cortisol (Table 4). Dettenborn et al. (2012a) evaluated the effect of sex stratified by age and found no sex difference for those aged 10 to 17 years or 50 to 91 years, but hair cortisol concentrations were higher in males than females in 1- to 9-year-olds and 18- to 49-year-olds. Staufenbiel et al. (2015) found adult males had higher hair cortisol using a partially adjusted regression model, but this association did not remain significant in the mutually adjusted regression model. Finally, Feller et al. (2014) found that in adults aged 47 to 82 years, there was a difference by sex for those younger than 70-years-old, but not in those over 70-years-old.

When considering age, Noppe et al. (2014b) found that hair cortisol concentrations vary by age in children between the ages of 4 to 14 years. Dettenborn et al. (2012a) found hair cortisol to be higher in older adults (>70 years), as well as younger children, which declined by age ten (r = -0.428, p = 0.023, n = 28. In adults, two additional studies have found that older age was associated with higher hair cortisol (Staufenbiel et al., 2015; Stalder et al., 2013), but two other studies did not find the same result, which may be due to the cohorts only containing older adults aged 55 to 85 years (Saleem et al., 2013; Manenschijn et al., 2013). The majority of studies have not found age to be a factor, but all of these studies were conducted in adults aged 18 to 85 years, and it may be that older adults were not well represented (Manenschijn et al., 2011a & 2012b; Stalder et al., 2012; Wells et al., 2014; Wosu et al., 2015; Faresjö et al., 2013; Gerber et al., 2013).

Medication use, apart from corticosteroids, generally has not been found to affect hair cortisol concentrations (Dettenborn et al., 2012a; Stalder et al., 2012; Faresjö et al., 2013; Saleem et al., 2013; Manenschijn et al., 2012b; Karlén et al., 2011; Steudte et al., 2013). Though a few studies have shown no effect of oral contraceptives on hair cortisol concentrations (Dettenborn et al., 2012a; Stalder et al., 2012; Steudte et al., 2013), in one partially adjusted regression model for age and sex, oral contraceptives were associated with higher hair cortisol concentration (Staufenbiel et al., 2015), which is supported by the research showing their use increases serum cortisol concentrations (Ambroziak et al., 2015). Also, a recent study found the use of antidepressants was significantly associated with increased hair cortisol after adjusting for sub-study, sex, age, BMI, glucocorticoid use, and hair dying (βadj = 0.298, p = 0.000) (Wells et al.,
2014), although this may possibly reflect what has already been observed for those with depression or anxiety, \(i.e.,\) a disease effect (Table 4). Meanwhile, a previous study did not find any association with antidepressants or atypical depression (Hinkelmann et al., 2013).

Only in one instance was natural hair colour shown to have any influence. In a regression model adjusted only for age and sex, people with black hair were found to have increased hair cortisol. However, this association did not remain significant in the mutually adjusted regression model (Staufenbiel et al., 2015). Furthermore, hair products appear to have no effect in either children or adults (Noppe et al., 2014b; Manenschijn et al., 2011a; Stalder et al., 2012; Staufenbiel et al., 2015).

In very recent research, seasonal variations in hair cortisol concentrations have been reported in the literature. Braig et al. (2015) found seasonal variations with higher cortisol levels for pregnant women in third trimester from June to November and Staufenbiel et al. (2015) found hair cortisol in adults was the lowest in the winter with all other seasons significantly higher. Additionally, Boesch et al. (2015) found hair cortisol decreased with increasing ambient temperature and increased with increasing humidity.
Table 4. References for research investigating factors affecting hair cortisol.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Increase</th>
<th>Decrease</th>
<th>No change</th>
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<tr>
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<td>Male sex</td>
<td>Dettenborn et al. (2012a); Feller et al. (2014); Manenschijn et al. (2013); Skoluda et al. (2012); Wells et al. (2014)</td>
<td>Dettenborn et al. (2010); Faresjö et al. (2013); Gerber et al. (2013); Hinkelmann et al. (2013); Karlén et al. (2013); Manenschijn et al. (2011a); Manenschijn et al. (2012b); Noppe et al. (2014b); Stalder et al. (2012); Wosu et al. (2015)</td>
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<tr>
<td>Age</td>
<td>Dettenborn et al. (2012a), higher in children and older adults Noppe et al. (2014b), higher in younger children decreases to age 10 years; Stalder et al. (2013), higher in older adults; Staufenbiel et al. (2015), higher in older adults</td>
<td>Faresjö et al. (2013); Gerber et al. (2013); Manenschijn et al. (2011a); Manenschijn et al. (2012b); Stalder et al. (2012); Wells et al. (2014); Wosu et al. (2015)</td>
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<tr>
<td>Natural hair colour</td>
<td></td>
<td>Boesch et al. (2015); Braig et al. (2015); Dettenborn et al. (2012a); Manenschijn et al. (2011a); Noppe et al. (2014b); Sauvé et al. (2007); Wells et al. (2014)</td>
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<td>Hair washing</td>
<td>Dettenborn et al. (2012a), lower for 6-9 cm segment; Hamel et al. (2011), <em>in vitro</em>; Li et al. (2012), <em>in vitro</em>; Staufenbiel et al. (2015); Wosu et al. (2015)</td>
<td>Braig et al. (2015); Dettenborn et al. (2012a), for the first 6 cm; Kirschbaum et al. (2009); Manenschijn et al. (2011a); Noppe et al. (2014b); Stalder et al. (2012); Stalder et al. (2013)</td>
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<td>Hair products</td>
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<td>Manenschijn et al. (2011a); Noppe et al. (2014b); Stalder et al. (2012); Staufenbiel et al. (2015)</td>
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<td>Factor</td>
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<td>Chemical treatment</td>
<td>Karlén et al. (2011); Manenschijn et al. (2012a); Sauvé et al. (2007);</td>
<td>Wosu et al. (2015), for relaxers/perms used by black women only</td>
<td>Braig et al. (2015); Dowlati et al. (2010); Faresjö et al. (2013);</td>
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<td></td>
<td>Stalder et al. (2013); Wells et al. (2014)</td>
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<td>Karlén et al. (2011); Manenschijn et al. (2011a); Stalder et al. (2012);</td>
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<td>Staufenbiel et al. (2015)</td>
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<td>Age of the hair sample</td>
<td>Dettenborn et al. (2010); Dettenborn et al. (2012a); Krumbholtz et al. (2013); Li et al. (2012), <em>in vitro</em> UV exposure; Luo et al. (2012); Skoluda et al. (2012); Steudte et al. (2011b); Xie et al. (2011)</td>
<td>Manenschijn et al. (2011a); Manenschijn et al. (2012a); Noppe et al. (2014b), within 6 months; Thomson et al. (2010)</td>
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<td>Higher BMI</td>
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<td>Stalder et al. (2012); Stalder et al. (2013); Wells et al. (2014)</td>
<td>Manenschijn et al. (2012b); Manenschijn et al. (2013); Noppe et al. (2014b)</td>
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<td>Exercise/frequent sweating</td>
<td>Gerber et al. (2013); Russell et al. (2014a), <em>in vitro</em>; Skoluda et al. (2012), both for intense exercise;</td>
<td>Grass et al. (2015); Noppe et al. (2014b), in children; Stalder et al. (2013); Wosu et al. (2015)</td>
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<td>Perceived stress</td>
<td>Wells et al. (2014), until the highest level of perceived stress</td>
<td>Karlén et al. (2011); Wells et al. (2014), highest level of perceived stress</td>
<td>Boesch et al. (2015); Dettenborn et al. (2010); Dowlati et al. (2010); Gerber et al. (2013); Saleem et al. (2013); Skoluda et al. (2012); Stalder et al. (2010)</td>
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<td>Depression/Anxiety</td>
<td>Dettenborn et al. (2012b); Faresjö et al. (2013), depression only</td>
<td>Gerber et al. (2013); Manenschijn et al. (2012b), for bipolar with co-morbid panic disorder; Steudte et al. (2011b)</td>
<td>Dowlati et al. (2010); Hinkelmann et al. (2013); Saleem et al. (2013); Stalder et al. (2010); Wells et al. (2014)</td>
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<td>Traumatic event</td>
<td>Karlén et al. (2011); Luo et al. (2012); Steudte et al. (2011a), for lifetime # of traumas</td>
<td>Steudte et al. (2011a), PTSD higher than traumatized; Steudte et al. (2013), for PTSD, trauma, and lifetime # of traumas</td>
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<td>Smoking</td>
<td>Braig et al. (2015); Feller et al. (2014); Wells et al. (2014); Wosu et al. (2015)</td>
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<td>Boesch et al. (2015); Dettenborn et al. (2012a); Faresjö et al. (2013); Hinkelmann et al. (2013);</td>
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<tr>
<td>Lower education</td>
<td>Boesch et al. (2015)</td>
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<td>Braig et al. (2015); Saleem et al. (2013)</td>
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<td>Ethnicity</td>
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<td>Medications, other than corticosteroids</td>
<td>Staufenbiel et al. (2015), for oral contraceptives; Wells et al. (2014), for antidepressants</td>
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<td>Dettenborn et al. (2012a); Faresjö et al. (2013); Hinkelmann et al. (2013); Karlén et al. (2011); Manenschijn et al. (2012b); Saleem et al. (2013); Stalder et al. (2012); Steudte et al. (2013)</td>
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<td>Winter season</td>
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Chapter 3
Methods

3.1 The Drug Safety and Effectiveness Network

The Drug Safety and Effectiveness Network (DSEN) was established and funded by the Canadian Institutes for Health Research to gather and disseminate information on the safety and effectiveness of pharmaceuticals beyond that determined in clinical trials. The Network is a collaboration of numerous researchers, healthcare providers, government officials, and graduate and postdoctoral trainees across Canada, in 13 adult and 13 pediatric hospitals (Figure 8). The Canadian Pharmacogenomics Network for Drug Safety (CPNDS), a DSEN grant recipient, created DSEN-Surveillance and Evaluation of Adverse Reactions in Canadian Healthcare in 2005 to focus on adverse drug reactions (ADRs). Their main objective is early detection of ADRs to improve the health, social, and economic burdens that result from ADRs. In their efforts, they focus on patient populations that are commonly excluded from clinical trials (e.g., pregnant women and children), and evaluate medications that may be in use "off-label" in these populations. The research contained in this thesis is one part of the DSEN-SEARCH demonstration project. The overall goal of the demonstration project is to assess the safety of inhaled corticosteroid use in pregnant women and children with asthma.

Figure 8. Map of the DSEN-SEARCH network. (Image courtesy of CPNDS, used with permission.)
3.2 Study design, ethics, and participants

All studies included in this thesis are retrospective observational cohort studies. Children and pregnant women were recruited for voluntary participation in the study from June 2012 to December 2014. One study was a pilot performed with a small sample of children that acted as their own control group. Otherwise, the cohorts were divided into three groups: participants with ICS-treated asthma (ICS Treated), and two comparison groups consisting of participants without asthma (Controls) and a disease-matched group with asthma not treated with ICS during their pregnancy or within the last five months for the children (No ICS). The sample size for each cohort was not formally determined prior to recruitment due to the unavailability of data on hair cortisol concentrations in pregnant women and children with and without asthma when the protocol was created. Therefore, the sample size was chosen based on convenience and projected recruitment potential within a two-year time limit. The goal was 750 children and 225 pregnant women.

Pregnant women were recruited at DSEN-SEARCH network hospitals including the British Columbia Women's Hospital and Health Centre in Vancouver, British Columbia; St. Michael's Hospital in Toronto, Ontario; and the Hospital for Sick Children in Toronto, Ontario. Recruitment occurred in person in obstetric clinics or through the Hospital for Sick Children's Motherisk Program teratology information service via telephone and mail. Recruitment of pregnant women was also attempted through social media, but efforts were unsuccessful. Children were recruited at an additional DSEN-SEARCH location at the Winnipeg Children's Hospital in Winnipeg, Manitoba. The children were recruited through hospital or community asthma clinics associated with the hospital.

All participants for all studies were eligible provided they could read and understand English, did not use any corticosteroid products on their scalp, or have any known medical conditions characterized by high endogenous cortisol concentrations such as Cushing's syndrome. Children were recruited if they were 0- to 18-years-old. Pregnant women were recruited at any time during pregnancy up to six months postpartum. All participants with asthma were diagnosed by a physician, either currently, as for the children attending an asthma clinic, or at some time in their past, which is mainly the case for the pregnant women. Research ethics
board approval was obtained from each institution and informed written consent and assent was obtained from all participants, as appropriate.

### 3.3 Data collection

Relevant clinical information for all participants of all studies was obtained through medical record review and/or participant interview using the DSEN-SEARCH questionnaire developed for this project (Appendix A). The data included medical history, asthma and ICS treatment history, concomitant medications, and pregnancy outcomes, if applicable. Specifically, the questionnaire sought to collect information such as:

- a) the patients’ asthma medication history from first recollection of the onset of the disease;
- b) their lifetime ICS medication use;
- c) if they stopped using their ICS, and why, at any time in the last 12 months;
- d) any medical conditions experienced during their lifetime and pregnancy related to the documented ADRs associated with ICS use or uncontrolled asthma to include the timing of the event, if the patient was hospitalized, and the type and timing of any treatment received;
- e) the timing and dosage of all other medications used within the last 12 months or during their pregnancy; and
- f) the timing, number, and treatment method of asthma exacerbations within the last 12 months.

A questionnaire supplemental was also created to collect the participants' height and weight for BMI calculation (Appendix A). Hair grooming factors reported to affect hair cortisol levels, such as frequency of hair washing, days since last washing and chemical treatment (color or relaxer), were also collected using a Hair Sample Collection Form, which was developed for this thesis study (Appendix B) (Wosu et al., 2013). Additionally, ethics approval to administer the Perceived Stress Scale (PSS, Appendix C), a validated tool to assess stress levels experienced in the previous month (Cohen et al., 1988), was only obtained for use with participants enrolled in Ontario.
3.4 Hair cortisol analysis

Hair cortisol analysis was carried out at two of the DSEN-SEARCH laboratories. The children's pilot study samples were analyzed in the laboratory at Western University in London, Ontario. The remainder of the testing was performed at the Hospital for Sick Children in Toronto, Ontario. A laboratory technician provided assistance with segmentation and washing of the samples for the large cohort of children and two summer students, supervised by the doctoral candidate, provided further assistance washing and processing the samples for cortisol extraction of both the children and pregnant women cohorts. The doctoral candidate completed all other aspects of the analysis.

3.4.1 Hair sample collection

A lock of ≥ 40 strands of hair was collected non-invasively from the vertex posterior region of each participant's head by cutting close to the scalp with scissors. The collection was performed by the study personnel or by the participant (i.e. the pregnant women participating by mail) following simple instructions for proper hair sample collection provided to them on the Hair Sample Collection Form and in a separate instruction document (Appendix D). The rationale for collection from this scalp location stems from evidence that hair growing at the vertex posterior of the head was found to have the least variation in growth rate (Cooper et al., 2012), the highest cortisol concentrations, and the lowest intra-individual variation of cortisol concentration (16-26%) of five different scalp locations (Li et al., 2012; Sauvé et al., 2007). After collection, the hair was affixed with tape to the Hair Sample Collection Form keeping the cut ends aligned as well as possible (Plate 1), put in an envelope, shipped, and stored at room temperature until analysis was performed.
3.4.2 Hair sample segmentation

Because hair grows on average 1 cm/mo, it allows for temporal analysis (Kintz (Ed.), 2007), which was important for the children's pilot study and to capture the women's pregnancy. Taking into consideration that 14 days of hair resides below the scalp, participants' hair samples were accurately mapped by the study personnel. The maps provided a visual representation of which ICS were used, when they were used, changed, or discontinued, if the ICS dose changed, when oral corticosteroids were given, and, for the pregnant women, the pregnancy trimesters (see Appendix E for sample hair maps). Thus, the map acted as a guide for the hair segmentation and data interpretation. For the children's pilot study, hair samples were chosen from children with sufficiently long hair to capture a period prior to the initiation of their ICS therapy for comparison to hair during their ICS therapy. Each hair sample was cut into 2 cm segments for

Plate 1. Photo showing a hair sample affixed to the Hair Sample Collection Form. Two samples are shown. Note how the cut ends of the hair have been kept aligned. The sample at the bottom has had a 6 cm section removed for segmentation.
analysis of the full length of the hair. For the large cohort of children, the 3 cm proximal hair segment was used in the analysis.

The segmentation for the pregnant women required the greatest attention to detail and precision. The conception date was calculated from the due date with subsequent accurate calculation and measurement of where to cut the hair to capture the periods of three months prior to conception (preconception, PC), first, second and third trimesters (T1, T2, T3), and postpartum (PP). The majority of segments were 3 cm long except for a few scenarios. For example, when women experienced preterm birth or surpassed their due date, it necessitated cutting a shorter-than or longer-than 3 cm segment to properly represent their T3. Additionally, because women were recruited at various stages of pregnancy and their hair varied in length, some women did not have a full 3 cm segment for each period. Segments were not used if they were less than 2 cm; therefore, the hair samples for the pregnant women ranged from 2-3.6 cm.

### 3.4.3 Hair cortisol measurement by enzyme immunoassay

Many leading groups in hair cortisol research have used the method for hair sample preparation and cortisol measurement used in this thesis, which is based on the work of Sauvé et al. (2007), with slight variations (Davenport et al., 2006; Dettenborn et al., 2010; Kirschbaum et al., 2009; Manenschijn et al., 2011a & 2011b; Pereg et al., 2011). For this thesis work, the method varied slightly between the children's pilot study and the other two cohorts, the large cohort of children and the pregnant women cohort, for which the minor modifications will be described. After segmentation, hair samples of 10-25 mg were accurately weighed on an analytical balance, washed twice with 3 mL of isopropanol, and allowed to air dry for at least five hours, but usually overnight. Next, the hair samples were finely cut with scissors (Plate 2), extracted in 1 mL of methanol, incubate overnight for 16 h at 50 ºC while rotated at 100 rpm. The supernatant was removed and dried under N\textsubscript{2} at 50 ºC (37 ºC for the large cohort of children and the pregnant women cohort), reconstituted in 250 µL of phosphate buffered saline (PBS) (at pH 8.0, for the children's pilot study, or pH 7.2-7.4 for the other two cohorts), and vortexed for one minute.
Samples were analyzed using a commercially available saliva cortisol competitive enzyme immunoassay following the manufacturers' instructions. The ALPCO Diagnostics (Salem, NH, USA) salivary cortisol kit was used for the children's pilot study, whereas the Salimetrics High Sensitivity Salivary Cortisol Enzyme Immunoassay (EIA) kit (Salimetrics, Philadelphia, PA) was used for the other two cohorts. Due to differences in performance between the two kits, when the pregnant women cohort samples were reconstituted with 250 µL of PBS, it was quickly discovered some samples were below the quantitation limit of the Salimetrics kit (0.12 ng/mL or 0.33 nmol/L). Therefore, the method was altered so all subsequent pregnant women and children cohort samples were reconstituted with 125 µL of PBS, thus increasing the concentration of cortisol in the sample. Because EIA analysis requires all samples to be run in duplicate, any duplicates with a coefficient of variation greater than 15% or 20% were reanalyzed or reprocessed based on sample availability. Final hair cortisol concentrations are calculated and reported as a ratio to the hair sample weight in units of ng/g by converting the ng/µL result using the reconstitution volume and weight of the hair sample in the following manner (multiply by 2.759 for pmol/g):

\[
\text{Hair cortisol in ng/g = } \frac{\text{Cortisol result in ng/µL} \times \text{reconstitution volume (µL)}}{\text{weight of hair (mg)} \times (0.001 \text{ g/mg})}
\]
3.4.4 Hair extraction and enzyme immunoassay performance characteristics

The average cortisol extraction recovery from hair matrix spiked with a range of cortisol standards using the same or similar hair extraction method has been previously published and was reported as 88.1% by Sauvé et al. (2007) and 84.5% by Manenschijn et al. (2011a). The limit of quantitation for the ALPCO kit is 1.0 ng/mL (2.8 nmol/L) and 0.12 ng/mL (0.33 nmol/L) for the Salimetrics kit. The cross-reactivity with other endogenous and exogenous corticosteroids reported by the manufacturer for the ALPCO kit includes prednisolone 13.6%, corticosterone 7.6%, deoxycorticosterone 7.2%, progesterone 7.2%, cortisone 6.2%, deoxycortisol 5.6%, prednisone 5.6%, and dexamethasone 1.6%. For the Salimetrics kit, all of the cross-reactivity reported by the manufacturer, except one, is < 0.6%, which includes prednisolone, prednisone, cortisone, 11-deoxycortisol, 21-deoxycortisol, 17α-hydroxyprogesterone, triamcinolone, corticosterone, progesterone, 17β-estradiol, dehydroepiandrosterone, testosterone, transferrin, and aldosterone; the cross-reactivity for dexamethasone was 19.2%. Further cross-reactivity of the Salimetrics kit was determined for each of the five ICS available in Canada using an 8 000 ng/mL solution run six times on two different EIA plates. The cross-reactivity was not detectable for fluticasone propionate, budesonide and ciclesonide, 0.01% for beclomethasone dipropionate, and 0.03% for mometasone furoate. Also, to ascertain potential effects of the hair matrix on the immunoassay results, four hair samples, determined to have a cortisol concentration close to the highest cortisol standard (30 ng/mL or 82.8 nmol/L), were diluted and analyzed to see if they deviated from the calibration curve. The analysis showed there was no effect of hair matrix on the measured concentration (Figure 9). Comparison of the curves indicated that they were not significantly different from one another ($F_{(4,56)} = 1.54$, $p = 0.20$) and the average $R^2$ was 0.998, which is almost exactly the same result reported by Davenport et al. (2006) for rhesus macaque hair.
Figure 9. Graph of the sample dilution study comparing four diluted hair cortisol samples to the enzyme immunoassay cortisol standards. The graphs were "nudged," ±0.02 and ±0.04 data units, to enhance the visibility of each sample and the standards due to significant overlap with the each other. The original graph is shown in the insert.

The kit quality control, as well as a pooled in-house hair quality control sample, was run with each EIA plate as directed by the manufacturer. For the testing performed in Toronto, the results of the pooled hair quality control were plotted on a Levey-Jennings chart and evaluated using Westgard Rules for acceptance or rejection (Westgard QC, 2009). The results of the EIA were considered acceptable if two of three control values were within expected range. For the Salimetrics kit, based on the coefficients of variation for the participant samples, the average intra-day coefficient of variation was 6.3%. The average inter-day coefficients of variation were 1.5% and 4.5% for the high and low quality control, respectively. The inter-day coefficient of variation for the pooled hair quality control was 13.4% based on 40 runs, from October 2014 to August 2015, using two different kit lot numbers. The intra-day and inter-day coefficients of variation for the ALPCO kit have previously been reported as 3.8% and 8%, respectively (Pereg et al., 2011).
Russell et al. (2014b) published an important comparison of different hair cortisol detection methods. Hair samples (n = 15) were randomized and shared with four different, blinded research groups who frequently perform cortisol analysis in hair. The cortisol concentrations were determined using their respective methods, which included four different immunoassays and two liquid chromatography-mass spectrometry (LC-MS) methods. The ALPCO and Salimetrics kit hair cortisol concentration were significantly correlated with the average concentration of the LC-MS methods, which is generally considered a "gold standard" method, $R^2 = 0.89$ ($p < 0.0001$) and $R^2 = 0.98$ ($p < 0.0001$), respectively. The ALPCO kit produced the highest hair cortisol results with values ~28-times higher than the LC-MS result, whereas the Salimetrics kit results were only ~2.6-times higher than the LC-MS result.

Of note, many of the performance characteristics presented herein are from previously published research or from the EIA kit manufacturer's package insert. Due to the exploratory nature of this research, a thorough evaluation of the EIA method, for example, linearity, quantitation limit, extraction recovery, and cross-reactivity, was not performed. However, to utilize this method for clinic service would require such an evaluation to be performed using hair matrix and development and rigorous testing of an LC-MS method is recommended instead.

### 3.5 Statistical analysis

Many research studies have excluded hair cortisol values higher than 2 or 3 standard deviations (SD) from their statistical analysis, which removed approximately 2-12% of the participants from the study groups (Boesch et al., 2015; Dettenborn et al., 2012a; Faresjö et al., 2013; Feller et al., 2014; Gow et al., 2011; Grass et al., 2015; Heijmans et al., 2011; Karlén et al., 2011; Saleem et al., 2013; Stalder et al., 2012 & 2013; Steudte et al., 2011a, 2011b & 2013; Wells et al., 2014; Wosu et al., 2015). This approach was not employed in this research because, although there were individuals with outlying hair cortisol concentrations, there was no indication the results were falsely elevated or due to a known medical condition. More appropriately, Wosu et al. (2015) performed calculations with and without the 8 "outliers" of the 100 participants in their study and Karlén et al. (2013) included the high results in calculations using log transformation. A similar approach is applied to the data in this thesis and, since outlying results are potentially confounding, the statistical analyses for the pregnant women cohort were performed and reported with and without the high hair cortisol values. For the large
cohort of children, a regression method able to robustly handle any "outliers" was used, which will be described in section 3.5.2.

All statistical analyses were performed on an Apple MacIntosh computer using Graphpad Prism software version 5.0c (Graphpad Software, Inc., La Jolla, CA), SPSS versions 20.0 or 22.0 (IBM Corporation, New York, NY), and RStudio version 0.99.486 (RStudio, Inc., Boston, MA) running R version 3.2.2 (The R Foundation for Statistical Computing, Vienna, Austria).

For all analyses, two-tailed p-values were calculated and considered significant if $\leq 0.05$.

### 3.5.1 Statistical analysis for the pregnant women cohort

Comparisons between the groups for hair cortisol concentrations, demographic information, and clinical information were performed as appropriate for normally distributed, not normally distributed, and categorical data using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, the Kruskal-Wallis test with Dunn's multiple comparison test, and Chi-square test or Freeman-Halton extension of the Fisher's exact probability test. Further post hoc analyses were performed using the unpaired t-test with Welch's correction, and Mann-Whitney U test with Bonferroni correction for multiple comparisons to calculate the adjusted p-values ($p_{adj}$), as needed.

The Friedman test was used to compare hair cortisol concentrations for the five time points within each group. Because most women did not have results for all five time points, post hoc analysis for the Friedman test was performed using the Wilcoxon matched-pairs signed rank test. A natural log transformation was required for the hair cortisol data prior to performing the Wilcoxon tests to best satisfy statistical assumptions. The Holm-Bonferroni correction was applied to p-values to correct for multiple comparisons. Additionally, the linear regression for median hair cortisol concentrations from PC to T3 for each group were compared using analysis of covariance (ANCOVA) with the Bonferroni correction for multiple comparisons to calculate the adjusted p-values. Univariate and multiple linear regressions were performed with the variables "consistent ICS use" (defined as use of ICS for $\geq$ 5 doses per week), "intranasal corticosteroid use" (yes/no), and "asthma" (yes/no) for T3 to determine if there was any influence of these variables on hair cortisol concentrations in that trimester.
Potential type and dose effects of the ICS were evaluated, but only for fluticasone, budesonide, or multiple ICS since they were the only ICS types used during pregnancy in this study cohort. The type effects were evaluated by calculating the estimated marginal mean of the natural logarithm (ln) transformed hair cortisol concentrations adjusted for daily ICS dose. Bootstrapping was performed up to 1,000 samples, but was lower than 1,000 if the original sample size was small, for example multiple ICS users \( n = 2 \). The inverse ln was taken to provide results in the original units of ng/g for graphical representation. The women's average daily ICS dose over the course of pregnancy was determined based on their prescribed ICS dose in beclomethasone-HFA equivalents (see Table 1, p. 16), self-reported frequency, and periods of use. The dose categories were chosen post hoc to provide the best distribution of women in each category. The dose categories in beclomethasone-HFA equivalents are none (for the Controls and No ICS), 0-250 µg/d, and >250 µg/d. The comparisons of hair cortisol concentrations according to the ICS type or dose category were performed using the Kruskal-Wallis test.

Spearman correlations were calculated for the hair cortisol results with previously published confounders, including the age of hair sample, body mass index (BMI) (pre-pregnancy and at time of hair collection), number of hair washes per week, days since last washing, and PSS score. Because hair chemical treatment is a binary outcome, a point biserial correlation was performed using ln-transformed hair cortisol concentrations, which was unsuccessful for the PC concentrations in the treated asthma group. Unfortunately, the concentrations ≥100 ng/g (≥276 pmol/g) had to be removed for the biserial correlation to best satisfy the assumption of a normal distribution. Correlations with the hair segment length were added post hoc when the segment lengths were finalized and some deviated from 3 cm.

### 3.5.2 Statistical analysis for the children studies

For the children's pilot study, the average monthly hair cortisol for a maximum 6-mo period (6 cm of hair) prior to ICS use and minimum 2-mo period (2 cm of hair) during ICS use was determined for each participant. The 2-cm hair segment corresponding to the period when ICS use was initiated was not included in the analysis to avoid variability in cortisol hair levels during the period of ICS buildup and to reduce the influence of systemic corticosteroid (oral and/or intravenous) exposure, which was frequently given at the time of starting ICS therapy. Because the average hair cortisol concentrations were not normally distributed, the median of the
average monthly hair cortisol concentrations prior to and during the use of ICS was compared using the Wilcoxon matched-pairs signed-rank test (Smy et al., 2015).

For the large cohort of children, the comparisons of hair cortisol concentrations, demographic information, and clinical information among the groups were performed using the same statistical tests as described for the pregnant women cohort in section 3.4.1, with the addition of the Welch's ANOVA for the PSS score. The hair cortisol concentration data was not normally distributed and it could not be normalized by transformation due to the large proportion of samples with high concentrations. Therefore, to determine the potential effects of the variables and correct for possible confounders, a more robust regression method, quantile regression, was used (Koenker & Hollack, 2001). Quantile regression is based on linear regression mathematics and based on linear regression principles but is not constrained by the same assumptions. At each specified percentile, quantile regression takes into consideration the complete dependent variable distribution (thus, there is no sample size associated with each percentile), adjusts for all covariates specified in the model, and minimizes the error in an asymmetric fashion by calculating the upper and lower limits using rank inversion (SAS Institute Inc., 2011). The calculations for each quantile/percentile are determined by minimizing the sum of asymmetrically weighted absolute residuals for all data points in the distribution (Pescher et al., 2013). In the words of Cade et al. (2003), "Quantile regression estimates multiple rates of change (slopes) from the minimum to maximum response, providing a more complete picture of the relationships between variables missed by other regression methods." The quantiles/percentiles that were calculated for this research include 5th, 15th, 25th, 35th, 45th, 50th, 55th, 65th, 75th, 85th, and 95th. The results of the quantile regression were compared to those of ordinary least squares (OLS) regression of the original hair cortisol concentration data and rank transformed data, which was chosen as a potentially acceptable transformation of non-parametric data, to assess the method suitability.

Two models were created to assess ICS type and dose effects separately since they could not be included in the same model due to matrix collinearity. Model 1 evaluated the ICS type and Model 2 evaluated the average daily ICS dose. The variables common to each model are age, sex, use of intranasal corticosteroids, asthma (yes or no), BMI, recruitment location, hair chemical treatment (which includes hair colour or other chemical treatments such as frequent chlorine exposure from swimming, if known), and lifetime duration of ICS use. Children were
not included in the models if they had any missing values for the variables. Age in the models was centred to 7.48 years and the BMI was centred to the median BMI for a 7.48 year-old, which is 15.5 kg/m$^2$. The common variables were chosen to adjust the models appropriately for confounding factor(s) that:

a) had a significant effect on the hair cortisol concentrations when evaluated individually, such as age, sex, BMI, and intranasal corticosteroid use;

b) were significantly different among the groups, such as recruitment location and chemical treatment of the hair; or

c) specifically related to ICS therapy, e.g., the lifetime duration of ICS use.

Dummy variables were created for the locations, ICS types, and dose categories in order to include them in the models. The average daily ICS dose in µg/kg/d was determined using the child's prescribed ICS dose in beclomethasone-HFA equivalents (Table 1), the self-reported frequency and periods of use, and their weight. The categories were assigned post hoc and include none (for the Controls and No ICS), > 0-1.0 µg/kg/d, 1.01-2.5 µg/kg/d, and > 2.5 µg/kg/d.

The unstandardized regression coefficients for Model 2, ICS dose, were graphed and presented in table form. Conversion of unstandardized regression coefficients to standardized coefficients allows for comparison of the effect on the dependent variable by independent variables with differing units of measure because it produces a coefficient that is in relation to the change per 1 standard deviation of the independent variables, i.e. the unit of measure for the independent variables is standard deviation (Rockefeller College University at Albany, 2004; Williams, 2004). Therefore, calculation of the standardized coefficients was performed using the equation:

$$\beta_{\text{standardized}} = \frac{\beta_{\text{unstandardized}} \times s_x}{s_y}$$

where $\beta$ is the quantile regression coefficient in the standardized or unstandardized form, as indicated, and 's' is the standard deviation of the independent variable ($s_x$) or dependent variable ($s_y$) (Rockefeller College University at Albany, 2004; Williams, 2004). However, since $s_y$ is common to all independent variables, because in the quantile regression performed in this
research it is the standard deviation of the hair cortisol concentrations, this variable was eliminated (Williams, 2015). This elimination also allows the reported standardized coefficients to be in reference to the change in hair cortisol concentration in original units, ng/g, which is more intuitive to interpret rather than in units related to a 1-standard deviation change in hair cortisol (Williams, 2015).
Chapter 4
Results

4.1 Hair cortisol as a potential biomarker of the hypothalamic-pituitary-adrenal axis in pregnant women with asthma

4.1.1 Participant results and demographics

Hair samples were analyzed for 118 pregnant women, consisting of 31 Controls, 31 No ICS, and 56 ICS Treated. There were fourteen hair samples from the ICS Treated group that could not be analyzed due to insufficient quantity, inaccurate segmentation, ICS being used to treat a condition other than asthma, or hair being collected too early in T1 (i.e. if the hair segment was less than 2 cm) or > 6 months postpartum. Before altering the reconstitution volume from 250 µL to 125 µL, some samples cortisol concentrations were below the quantitation limit (0.12 ng/L or 0.33 nmol/L), but, unfortunately, could not be repeated due to insufficient quantity of the original hair sample. Therefore, these results were recalculated substituting the quantitation limit for the original measured results. The recalculation predominantly affected the No ICS group (n = 5 patients/12 segments), but also included some samples in the Controls (n = 3 patients/3 segments) and ICS Treated (n = 2 patients/3 segments).

Comparisons of demographics, hair variables, and medication use among the groups are listed in Table 5. Overall, there were no significant differences among the three groups except for their use of intra-nasal corticosteroids and beta-agonists, which, as would be expected, were more frequent among women with asthma (Table 5). Associations between the hair cortisol results and factors previously reported to affect hair cortisol concentrations were further explored but no significant confounding effects were found (see Table 6). Therefore, the test statistics were not adjusted for any of these factors. Of all the women recruited postpartum or in T3, only one woman in the No ICS group received corticosteroids due to threatened preterm labor during the period captured by her hair sample. It is unknown whether any of the women received hydrocortisone as a stress dose treatment during labor.
Table 5. Comparison of demographics, hair sample variables, and medication use among the three groups of pregnant women with and without asthma.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Controls (n = 31)</th>
<th>No ICS (n = 31)</th>
<th>ICS Treated (n = 56)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, mean (SD)</td>
<td>33.8 (4.3)</td>
<td>31.6 (6.0)</td>
<td>33.3 (5.5)</td>
<td>0.240^k</td>
</tr>
<tr>
<td>BMI, kg/m^2, median (IQR, n)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant</td>
<td>28.8 (25.4-32.5, 31)</td>
<td>28.2 (25.0-34.2, 25)</td>
<td>27.8 (24.7-32.2, 41)</td>
<td>0.716^i</td>
</tr>
<tr>
<td>Pre-Pregnancy</td>
<td>24.6 (20.9-27.2, 31)</td>
<td>25.8 (22.8-31.3, 25)</td>
<td>24.8 (21.8-29.4, 42)</td>
<td>0.554^l</td>
</tr>
<tr>
<td>PSS Score, mean (SD, n)</td>
<td>12 (5-26)</td>
<td>15 (7-9)</td>
<td>15 (6-23)</td>
<td>0.210^k</td>
</tr>
<tr>
<td>Birth Data^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age, weeks, median (IQR, n)</td>
<td>39.6 (39.0-40.9, 30)</td>
<td>39.1 (37.6-40.6, 28)</td>
<td>39.1 (38.1-40.3, 51)</td>
<td>0.155^l</td>
</tr>
<tr>
<td>Birth weight, kg, median (IQR, n)</td>
<td>3.41 (3.11-3.65, 31)</td>
<td>3.37 (2.76-3.64, 30)</td>
<td>3.31 (2.89-3.63, 52)</td>
<td>0.701^l</td>
</tr>
<tr>
<td>Hair Sample Variables</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample age, days^b, mean (SD)</td>
<td>334 (82)</td>
<td>325 (93)</td>
<td>315 (103)</td>
<td>0.661^k</td>
</tr>
<tr>
<td># Washes per week, median (IQR, n)</td>
<td>3.5 (2.5-4.5, 31)</td>
<td>3.0 (2.5-5.5, 24)</td>
<td>4.0 (3.6-6.6, 42)</td>
<td>0.141^l</td>
</tr>
<tr>
<td># Days since last washed, median (IQR, n)</td>
<td>1 (0-2, 29)</td>
<td>1 (0-1, 23)</td>
<td>1 (0-1, 37)</td>
<td>0.375^l</td>
</tr>
<tr>
<td>Chemical treatment^c, n (%)</td>
<td>19 (61.3)</td>
<td>17 (of 26, 65.4)</td>
<td>25 (of 47, 53.2)</td>
<td>0.563^m</td>
</tr>
<tr>
<td>Medication Use During Pregnancy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhaled corticosteroid use^d, Yes/No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td>Oral corticosteroid use^e, n (%)</td>
<td>0 (0)</td>
<td>3 (9.7)</td>
<td>4 (7.1)</td>
<td>0.280^m</td>
</tr>
<tr>
<td>Intranasal corticosteroid use^f, n (%)</td>
<td>3 (9.7)^b</td>
<td>2 (6.5)^b</td>
<td>16 (28.6)</td>
<td>0.014^m</td>
</tr>
<tr>
<td>Topical corticosteroid use^g, n (%)</td>
<td>5 (16.1)</td>
<td>2 (6.4)</td>
<td>7 (12.5)</td>
<td>0.343^m</td>
</tr>
<tr>
<td>Other steroid hormone use (e.g., progesterone)^h, n (%)</td>
<td>3 (9.7)</td>
<td>4 (12.9)</td>
<td>6 (10.7)</td>
<td>0.867^m</td>
</tr>
<tr>
<td>Chemical treatment^i, n (%)</td>
<td>2 (6.4)^j</td>
<td>24 (77.4)^j</td>
<td>54 (96.4)</td>
<td>&lt; 0.001^m</td>
</tr>
<tr>
<td>Number of other classes of medications used^j, median (IQR)</td>
<td>2 (1-3)</td>
<td>2 (0-4)</td>
<td>2 (1-3)</td>
<td>0.650^l</td>
</tr>
</tbody>
</table>

BMI = body mass index, PSS = perceived stress scale, IQR = interquartile range, SD = standard deviation

^aInclusive of data for twin births. The frequency of which was not significantly different among the groups. ^bThe age is calculated to the oldest part of the hair sample at the beginning of the preconception segment. ^cFor No ICS and ICS Treated, information regarding chemical treatment (color or relaxer) was not available for all women. The total number of women is indicated in the parentheses. ^dInhaled corticosteroid use includes any use within the time captured by the tested hair segment, regardless of frequency or duration. ^eOral corticosteroid use is reported if within one month prior to the tested hair segment. ^fUse within the last 12 months, which may not be during pregnancy. ^gIncludes beta-agonist drugs that are short and long-acting, including use of combination inhaler products. ^hSignificantly different from ICS Treated, p < 0.05. ^iSignificantly different from both No ICS and ICS Treated, p < 0.0001. ^jSignificantly different from ICS Treated p < 0.01. ^kOne-way analysis of variance with Tukey's multiple comparison test. ^lChi-squared or Freeman-Halton extension of the Fisher's exact probability test, as appropriate.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>No ICS</th>
<th></th>
<th></th>
<th>ICS Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC</td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td>PP</td>
<td>PC</td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
</tr>
<tr>
<td>Segment length</td>
<td>rho</td>
<td>-0.248</td>
<td>-0.279</td>
<td>-0.257</td>
<td>0.739</td>
<td>0.283</td>
<td>-0.155</td>
<td>-0.102</td>
<td></td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.194</td>
<td>0.176</td>
<td>0.446</td>
<td>0.262</td>
<td>0.129</td>
<td>0.492</td>
<td>0.795</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>29</td>
<td>30</td>
<td>25</td>
<td>11</td>
<td>4</td>
<td>29</td>
<td>30</td>
<td>22</td>
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<tr>
<td>Hair sample age</td>
<td>rho</td>
<td>-0.317</td>
<td>-0.376</td>
<td>-0.392</td>
<td>-0.082</td>
<td>-0.201</td>
<td>-0.268</td>
<td>-0.393</td>
<td>-0.833</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.094</td>
<td>0.04</td>
<td>0.055</td>
<td>0.811</td>
<td>0.295</td>
<td>0.152</td>
<td>0.071</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>29</td>
<td>30</td>
<td>25</td>
<td>11</td>
<td>4</td>
<td>29</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td>Pregnant BMI</td>
<td>rho</td>
<td>-0.325</td>
<td>-0.131</td>
<td>-0.112</td>
<td>-0.497</td>
<td>0.069</td>
<td>-0.015</td>
<td>0.12</td>
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<td></td>
<td>p</td>
<td>0.085</td>
<td>0.491</td>
<td>0.593</td>
<td>0.12</td>
<td>0.2</td>
<td>0.755</td>
<td>0.944</td>
<td>0.646</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>29</td>
<td>30</td>
<td>25</td>
<td>11</td>
<td>4</td>
<td>23</td>
<td>24</td>
<td>17</td>
</tr>
<tr>
<td>Pre-pregnancy BMI</td>
<td>rho</td>
<td>-0.19</td>
<td>-0.014</td>
<td>-0.147</td>
<td>-0.664</td>
<td>0.196</td>
<td>0.149</td>
<td>0.24</td>
<td>0.286</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.323</td>
<td>0.941</td>
<td>0.483</td>
<td>0.026</td>
<td>0.37</td>
<td>0.488</td>
<td>0.353</td>
<td>0.535</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>29</td>
<td>30</td>
<td>25</td>
<td>11</td>
<td>4</td>
<td>23</td>
<td>24</td>
<td>17</td>
</tr>
<tr>
<td>PSS score washes</td>
<td>rho</td>
<td>0.123</td>
<td>0.241</td>
<td>0.081</td>
<td>-0.546</td>
<td>0.563</td>
<td>0.707</td>
<td>0.371</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.559</td>
<td>0.246</td>
<td>0.72</td>
<td>0.102</td>
<td>0.146</td>
<td>0.05</td>
<td>0.468</td>
<td>0.624</td>
</tr>
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<td>10</td>
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<td>8</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>No. of hair washes</td>
<td>rho</td>
<td>-0.333</td>
<td>-0.277</td>
<td>-0.276</td>
<td>0.038</td>
<td>0.738</td>
<td>0.178</td>
<td>0.257</td>
<td>0.395</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.077</td>
<td>0.138</td>
<td>0.181</td>
<td>0.911</td>
<td>0.262</td>
<td>0.428</td>
<td>0.236</td>
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<td>25</td>
<td>11</td>
<td>4</td>
<td>22</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>No. of hair since</td>
<td>rho</td>
<td>0.365</td>
<td>0.175</td>
<td>0.03</td>
<td>0.103</td>
<td>0.775</td>
<td>-0.019</td>
<td>0.139</td>
<td>0.201</td>
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<tr>
<td></td>
<td>p</td>
<td>0.056</td>
<td>0.374</td>
<td>0.885</td>
<td>0.763</td>
<td>0.225</td>
<td>0.935</td>
<td>0.538</td>
<td>0.473</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>28</td>
<td>28</td>
<td>25</td>
<td>11</td>
<td>4</td>
<td>21</td>
<td>22</td>
<td>15</td>
</tr>
<tr>
<td>Chemical treatment</td>
<td>rho</td>
<td>-0.168</td>
<td>-0.171</td>
<td>-0.171</td>
<td>-0.331</td>
<td>-0.327</td>
<td>-0.318</td>
<td>-0.267</td>
<td>-0.152</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.383</td>
<td>0.365</td>
<td>0.413</td>
<td>0.320</td>
<td>0.673</td>
<td>0.130</td>
<td>0.197</td>
<td>0.547</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>29</td>
<td>30</td>
<td>25</td>
<td>11</td>
<td>4</td>
<td>24</td>
<td>25</td>
<td>18</td>
</tr>
</tbody>
</table>

PC = preconception, T1 = first trimester, T2 = second trimester, T3 = third trimester, PP = postpartum, BMI = body mass index, PSS = perceived stress scale.

*Results listed top to bottom are the correlation coefficient (rho or r), p-value/, and n.

*p-values are not adjusted for multiple comparisons.

/Color or relaxer. All hair cortisol concentrations were natural log transformed and outliers were removed to normalize the data (which was unsuccessful for the ICS Treated PC results) in order to perform the point biserial correlation.
4.1.2 Hair cortisol concentrations during pregnancy

The hair cortisol concentrations for each period for each group of pregnant are shown in Figure 10. A similar increase in cortisol over the course of pregnancy from PC to T3, followed by a decline PP, is evident for all three groups, although this trend is less pronounced for the two groups of women with asthma. Seven patients (five ICS Treated and two No ICS) had hair cortisol concentrations that declined from PC to T2. Additionally, there was some noteworthy variability due to a subgroup of three women (11 samples) with hair cortisol concentrations ≥ 100 ng/g consisting of one Control for segments PC to PP, and two ICS Treated for segments PC to T3 and T3 to PP (Figure 10). This variability is reflected in the wide IQRs reported in Table 7 for a few of the time points. The cortisol concentrations for the Controls differed significantly across all time points ($\chi^2(4) = 9.6$, $p = 0.028$, $n = 4$, see Table 7 for the values) and increased from PC to T3. The ICS Treated group also showed overall significant changes in cortisol ($\chi^2(4) = 21.4$, $p < 0.001$, $n = 7$) as well as an increase between PC to T3. The No ICS group was the only group that did not have a significant overall change in cortisol across all time points ($\chi^2(4) = 2.1$, $p = 0.768$, $n = 3$) but did show a similar, although not statistically significant ($\chi^2(4) = 2.0$, $p = 0.583$, $n = 8$), trend of increasing cortisol from PC to T3. Generally, post hoc analyses revealed significant differences over the course of pregnancy between hair cortisol concentrations during PC and T1 compared to T2 and onward for the Controls and ICS Treated (Figure 10).

Table 7. Hair cortisol results for each time point for the pregnant women cohort.

<table>
<thead>
<tr>
<th>Group</th>
<th>PC</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>PP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>2.9 (1.4-6.1, 29)</td>
<td>3.9 (2.0-9.2, 30)</td>
<td>5.9 (3.3-9.9, 25)</td>
<td>7.7 (5.3-11.2, 11)</td>
<td>6.4 (3.2-90.3, 4)</td>
</tr>
<tr>
<td>No ICS</td>
<td>3.0 (1.7-4.5, 29)</td>
<td>3.6 (2.6-4.8, 30)</td>
<td>4.7 (3.1-5.7, 22)</td>
<td>4.7 (2.4-7.4, 9)</td>
<td>2.7 (2.5-3.4, 3)</td>
</tr>
<tr>
<td>ICS Treated</td>
<td>2.9 (1.9-5.1, 50)</td>
<td>3.9 (2.5-5.9, 54)</td>
<td>4.1 (3.0-8.0, 38)</td>
<td>5.1 (3.7-7.9, 19)</td>
<td>4.4 (3.6-6.4, 10)</td>
</tr>
</tbody>
</table>

IQR = interquartile range
Figure 10. Scatter plots of median hair cortisol concentrations for the pregnant women cohort. Median hair cortisol concentrations (horizontal bar) in ng/g of hair are shown for each group of pregnant women (A - Controls, B – No ICS, C – ICS Treated) by pregnancy time point consisting of preconception (PC), first trimester (T1), second trimester (T2), third trimester (T3), and postpartum (PP). Hair cortisol is plotted on a log10 y-axis. Sample sizes for each time point are shown below the x-axis. The change in cortisol over the five time points was significant for the Controls and ICS Treated. Post hoc analysis showed significant differences between time points as indicated in the figure. When analyses were repeated with all hair cortisol concentrations ≥ 100 ng/g removed, the results were not greatly changed. The alternate p-values are shown in parentheses, ( ). Holm-Bonferroni correction for multiple comparisons was applied to all p-values. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001

4.1.3 Hair cortisol concentration in pregnant women with asthma

The median hair concentrations from PC to T3 were plotted for each group to determine the slopes of the regression lines for the change in cortisol during pregnancy. There was a significant difference among the three slopes (F(2,6) = 14.8, p = 0.005) (Figure 11A). Comparing the slopes to one another, the change in median hair cortisol concentrations from PC to T3 for the Controls was significantly different from the ICS Treated (F(1,4) = 22.6, p_adj = 0.026) and No
ICS ($F_{(1,4)} = 20.2, \ p_{adj} = 0.033$), whereas there was no significant difference between the No ICS and ICS Treated ($F_{(1,4)} = 0.1, \ p_{adj} = 2.29$). When the subgroup of women with samples with concentrations $\geq 100$ ng/g were excluded as potential confounders, the comparison between the Controls and No ICS was no longer significant ($F_{(1,4)} = 13.5, \ p_{adj} = 0.064$)(Figure 11B).

**Figure 11.** Comparison of the change in hair cortisol concentrations during pregnancy. The linear regressions for median hair cortisol concentrations for each group of pregnant women from PC to T3 are shown. The similarity of the slopes for the No ICS and ICS Treated groups can be seen. The difference of those slopes from the slope for the Controls is significant if the samples with concentrations $\geq 100$ ng/g are included (A), but when they are excluded (B), the comparison between the Controls and No ICS is no longer significant. PC = preconception, T1 = first trimester, T2 = second trimester, T3 = third trimester.

All groups of women had a similar median hair cortisol concentration at PC and T1 with differences becoming apparent in T2 and T3 (Figure 12 A, B). When cortisol levels for each individual time point were compared among the groups, although there was a visible difference between the Controls and two asthma groups for T3, the results were not significant ($\chi^2_{(2)} = 5.1, \ p = 0.078$) (Figure 12A) and performing the statistical analyses with and without the concentrations $\geq 100$ ng/g yielded similar results. However, when the cortisol results for all women with asthma were combined, the mean T3 cortisol concentration was significantly lower compared to the Controls ($t_{(34)} = 2.189, \ p = 0.036$). Moreover, when women who reported ICS use $\leq 1$ dose per
day on < 5 days per week (i.e. < 5 doses per week) and concentrations ≥ 100 ng/g were excluded as potential confounders, the T3 median hair cortisol concentration for the ICS Treated group was significantly lower (47%) than the Controls (7.2 ng/g vs. 3.8 ng/g, U = 9, \( p_{adj} = 0.029 \)) (Figure 12B). Further, univariate and multiple linear regression analysis to determine the influence of consistent ICS use, intranasal corticosteroids, or asthma on the T3 hair cortisol concentration showed that asthma was the only significant factor (\( F(1, 25) = 9.7, p = 0.005, R^2_{adj} = 0.257 \)). When the samples with concentrations ≥ 100 ng/g were included (with ICS use still restricted to ≥ 5 doses per week on ≥ 5 days per week), the median hair cortisol concentrations at T3 for the ICS Treated group was still 48% lower than the Controls, but the difference was no longer significant (7.7 ng/g vs. 4.0 ng/g, U = 29, \( p_{adj} = 0.386 \)) (Figure 12C).

**Figures 12.** Bar graphs comparing median hair cortisol concentrations for the pregnant women cohort. The median hair cortisol concentrations (y-axis) at each time point (x-axis) for Controls, No ICS, and ICS Treated are shown. Sample sizes for each time point are shown below the x-axis. A. Comparison inclusive of all women in the ICS Treated group who reported ICS use captured in the hair sample, regardless of frequency or duration, showing no significant difference among the three groups. B) Comparison excluding women with hair cortisol concentrations ≥ 100 ng/g and women in the ICS Treated group who reported ICS use ≤ 5 doses per week showing a significant difference between Controls and ICS Treated women in T3. However, when the samples with concentrations ≥100 ng/g were included, the comparison was no longer significant (C). *\( p \leq 0.05 \), error bars indicate the interquartile range, PC = preconception, T1 = first trimester, T2 = second trimester, T3 = third trimester, PP = postpartum.
4.1.4 Evaluation of inhaled corticosteroid type- and dose-dependent effects

In addition to comparison between the groups, an evaluation for any type- or dose-effects of the ICS on the HPA axis was performed with the recognition that the sample sizes were small for T3 where the difference among the groups was notable. The ICS most prescribed for the women was fluticasone propionate ($n = 38$), followed by budesonide ($n = 16$). The remaining two women used more than one ICS from PC to PP. The results of the estimated marginal means comparisons, adjusted for dose, are presented in Figure 13. The Budesonide group had significantly lower hair cortisol than the Fluticasone and Controls groups in T2. Additionally, the Budesonide group significantly differed from the Fluticasone group in PC and T1. No significant difference between the Controls and the Fluticasone group was evident for any period. The women who used multiple ICS had the lowest hair cortisol concentrations, but the sample consisted of only two women; therefore, the results should be interpreted with caution. When the analysis was performed without bootstrapping, the comparisons for T2 between the Controls and Fluticasone groups versus the Budesonide group remained significant, $p = 0.024$ and $p = 0.011$ (after Bonferroni correction), respectively.
Figure 13. Bar graph comparing the estimated marginal means of hair cortisol concentrations for pregnant women by ICS type after adjustment for the average daily dose (in beclomethasone-HFA equivalent). The hair cortisol estimated marginal mean is shown on the y-axis and the pregnancy time point is shown on the x-axis. The sample size for each group is also shown under the x-axis. Participants with results $\geq$ 100 ng/g were excluded in order to have a normal distribution for the analysis of covariance analysis. Significant differences, based on up to 1000 bootstrapped samples, are indicated * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PC = preconception, T1 = first trimester, T2 = second trimester, T3 = third trimester, PP = postpartum.

Finally, the investigation for any difference based on dose did not produce any significant results (Figure 14). The difference between the groups for T3 is visible but was not significant likely due to the small sample sizes when the women were divided into the average daily ICS dose groups. Interestingly, the women taking ICS doses $> 251$ µg/d had higher hair cortisol in T2 to PP than the No ICS and 0-250 µg/d groups (Figure 14A,B).
Figure 14. Graphs comparing the median hair cortisol concentrations for pregnant women by average daily ICS dose. Hair cortisol is on the y-axis and pregnancy time point is on the x-axis with the sample size for the groups shown below. A) Inclusive of women with hair cortisol concentrations ≥ 100 ng/g. B) Excluding women with hair cortisol results ≥ 100 ng/g. Average daily ICS dose is in beclomethasone-HFA equivalents. PC = preconception, T1 = first trimester, T2 = second trimester, T3 = third trimester, PP = postpartum.

4.2 Hair cortisol as a potential biomarker of hypothalamic-pituitary-adrenal axis suppression by inhaled corticosteroids in children with asthma

4.2.1 Children – pilot study


Hair samples from 23 children with asthma were analyzed. The hair samples from five of these patients did not include the time prior to ICS therapy; therefore, their results were not included in the analysis. The patient demographics and description of ICS and concomitant
steroid use are listed in Table 8. Further details for each individual patient regarding the type and duration of ICS use and the associated relative change in hair cortisol concentration are depicted in Figure 15. The median hair cortisol concentration was twofold lower during periods of ICS exposure compared with the control period with no exposure to ICS (89.8 ng/g (IQR, 24.5–256.5) vs. 198.2 ng/g (IQR, 56.2–798.0); \( p = 0.0015 \), Figure 16).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years, median (range)</td>
<td>4.1 (1.4 – 8.8)</td>
</tr>
<tr>
<td>Gender, F (M)</td>
<td>15 (3)</td>
</tr>
<tr>
<td>Duration of ICS use, median (range), months</td>
<td>5.1 (2.3 – 20.7)</td>
</tr>
<tr>
<td>ICS dose,(^a) number of patients</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>7</td>
</tr>
<tr>
<td>Low/moderate</td>
<td>9</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>ICS frequency of use,(^a) number of patients</td>
<td></td>
</tr>
<tr>
<td>Intermittent</td>
<td>1</td>
</tr>
<tr>
<td>Intermittent/Continuous</td>
<td>8</td>
</tr>
<tr>
<td>Continuous</td>
<td>9</td>
</tr>
<tr>
<td>Switched from one type of ICS to another during therapy, number of patients</td>
<td>9</td>
</tr>
<tr>
<td>Types of ICS used, number of patients(^b)</td>
<td></td>
</tr>
<tr>
<td>Ciclesonide</td>
<td>14</td>
</tr>
<tr>
<td>Fluticasone propionate</td>
<td>9</td>
</tr>
<tr>
<td>Beclomethasone</td>
<td>1</td>
</tr>
<tr>
<td>Combination, total (mometasone / fluticasone propionate)</td>
<td>3 (2 / 1)</td>
</tr>
<tr>
<td>Use of other corticosteroids during period assessed by hair cortisol results, number of patients</td>
<td></td>
</tr>
<tr>
<td>Systemic(^c) - total</td>
<td>7</td>
</tr>
<tr>
<td>• prior to starting ICS</td>
<td>2</td>
</tr>
<tr>
<td>• after starting ICS</td>
<td>6</td>
</tr>
<tr>
<td>• &gt;1 course</td>
<td>1</td>
</tr>
<tr>
<td>Intranasal</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^a\) See thesis section 2.3.1, Table 1, p. 16 for definitions.
\(^b\) Results equal >18 due to patients using more than one ICS type captured by the hair analysis.
\(^c\) Count restricted to patients who received systemic corticosteroids during the periods captured by the hair segments included in the statistical analysis.
Figure 15. Individual patient ICS therapy duration and associated percent change in hair cortisol before ICS therapy compared to during ICS therapy. Stacked bar graph displays the data for each of the 18 individual participants along the x-axis from left to right. Each participant's percent change in hair cortisol is indicated using the right y-axis and the white bar with the majority of children showing a negative change in hair cortisol. The duration of ICS use in months is indicated along the left y-axis with the bars extending in the positive direction. Each bar is coded to indicate the specific ICS used by the patient (see legend). Nine patients switched the type of ICS used, as shown by the change in color or pattern of the bar, and the hair segments that were analyzed for cortisol captured this switch. The bars for duration of ICS use also illustrate the length of the hair that was segmented for cortisol analysis during ICS therapy based on the average growth rate of 1 cm/mo, but it is an overestimation to a varying degree for each patient due to the exclusion of the hair segment when ICS was initiated.
4.2.2 Children – large cohort

4.2.2.1 Participant demographics

A total of 586 children were enrolled in the large cohort of children. Of those, 460 samples were suitable for analysis. The remaining hair samples had either already been used in the children's pilot study (n = 23) or could not be tested because they were not of sufficient quantity (n = 20), less than 3 cm long (n = 76), too curly for accurate segmentation (n = 2), mislabelled (n = 2), or the participant had a condition that was not asthma (n = 2) or was associated with elevated endogenous cortisol (n = 1). Table 9 summarizes the cohort demographics, hair sample variables, and medication use. The three groups of children differed in the ratio of males to females, the location of recruitment, whether their hair was chemically treated, and in all aspects of medication use except the use of topical corticosteroids and/or other hormones. For the children on ICS therapy, 33.6% used fluticasone, 36.7% ciclesonide, 8.1% beclomethasone, 8.1% budesonide, 3.3% mometasone, 10.0% multiple ICS, and for one child (0.2%) the ICS use was unclear. For each dose category, there were 118 taking 0-1.0 μg/kg/d, 139 taking >1.0-2.5 μg/kg/d, 91 taking >2.5 μg/kg/d, 11 children did not have their weight recorded to determine their ICS dose category, and 1 child did not have their ICS dose recorded.

**Figure 16.** Children's pilot study comparison of the median average monthly hair cortisol concentration before and during ICS therapy. Tukey boxplot comparison of the participants’ median average monthly hair cortisol results for a maximum 6-mo period prior to ICS therapy and minimum 2-mo period during ICS therapy (n = 18). *p = 0.0015.
Table 9. Comparison of demographics, hair sample variables, and medication use among the three groups of children with and without asthma.

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Controls (n = 53)</th>
<th>No ICS (n = 47)</th>
<th>ICS Treated (n = 360)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, years, median (IQR)</strong></td>
<td>7.7 (3.1-12.3)</td>
<td>7.9 (4.2-12.0)</td>
<td>6.3 (3.7-10.1)</td>
<td>0.236</td>
</tr>
<tr>
<td><strong>BMI, kg/m², median (IQR, n)</strong></td>
<td>17.6 (15.6-20.7, 51)</td>
<td>17.5 (16.3-19.6, 46)</td>
<td>16.8 (15.5-19.2, 349)</td>
<td>0.061</td>
</tr>
<tr>
<td><strong>Male:Female, n</strong></td>
<td>22:31</td>
<td>28:19</td>
<td>213:147</td>
<td>0.050</td>
</tr>
<tr>
<td><strong>Toronto:Vancouver:Winnipeg, n</strong></td>
<td>50:3:0</td>
<td>24:19:4</td>
<td>66:229:65</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>PSS Score, mean (SD, n)</strong></td>
<td>15.7 (8.0, 22)</td>
<td>13.7 (4.1, 17)</td>
<td>15.4 (6.9, 11)</td>
<td>0.537</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hair Sample Variables</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong># Washes per week, median (IQR, n)</strong></td>
<td>3.5 (2.0-4.9, 52)</td>
<td>3.5 (2.0-7.0, 47)</td>
<td>3.5 (2.4-5.0, 353)</td>
<td>0.663</td>
</tr>
<tr>
<td><strong># Days since last washed, median (IQR, n)</strong></td>
<td>2.0 (1.0-2.0, 51)</td>
<td>1.0 (0.8-2.0, 42)</td>
<td>1.0 (1.0-2.0, 304)</td>
<td>0.162</td>
</tr>
<tr>
<td><strong>Chemical treatment, n (%)</strong></td>
<td>8 (15.1)</td>
<td>2 (4.3)</td>
<td>17 (of 354, 4.8)</td>
<td>0.010</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medication Use</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhaled corticosteroid use, Yes/No</strong></td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td><strong>Lifetime duration of ICS use, years, median (IQR)</strong></td>
<td>0 (0-0)</td>
<td>0.9 (0.0-4.5)</td>
<td>2.3 (0.9-5.2)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Systemic corticosteroid use, n (%)</strong></td>
<td>1</td>
<td>4</td>
<td>129</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Intranasal corticosteroid use, n (%)</strong></td>
<td>4</td>
<td>10</td>
<td>106</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Topical corticosteroid and/or other hormone use, n (%)</strong></td>
<td>10</td>
<td>8</td>
<td>69</td>
<td>0.940</td>
</tr>
<tr>
<td><strong>Beta-agonist, n (%)</strong></td>
<td>3</td>
<td>35</td>
<td>355</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td><strong>Number of other classes of medications used, median (IQR)</strong></td>
<td>2.0 (1.0-2.0)</td>
<td>2.0 (1.0-3.0)</td>
<td>1.0 (1.0-2.0)</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

BMI = body mass index, PSS = perceived stress scale; IQR = interquartile range, SD = standard deviation

*Inhaled corticosteroid use includes any use within the time captured by the tested hair segment, regardless of frequency or duration.

*Systemic corticosteroid use is reported if within two months prior to the tested hair segment.

*Use within the last 12 months.

*Includes beta-agonist drugs that are short and long-acting, including use of combination inhaler products.

*Significantly different from ICS Treated, p < 0.05.

*Significantly different from ICS Treated, p < 0.001.

*Significantly different from ICS Treated, p < 0.01.

*Significantly different from each other, p < 0.001.

*Significantly different from No ICS only, p < 0.01.

*One-way analysis of variance with Tukey's multiple comparison test.

*Kruskal-Wallis test with Dunn's multiple comparison test.

*Chi-squared test. Note: Chemical treatment had greater than 80% with expected value <5.

*Welch's analysis of variance.
4.2.2.2 Comparison of hair cortisol concentrations among the groups

The median hair cortisol concentrations did not differ among the three groups of children with and without asthma and are shown in Figure 17. The median and range for each group is: Controls 5.8 ng/g (IQR 4.6-16.7 ng/g), No ICS 6.7 ng/g (IQR 3.7-9.8 ng/g), and ICS Treated 6.5 ng/g (IQR 3.8-14.3 ng/g). A rank transformation of the data improved, but did not fully normalize, the distributions. The comparison of the median rank hair cortisol concentrations was also not significant.

Upon visual inspection, there appears to be subpopulations present. The distributions for the Controls and No ICS appear bimodal with a hair cortisol concentration upper limit of 40 ng/g for participants around the median (Figure 17). Using this cut-off, the subpopulation of children in each group with hair cortisol concentrations > 40 ng/g was not significantly different with 11%, 13%, and 14% in the Controls, No ICS, and ICS Treated, respectively. In the ICS Treated group, 5 children also had hair cortisol concentrations > 1000 ng/g. Similarly, a lower limit for hair cortisol concentration of 2.0 ng/g was notable given that none of the children in the control groups had a concentration below this level. Therefore, of note is the subpopulation of 19 children (5.3%) in the ICS Treated group with HCC <2.0 ng/g.

**Figure 17.** Scatterplot of hair cortisol concentrations by group for children in the large cohort. Hair cortisol is shown on the y-axis with a logarithmic scale and the groups are indicated on the x-axis. The red bars indicate the median hair cortisol concentrations for each group. The black dotted lines indicate the 2 and 40 ng/g concentrations.
4.2.2.3 Age and sex effects on hair cortisol concentrations

The change in hair cortisol concentration was evaluated by age and sex in the large cohort of children inclusive of healthy and children with asthma (Figure 18), even though there was not a significant difference among the groups for the participants' age. Age was included because it is known that children will generally have age dependent reference intervals established for clinical laboratory tests and previous research has found hair cortisol concentrations will vary by age (see Table 4).

**Figure 18.** Scatterplot of hair cortisol concentration versus age and sex for the large cohort of children. Hair cortisol concentration is indicated on the y-axis with a logarithmic scale. The age is shown on the x-axis. The children's sex is indicated by the different colour dots as shown in the legend. The linear regressions comparing hair cortisol concentration by sex for children aged 1- to 8-years and > 8- to 18-years are shown in the left and right insets, respectively, with the log\(_{10}\) hair cortisol concentration plotted on the y-axis.
Younger children tend to have higher hair cortisol than older children. Linear regression showed that hair cortisol concentration significantly decreases from 1-year-old until approximately 8-years-old but does not significantly differ between the sexes (slope_{males&females} = -0.09 ng/g; slope_{males} = -0.08 ± 0.03 ng/g, p = 0.004; slope_{females} = -0.10 ± 0.03 ng/g, p = 0.006) (Figure 18, left inset). From 8- to 18-years of age, the regression lines suggest that hair cortisol concentration increases with age for females and stay approximately the same for males, but this was not a significant difference (slope_{males} = 0.01 ± 0.02 ng/g vs. slope_{females} = 0.03 ± 0.01 ng/g, F_{(1,170)} = 1.9, p = 0.17). However, males have a significantly higher hair cortisol in adolescence, around 8-years-old, that diminishes with age for this cohort due to that slight increase in hair cortisol seen for females (comparison of y-intercept: y-intercept_{male} = 0.70 ± 0.20 ng/g vs. y-intercept_{female} = 0.21 ± 0.15 ng/g, F_{(1,171)} = 8.3, p = 0.004) (Figure 18, right inset). Because of the variations in hair cortisol concentration by age and sex, these variables were included in the quantile regression, the results of which are presented below.

4.2.2.4 Quantile regression analysis of factors affecting hair cortisol concentrations

A regression analysis was performed to specifically determine any ICS type- or dose-dependent effects on hair cortisol concentrations in children with and without asthma while adjusting for other variables with potentially significant effects. Quantile regression was performed because the hair cortisol data for the large cohort of children (n = 443 for Model 1, n = 442 for Model 2) was not normally distributed and could not be corrected by transformation. The outcome was compared to the results of OLS regression of the original data and rank transformed data, which was chosen as a transformation that may normalize the data. The results of Models 1 (ICS type) and 2 (ICS dose) are summarized for the 5th, 25th, 50th, 75th, and 95th quantiles (percentiles) of hair cortisol concentration in Tables 10-12, with Table 12 containing the standardized regression coefficients for the significant variables only. Figure 19 shows the graphs for Model 2 (Model 1 graphs are not shown) to illustrate the outcome of the quantile regression. Similar to linear regression, the coefficient is significant if it is non-zero and the confidence interval (shaded in grey in Figure 19) does not include zero. The variable age was centred to the average age of the large cohort of children (7.48 years), and the variable BMI was centred to the average BMI for a child aged 7.48 years, which is 15.5 kg/m². Therefore, the reference group for Models 1 and 2 is a Control male, living in Toronto, aged 7.48 years with a
BMI of 15.5 who does not have chemically treated hair and does not use intranasal corticosteroids or ICS.

Upon reviewing the graphs, a few observations are immediately obvious. First, the OLS regression of the original hair cortisol concentrations does not perform well due to the influence of high hair cortisol concentrations as indicated by the significant difference of the coefficient (red line in Figure 19) from the quantile regression coefficients. Additionally, the confidence intervals for this OLS are extremely wide and are not shown because the values are beyond the scale of the y-axes, which also denotes that the outcomes are not significant. Second, the OLS regression of the ranked hair cortisol concentrations fared better, but still overestimated the effect of the significant variables, such as the intercept, age, BMI, sex, and intranasal corticosteroid use. Third, for all but one of the variables included in the models, confounding due to the extremely high hair cortisol concentrations is evidenced by the wide confidence intervals at the highest percentiles of hair cortisol concentrations beginning at the 75th to 85th percentile and up, depending on the variable. This occurs in all graphs for both models, except for age in Model 2 (Figure 19). Finally, there are variables with confidence limits that include infinity for the lowest and highest percentiles, such as chemical treatment of the hair, beclomethasone use, and mometasone use, which is likely due to underrepresentation of those variable in those percentiles.

The quantile regression results were not significant for living in Winnipeg, lifetime duration of ICS use, and, ICS type. Additionally, the ICS dose did not have significant results with a couple of exceptions. Using ICS at doses >2.5 µg/kg/d had a diverging effect. Of note, for children in 5th percentile of hair cortisol concentration, the high dose was associated with lower hair cortisol concentrations (unstandardized regression coefficient (β) = -0.7 ng/g, CI95% -1.0 to -0.04 ng/g, Table 11), but for children in the 55th percentile, that dose was associated with higher hair cortisol concentrations (β = 3.8 ng/g, CI95% 0.4-5.6 ng/g). Further, in Model 1, there was a negative effect at the 5th percentile for children who live in Vancouver and an increase in hair cortisol for those who chemically treat their hair at the 50th and 75th percentiles (Table 10). In Model 2, for children in the 15th percentile, having asthma decreased their hair cortisol (β = -1.0 ng/g, CI95% -2.1 to -0.4 ng/g) and chemically treated hair was associated with increased hair cortisol (β = 0.5 ng/g, CI95% 0.1-1.2 ng/g). However, an interpretation of variables with significance at only one or two percentiles may be over-interpretation (Good and Hardin, 2012).
The quantile regression did reveal variables with a significant effect on hair cortisol concentrations. For the intercept, the coefficients in Models 1 and 2 are significant for all of the percentiles until confounding by high cortisol concentrations takes effect at the 75\textsuperscript{th} percentile. The quantile regression results for the intercepts in both models correspond to the hair cortisol concentrations of the Control group and the significant coefficients fall within the interquartile range (IQR) for the Controls' median hair cortisol concentration. The variables with significant effects on hair cortisol concentrations in both models include age, sex, BMI, and intranasal corticosteroid use (Figure 219, Tables 10-12). Age had a significant and increasingly negative effect on hair cortisol concentrations as the percentile increased in Models 1 and 2, but only for the 5\textsuperscript{th} to 85\textsuperscript{th} percentiles in Model 1. Being female slightly, but significantly, lowers hair cortisol concentrations for children in percentiles 15 to 55 in Model 1 and 5 to 25 in Model 2. Intranasal corticosteroid use was also negatively associated with hair cortisol concentrations. In Model 1, their use was significant for children in the 15\textsuperscript{th} to 65\textsuperscript{th} percentiles and in Model 2 for those in the 25\textsuperscript{th} to 75\textsuperscript{th} percentiles. In contrast, having a higher BMI was associated with higher hair cortisol concentrations for children in the 5\textsuperscript{th} to 50\textsuperscript{th} percentiles in Model 1 and the 5\textsuperscript{th} to 75\textsuperscript{th} percentiles in Model 2. The standardized coefficients for these significant variables in Models 1 and 2 are summarized in Table 12. To summarized the results, age was the most significant factor affecting the hair cortisol concentration, followed next by BMI and intranasal corticosteroid use, and being female had the least effect on hair cortisol concentrations.
Figure 19. Quantile regression graphs for each variable included in Model 2 to evaluate ICS dose-dependent effects on hair cortisol concentrations in the large cohort of children (see next page for figure and legend continuation).
Figure 19, continued. Quantile regression graphs for each variable included in Model 2 to evaluate ICS dose-dependent effects on hair cortisol concentrations in the large cohort of children. Figure continued from previous page. The unstandardized coefficient is plotted on the y-axis and the quantile (or percentile) is plotted on the x-axis of each graph. The zero y-intercept is indicated by the black dotted line in each graph for reference. The solid black line indicates the coefficient and the grey shaded area indicates the CI_{95%}. The OLS regression coefficients and CI_{95%} of the original and rank transformed hair cortisol concentrations are shown by the solid and dashed red and green lines, respectively. The dashed lines are not shown if the values are beyond the y-axis scale.
<table>
<thead>
<tr>
<th>Variable</th>
<th>0.05</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>0.95</th>
<th>OLS</th>
<th>Rank OLS†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>5.8</td>
<td>9.1</td>
<td>14.0</td>
<td>13.2</td>
<td>-452.8</td>
<td>54.0</td>
<td>258.2</td>
</tr>
<tr>
<td>Age (years, centred to 7.48)</td>
<td>-0.2</td>
<td>-0.3</td>
<td>-0.5</td>
<td>-1.3</td>
<td>-10.9</td>
<td>-16.6</td>
<td>-12.5</td>
</tr>
<tr>
<td>Female</td>
<td>-0.6</td>
<td>-1.2</td>
<td>-1.3</td>
<td>4.1</td>
<td>8.3</td>
<td>-33.6</td>
<td>-31.5</td>
</tr>
<tr>
<td>BMI (kg/m², centred to 15.5)</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>3.6</td>
<td>-4.5</td>
<td>3.4</td>
</tr>
<tr>
<td>Lifetime duration of ICS use (years)</td>
<td>0.04</td>
<td>0.00</td>
<td>0.1</td>
<td>0.1</td>
<td>-2.6</td>
<td>-12.4</td>
<td>1.1</td>
</tr>
<tr>
<td>Intranasal corticosteroid use</td>
<td>0.1</td>
<td>-0.9</td>
<td>-1.9</td>
<td>-3.2</td>
<td>8.3</td>
<td>192.6</td>
<td>-40.8</td>
</tr>
<tr>
<td>Living in Vancouver</td>
<td>-0.4</td>
<td>0.03</td>
<td>0.5</td>
<td>3.1</td>
<td>204.1</td>
<td>65.2</td>
<td>36.6</td>
</tr>
<tr>
<td>Living in Winnipeg</td>
<td>-0.2</td>
<td>-0.3</td>
<td>-0.9</td>
<td>4.0</td>
<td>47.1</td>
<td>24.3</td>
<td>-24.4</td>
</tr>
<tr>
<td>Chemically treated hair</td>
<td>-0.6</td>
<td>0.9</td>
<td>1.6</td>
<td>4.7</td>
<td>15.7</td>
<td>58.1</td>
<td>42.2</td>
</tr>
<tr>
<td>Having asthma</td>
<td>-0.9</td>
<td>-0.6</td>
<td>0.2</td>
<td>5.8</td>
<td>318.7</td>
<td>70.3</td>
<td>5.9</td>
</tr>
<tr>
<td>Use of fluticasone</td>
<td>-0.6</td>
<td>1.1</td>
<td>2.2</td>
<td>-6.4</td>
<td>25.7</td>
<td>60.3</td>
<td>123.3</td>
</tr>
<tr>
<td>Use of beclomethasone</td>
<td>-0.8</td>
<td>-0.2</td>
<td>-0.6</td>
<td>5.8</td>
<td>318.7</td>
<td>70.3</td>
<td>5.9</td>
</tr>
<tr>
<td>Use of udesonide</td>
<td>-0.4</td>
<td>0.4</td>
<td>0.9</td>
<td>2.0</td>
<td>201.5</td>
<td>105.1</td>
<td>24.3</td>
</tr>
<tr>
<td>Use of mometasone</td>
<td>0.7</td>
<td>0.6</td>
<td>-0.4</td>
<td>5.8</td>
<td>101.5</td>
<td>105.1</td>
<td>24.3</td>
</tr>
<tr>
<td>Use of ciclesonide</td>
<td>-0.5</td>
<td>0.2</td>
<td>-0.2</td>
<td>0.5</td>
<td>52.4</td>
<td>311.7</td>
<td>-2.7</td>
</tr>
<tr>
<td>Use of multiple ICS</td>
<td>0.2</td>
<td>-0.7</td>
<td>-1.0</td>
<td>-2.6</td>
<td>-60.2</td>
<td>26.6</td>
<td>5.2</td>
</tr>
</tbody>
</table>

**Table 10.** Comparison of the coefficients* for quantile regression Model 1 investigating ICS type to the OLS regression of original and rank transformed hair cortisol concentrations for the large cohort of children.

*All coefficients are unstandardized and reported with the lower and upper limits in parentheses. †Rank OLS coefficients are for change in rank hair cortisol concentration (ranked from 1-460), not the actual hair cortisol concentration. Results significantly different from 0 are shown in bold. Quantile results are significantly different from the OLS results if the OLS coefficient is not contained within the range shown for the quantile regression.

OLS = ordinary least squares
Table 11. Comparison of the coefficients* for quantile regression Model 2 investigating ICS dose to the OLS regression of original and rank transformed hair cortisol concentrations for the large cohort of children.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Quantile (Percentile)</th>
<th>OLS</th>
<th>Rank OLS†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>Intercept</td>
<td>6.0</td>
<td>9.4</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>(4.0, 7.4)</td>
<td>(6.4, 11.9)</td>
<td>(0.8, 17.7)</td>
</tr>
<tr>
<td>Age (years, centred to 7.48)</td>
<td>-0.2</td>
<td>-0.2</td>
<td>-0.4</td>
</tr>
<tr>
<td></td>
<td>(-0.3, -0.1)</td>
<td>(-0.4, -0.1)</td>
<td>(-0.6, -0.2)</td>
</tr>
<tr>
<td>Female</td>
<td>-0.5</td>
<td>-0.9</td>
<td>-1.1</td>
</tr>
<tr>
<td></td>
<td>(-0.8, -0.01)</td>
<td>(-1.4, -0.3)</td>
<td>(-2.6, 0.5)</td>
</tr>
<tr>
<td>BMI (kg/m², centred to 15.5)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>(0.05, 0.13)</td>
<td>(0.07, 0.2)</td>
<td>(0.04, 0.3)</td>
</tr>
<tr>
<td>Lifetime duration of ICS use</td>
<td>0.04</td>
<td>0.00</td>
<td>-0.01</td>
</tr>
<tr>
<td></td>
<td>(-0.04, 0.08)</td>
<td>(-0.06, 0.1)</td>
<td>(-0.2, 0.2)</td>
</tr>
<tr>
<td>Intranasal corticosteroid use</td>
<td>0.1</td>
<td>-1.2</td>
<td>-1.7</td>
</tr>
<tr>
<td></td>
<td>(-0.5, 0.2)</td>
<td>(-1.7, -0.4)</td>
<td>(-3.1, -0.8)</td>
</tr>
<tr>
<td>Living in Vancouver</td>
<td>-0.2</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>(-0.7, 0.1)</td>
<td>(-0.9, 1.1)</td>
<td>(-2.2, 1.9)</td>
</tr>
<tr>
<td>Living in Winnipeg</td>
<td>0.07</td>
<td>-0.3</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>(-0.8, 0.6)</td>
<td>(-1.2, 0.6)</td>
<td>(-2.3, 2.1)</td>
</tr>
<tr>
<td>Chemically treated hair</td>
<td>1.0</td>
<td>-0.2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>(-infinity, 1.3)</td>
<td>(-0.8, 1.7)</td>
<td>(-1.0, 3.0)</td>
</tr>
<tr>
<td>Having asthma</td>
<td>-1.0</td>
<td>-0.7</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>(-1.6, 0.2)</td>
<td>(-2.3, 0.5)</td>
<td>(-4.0, 1.8)</td>
</tr>
<tr>
<td>Average daily dose &gt;0-1.0 µg/kg/d</td>
<td>-0.6</td>
<td>0.1</td>
<td>-0.8</td>
</tr>
<tr>
<td></td>
<td>(-1.0, 0.6)</td>
<td>(-1.1, 1.5)</td>
<td>(-2.3, 1.1)</td>
</tr>
<tr>
<td>Average daily dose 1.01-2.5 µg/kg/d</td>
<td>-0.6</td>
<td>-0.4</td>
<td>-0.4</td>
</tr>
<tr>
<td></td>
<td>(-1.3, 0.1)</td>
<td>(-1.7, 0.9)</td>
<td>(-2.1, 1.4)</td>
</tr>
<tr>
<td>Average daily dose &gt;2.5 µg/kg/d</td>
<td>-0.7</td>
<td>-0.5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>(-1.0, -0.04)</td>
<td>(-1.9, 1.1)</td>
<td>(-1.3, 5.9)</td>
</tr>
</tbody>
</table>

OLS = ordinary least squares

*All coefficients are unstandardized and reported with the lower and upper limits in parentheses.

†Rank OLS coefficients are for change in rank hair cortisol concentration (ranked from 1-460), not the actual hair cortisol concentration. Results significantly different from 0 are shown in **bold**. Quantile results are significantly different from the OLS results if the OLS coefficient is not contained within the range shown for the quantile regression.
Table 12. Summary of standardized quantile regression coefficients for significant independent variables in Models 1 and 2 for the large cohort of children

<table>
<thead>
<tr>
<th>Variable</th>
<th>0.05</th>
<th>0.25</th>
<th>0.5</th>
<th>0.75</th>
<th>0.95</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years, centred to 7.48)</td>
<td>-1.0</td>
<td>-1.3</td>
<td>-2.3</td>
<td>-5.9</td>
<td>-48.5</td>
</tr>
<tr>
<td></td>
<td>(-1.4, -0.4)</td>
<td>(-1.7, -0.8)</td>
<td>(-3.0, -1.6)</td>
<td>(-7.7, -4.2)</td>
<td>(-68.9, 22.1)</td>
</tr>
<tr>
<td>Female</td>
<td>-0.3</td>
<td>-0.6</td>
<td>-0.6</td>
<td>-2.0</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>(-0.35, 0.01)</td>
<td>(-0.8, -0.2)</td>
<td>(-1.1, -0.1)</td>
<td>(-3.0, 1.1)</td>
<td>(-61.6, 69.6)</td>
</tr>
<tr>
<td>BMI (kg/m², centred to 15.5)</td>
<td>0.5</td>
<td>0.7</td>
<td>0.6</td>
<td>0.5</td>
<td>-15.5</td>
</tr>
<tr>
<td></td>
<td>(0.3, 0.6)</td>
<td>(0.4, 1.0)</td>
<td>(0.03, 1.1)</td>
<td>(-1.0, 1.8)</td>
<td>(-23.2, 2.1)</td>
</tr>
<tr>
<td>Intranasal corticosteroid use</td>
<td>0.1</td>
<td>-0.4</td>
<td>-0.9</td>
<td>-1.4</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>(-0.1, 0.2)</td>
<td>(-0.7, -0.2)</td>
<td>(-1.4, -0.4)</td>
<td>(-3.5, 0.04)</td>
<td>(-50.2, 36.9)</td>
</tr>
<tr>
<td><strong>Model 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years, centred to 7.48)</td>
<td>-0.9</td>
<td>-1.1</td>
<td>-1.8</td>
<td>-5.2</td>
<td>-88.0</td>
</tr>
<tr>
<td></td>
<td>(-1.5, -0.3)</td>
<td>(-1.8, -0.6)</td>
<td>(-2.8, -1.1)</td>
<td>(-6.5, -3.9)</td>
<td>(-127.7, -14.4)</td>
</tr>
<tr>
<td>Female</td>
<td>-0.3</td>
<td>-0.5</td>
<td>-0.6</td>
<td>-1.2</td>
<td>26.7</td>
</tr>
<tr>
<td></td>
<td>(-0.4, 0.0)</td>
<td>(-0.7, -0.1)</td>
<td>(-1.3, 0.2)</td>
<td>(-2.7, 0.4)</td>
<td>(-28.0, 93.2)</td>
</tr>
<tr>
<td>BMI (kg/m², centred to 15.5)</td>
<td>0.4</td>
<td>0.6</td>
<td>0.7</td>
<td>1.0</td>
<td>-14.9</td>
</tr>
<tr>
<td></td>
<td>(0.2, 0.6)</td>
<td>(0.3, 0.9)</td>
<td>(0.2, 1.4)</td>
<td>(0.1, 1.9)</td>
<td>(-42.6, 8.2)</td>
</tr>
<tr>
<td>Intranasal corticosteroid use</td>
<td>0.03</td>
<td>-0.5</td>
<td>-0.8</td>
<td>-2.2</td>
<td>-7.9</td>
</tr>
<tr>
<td></td>
<td>(-0.2, 0.1)</td>
<td>(-0.7, -0.2)</td>
<td>(-1.4, -0.3)</td>
<td>(-2.8, -0.3)</td>
<td>(-47.9, 51.5)</td>
</tr>
</tbody>
</table>

*Coefficients were standardized using the equation reported in the Methods and indicate the change in hair cortisol concentration in ng/g per 1 standard deviation change of the independent variable. Significant results are in **bold.**
Chapter 5
Discussion

5.1 Hair cortisol as a potential biomarker of the hypothalamic-pituitary-adrenal axis in pregnant women with asthma

5.1.1 Hair cortisol during pregnancy

This study investigated the potential of hair cortisol as a biomarker to assess asthma and the possible effects of ICS on the HPA axis in pregnant women. The present findings, which showed an increase over the course of pregnancy for all groups, are in line with previous hair cortisol research (D’Anna-Hernandez et al., 2011; Karlén et al., 2013; Kirschbaum et al., 2009; Krumbholz et al., 2013). D’Anna-Hernandez et al. (2011), who analyzed the most proximal 3 cm of hair in pregnant women during each trimester found a similar relationship between the trimesters as presented in this study with cortisol increasing from T1 to T3 and dropping PP, and T3 was significantly higher than T1 and PP but not T2. Additionally, the 2- to 3-fold increase in hair cortisol concentrations for the Controls from PC to T3 corresponds to the documented 2- to 3-fold increase in serum and salivary cortisol over the course of a normal pregnancy compared to a non-pregnant state (Kamoun et al., 2014; Mikkelsen et al., 1984; Suri et al., 2006). Moreover, this study also used hair cortisol measurements to assess the HPA axis in women with asthma and found significantly lower hair cortisol concentrations over the course of pregnancy in women with asthma compared to women without asthma regardless of ICS use, most notably in T3, suggestive of adrenal suppression.

The present results are in contrast to findings reported previously that did not find a significant difference in serum cortisol concentrations among pregnant women with asthma, with and without ICS treatment, and pregnant women without asthma (Hodyl et al., 2011). However, there are a few limitations of the previous study. The serum cortisol was only assessed on one occasion per trimester for each participant in a 3-hour time window, and the sample collection timing varied over a 4- to 9-week period of each trimester. The sample collection for T3 occurred during 25-34 weeks gestation, which is considered late T2 or early T3. This sampling pattern may not have fully captured the dynamic changes in cortisol that occur during pregnancy, including the rise in cortisol during T3 as seen in the current study. Finally, the serum cortisol measurement was of total cortisol and, therefore, subject to the influence of inter-individual
variation for changes in timing and quantity of corticosteroid-binding globulin production, whereas hair cortisol concentrations represent free cortisol concentrations. Thus, the present results suggest that using hair as the sample matrix, which is representative of the average active cortisol concentrations for the entire trimester, may be more sensitive for detecting changes in cortisol between each trimester and is a significant advantage of this research.

5.1.2 Pregnancy outcomes for women with adrenal insufficiency compared to women with asthma

The prevalence of clinically significant adrenal suppression, in pregnancy is currently not well documented. This is in part due to the dynamic and significant increases of cortisol during pregnancy potentially masking any deficit, and a lack of reference intervals specific to pregnancy (Kamoun et al., 2014). Previous research has largely focused on the fetal and perinatal outcomes of fetal exposure to increased cortisol concentrations (Moisiadis & Matthews, 2014a & 2014b), leaving a large gap in knowledge on the potential adverse effects of decreased cortisol concentrations. The present study suggests that women with asthma during pregnancy experience adrenal suppression, which may represent adrenal hypo-functionality that is less severe than that of adrenal insufficiency but could potentially still impact pregnancy outcomes and normal fetal maturation. Research shows that women with asthma, with or without ICS treatment, have similar perinatal outcomes to women with autoimmune adrenal insufficiency in that both groups are more likely to require a Cesarean section or have a preterm birth (Björnsdottir et al., 2010; Murphy et al., 2011; Wen et al., 2001), potentially indicating a link with decreased cortisol levels. The current data do not suggest women with asthma experience preterm birth (Table 1), but the sample size may be too small to detect a significant difference. However, the results of a recent publication investigating the determinants of maternal hair cortisol at delivery support an association between preterm birth and decreased cortisol. Although they did not take asthma or other diseases into consideration, Braig et al. (2015) found that women who had premature babies (≤ 37 weeks of gestation) had significantly lower hair cortisol levels in the three months prior to delivery ($\beta = -0.16$, $p = 0.029$). However, this significance did not remain when the regression model was adjusted for all other significant variables considered by the researchers (mutually adjusted $\beta = -0.10$, $p = 0.157$) (Braig et al., 2015). Nevertheless, the research contained in this thesis and that of Braig et al. (2015) supports the premise that hair cortisol is a useful tool and could be used in research to ascertain whether there is an association between decreased
maternal cortisol and pregnancy outcomes. Further research reporting on pregnancy outcomes for women with lower-than-normal cortisol production could not be found suggesting that the condition has possibly gone undetected until now, thus indicating an advantage of hair cortisol as a biomarker of adrenal function during pregnancy.

5.1.3 Hypothalamic-pituitary-adrenal axis fatigue

Based on previous reports of the association between decreased cortisol production and ICS therapy (Smy et al., 2015; Todd et al., 2002), it was anticipated that pregnant women using ICS might have decreased cortisol concentrations compared to both comparison groups. A significant decrease was apparent for women who used more than five ICS doses per week throughout pregnancy, but only in T3 when compared to Controls and if the three women with hair cortisol concentrations ≥ 100 ng/g were excluded. Unexpectedly, the No ICS group showed a diminished adrenal response over the course of pregnancy from PC to T3 similar to the ICS Treated group. One possible explanation for the lower hair cortisol concentrations in women with asthma may be sustained overwork and resultant fatigue of the HPA axis (Tian et al., 2014). Research shows that the initial response to stress is increased cortisol production with decreased expression of pro-inflammatory cytokines; however, with chronic exposure to stress hormones there is a decrease in immune system sensitivity and response to cortisol, ultimately resulting in decreased cortisol and increased pro-inflammatory cytokines (Tian et al., 2014). In preliminary results, pregnant women experiencing higher stress had lower morning cortisol levels than normal or low stressed pregnant women and the authors proposed there may be a protective effect for hypocortisolism in pregnancy by mitigating any potential stimulatory effects of increased maternal cortisol levels on placental CRH (Fries et al., 2005). Other examples of decreased cortisol in individuals experiencing stress include the work of Faresjö et al. (2013), who found the hair cortisol of Greek citizens was significantly lower than that of Swedish citizens during the recent world economic crisis, and Steudte et al. (2011a & 2013) who found an association between decreased hair cortisol concentrations and PTSD. Furthermore, Wells et al. (2014) found participants scoring in the first three quintiles for the PSS had increasing hair cortisol concentrations; but, for individuals scoring at the highest quintile, hair cortisol concentrations fell between that of individuals with scores in the first and second quintiles, thus supporting the potential for HPA axis fatigue with higher stress.
Pregnancy generates an inflammatory state and approximately one-third of women experience increased asthma symptoms when pregnant (Clifton & Murphy, 2004). It is possible that the added physiological stress of pregnancy in combination with asthma, or a woman's chronic state or severity of asthma, exacerbates HPA axis fatigue through chronic exposure to stress hormones, and ultimately leads to a decreased cortisol response regardless of ICS use. This theory is supported by the linear regression analysis of the data for T3 in this study that indicates, between consistent ICS use, intranasal corticosteroid use and asthma, asthma accounted for approximately 26% of the decrease observed in hair cortisol. This finding possibly indicates that the severity of asthma may be a significant factor since women with more severe asthma would also be likely to take their ICS with regularity. Also, in support of the current findings, patients with chronic asthma were previously found to have a decreased response to ACTH stimulation, but research is limited in this area (Robson & Kilborn, 1965). Alternatively, the less-pronounced increase in hair cortisol during pregnancy in women with asthma may be due to decreased cortisol sensitivity from a reduction in glucocorticoid receptors (Tian et al., 2014), as was found in children with asthma who were shown to have a 5.5-fold decrease in expression of the glucocorticoid receptor (Miller & Chen, 2006). Further research comparing hair cortisol concentrations in healthy adults to those with asthma, with and without ICS treatment, may confirm whether the observed difference in cortisol concentrations is due to HPA axis fatigue from the combined effect of the physiological stress of pregnancy with asthma rather than solely asthma chronicity or severity.

5.1.4 Potential role for cortisol in observed medical conditions of children born to women with asthma

The surges in cortisol from fetal and maternal sources during pregnancy both likely contribute to fetal maturation. Multiple mechanisms, such as the release of placental CRH and ACTH, result in increased maternal cortisol as pregnancy progresses, and the surge in cortisol in late pregnancy is involved in epigenetic processes that program fetal cardiovascular, neurologic, endocrine, and metabolic systems (Kamoun et al., 2014; Moisiadis & Matthews, 2014b). If the required surge in cortisol is diminished, as was evident in this study in women with asthma, organ systems that rely on cortisol for maturation and programming may be adversely affected. Only one study thus far has examined the long-term effects of asthma and asthma treated with ICS on childhood disease. The Danish National Birth Cohort followed children to 6 years of age.
and found that children exposed to ICS in utero were more likely to experience ‘endocrine, metabolic disorders’ (hazard ratio (HR) = 1.84, CI\textsubscript{95\%} 1.13-2.99) and digestive system diseases (HR = 1.54, CI\textsubscript{95\%} 1.18-2.02) (Tegethoff et al., 2012). Secondary analyses assessing the effects of maternal asthma, combining those with and without ICS treatment, revealed an increased risk of diseases of the respiratory system (HR\textsubscript{adj} = 1.43, CI\textsubscript{95\%} 1.34-1.52), nervous system (HR\textsubscript{adj} = 1.43, CI\textsubscript{95\%} 1.18-1.73), and digestive system (HR\textsubscript{adj} = 1.17, CI\textsubscript{95\%} 1.04-1.32) (Tegethoff et al., 2013), all of which rely on cortisol for proper fetal maturation or programming (Liggins, 1994; Moisiadis & Matthews, 2014a & 2014b).

The research in this thesis also found that pregnant women taking budesonide had significantly lower hair cortisol concentrations than the Controls or women taking fluticasone propionate in T2 (bootstrapped results). This is an unexpected finding given that fluticasone is more commonly associated with cases of adrenal insufficiency or suppression and has a greater suppressive effect on morning serum cortisol concentrations in healthy subjects than budesonide when given in equivalent doses (Derom et al., 2009; Drake et al., 2002; Todd et al., 2002; Wales et al., 1999). The greater decrease in hair cortisol for budesonide users is also interesting in relation to the findings reported by Tegethoff et al. (2012) already mentioned above. As part of their analysis of the effects of in utero exposure to ICS, the researchers restricted their analysis to children only exposed to budesonide (n = 983 (79.9%)) compared to women with asthma who did not use ICS (n = 2852). They found that the risk of endocrine and metabolic disease was still significant (HR = 1.72, CI\textsubscript{95\%} 1.07-2.77) and not greatly different from the HR when all ICS were considered together (see above). Unfortunately, the authors were unable to perform the same analysis focusing on women who used fluticasone or beclomethasone due to the small sample sizes for those groups (n = 217 (17.6%) and 67 (5.4%), respectively). Therefore, comparing the effect of fluticasone on hair cortisol concentrations in this thesis research to that of fluticasone on congenital diseases and associating it with the potential relationship found for budesonide is not possible. Although this present study was not designed to draw conclusions on the effect of decreased cortisol concentrations and disease risk in children born to women with asthma, the growing evidence may warrant further research in this area for which hair cortisol measurement could be a useful assessment tool.

Although a comparison of hair cortisol concentrations by dose group did not reveal any significant differences, likely due to the small sample sizes, the findings are consistent with what
was observed when comparing Controls, No ICS, and ICS Treated women who used ICS for ≥ 5 doses per week on ≥ 5 days per week. Lower hair cortisol concentrations in T3 were noted for the No ICS and ICS Treated women taking 0-250 µg/d. Interestingly, the ICS Treated women using > 250 µg/d had higher than expected hair cortisol concentrations in T3. One possible explanation for this is increased levels of cortisol due to the added physiological stress of their asthma severity. This theory suggests that the adrenal glands of these women were able to respond when stimulated, but only partially since the median hair cortisol concentration of the ICS Treated women taking > 250 µg/d is still lower than the Controls in T3 (when the women with results ≥ 100 ng/g were excluded, Figure 15B). A larger study comparing HPA axis stimulation tests and hair cortisol concentrations during pregnancy could address the validity of this theory. The normal response for pregnant women to the ACTH stimulation test has been reported and could be used as a point of comparison for such a study (Bancos et al., 2015; Suri et al., 2006).

5.1.5 Study strengths and limitations

Limitations of this study include its relatively small sample size and possible discrepancies in how the hair was collected (e.g., scalp location, distance from scalp). Given the observed expected change in hair cortisol concentrations over the course of pregnancy, any inaccuracy due to improperly collected samples is not obvious, nor expected to be significant. Additionally, all medication use was self-reported, either to the study personnel or a healthcare provider. Generally, women tend to reduce their ICS use during pregnancy (Enriquez et al., 2007; Schatz & Liebman, 2005), and some women in this study reported that they did so, but issues with recall may affect the reporting of the use of ICS and other medications.

For the hair cortisol measurements, a positive bias could have occurred for the cortisol concentrations below the method quantitation limit that were recalculated using the quantitation limit. This predominantly affected the No ICS group for PC, T1, and T2 and may have reduced potential differences between this group and the Controls. Although the degree of bias cannot definitively be determined, future studies involving a larger number of women would be beneficial and may help resolve any uncertainty. Even with a positive bias present for the recalculated results, hair cortisol analysis clearly showed the change in cortisol over the course of pregnancy and identified a difference between the Controls and No ICS or ICS Treated.
Further confounding may be present due to the inter-individual variability in asthma severity or difference between the No ICS and ICS Treated groups for asthma severity. Unfortunately, a reliable measure of asthma severity was not included in the data collection. Also, the groups did not differ in perceived stress as indicated by the PSS score and, therefore, it was not included in any analysis. However, this does not rule out other forms of stress that may have been detected and possibly different among the groups if other tests of stress were performed.

For the evaluation of ICS type and dose effects, a linear statistical model was used for the data, which could not be fully normalized even after natural log transformation and required removal of the hair cortisol results ≥ 100 ng/g. Bootstrapping was used to improve the distribution to better meet the assumption of a normal distribution. Also, a linear relationship between the dose covariate and hair cortisol concentrations was not met. Taken together, the results should be considered with caution and a larger study to contribute further evidence on effects of ICS type and dose on hair cortisol concentrations would be beneficial.

Finally, there are reports of decreased hair cortisol concentrations ranging from 16-41% with increasing age of the hair (i.e. hair further from the scalp), especially more than six months, but this is not supported by all hair cortisol research (Table 4). One major advantage of measuring hair cortisol is the ability to determine historic hair cortisol concentrations. If there is a decline in hair cortisol with hair age, this may reduce the number of significant differences between the time points shown in Figure 10 for the Controls. Even with fewer significant differences, it is expected that the results would resemble the significant differences for pregnant women found by D’Anna-Hernandez et al. (2011). The No ICS group would possibly not have an increase in hair cortisol over the course of pregnancy and the ICS Treated may also have fewer significant differences between time points. However, this does not alter the fact that, in the current study, the women with asthma had lower hair cortisol concentrations than the Controls. In addition, each of the groups was likely affected equally since the average age of the hair samples, as determined by the PC segment, did not differ between the groups (Table 1) and, therefore, comparisons among the groups would not be greatly impacted.
5.2 Child cohort – hair cortisol as a novel biomarker of hypothalamic-pituitary-adrenal axis suppression by inhaled corticosteroids

5.2.1 Children – pilot study


5.2.1.1 Concerns surrounding long-term inhaled corticosteroid use in children

Despite the perception that there is low systemic bioavailability of ICS compared with oral or intravenous steroids, there is evidence of unexpected ICS-induced adverse effects in children such as height deficits (Sorkness, 1998; Loke et al., 2015), which appears to persist into adulthood with long-term budesonide use according to one strong randomized controlled trial (Mean reduction = -1.20 cm/year, CI95% -1.90 to -0.50 cm/year) (Kelly et al., 2012). However, in their 2015 meta-analysis, Loke et al. report that the growth reduction for children was most prominent in the first year of therapy, based on studies following children for 24-36 months. They further discuss that the reduction in height must take into account the average height of the population concerned to determine if the reduction would be considered clinically significant.

Glucocorticoids, endogenous and exogenous, affect a variety of biological systems involved with height, for example, increased somatostatin release, inhibition of growth hormone secretion, insulin-like growth factor-I activity, and cartilage synthesis (Allen et al., 1998). Administration of growth hormone to children with a variety of diseases requiring treatment with exogenous oral corticosteroids increases the yearly growth rate, most notably for those with asthma (pretreatment 2.5 cm/y vs. 1 year post-treatment 6.7 cm/y) (Allen et al., 1998).

In addition to height deficits, children have been frequently documented to experience adrenal suppression, insufficiency, or crisis while receiving ICS (Drake et al., 2002; Heijmans et al., 2011; Molimard et al., 2008; Todd et al., 2002). These adverse effects depend on the dose, duration of therapy, and age at the initiation of therapy. In addition, it is likely that the different ICS pharmacokinetic characteristics are a determinant of adverse effects given the impact of pharmacokinetic properties on systemic activity (Kelly, 2009). The concern regarding long-term effects may escalate with increased education regarding inhaler technique and the use of a spacer.
device as well as the recent change in formulation of metered dose inhaler propellants from chlorofluorocarbons to HFA, which may increase or decrease the dose reaching the lungs depending on the solubility of the corticosteroid in HFA. For example, the change in formulation had resulted in reduced systemic exposure for beclomethasone, which is soluble in HFA, by delivering more to the lungs rather than depositing it in the oropharynx where absorption was high (Table 2) (Kelly, 2009). For fluticasone, less of the dose is delivered to the lungs in HFA, but when paired with a spacer device, there is better delivery of fluticasone to the lungs where it is more readily absorbed compared to its oral bioavailability (Table 2) (Kelly, 2009). Pair this with the fact that ICS are being used off-label in young children with 5.6% of children ≤ 5-years-old and 10% of children 5- to 11-years-old prescribed high doses for their age group (Thomas et al., 2006). A simple method for evaluating systemic effects of ICS on the HPA axis would be attractive and the results of this study suggest hair cortisol may be an effective biomarker for that purpose.

5.2.1.2 Hair cortisol as a potential biomarker of the hypothalamic-pituitary-adrenal axis in children treated with inhaled corticosteroids

The measurement of hair cortisol has been shown to be a sensitive biomarker of Cushing syndrome (Manenschijn et al., 2012a; Thomson et al., 2010), as well as stress associated with chronic pain, myocardial infarction, congestive heart failure, and numerous other conditions (Russell et al., 2012). Changes in cortisol can be detected in hair within two months of the corresponding changes in serum/plasma cortisol and reflect the average endogenous cortisol concentrations. The potential for hair cortisol to serve as a biomarker of systemic suppression of the HPA axis may offer clinicians and researchers a time-sensitive tool to monitor the suppression of cortisol secretion in children with asthma who receive ICS, as well as in patients with other causes of decreased cortisol secretion.

The results of a recent study analyzing hair cortisol in children with asthma receiving ICS were similar to the current findings with reported cortisol concentrations approximately 50% lower than those in children without asthma (Kamps et al., 2014). However, it was unknown whether the decrease in cortisol was due to the inflammatory condition itself or due to the effect of ICS. The present study addresses this issue by having the same children acting as the control group, which makes it less likely that the changes are solely due to the disease itself. As this study is a pilot investigation, it lacks the statistical power to discern any dose- or ICS type-
dependent differences. Regardless, the results show that hair cortisol may be an effective biomarker of HPA suppression by ICS with respect to cortisol production.

5.2.1.3 Ciclesonide and hypothalamic-pituitary-adrenal axis suppression

Preliminary peer reviewed reports suggest that ciclesonide may be associated with fewer adverse effects on growth in children (Skoner et al., 2008), and adrenal suppression in adults (Lipworth et al., 2005; Takeda Canada Inc., 2012). Fewer observed adverse effects are believed to occur because ciclesonide is administered as a prodrug and activated by esterases in the lung, and because of its low oral bioavailability, rapid elimination, and high plasma protein binding (Takeda Canada Inc., 2012). The authors of one article that described four case studies with normalized adrenal cortisol release after switching from fluticasone to ciclesonide even suggest that ciclesonide use should be considered to potentially reverse adrenal suppression occurring secondary to the use of other ICS (Kupfert Heller et al., 2010). Additionally, Skoner et al. (2008) did not find a difference in 24-h or 10-h urinary free cortisol concentrations at baseline or at the end of the study between children treated with ciclesonide and those given placebo. With 14 out of 18 children in this pilot study using ciclesonide, 6 of whom have never used any other ICS, the current findings contradict the previous findings and, instead, suggest that possible HPA axis suppression can occur with the use of ciclesonide. The ability to assess the overall average endogenous cortisol response using hair cortisol measurements may indicate that the sensitivity of hair cortisol for detecting adrenal suppression is better than morning serum/plasma cortisol or urine free cortisol concentrations, but this remains to be investigated further.

5.2.1.4 Study strengths and limitations

The use of intranasal steroids and systemic steroids by some of the children is a possible confounder. However, intranasal corticosteroid use has not been shown to decrease saliva cortisol concentrations more than the use of ICS alone (Heijsman et al., 2011); and short-course systemic corticosteroid therapy for the treatment of asthma, as recommended by asthma treatment guidelines (Lougheed et al., 2010), was not associated with long-term suppression of adrenal function (Ducharme et al., 2003; Heijsman et al., 2011). Furthermore, hair cortisol analysis has the advantage of representing an integrated, long-term picture of adrenal cortisol production and function of the HPA axis, rather than indicating acute or short-term fluctuations. Therefore, the results are less likely to be affected by short-course systemic corticosteroid
therapy. Additional confounding may be present with the analysis of older hair segments. While the majority of studies have not found an association with the frequency of hair washing, some have reported declining hair cortisol in older hair segments (Wosu et al., 2013). This may be in part due to repeated shampooing as found by Hamel et al. (2011). Should such confounding be present in this study, the results would underestimate any decrease or overestimate any increase observed when comparing the hair cortisol concentrations prior to and during ICS therapy.

5.2.2 Child cohort large study

5.2.2.1 Results of hair cortisol analysis in relation to previous research on adrenal suppression in children with asthma treated with inhaled corticosteroids

The initial data comparison for this larger cohort of children did not show a significant difference in median hair cortisol concentrations among the Controls, No ICS, and ICS Treated groups. However, the comparison could not be adjusted for covariates because the data was not normally distributed. A comparison of rank transformed data also produced insignificant results. For a thorough evaluation of the data, a more robust statistical analysis was required, quantile regression, which will be discussed in section 5.2.2.3.

These non-significant results may not be surprising when considering the conflicting results of previous research in the literature. Kannisto et al. (2000) determined the adrenal status of children with asthma after taking budesonide, fluticasone propionate, and cromolyn or nedocromil over 6 months using serum cortisol and the low-dose ACTH stimulation test. The serum cortisol concentrations were normal at the 2-, 4- and 6-month evaluation points. The ACTH stimulated cortisol concentrations decreased significantly over the 6 months for the budesonide and fluticasone propionate groups, but none of the mean values were low enough to meet the threshold for adrenal suppression defined by the authors. Similar findings for basal and stimulation test cortisol results occurred in an earlier study comparing children with severe persistent asthma taking high doses of budesonide and beclomethasone for more than 2 years, with the exception that the morning cortisol concentrations for the children using ICS were significantly lower than the controls, but they were still within the normal range (Ninan et al., 1993). In another study, children aged 7- to 12-years with asthma taking no ICS or low-dose ICS were found to have similar morning and evening salivary cortisol concentrations compared to controls (the morning salivary cortisol results were 9.0 nmol/L ($n = 24$) and 9.9 nmol/L ($n = 14$) vs. 10.4 nmol/L ($n = 52$), respectively) (Bakkeheim et al., 2010).
On the other hand, the children taking moderate to high doses of ICS in the Bakkeheim et al. (2010) study had significantly lower morning and evening salivary cortisol levels (morning salivary cortisol = 6.4 nmol/L). Furthermore, and surprisingly, the findings of this large cohort of children contradict the results of the pilot study contained in this thesis and the small study by Kamps et al. (2014) who found a significant difference in hair cortisol between 10 children with asthma compared to 10 healthy control children. It may be that the sample size for the Kamps et al. (2014) study was not large enough to capture the wide inter-individual variation in cortisol. Previous research also found salivary cortisol to be significantly lower in children taking ICS with \((n = 22)\) or without \((n = 41)\) intranasal corticosteroids when compared to healthy controls \((n = 18)\) (Heijsman et al., 2011). Collectively, the literature suggests that for many children using ICS adrenal suppression may not be chronic but rather sub-clinical and only realized when there is an acute stress applied requiring cortisol production. For this reason, hair cortisol analysis would not be sensitive enough to detect the suppression since it represents the average over a long duration rather than of an acute response. Therefore, because of the lack of sensitivity for acute responses and the great inter-individual variation in cortisol concentrations, an intra-individual comparison (as was done in the children's pilot study of this thesis) may be a more sensitive way to detect changes in adrenal function that could be clinically significant. Further research is needed to determine what is a clinically significant change in hair cortisol concentration.

5.2.2.2 Hair cortisol and identification of subpopulations

Although there was not a significant difference in median hair cortisol concentrations among the three groups of children, the variance differed significantly among all of the groups and the ICS Treated children had a wider range of hair cortisol concentrations from very low to extremely high. While the majority of research investigating adrenal suppression due to ICS use has incorporated the use of an HPA axis test, such as the ACTH stimulation test, the wide variation in hair cortisol concentrations of the ICS Treated group may indicate distinct subpopulations within this group. The children with very low hair cortisol concentrations \(< 2.0 \text{ ng/g}\) in the ICS Treated group \((19 \text{ of 360 children or } 5.3\%)\) compared to none in the control groups may represent a population of children with or at risk of having adrenal suppression or insufficiency due to ICS therapy, similar to diagnosing those with morning serum cortisol \(< 85-112 \text{ nmol/L}\) as having adrenal insufficiency (Ahmet et al., 2011). This theory is supported by the
results of the low-dose ACTH stimulation test performed by Kannisto et al. (2000). The mean result of the ACTH stimulation test did not meet the threshold to define adrenal insufficiency for their budesonide and fluticasone groups, thus indicating adrenal suppression. However, the authors recognized there was a subgroup of study participants with stimulation test concentrations low enough to indicate adrenal insufficiency, which comprised 23% of all the steroid users. Further support may be provided by the recent meta-analysis by Broersen et al. (2015) that examined 27 asthma studies including patients ≥ 12-years-old with ICS-induced adrenal insufficiency diagnosed by one of the recognized stimulation tests (ACTH stimulation test, metyrapone, etc.). They determined that 11.1% (CI_{95%} 6.8-17.7) of participants experienced adrenal insufficiency due to corticosteroid use (inclusive of inhaled and other administration forms). The percentage dropped to 6.8% (CI_{95%} 3.8-12.0) for participants with asthma when the analysis was restricted to those who used only ICS (which is reassuring because it suggests that systemic exposure to corticosteroids is decreased with ICS use). Similarly, in children aged 0-years and up, prevalence rates for adrenal insufficiency determined by the low-dose ACTH stimulation test was 9.3% (CI_{95%} 5.3-13.4%) (Smith et al., 2012), and 7.7% (CI_{95%} 3.5-15.3%) in children taking moderate to high doses of ICS (Cavkaytar et al., 2015). Finally, in a study by Zollner et al. (2012), 6.1% (CI_{95%} 1.8-10.5) of children aged 5 to 17 years with asthma using ICS with or without intranasal corticosteroids had adrenal suppression based on decreased morning blood cortisol. When all forms of HPA axis suppression were considered as determined by the measurement of blood cortisol, 11-deoxycortisol, and ACTH after the overnight metyrapone test, the majority of the children demonstrated suppression of the HPA axis (65%, CI_{95%} 56.5-72.9%).

The similarity in percentages of adults and children with adrenal insufficiency identified by these previous studies, using traditionally accepted methods, to the percentage of children with hair cortisol < 2.0 ng/g in this thesis research is noteworthy and suggests hair cortisol may be useful biomarker for detecting adrenal suppression and insufficiency. A strength of hair cortisol measurement is that it is an easy and non-invasive test that could potentially identify the at-risk population more sensitively than basal saliva or blood cortisol measurement due to its temporal nature and without having to perform an invasive stimulation test, potentially reducing the number of indeterminate cases requiring an invasive test. To verify this theory, further research is required to determine hair cortisol concentration cut-offs that are indicative of possible and probable adrenal insufficiency. Therefore, research combining hair cortisol
measurements and ACTH stimulation tests would be beneficial for determining if a hair cortisol concentration cut-off can be identified similar to that for serum cortisol.

Analogous to the ICS Treated children, the Controls and No ICS had a subpopulation of children with high hair cortisol concentrations. A similar finding is seen in research published by Karlén et al. (2013) in a population of 100 children followed from birth to 8-years-old. Although there is not a clear or known cause for these elevated results, many hair cortisol studies (and one saliva cortisol study) have excluded the subjects with extremely high values identifying them as outliers (Boesch et al., 2015; Dettenborn et al., 2012a; Faresjö et al., 2013; Feller et al., 2014; Gow et al. 2011; Grass et al., 2015; Heijisman et al., 2011; Karlén et al., 2011; Saleem et al., 2013; Stalder et al., 2012 & 2013; Steudte et al., 2011a, 2011b & 2013; Wells et al., 2014; Wosu et al., 2015). This has resulted in ~2-12% of participants in the study groups being excluded from the analysis for those studies. If the children with high results were excluded from this thesis research, ~13% of participants would have been excluded. This population of individuals with high hair cortisol concentrations is present and persisting but currently without explanation. Even though they are statistical outliers, unless there is a known reason for the aberrant observation, the data probably should not be ignored or excluded as Dettenborn et al. (2012a) did for "no clear causes" or as Boesch et al. (2015) and Saleem et al. (2013) did assuming Cushing's syndrome or glucocorticoid contamination. Instead, the data should be included because the affected individuals are present in all study groups including the "healthy" controls, as seen in this large cohort of children (Faresjö et al., 2013; Gow et al., 2011; Heijisman et al., 2011; Steudte et al., 2013). Moreover, non-parametric statistical tests and other robust statistics are not affected greatly by "outliers." Future research to determine if there is immunoassay interference, such as human anti-animal antibodies or lipemia of the extracted hair sample (Tate and Ward, 2004), or if these individuals have a chronic non-pathologic or pathologic hypercortisolemia (possibly associated with asthma severity) would provide an enriched examination of hair cortisol as a potential biomarker of endogenous cortisol concentrations and associated conditions. Hypercortisolemia may be due to chronic stress from accidents, surgery, illness, or disease (severe asthma?), or a pathological condition, such as Cushing syndrome as already reported (Manenschijn et al., 2012a; Thomson et al., 2010). There may be hair factors that have not yet been identified in the literature to explain why a consistent percentage of individuals have increased hair cortisol concentrations. The literature suggests that intense exercise and sweat may result in elevated hair cortisol concentrations (Gerber et al., 2013; Russell et al., 2014; Skoluda et
al., 2012). This would not likely be the explanation for the young children in this study, as supported by Noppe et al. (2014), or for pregnant women in T3 or PP as presented earlier in this thesis (section 5.1). But, because those studies were not available when this research protocol was developed, data specific to exercise and sweating to definitively answer this question was not collected.

5.2.2.3 Age, sex, and cortisol concentrations

Previous hair cortisol research has suggested that age and sex affect hair cortisol concentrations (see Table 4), but the literature is full of contradictions. Therefore, a specific analysis of the hair cortisol results in this study for any age or sex effects was performed. Overall, the results showed that hair cortisol concentrations were affected most by age, but not significantly different between males and females. Two sub-analyses were performed. The linear regression sub-analysis of children aged 1- to < 8-years showed a significant decline in hair cortisol with age that was not affected by sex. The second sub-analysis, of children aged 8- to 18-years, found that adolescent boys tend to have higher hair cortisol than adolescent girls and the difference between the sexes decreases with age due to a steady, but insignificant, increase in hair cortisol concentration seen for females.

Looking at previously published research, researchers measuring morning saliva cortisol concentrations in 286 children aged 7- to 15-years-old found sex did not influence the result except for at certain specific ages, e.g., 7-years-old (Törnhage, 2002). Similar to the findings of this research, children aged 7- to 9-years-old had lower salivary cortisol than those aged 10- to 12-years-old (Törnhage, 2002). In contrast, a study evaluating serum cortisol concentrations in 235 children aged 2- to 18-years did not find a correlation with age or sex, but similar to the hair cortisol concentrations for this large cohort of children, they did have a wide range of cortisol concentrations (100-510 nmol/L), albeit not as wide as this study (Knutsson et al., 1997).

The CALIPER study, the largest study ever performed to determine pediatric reference intervals for numerous biochemical and immunochemical analytes, such as glucose, albumin, ferritin, and cortisol, etc., found that age-related differences in analytes were more commonly seen than differences due to sex (Colantonio et al., 2012). A comparison of the results of this study to their results for cortisol revealed contradictory findings. Unlike the findings in this thesis, CALIPER did not find a difference between the sexes for cortisol for the six different age
ranges reported, and the reference intervals showed an increase in cortisol with ages ranging from 2-days-old to 19-years-old (Bailey et al., 2013). However, the scatter plot of cortisol concentration versus age showed a similarity in shape between the two studies. The CALIPER scatterplot and that of the large cohort in the current study both show a number of children 1-year of age with higher cortisol concentrations, and cortisol appears to decline until adolescence. Bailey et al. (2013) classified cortisol under "analytes with high variance at birth that decreased abruptly around 1 year of age and increased again in adolescence." Factors that may account for the differences between this study and the CALIPER study include the sample type, varied sample collection time in the CALIPER study, or the population of children studied (e.g., healthy community children in the CALIPER study vs. a majority of children with asthma in this cohort, or inclusion of children < 1-year-old in the CALIPER study who had significant variance in serum cortisol).

When seeking to compare the results from this study to other hair cortisol studies, only a few have studied children. Dettenborn et al. (2012a) found age and sex differences, but the sex differences did not appear in all age groups, only in those 1- to 9-years-old (which is contrary to the findings of this thesis research) and 18- to 49-years-old. Noppe et al. (2014b) found hair cortisol increased from age 5-years to 10-years. Most similar to the results seen in this research, Kalén et al. (2013) measured the hair cortisol in 100 children that were followed from birth until 8-years-old and found that the children's hair cortisol decreased with age and suggested this may be due to maturation of the stress response. Because the literature is contradictory, age and sex were included as covariates in the quantile regression models assessing which factors significantly affect hair cortisol concentrations.

5.2.2.4 Factors affecting hair cortisol concentrations in children with asthma as revealed by quantile regression

Because of the non-normally distributed data observed for this large cohort of children, two quantile regression models were generated to determine which factors affect hair cortisol concentrations, specifically trying to discover if any ICS type- or dose-dependent relationships exist. Unfortunately, the model evaluating the ICS type could not be adjusted for dose due to collinearity of those variables. By using quantile regression, the "outlier" hair cortisol concentrations were preserved in the analysis, but the results show that the "outliers" do prevent influential factors from being identified for hair cortisol concentrations in the upper percentiles.
(around the 75th to 85th percentile and up, depending on the variable). Hence, none of the regression methods (OLS or quantile) were immune to the confounding of extremely high results for the dependent variable, but quantile regression mitigated the confounding for the lower percentiles while considering the whole distribution in the calculation, making it the best choice of regression method for the data of the large children cohort.

The results of the quantile regression using Model 1 for any ICS type-dependent effects was negative for all ICS types, which appears to be in contrast to some previous research. A few studies have found that fluticasone is associated with adrenal insufficiency or crisis more often or produces a greater degree of adrenal suppression than the other ICS (Clark & Lipworth, 1997; Derom et al., 2009; Drake et al., 2002; Todd et al., 2002). However, the study by Clark and Lipworth (1997) used equivalent microgram doses of budesonide and fluticasone in their comparison, which did not take into account that fluticasone is approximately twice as potent as budesonide. Other studies used equipotent but high doses for comparison, e.g., 500 µg/d or 1000 µg/d of fluticasone for adults (Derom et al., 2009). Conversely, one study found ICS type was not a significant factor (Smith et al., 2012), and another study found that budesonide had a more suppressive effect on adrenal function than fluticasone in children who took the ICS for 6 months (Kannisto et al., 2000). Importantly, all groups in these studies had serum cortisol concentrations within the normal range for the duration of the study (Clark & Lipworth, 1997; Derom et al., 2009, Kannisto et al., 2000). While the different ICS types are associated with adrenal suppression when tested by an HPA axis stimulation test, they do not generally result in abnormal serum cortisol concentrations. Therefore, it is understandable that hair cortisol measurement may not be sensitive enough to detect adrenal suppression due to different ICS because hair cortisol predominantly reflects the average serum cortisol concentrations.

Similar to Model 1, the quantile regression results for Model 2 evaluating ICS dose was not significant for any of the dose categories. While ICS dose-response adverse effects may vary from person to person due to differences in enzyme kinetics (National Heart Lung and Blood Institute, 2007), more than one study has found that children on moderate or high dose ICS have adrenal suppression or insufficiency (Bakkeheim et al., 2010; Cavkaytar et al., 2015; Kannisto et al., 2000; Molimard et al., 2008; Ninan et al., 1993; Smith et al., 2012; Zollner et al., 2012). The meta-analysis by Broersen et al. (2015) revealed the percentage of patients with a positive HPA axis stimulation test increased in a dose-dependent manner. The percentages for low, medium,
and high doses of ICS were 1.5%, 5.4%, and 18.5%, respectively. Derom et al. (2009) reported that subjects taking 320 µg/d and 640 µg/d of ciclesonide for 7 days had a mean serum cortisol mesor reduction of 6.1% and 7.9%, respectively, when compared to the placebo group. Their results for fluticasone showed an even greater reduction in the mean serum cortisol mesor (10.3% for 500 µg/d vs. 19.8% for 1000 µg/d). However, the serum cortisol concentrations for all of the groups in their study still fell within the normal range and this may explain why the results of the quantile regression found a limited effect on hair cortisol concentrations with respect to dose in Model 2. There was a negative effect for the highest ICS dose of > 2.5 µg/kg/d, but it was only for children with hair cortisol concentrations at the 5th percentile. As previously mentioned, hair cortisol predominantly represents the average serum cortisol concentrations over time and since the serum cortisol concentrations were normal for the groups in other studies, it is not surprising that a general dose-dependent relationship was not observed. Likewise, observing a dose-response for the children in the 5th percentile is also not surprising for they may belong to the percentage of the population that has low basal cortisol concentrations indicative of adrenal insufficiency, as discussed in section 5.2.2. It is more difficult to provide a possible explanation for the increased hair cortisol associated with the > 2.5 µg/kg/d dose for the children in the 55th percentile and it may be over interpreting the results to try (Good & Hardin, 2012). However, one hypothesis is that these children may be experiencing and exhibiting increased cortisol concentrations due to physiological stress from severe or uncontrolled asthma requiring the use of higher ICS doses in an attempt to control their disease.

There is conflicting literature as to whether ICS duration is associated with adrenal suppression. The moderately sized study by Smith et al. (2012) did not find an association with duration of ICS use in children aged 0- to 18-years-old. On the other hand, the meta-analysis by Broersen et al. (2015), with 1317 participants ≥ 12-years-old with ICS-treated asthma, found an increased incidence of adrenal insufficiency in participants as the duration of ICS use increased from short-term to long-term (1.3% to 20.3%). In this large cohort of children, the ICS duration was closely correlated to age, although not strongly enough to be collinear. ICS duration was included in the regression models to ensure adjustment of the models because previous research had identified it as a significant variable. The reason it may not have been significant for this cohort is that this is a cohort of children with a wide range of ages (1 to 18 years), similar to Smith et al. (2012), and cortisol varies as age changes, whereas Broersen et al.’s (2015) study included older children and adults in whom cortisol varies less. Therefore, for children in this
study, other factors such as age may have a greater impact than ICS duration, or confound the effect of ICS duration, on hair cortisol concentrations.

In Models 1 and 2, significant factors affecting hair cortisol concentrations included age, sex, BMI, and intranasal corticosteroid use. The research regarding age and sex have already been discussed in section 5.2.2.3. The quantile regression results support the observed relationship in the scatterplot of hair cortisol concentrations versus age. In the higher percentiles, age has a negative association, which supports the observation that the youngest children had some of the highest hair cortisol concentrations. The significance of BMI has varied in hair cortisol studies. It has been associated with increased hair cortisol concentrations (see Table 4), but this did not hold true for older adults, possibly due to changes in body composition with a higher body fat content and lower muscle mass (Manenschijn et al., 2013). The BMI in the quantile regression was centered and, thus, the results indicate that hair cortisol increases slightly with every 1 kg/m² above 15.5 kg/m². This outcome is best explained by the fact that older children tend to have higher BMIs and they also have gradually increasing hair cortisol concentrations as seen in the scatterplot of hair cortisol concentration versus age (Figure 19). In adults, the positive association may be related to metabolic syndrome (Manenschijn et al., 2011b; Stalder et al., 2013). Finally, with respect to intranasal corticosteroid use, the quantile regression showed a negative association for their use and hair cortisol concentrations. In support of this result, the meta-analysis by Broersen et al. (2015) determined that 4.2% (CI₉₅% 0.5-28.9) of individuals using intranasal corticosteroids had adrenal insufficiency. In this large cohort of children, a child in the 50th percentile using intranasal corticosteroids would have a hair cortisol concentration ~1.8 ng/g lower than children in that percentile not using intranasal corticosteroids possibly indicating there is slight adrenal suppression with their use. However, children using ICS alone compared to ICS and intranasal corticosteroids did not have significantly different morning salivary cortisol concentrations (Heijsman et al., 2011). Again, hair cortisol concentrations may be more sensitive at detecting chronic adrenal suppression than morning saliva cortisol because it represents the average cortisol concentrations over a longer period. In Bakkeheim et al.'s (2010) regression model, which included the variables low-dose ICS use, allergic rhinitis, and asthma, and was adjusted for age, gender, and month of sampling, only allergic rhinitis remained significant. Their results suggested that rhinitis rather than the use of intranasal corticosteroids may be associated with lower morning salivary cortisol concentrations because only 2 children of the 23 with allergic rhinitis used intranasal corticosteroids. Future
research comparing the hair cortisol concentrations of individuals with and without treated allergic rhinitis may provide further insight.

For the variables that showed limited significant effects on hair cortisol concentrations, such as having asthma (yes/no) or chemically treated hair, the results should be considered with caution. The contradiction with previous research for chemical treatment may indicate the variable was not properly represented in the large cohort of children for accurate analysis and other factor(s) had a greater influence. This may be true since only 27 of the 460 children had chemically treated hair. Alternatively, the hair colour used by the children may be a different formulation that does not damage the hair to the same degree as other formulations. Some of the colours used by the children were different than those used by the pregnant women, e.g., blue, green, and pink/purple as compared to brown and blonde. The significant association for living in Vancouver at the 5th percentile is not surprising since the majority of children with asthma were recruited in Vancouver and, therefore, children from Vancouver may be represented more in the 5th percentile.

5.2.2.5 Study strengths and limitations

This study included a large population of children treated with ICS that allowed for the determination of associations for numerous variables with hair cortisol concentrations and potentially identified a population of children at risk of adrenal insufficiency with hair cortisol concentrations < 2.0 ng/g. If confirmed by future research, the ability to identify children with adrenal insufficiency using hair cortisol measurement would indicate that hair cortisol may be a more sensitive test than other tests of basal adrenal function, such as morning serum or saliva cortisol. An added strength is the easy and non-invasive nature of collecting and storing a hair sample compared to performing an invasive HPA axis stimulation test.

Another strength of this study was the use of quantile regression for the data analysis, which allowed all children to be represented in the analysis rather than excluding the children with high values as "outliers." The quantile regression indicated that the type of ICS did not have an effect on hair cortisol results and ICS dose had a minimal effect. Unfortunately, some variables were underrepresented in the hair cortisol concentration distribution of this large cohort of children to properly determine their relationship with hair cortisol concentrations. This was indicated by the variables with "infinity" as part of the confidence interval, such as chemical
treatment, use of mometasone, or use of beclomethasone. Future studies specifically targeting mometasone and beclomethasone users may be beneficial. Mometasone is a relatively new ICS that may become more frequently prescribed by physicians with time, education, and more research to understand this ICS. A larger study would also likely capture a higher percentage of mometasone and beclomethasone users.

The use of oral corticosteroids was not found to be a significant factor in univariate quantile regression (data not shown) and, therefore, was not included in Models 1 or 2. Yet, the administration of systemic dexamethasone may be a confounding factor because the cross-reactivity of dexamethasone with the enzyme immunoassay was 19.2%. In the ICS Treated group, 60 children were given dexamethasone during the 3-month period represented by the hair sample. The potential for confounding assumes that dexamethasone is incorporated into hair in a similar fashion to cortisol, which is likely but unknown. With the hair analysis performed for this research, it is not possible to distinguish whether dexamethasone was present in the hair and measured as cortisol or if the participant's cortisol was decreased due to dexamethasone use; however, the correlation for dexamethasone use and hair cortisol concentration was not significant (Spearman correlation, rho = 0.066, \( P = 0.156 \)). On one hand, the hair cortisol concentration may be falsely elevated due to the cross-reactivity, but, on the other hand, the concentration may be decreased due to the endogenous suppressive effect of dexamethasone on cortisol secretion. With this cohort, dexamethasone does not appear to be associated with a trend for lower or higher hair cortisol concentrations given that the range of hair cortisol concentrations for the 60 children is 1.7–671.6 ng/g.

Further confounding may be present due to the inter-individual variability in asthma severity or difference between the No ICS and ICS Treated groups for asthma severity. Unfortunately, a reliable measure of asthma severity was not included in the data collection. Also, the groups did not differ in perceived stress as indicated by the PSS score and, therefore, it was not included in any analysis. However, this does not rule out other forms of stress that may have been detected and possibly different among the groups if other tests of stress were performed.
Chapter 6  
Overall Summary and Conclusions

6.1 Summary and Conclusions

Hair cortisol analysis is a unique, easy, and non-invasive means of measuring endogenous cortisol levels. Hair grows on average 1 cm/mo, thus allowing for determination of historic cortisol concentrations that can be used to detect changes over time, which may be of great benefit to endocrinologists. A hair sample is easy and non-invasive because it is collected by simply cutting a lock of hair with scissors from an inconspicuous location at the back of the head.

The research contained in this thesis has shown that hair cortisol is significantly lower in those with ICS therapy. This was observed in two different populations, children and pregnant women. In children, hair cortisol concentrations were 50% lower in those with ICS use when comparing the intra-individual hair cortisol concentrations before and during ICS therapy. For this reason, it has the potential to be a sensitive biomarker of toxicity to monitor patients for possible adrenal suppression due to ICS therapy, maybe even as a pharmacodynamic biomarker to monitor adherence to therapy since a decreased cortisol concentration is probable with ICS use. The ability to monitor ICS therapy would be a significant outcome of establishing hair cortisol as a biomarker because, according to Perlis (2011), "a test that improves the patient’s adherence—even if it does not change treatment strategy—might still have value, because it changes the patient’s behavior."

A comparison of median hair cortisol concentrations among the groups of children in the large cohort was not significant. This outcome is not surprising given that hair cortisol reflects the average endogenous cortisol concentration over time and many studies investigating adrenal insufficiency in children due to ICS therapy found that children tend to have normal morning serum cortisol concentrations and normal or abnormal HPA axis stimulation tests, indicating adrenal suppression rather than overt insufficiency for some children. However, the children in the 5th percentile of hair cortisol concentration may be analogous to patients with very low morning serum cortisol, which is considered diagnostic of adrenal insufficiency. These results suggest hair cortisol is a potential biomarker that is as sensitive as currently accepted biomarkers.
for diagnosing adrenal insufficiency. Future research is required to confirm this theory. However, compared to the other sample matrices, hair has a temporally integrated nature and its non-invasive, easy collection makes it well suited to children. While quantile regression revealed ICS type and dose did not significantly affect hair cortisol concentrations in the children with asthma, there were a few factors associated with hair cortisol, such as age, sex, BMI, and use of intranasal corticosteroids. Consequently, adjustment for these factors is recommended for future research involving hair cortisol analysis in children.

The hair cortisol analysis in this thesis also indicated that pregnant women with asthma have lower cortisol production over the course of pregnancy than healthy control pregnant women, regardless of ICS use, which was previously unknown. Supplementary analysis for ICS type-dependent effects suggested that budesonide might be associated with significantly lower hair cortisol concentrations in T3. Future research to determine if any perinatal outcomes associated with maternal asthma are associated with decreased maternal cortisol would be advisable since, theoretically, suboptimal cortisol concentrations may adversely impact fetal maturation and epigenetic programming. Building upon this current work and studies by others, hair cortisol appears to be a useful biomarker of the HPA axis function during pregnancy and, accordingly, could be a useful tool for future research correlating hair cortisol concentrations with pregnancy outcomes.

Overall, with many people experiencing asymptomatic adrenal suppression (Broersen, Pereira, Jørgensen, & Dekkers, 2015), finding a simple, non-invasive method to detect those at risk is important. Better detection will reduce the risk of adrenal crisis or acute insufficiency; while these may be relatively rare phenomena, they are associated with high morbidity and mortality. Using hair cortisol as a non-invasive means of routine monitoring for adrenal suppression and adherence to ICS therapy may be beneficial to reduce the incidence of morbidity and mortality still occurring and of concern to the medical community (Shulman, Palmert, & Kemp, 2007). Hair cortisol could potentially also be used to monitor the adrenal effects of glucocorticoids administered in other forms, e.g., intraocular or oral, as already shown by Gow et al. (2011) and Noppe et al. (2014). Given the results in this thesis for the cohort of children, when monitoring therapy it would be best to compare hair cortisol concentrations within the individual rather than to established reference ranges because of the large inter-individual variability of endogenous cortisol concentrations. Manenschijn et al. (2012a) also suggested
intra-individual comparison for patients with cyclic Cushing syndrome. Nonetheless, the results in this thesis support that hair cortisol analysis may be useful in the investigation of other conditions associated with reduced cortisol secretion, but larger studies are required to confirm and extend these findings.

6.2 Challenges of establishing hair cortisol as a "biomarker"

Challenges exist when trying to establish any "biomarker" and they vary depending on its intended use. For hair cortisol to be a biomarker of toxicity, it must go through the process of validation by showing method sensitivity, specificity, and reproducibility, which requires extensive studies. The research in this thesis contributes to the literature working to establish hair cortisol as a biomarker of toxicity.

In general, there needs to be "Analytical validation – can the biomarker be measured accurately?" (Amur et al., 2015). Research has been carried out previously indicating that hair cortisol can be measured accurately and precisely, especially the international round robin by Russell et al. (2014), which showed measurements made by different researchers using different EIA and LC-MS methods compared well. Hair cortisol researchers have generally accepted that hair cortisol can be measured accurately and are now focusing on utilizing hair cortisol to evaluate populations with different conditions and diseases. However, before using hair cortisol analysis in a clinical laboratory, development and rigorous testing of an LC-MS method would be necessary.

Another consideration is qualification: "is the biomarker associated with the clinical endpoint of concern?" (Amur et al., 2015). This thesis research suggests that hair cortisol concentration is able to identify individuals with significantly lower concentrations when compared intra-individually in the children or to healthy controls for the pregnant women. Hair cortisol as a biomarker bears a similarity to the current tests of basal adrenal function, such as a morning serum, in that it may be able to identify individuals with significantly decreased cortisol, suggestive of adrenal suppression. For example, this is possibly the case for the 5.3% of ICS Treated children who had hair cortisol concentration < 2.0 ng/g compared to none of the children in the control groups. However, unlike serum cortisol, hair analysis is unable to detect an acute change in cortisol, e.g., after an HPA axis stimulation test. Hair cortisol reflects the average cortisol concentration over a longer period, which may be more sensitive and clinically
relevant than the current basal cortisol tests in certain circumstances, as suggested by this thesis research in pregnant women with asthma. Hair cortisol is not expected to replace the current tests available, but as a potentially new biomarker of adrenal suppression, it may have a place among the current biomarkers. This is due to its ease of collection and storage and that it can provide new information on average cortisol concentration over time currently unavailable through measurement of cortisol in serum. With further research, cut-offs for hair cortisol concentrations might be established to identify those with known adrenal suppression and those who require follow-up with an HPA axis stimulation test for confirmation, as has been determined for morning serum cortisol concentrations. Also, research remains to be performed to discern why a consistent percentage of people have an increased hair cortisol concentration. Previous hair cortisol research has documented a wide range of factors that can affect hair cortisol (Table 4). Without a better understanding of the factor(s) contributing to this phenomenon observed in the control groups, as well as the case groups, hair cortisol as a biomarker will have limitations.

Finally, there is utilization: "what is the specific [context of use] for biomarker qualification?" (Amur et al., 2015). The children's pilot study in this thesis suggests that hair cortisol may be an effective biomarker for possible adrenal suppression due to ICS therapy, which may also become a means to monitor adherence to therapy. The caveat to using hair cortisol analysis in this way is that the comparison must be intra-individual and only a small group of children were involved in the pilot study. This current research adds information regarding hair cortisol as a potential biomarker of toxicity, but is a good example of caution needed regarding generalizability. The results showed hair cortisol may be an effective biomarker of toxicity due to ICS therapy in children but not in pregnant women unless they took ICS ≥ 5 doses per week. In pregnant women, hair cortisol appears to be a more successful biomarker of the effects of asthma on the HPA axis function. Furthermore, while the current research applies to children and pregnant women with asthma, the generalizability of this research to other populations, such as all adults with asthma or other chronic conditions such as rheumatoid arthritis, is not known and requires further research with larger sample sizes. However, previous research and this thesis research is a foundation that future research can build upon.

When it comes to transitioning hair cortisol from a potential biomarker of toxicity to a surrogate endpoint for toxicity, even more work is required. The evidence required to confirm a
surrogate endpoint must be of the highest quality because of their use in clinical and policy decisions (Amur et al., 2015). Although this current research indicates hair cortisol is a potentially useful biomarker of ICS toxicity, and detecting cortisol suppression has frequently been used as a surrogate endpoint in clinical trials of ICS (Buchwald, 2008; Kelly, 2003; Nieto et al., 2007), only further research will determine if hair cortisol can effectively be used in this context.
Chapter 7
Future Studies

Future studies incorporating hair cortisol as a potentially useful biomarker can take many directions. To build upon the work presented in this thesis, further validation of hair cortisol concentration as a biomarker of the HPA axis in pregnant women with asthma would be beneficial. A study enrolling more women to achieve larger sample sizes and focusing on the later trimester(s) could be performed. One suggested study is to investigate any potential relationship between hair cortisol concentration and the perinatal outcomes associated with asthma. As long as a control group is included as part of the comparison, such a study may confirm or refute the differences among the groups revealed in this research for T3 while beginning to answer whether decreased cortisol concentrations are associated with certain perinatal outcomes for these women.

A highly informative study, and next logical step from this thesis work, would be to correlate hair cortisol concentration with the results of HPA axis stimulation tests, such as the ACTH stimulation test, which would also include the morning serum cortisol. This comparison could be performed as part of randomized controlled trials of ICS, in children or adults. Alternatively, the comparison does not need to be restricted to individuals with asthma but could easily be performed in collaboration with a pediatric or adult endocrinology department for all patients undergoing an HPA axis stimulation test. The research in this thesis did not include an HPA axis stimulation test because, when the protocol was created, previous hair cortisol studies had not used hair cortisol to detect lower cortisol concentrations associated with confirmed adrenal suppression. Since hair cortisol's potential for the detection of adrenal suppression was unknown, evaluating the utility of hair cortisol for this purpose before performing an invasive test was desirable, especially since the research involved children. However, now that this thesis research has shown hair cortisol concentration identifies individuals who may have or be at risk of adrenal suppression, further research to correlate the hair cortisol concentrations to abnormal HPA axis stimulation test results is warranted. Such research may be able to identify hair cortisol concentration cut-offs for those with adrenal suppression (those with a normal HPA axis stimulation test result but a result significantly lower than controls) or adrenal insufficiency (those with an abnormal morning serum cortisol or HPA axis stimulation test).
Results of this thesis possibly indicate that some people with more severe asthma have a higher hair cortisol concentration (e.g., the children and women with higher cortisol concentrations despite using a high-dose ICS). Performing a study comparing the hair cortisol concentrations of individuals with poorly controlled asthma to individuals with well-controlled asthma, regardless of ICS use, may be advantageous. It could potentially rule out or confirm if the physiological stress due to the disease is a potential factor influencing the hair cortisol concentration or if there is a correlation with glucocorticoid resistance.

To further evaluate hair cortisol as a potential pharmacodynamic biomarker, performing a larger study specifically aimed at monitoring ICS therapy would indicate if measuring hair cortisol concentration would be a useful tool to monitor for decreased cortisol concentrations as a possible indicator of adrenal suppression or ICS adherence. Since the comparisons for these studies would be intra-individual, when monitoring for adrenal suppression, the addition of an HPA axis suppression test would be beneficial to determine what percentage decrease in hair cortisol indicates clinically significant adrenal suppression. Should the outcome of this research be informative, the data could subsequently be used in a study to correlate ICS prescribed dose and asthma control to determine when add-on therapy or alternate therapies should be considered. When evaluating adherence, collecting data as to whether adherence was improved if participants knew they were being monitored would be informative because, as previously mentioned, a biomarker that improves adherence is also valuable, especially one that is non-invasive. A supplementary means of evaluating adherence would be to determine if, in addition to cortisol, the ICS could be detected in hair by developing a liquid chromatography-mass spectrometry method. If successful, this would not only provide a new means for therapeutic drug monitoring, but also speak to the systemic distribution of the ICS since it is likely the ICS will more readily incorporate into hair given the lipid content compared to the aqueous environment of serum.

As an example of how the results of this research may be beneficial in other areas of research, hair cortisol could be used in growth studies of those with adrenal disease. Mazziotti and Giustina (2013) mention in their study, "the effects of untreated primary adrenal failure on [growth hormone] secretion have not been studied owing to the clinical severity of this clinical condition and the need to start glucocorticoid replacement therapy immediately after the diagnosis." Hair testing for cortisol would be beneficial in this kind of research because of the
temporal nature and ability to measure historic concentrations. Taking it one step further, studies would be enhanced by also investigating whether growth hormone can be measured in hair.
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Appendix A

Study ID: ____________________________

Safety of Inhaled Corticosteroid Use in Pregnant Women and in Children
ICS – Data Collection Form

Data to be collected by DSEN SEARCH trained research staff/students either at the time of enrollment or by telephone follow-up. □ In Person □ Telephone

Date of questionnaire: ____________________________

Date of Birth: _____MM _____YYYY Date of hair collection: ___________________ N/A_____

For pregnant women: Weeks Pregnant_______________ Expected due date_____________________

1. Which of the following ICS medications has the subject ever taken?
   □ Beclomethasone (Beclovent, Vanceril, Qvar)
   □ Budesonide (Pulmicort)
   □ Ciclesonide (Alvesco)
   □ Fluticasone (Flovent)
   □ Mometasone (Asmanex Twisthaler)
   □ Combination products (Symbicort, Advair)
   □ Subject cannot recall [DISCONTINUE]
   □ Not applicable (continue to #7)

2. Age when subject first began taking ICS medication?

   Age ___________ Years

   □ Subject cannot recall

3. How long has subject been taking ICS medication?

   ___________ Years

   □ Subject cannot recall
Appendix A

Study ID: __________________________

4. Start and stop dates?
When did subject start and stop taking medication(s)
[IF not yet stopped their ICS medication; draw a line through the stop date box]:

<table>
<thead>
<tr>
<th>ICS Drug (dose + frequency)</th>
<th>Delivery System</th>
<th>Start date 1 MM/YYYY</th>
<th>Stop date 1 MM/YYYY</th>
<th>Start date 2 MM/YYYY</th>
<th>Stop date 2 MM/YYYY</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
</tbody>
</table>

1 = Meter Dose Inhaler; 2=Dry Powder; 3=Nebulized; 4=other

5. Times subject may have stopped taking ICS [insert name of ICS medication stopped] and the Reason for this during past 12 months. Include seasonal or other drug holidays.

☐ Did not stop at any time
☐ Did stop at times (details below)
☐ Subject cannot recall
☐ Subject has not taken the medication in the last 12 months

<table>
<thead>
<tr>
<th>Name of drug (brand name if possible)</th>
<th>Months or days drug not taken</th>
<th>Reason for not taking (5-9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

5= No asthma during season; 6= not medically needed; 7=forget; 8= adverse reactions; 9=other (specify)

6. Does the subject use a spacer device?
☐ Yes
☐ No
### 7a. Adverse reaction to ICS? (Describe below)  *Reactions as defined by AHFS

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Date of reaction (MM/YYYY)</th>
<th>Reaction required hospitalization?</th>
<th>Received treatment from Dr.</th>
<th>Type of treatment/Date</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adrenocortical Insufficiency</strong> (HPA axis suppression) (e.g. hypotension, hypoglycemia, dehydration, disorientation, weakness, dizziness, tiredness, irritability)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>Musculoskeletal Effects</strong> (e.g. muscle weakness, muscle pain, osteoporosis/bone fractures, growth retardation)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>Increased Susceptibility to Infections</strong> (e.g. viral, bacterial, fungal, protozoan, helminthic - in any organ)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>Fluid and Electrolyte Disturbance</strong> (e.g. sodium retention, edema, weight gain, congestive heart failure)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>Ocular Effects</strong> (e.g. cataracts/glucoma)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>Endocrine Effects</strong> (e.g. cushingoid state, amenorrhea or menstrual difficulties, hyperglycemia)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>GI Effects</strong> (e.g. nausea, vomiting, anorexia, increased appetite, diarrhea, constipation, indigestion, peptic ulcer)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>Nervous System Effects</strong> (e.g. headaches, vertigo, insomnia (sleep disturbances), restlessness, EEG abnormality, seizures, mental disturbances - mood swings, euphoria, depression, psychoses)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>Dermatologic Effects</strong> (e.g. delayed wound healing, skin thinning, acne, increased sweating, hirsutism, easy bruising)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
</tbody>
</table>
7b. Adverse reaction to ICS? (Describe below)

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Date of reaction (MM/YYYY)</th>
<th>Reaction required hospitalization?</th>
<th>Received treatment from the physician</th>
<th>Type of treatment/ Date</th>
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<tbody>
<tr>
<td>Gestational diabetes/hyperglycemia</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td>Preeclampsia</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
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<tr>
<td>(e.g. hypertension, proteinuria)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased susceptibility to infections</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td>(e.g. viral, bacterial, fungal, protozoan, helminthic - in any organ)</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Infant malformation</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
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<tr>
<td>(e.g. cleft palate or other birth defects)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant hypo-hyperglycemia</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td>Infant infections</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td>ADDED DESCRIPTION</td>
<td></td>
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</tbody>
</table>

8. Birth outcome?

| Gestational Age | Birth Weight |
|-----------------|--------------|-------------|
|                 |              |             |
Appendix A

Study ID: ________________________________

9. Other medications taken within the past year (12 months)?

ANY other corticosteroids steroid medicines (e.g. oral prednisone, prednisolone, dexamethasone, or nasal preparations e.g. Flonase, Beclonase) used to control asthma exacerbations or nasal problems during the past 12 months.

Please include all tablets, liquids, sprays, inhalers, nebulized products.

Also include antibiotics and other drugs.

☐ Not taking any medicines/ did not take any medication in the past 12 months

☐ Taking one or more medicines (see below)

☐ Subject cannot recall

<table>
<thead>
<tr>
<th>Name of medicine</th>
<th>Dose and frequency (if known)</th>
<th>Reason for taking (10-13)</th>
<th>Start date</th>
<th>Stop Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

Reason for taking: 10=to alleviate asthma exacerbation; 11=for asthma prevention; 12=nasal rhinitis; 13=other (specify)

10. In the past 12 months how many asthma exacerbations did subject have?

Number ________________

☐ None

☐ Subject cannot recall

Details of the asthma exacerbations experienced in the past 12 months.

<table>
<thead>
<tr>
<th>Number of Exacerbation</th>
<th>Date (1st exacerbation)</th>
<th>Date (2nd exacerbation)</th>
<th>Date (3rd exacerbation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospitalized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ED visit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visit to Dr.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased Meds at home</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
11. **To be completed during post-delivery follow-up, if applicable** (information to be obtained from patient chart or via telephone/in person interview)

- [ ] From Chart
- [ ] In Person

**Date of follow-up:** ________________

---

**Birth outcome?**

- Gestational Age: ________________
- Birth Weight: ________________

---

**Adverse reaction to ICS?**

<table>
<thead>
<tr>
<th>Reaction Type</th>
<th>Date of reaction (MM/YYYY)</th>
<th>Reaction required hospitalization?</th>
<th>Received treatment from the physician</th>
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<td><em>Gestational diabetes/hyperglycemia</em></td>
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<td>YES / NO</td>
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</tr>
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<td><em>Pre-eclampsia</em> (e.g. hypertension, proteinuria)</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><em>Increased susceptibility to infections</em> (e.g. viral, bacterial, fungal, protozoan, helminthic - in any organ)*</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><em>Infant malformation</em> (e.g. cleft palate or other birth defects)*</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><em>Infant hypo-hyperglycemia</em></td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><em>Infant infections</em> (e.g. viral, bacterial, fungal, protozoan, helminthic - in any organ)*</td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><em>Other</em></td>
<td></td>
<td>YES / NO</td>
<td>YES / NO</td>
<td></td>
</tr>
<tr>
<td><strong>ADDED DESCRIPTION</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
**Hair Sample Collection Form**

Please fill out requested information. Thank you.

Participant ID #: ___ Date of Hair Collection: ________________

No. of times hair is washed per week: ___ Date of last wash: ________________

Is your hair chemically coloured? **Yes** **No**

Gestational Week at time of collection, if applicable: ________________ weeks

---

**Fold line 1**

Place scalp end of hair in this box. Affix with low-tack tape placed at least 2-4 cm from the scalp end. If the hair is shorter than 2 cm, place tape over one half of the hair leaving cut ends free.

DO NOT TAPE CHILD HAIR if <1 year-of-age. In that case, please put the hair in the centre of this paper, fold the paper four ways to contain the hair, and put in the “Hair Sample” envelope provided. Seal the envelope.

---

**Fold line 2**

After hair is attached to the paper, please fold as indicated by the numbered fold lines and place in the hair sample envelope provided, and seal the envelope.

**Version 2.0**

2013/05
Appendix C

ICS: Data Form/Questionnaire Supplemental
Version 1.0
August 2012

Safety of Inhaled Corticosteroid Use in Pregnant Women and in Children
ICS – Data Collection Form Supplemental

Data to be collected by DSEN SEARCH trained research staff/students either at the time of enrollment or by telephone follow-up.

A. Date of birth (MM/YYYY): __________________________

B. BMI determination

Height: __________________________ in / cm (circle one)

Weight: __________________________ lb / kg (circle one)

Calculated BMI: ________________

C. Perceived Stress Scale

Age: ____________________ Gender: M / F

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

1. In the last month, how often have you been upset because of something that happened unexpectedly?................................. 0 1 2 3 4

2. In the last month, how often have you felt that you were unable to control the important things in your life?................................. 0 1 2 3 4

3. In the last month, how often have you felt nervous and “stressed”? ............ 0 1 2 3 4

4. In the last month, how often have you felt confident about your ability to handle your personal problems?................................. 0 1 2 3 4

5. In the last month, how often have you felt that things were going your way?................................. 0 1 2 3 4

6. In the last month, how often have you found that you could not cope with all the things that you had to do?................................. 0 1 2 3 4

7. In the last month, how often have you been able to control irritations in your life?................................. 0 1 2 3 4
Appendix C

8. In the last month, how often have you felt that you were on top of things?.. 0 1 2 3 4

9. In the last month, how often have you been angered because of things that were outside of your control? .........................0 1 2 3 4

10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?.....................0 1 2 3 4

Score: _______ (Questions 4, 5, 7 and 8 are scored in reverse)
Hair Collection Instructions

Adult or Child (>1 year-of-age)

1. Obtain the Hair Sample Collection Form before starting. Ensure all necessary information is filled out on the form.
2. Obtain clean scissors, low-tack tape (normal household tape), and the Hair Sample Collection Form before starting. Scissors may be cleaned by wiping them thoroughly with a clean tissue soaked in rubbing alcohol, or washing with hot, soapy water and rinsing well.
3. Section hair at the back of the head just below the crown, known as the vertex posterior, to reveal an inconspicuous area for collection. A barrette may be helpful for those with long hair to secure hair not being cut out of the way. (Figures 1 and 2.)

![Figure 1](image1.png)  ![Figure 2](image2.png)

4. Using the tips of the scissors, isolate the hair to be sampled. The amount required for testing is approximately 40-60 strands, or about half the size of a pencil diameter. See Figures 3 and 4. It may be helpful to twist the hair, especially if the hair is shorter. See Figure 5.

![Figure 3](image3.png)  ![Figure 4](image4.png)  ![Figure 5](image5.png)

5. Cut the hair as close to the scalp as possible (within 2-4 mm), while not pulling the hair out from the root or cutting the skin. See Figures 6 and 7.

![Figure 6](image6.png)  ![Figure 7](image7.png)
6. Lay the hair on the Hair Sample Collection Form with the cut ends in the box and lined up with the line indicated. Fix the hair in place with the tape approximately 2-4 cm away from the scalp end of the hair. See Figures 8. If the hair is less than 2 cm long, place the tape over one half of the hair leaving the cut ends uncovered by tape.

**Please Note:** This image is for illustration of the proper way to fix the hair to the Hair Sample Collection Form. The sample form depicted is not the same as the one provided with this kit. The root end is the same as the cut end.

Figure 8

5. Fold the Hair Sample Collection Form following the numbered “fold” lines, and place it in the envelope provided labeled “Hair Sample”. It is okay if the hair gets folded. Seal the envelope and place it in the addressed, stamped, return envelope provided for mailing to the research facility.
## Length of Steroid Use in Hair Samples

### Symbol Legend:

<table>
<thead>
<tr>
<th>Drug</th>
<th>Contin. Use</th>
<th>Intermit. Use</th>
<th>Drug</th>
<th>Contin. Use</th>
<th>Intermit. Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciclesonide</td>
<td></td>
<td></td>
<td>Symbicort</td>
<td></td>
<td></td>
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<tr>
<td>Fluticasone</td>
<td></td>
<td></td>
<td>Budesonide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Advair</td>
<td></td>
<td></td>
<td>Zenhale/Mometasone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beclomethasone</td>
<td></td>
<td></td>
<td>Any systemic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- ? denotes dates of use are uncertain
- \( \uparrow \) denotes significant event(s), i.e. prednisone use, baby born or conceived (specify event)
- * denotes when questionnaire was completed
- \( \uparrow \) denotes end of hair sample

0 time point = 2 weeks prior to hair collection date

"Switch" date is approximate since one Rx may need to be finished prior to beginning the new Rx

### ID#: Sample child map 1

Total Length (cm): 30+

Date of hair collection: 2013/4/25

Date of switch to 100 mcg/d not definite. Dose increases from 100 to 200 mcg/d for colds.

### ID#: Sample child map 2

Total Length (cm): 9.5-10

Date of hair collection: 2013/9/10

Dates of fluticasone use unknown, but stated as intermittently. Unknown if ciclesonide used more than indicated.

### ID#: Sample child map 3

Total Length (cm): 3.5

Date of hair collection: 2013/4/28

Dose increased from 200 to 400 mcg/d for colds, until Feb. 2013 when it was 400 mcg/d daily.

### ID#: Sample pregnancy map 1

Total Length (cm): >38

Date of hair collection: 2013/11/9

Hair sample is thin

Baby born 2013/10/6

Conception ~ 2012/12/30

### ID#: Sample pregnancy map 2

Total Length (cm): 10

Date of hair collection: 2013/12/10

Thick hair sample, Feb-Mar 2013 used 4-5 times/week, Apr-July 1 every 2 weeks, only takes 1 puff once a day

Baby born 2013/10/11

Conception ~ 2013/1/11
Copyright Acknowledgements

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<th>Item</th>
<th>Publisher</th>
<th>License Number</th>
<th>License Date</th>
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<td>3767390204732</td>
<td>December 13, 2015</td>
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<tr>
<td>Figure 2</td>
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<td>3773950285799</td>
<td>December 21, 2015</td>
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<tr>
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<td>3816190070813</td>
<td>February 25, 2016</td>
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<td>Figure 7</td>
<td>Elsevier</td>
<td>3776750779256</td>
<td>December 26, 2015</td>
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<td>Figure 8</td>
<td>Canadian Pharmacogenomics Network for Drug Safety</td>
<td>Permission granted by Dr. Bruce Carleton, DSEN-SEARCH Principal Investigator</td>
<td>March 3, 2016</td>
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<td>Appendix D – Hair Collection Instructions Figures 1-4 and 6</td>
<td>lizgareriphotography.com</td>
<td>Used with permission from the Motherisk Drug Testing Laboratory</td>
<td>May 2012</td>
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