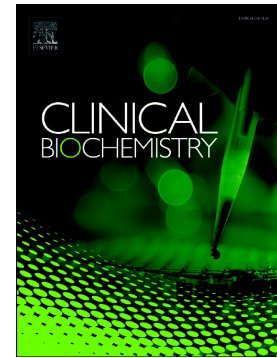


## Accepted Manuscript

Assessment of hair cortisol as a potential biomarker for possible adrenal suppression due to inhaled corticosteroid use in children with asthma: A retrospective observational study

Laura Smy, Kaitlyn Shaw, Ursula Amstutz, Michelle Staub, Shahnaz Chaudhry, Anne Smith, Bruce Carleton, Gideon Koren



PII: S0009-9120(17)31188-8  
DOI: doi:[10.1016/j.clinbiochem.2018.04.006](https://doi.org/10.1016/j.clinbiochem.2018.04.006)  
Reference: CLB 9758  
To appear in: *Clinical Biochemistry*  
Received date: 5 December 2017  
Revised date: 10 April 2018  
Accepted date: 10 April 2018

Please cite this article as: Laura Smy, Kaitlyn Shaw, Ursula Amstutz, Michelle Staub, Shahnaz Chaudhry, Anne Smith, Bruce Carleton, Gideon Koren , Assessment of hair cortisol as a potential biomarker for possible adrenal suppression due to inhaled corticosteroid use in children with asthma: A retrospective observational study. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Clb(2018), doi:[10.1016/j.clinbiochem.2018.04.006](https://doi.org/10.1016/j.clinbiochem.2018.04.006)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Assessment of hair cortisol as a potential biomarker for possible adrenal suppression due to inhaled corticosteroid use in children with asthma: a retrospective observational study**

Laura Smy<sup>a,b</sup>, Kaitlyn Shaw<sup>c,d</sup>, Ursula Amstutz<sup>c,d,e</sup>, Michelle Staub<sup>f</sup>, Shahnaz Chaudhry<sup>a</sup>, Anne Smith<sup>c,d,g</sup>,  
Bruce Carleton<sup>c,d,h,\*</sup>, Gideon Koren<sup>b,i</sup>

<sup>a</sup>Division of Clinical Pharmacology and Toxicology, The Hospital for Sick Children, Toronto, ON, Canada

<sup>b</sup>Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, ON, Canada

<sup>c</sup>Child & Family Research Institute, Vancouver, BC, Canada

<sup>d</sup>Division of Translational Therapeutics, Department of Pediatrics, University of British Columbia, Vancouver, BC, Canada

<sup>e</sup>University Institute of Clinical Chemistry, Inselspital Bern University Hospital, University of Bern, Switzerland

<sup>f</sup>Winnipeg Regional Health Authority, Pharmacy Department, Winnipeg, Manitoba, Canada

<sup>g</sup>Deceased

<sup>h</sup>Pharmaceutical Outcomes Programme, BC Children's Hospital, Vancouver, BC, Canada

<sup>i</sup>Maccabi-Kahn Research Institute, Tel Aviv, Israel

***\*Corresponding author and to whom reprints should be addressed:***

Bruce Carleton, PharmD

Pharmaceutical Outcomes Programme, Child & Family Research Institute,

950 W 28th Avenue,

Vancouver, BC, V5Z 4H4

Phone: 604-875-2179, Fax: 604-875-2494

e-mail: bcarleton@popi.ubc.ca.

## **Abstract**

### *Background*

Inhaled corticosteroids (ICS) are the recommended long-term control therapy for asthma in children. However, concern exists regarding potential adrenal suppression with chronic ICS use. Our pilot study reported that hair cortisol in children was 50% lower during ICS therapy than prior to therapy, suggestive of adrenal suppression.

### *Objective*

To evaluate hair cortisol concentration (HCC) as a potential biomarker for possible adrenal suppression from ICS use in children with asthma.

### *Methods*

A retrospective observational study was performed at asthma clinics in Vancouver, Winnipeg, and Toronto, Canada. Children ( $n=586$ ) were recruited from July 2012 to December 2014 inclusive of those without asthma, with asthma not using ICS, and with asthma using ICS. The most recent three-month HCC was measured by enzyme immunoassay and compared among the groups. Quantile regression analysis was performed to identify factors potentially affecting HCC.

### *Results*

The median HCC was not significantly different among the children: No ICS ( $n=47$ , 6.7 ng/g, interquartile range (IQR) 3.7-9.8 ng/g), ICS Treated ( $n=360$ , 6.5 ng/g, IQR 3.8-14.3 ng/g), and Controls ( $n=53$ , 5.8 ng/g, IQR 4.6-16.7 ng/g). 5.6% of the children using ICS had hair cortisol  $<2.0$  ng/g compared to none in the control groups ( $P<0.05$ , comparing ICS Treated (20/360) to all Controls combined (0/100)) and only half had been exposed to systemic corticosteroids. Age, sex, BMI, and intranasal corticosteroid use were significantly associated with HCC.

### *Conclusions*

Results suggest HCC may be a potential biomarker for adrenal suppression as a population of children using ICS with HCC  $<2.0$  ng/g was identified compared to none in the control groups. Further research is needed to determine if those children have or are at risk of adrenal suppression or insufficiency.

**Keywords/Phrases:** Hair cortisol, Asthma, Children, Inhaled corticosteroids, Biomarker

***Abbreviations***

ACTH = adrenocorticotrophic hormone; BMI = body mass index; EIA = enzyme immunoassay; HCC = hair cortisol concentration; HPA = hypothalamic-pituitary-adrenal; ICS = inhaled corticosteroids; IQR = interquartile range; INC = intranasal corticosteroid; OLS = ordinary least squares; PSS = perceived stress scale; SD = standard deviation

## 1.0 Introduction

Asthma is a common chronic respiratory condition affecting approximately 335 million people worldwide [1]. In 2008-2009, approximately 10% of Canadian children aged 2-7-years-old were diagnosed with asthma [2]. According to the Canadian Thoracic Society, the recommended therapy for long-term control of chronic asthma is inhaled corticosteroids (ICS) [3]. There are five ICS available in Canada: fluticasone, budesonide, beclomethasone, mometasone, and ciclesonide. Most ICS have not been properly evaluated for use in children <4-6-years-old with the exception of the fluticasone propionate metered dose inhaler, which is approved for children over 12-months-old [4-8]. Regardless, young children are prescribed ICS and Thomas *et al.* [9] reported that 5.6% of children <5-years-old and 10% of children 5-11-years-old were receiving high dose therapy (>400 µg/d for those age groups). Concern exists regarding adrenal suppression and insufficiency in children on normal and high dose ICS for chronic maintenance therapy [10-13].

Cortisol is a hormone released from the adrenal glands regulating multiple physiological functions including the stress response and inflammation. Adrenal suppression is characterized by a decreased but generally adequate production of cortisol, whereas adrenal insufficiency is a more serious condition characterized by chronically inadequate production of cortisol [10]. Individuals with adrenal suppression are at risk of experiencing adrenal insufficiency.

Adrenal insufficiency is diagnosed by clinical presentation and laboratory tests. The most common laboratory test is measurement of morning serum cortisol, which is diagnostic of adrenal insufficiency if <100 nmol/L and indeterminate if 100-500 nmol/L [14], possibly indicating adrenal suppression at the measurement time point. More recently, hair has been used in research as an alternative matrix to measure cortisol concentration. The benefits of a hair sample compared to serum include that it is painless, easy, and non-invasive to collect and, unlike serum and saliva, hair does not need to be collected at a specific time. Additionally, the constant growth rate of hair, approximately one centimetre per month on average [15], allows for the determination of the past, average, cortisol concentrations over time, not strictly at one point in time, and, thus, better able to detect chronic cortisol changes. All of these

qualities make it a potentially useful tool for cortisol assessment in children.

Research suggests that hair cortisol concentration (HCC) represents the average endogenous systemic cortisol concentration. HCC has been significantly correlated with a 24-hour urine sample, multiple saliva samples collected over three days in the course of a week, and the area under the concentration-time curve of saliva samples collected daily for one month [16–18]. In patients with Cushing's syndrome, the HCC was high, as expected, and the measurements were 86% sensitive and 98% specific for detecting cyclic Cushing's syndrome [19]. Additionally, HCC has been measured in many studies evaluating chronic stress [as reviewed by 20]. Only two small studies have evaluated HCC in children with asthma. One study compared the HCC of healthy children to children with asthma and the other was our pilot study comparing a child's HCC prior to and during ICS therapy as an indicator of possible adrenal suppression due to ICS use [21, 22]. Both studies reported approximately 50% lower HCC.

The objective of this study was to expand on our previous research assessing HCC as a potential biomarker of possible adrenal suppression due to ICS therapy in children with asthma and to determine if any effects are associated with ICS type or dose. We hypothesized that children with asthma on ICS therapy may have lower HCC than children not using ICS, either with or without asthma, and that HCC analysis may detect increased adrenal toxicity associated with the ICS type or dose.

## **2.0 Materials and Methods**

### *2.1 Study design, participants, and ethics*

This was a retrospective observational cohort study. Children aged 0-18-years-old were recruited through hospital asthma clinics or a hospital-associated community asthma clinic at three sites across Canada between July 2012 and December 2014. Participants were eligible provided they or their parents 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. Three groups of children were included: those without asthma not using ICS (Controls), those with asthma not using ICS (No ICS), and those with asthma using ICS (ICS Treated). Research ethics board approval was obtained

from each institution including: British Columbia Children's and Women's Health Centre in Vancouver, British Columbia; Hospital for Sick Children in Toronto, Ontario; and Winnipeg Children's Hospital in Winnipeg, Manitoba. Informed written consent from the parent, guardian or child, and assent from the child was obtained, as appropriate, for all participants.

## 2.2 *Data and Hair Sample Collection*

The participant's demographic and clinical information, such as their asthma and medication history inclusive of all corticosteroid use, was recorded after participant interview and/or medical record review. Children enrolled in Toronto also performed the Perceived Stress Scale (PSS) if they were capable of understanding the questions [23].

Hair was cut close to the scalp with scissors from the vertex posterior region of the head. The samples of  $\geq 40$  hair strands were affixed with tape to a hair sample collection form keeping the ends aligned and stored in an envelope at room temperature until analyzed. Factors potentially affecting HCC were recorded with the sample collection.

## 2.3 *Hair Cortisol Analysis*

The most proximal 3-cm segment of hair was analyzed for cortisol concentration. The method for cortisol extraction and analysis has previously been reported [24] and, with minor modifications, is described in brief in the Supplementary Materials. This extraction process recovery was reported as 84.5-88.9% from spiked hair samples [16, 25]. The cortisol concentration was measured using the Salimetrics High Sensitivity Salivary Cortisol Enzyme Immunoassay (EIA) kit (Salimetrics, Philadelphia, PA) following the manufacturer's instructions. The performance of this immunoassay has been examined by our group prior to using it in this research project [26]. We conducted a blinded comparison of hair samples ( $n=15$ ) spanning low, moderate, and high HCC analyzed by four immunoassays and two liquid chromatography-mass spectrometry (LC-MS) assays in an international round robin including four leading laboratories from Canada, Germany, the Netherlands, and the USA [26]. The Salimetrics kit comparison to the mean of both LC-MS concentrations had a Pearson correlation of  $r^2=0.98$  ( $P<0.0001$ ) and equation of  $y=0.3844x + 2.683$  for LC-MS vs. the Salimetrics immunoassay. The LC-MS methods

have reported intra-day coefficients of variation (CVs) between 1.9 and 8.4% and inter-day CVs between 4.7% and 14.5% for a range of cortisol concentrations in spiked and pooled hair samples [27, 28]. The average intra-day CV for this study using the Salimetrics immunoassay was 6.3% based on participant hair samples and the inter-day CVs were 1.5% and 4.5% for the high and low controls, respectively [29]. Additionally, a pooled hair quality control sample, extracted and analyzed with each run, had a CV of 13.4% over 40 runs. For a parallelism study, dilutions of four hair extracts were made spanning the analytical measurement range (AMR) at concentrations to mirror those of the calibrators and compared to the calibration curve. The curves for the hair extracts were not significantly different from the calibrator curve ( $F_{(4,56)}=1.54$ ,  $P=0.20$ , average  $R^2=0.998$ ). Since the hair extracts behaved the same as the calibrators across the AMR, the kit AMR was used for the hair analysis. Hair samples with concentrations beyond the AMR were diluted to obtain results within the AMR and the dilution factor applied to obtain the final concentration. The limit of quantitation was 0.12 ng/mL (0.33 nmol/L) as per the lowest kit calibrator. The manufacturer reported cross-reactivity is less than 0.6% for all other steroid hormones with the exception of dexamethasone, which is 19.2%. The cross-reactivity for each ICS was previously reported by our group as  $\leq 0.03\%$  [29]. The final HCC were calculated as the ratio of nanograms of cortisol per gram of hair (multiply by 2.759 for pmol/g).

#### 2.4 Statistical Analysis

Comparisons of HCC, demographic information, and clinical information were performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, the Kruskal-Wallis test with Dunn's multiple comparison test, Chi-square or Fisher's exact test, Welch's ANOVA, Mann-Whitney U test, and Spearman correlation, as appropriate. For all analyses, two-tailed  $P$ -values were calculated and considered significant if  $\leq 0.05$ .

All HCC were used in calculations; however, the HCC data were not normally distributed and could not be normalized by transformation. Therefore, quantile regression was used to determine the association of HCC with variables and correct for possible confounders [30]. The 5-95<sup>th</sup> quantiles, or percentiles, were calculated. To assess the method suitability, the results of the quantile regression were

compared to ordinary least squares (OLS) regression of the original and rank transformed HCC data, chosen as a potentially acceptable transformation of non-parametric data. Two models were used: Model 1 assessed ICS type and Model 2 ICS dose. Unstandardized and standardized regression coefficients were calculated. A full description of the regression models and the equation for calculation of standardized regression coefficients are provided in the Supplementary Materials.

All statistical analyses were performed using Graphpad Prism software version 5.0c (Graphpad Software, Inc., CA), SPSS version 22.0 (IBM Corporation, NY), and RStudio version 0.99.486 (RStudio, Inc., MA) running R version 3.2.2 (The R Foundation for Statistical Computing, Austria).

### 3.0 Results

Of 586 children enrolled, 460 hair samples were suitable for analysis. The remaining samples were: used in our children's pilot study ( $n=23$ ) [22], insufficient 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 other than asthma ( $n=3$ ). Table 1 summarizes the cohort's demographics, HCC, hair sample variables, and medication use. 33.6% of the children used fluticasone, 36.7% ciclesonide, 8.1% beclomethasone, 8.1% budesonide, 3.3% mometasone, 10.0% multiple ICS, and one child (0.2%) used an unknown ICS. The number of ICS Treated per dose category was 118 taking  $>0-1.0$   $\mu\text{g/kg/d}$ , 139 taking  $>1.0-2.5$   $\mu\text{g/kg/d}$ , 91 taking  $>2.5$   $\mu\text{g/kg/d}$ , 11 children did not have weight recorded, and 1 child did not have the ICS dose recorded.

The median HCC did not differ among the groups of children (Figure 1, Table 1), but upon visual inspection, there appear to be subpopulations present. The Controls and No ICS appear to have bimodal distributions with the division occurring at an HCC of 40 ng/g. The subpopulation of children in each group with HCC  $>40$  ng/g was not significantly different with 11%, 13%, and 14% in the Controls, No ICS, and ICS Treated, respectively. Similarly, a lower limit for HCC of 2.0 ng/g was notable given that 20 children (5.6%) in the ICS Treated group had an HCC below this concentration compared to none in the control groups. This is a significant difference when the controls groups are combined giving a

comparison of no ICS to ICS use (0/100 versus 20/360,  $P < 0.05$ ). Of note, 10 of those 20 children were only exposed to ICS and had not received systemic corticosteroids in the previous five months.

**Table 1.** Comparison of demographics, hair sample variables, and medication use among the three groups of children in the large cohort.

|                                                                         | Controls<br>(n = 53)  | No ICS<br>(n = 47)         | ICS Treated<br>(n = 360)   | P                        |
|-------------------------------------------------------------------------|-----------------------|----------------------------|----------------------------|--------------------------|
| <i>Demographics</i>                                                     |                       |                            |                            |                          |
| Age, years, median (IQR)                                                | 7.7 (3.1-12.3)        | 7.9 (4.2-12.0)             | 6.3 (3.7-10.1)             | 0.236 <sup>l</sup>       |
| BMI, kg/m <sup>2</sup> , median (IQR, n)                                | 17.6 (15.6-20.7, 51)  | 17.5 (16.3-19.6, 46)       | 16.8 (15.5-19.2, 349)      | 0.061 <sup>l</sup>       |
| Male:Female, n                                                          | 22:31                 | 28:19                      | 213:147                    | 0.050 <sup>m</sup>       |
| Toronto:Vancouver:Winnipeg, n                                           | 50:3:0                | 24:19:4                    | 66:229:65                  | <0.001 <sup>m</sup>      |
| PSS Score, mean (SD, n)                                                 | 15.7 (8.0, 22)        | 13.7 (4.1, 17)             | 15.4 (6.9, 11)             | 0.537 <sup>k</sup>       |
| <i>Hair Sample Variables</i>                                            |                       |                            |                            |                          |
| HCC, ng/g, median (IQR)                                                 | 5.8 (4.6-16.7)        | 6.7 (3.7-9.8)              | 6.5 (3.8-14.3)             | 0.532 <sup>l</sup>       |
| # Washes per week, median (IQR, n)                                      | 3.5 (2.0-4.9, 52)     | 3.5 (2.0-7.0, 47)          | 3.5 (2.4-5.0, 353)         | 0.663 <sup>l</sup>       |
| # Days since last washed, median (IQR, n)                               | 2.0 (1.0-2.0, 51)     | 1.0 (0.8-2.0, 42)          | 1.0 (1.0-2.0, 304)         | 0.162 <sup>l</sup>       |
| Chemical treatment, n (%)                                               | 8 (15.1) <sup>e</sup> | 2 (4.3)                    | 17 (of 354, 4.8)           | <b>0.010<sup>m</sup></b> |
| <i>Medication Use</i>                                                   |                       |                            |                            |                          |
| Inhaled corticosteroid use <sup>a</sup> , Yes/No                        | No                    | No                         | Yes                        | —                        |
| Lifetime duration of ICS use, years, median (IQR)                       | 0 (0-0) <sup>h</sup>  | 0.9 (0.0-4.5) <sup>h</sup> | 2.3 (0.9-5.2) <sup>h</sup> | <0.001 <sup>l</sup>      |
| Systemic corticosteroid use <sup>b</sup> , n (%)                        | 1 (1.9) <sup>f</sup>  | 4 (8.5) <sup>f</sup>       | 128 (35.6)                 | <0.001 <sup>m</sup>      |
| Intranasal corticosteroid use <sup>c</sup> , n (%)                      | 4 (7.5) <sup>g</sup>  | 10 (21.3)                  | 106 (29.4)                 | <b>0.002<sup>m</sup></b> |
| Topical corticosteroid and/or other hormone use <sup>c</sup> , n (%)    | 10 (18.9)             | 8 (17.0)                   | 69 (19.2)                  | 0.940 <sup>m</sup>       |
| Beta-agonist <sup>c,d</sup> , n (%)                                     | 3 (5.7) <sup>h</sup>  | 35 (74.5) <sup>h</sup>     | 355 (98.6) <sup>h</sup>    | <0.001 <sup>m</sup>      |
| Number of other classes of medications used <sup>c</sup> , median (IQR) | 2.0 (1.0-2.0)         | 2.0 (1.0-3.0)              | 1.0 (1.0-2.0) <sup>j</sup> | <0.001 <sup>l</sup>      |

BMI = body mass index; HCC = hair cortisol concentration; ICS = inhaled corticosteroids; IQR = interquartile range; PSS = perceived stress scale; SD = standard deviation. <sup>a</sup>Inhaled corticosteroid use includes any use within the time captured by the tested hair segment, regardless of frequency or duration. <sup>b</sup>Systemic corticosteroid use is reported if within five months of hair collection. <sup>c</sup>Use within the last 12 months. <sup>d</sup>Includes beta-agonist drugs that are short and long-acting, including use of combination inhaler products. <sup>e</sup>Significantly different from ICS Treated,  $P < 0.05$ . <sup>f</sup>Significantly different from ICS Treated,  $P \leq 0.001$ . <sup>g</sup>Significantly different from ICS Treated,  $P < 0.01$ . <sup>h</sup>All significantly different from each other,  $P < 0.001$ . <sup>j</sup>Significantly different from No ICS only,  $P < 0.01$ . <sup>k</sup>Welch's

analysis of variance. <sup>1</sup>Kruskal-Wallis test with Dunn's multiple comparison test. <sup>m</sup>Chi-squared test. Note, chemical treatment had >20% with expected values <5.

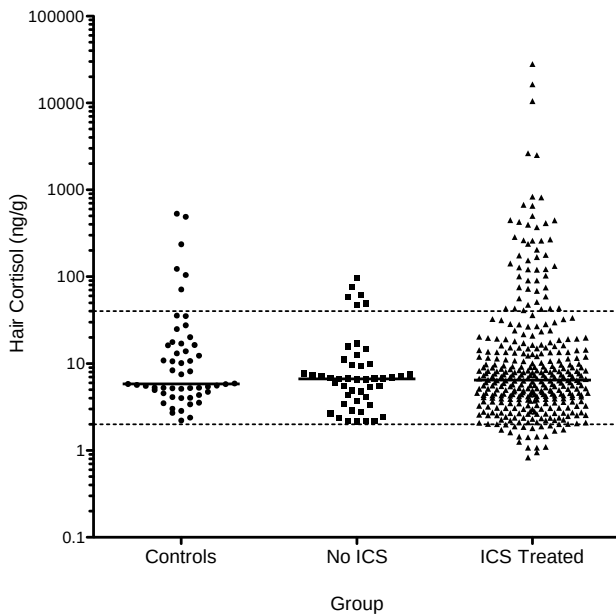


**Figure 1.** Scatterplot of hair cortisol concentrations by group. Hair cortisol concentration is shown on the y-axis with a logarithmic scale and the groups are indicated on the x-axis. The black bars indicate the median hair cortisol concentration for each group. The black dotted lines indicate the 2 and 40 ng/g concentrations.

Differences in HCC were evaluated for associations with age and sex (Figure 2). Younger children tended to have higher HCC than older children. Linear regression showed that HCC significantly decreased from 1-8-years-old but did not significantly differ between the sexes (comparison of slopes:  $\text{slope}_{(\text{males})} = -0.08 \pm 0.03 \text{ ng/g}$ ;  $\text{slope}_{(\text{females})} = -0.10 \pm 0.03 \text{ ng/g}$ ,  $F_{(1,282)} = 0.13$ ,  $P=0.72$ , Figure 2, left inset). From 8-18-years-old, HCC increased slightly with age for females, but the sexes were not significantly different from one another except at approximately 8-9-years-old where males had a significantly higher HCC than females (comparison of y-intercept  $F_{(1,171)}=4.7$ ,  $P=0.03$ , Figure 2, right inset).

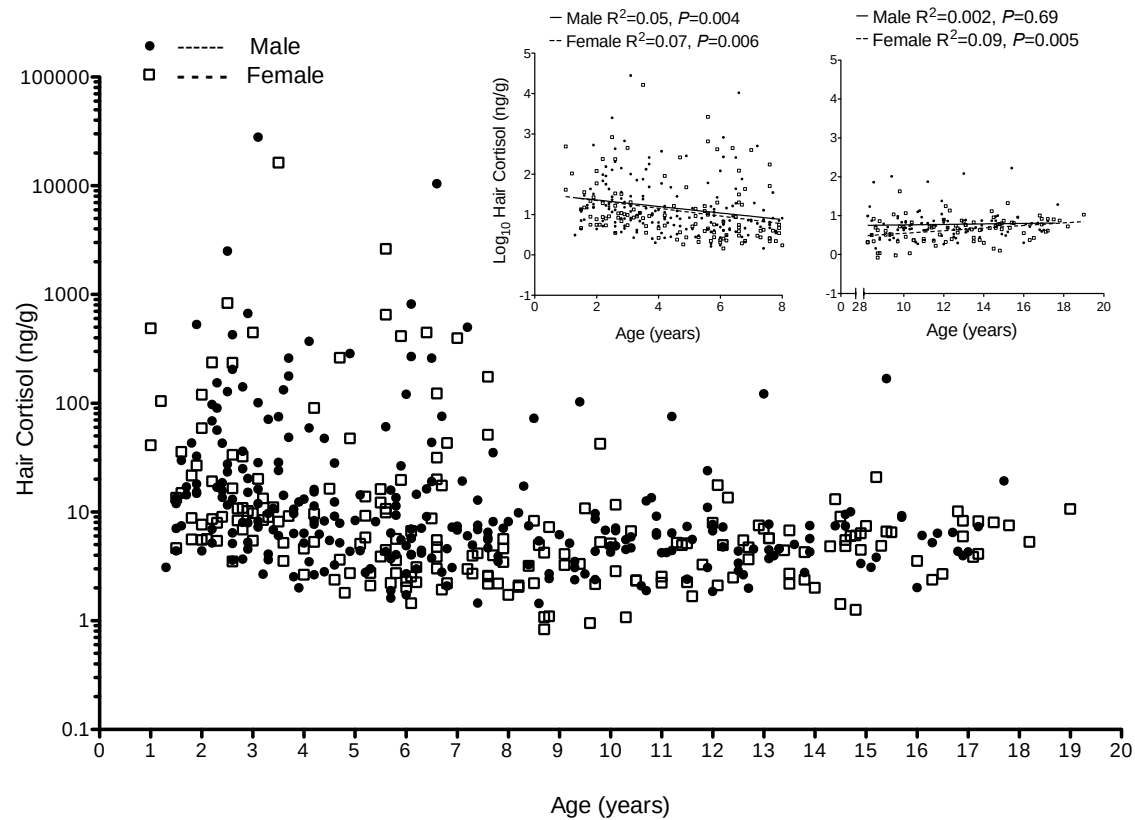


**Figure 2.** Scatterplot of hair cortisol concentration versus age and sex for the cohort of children. Hair cortisol concentration is indicated on the y-axis with a logarithmic scale. The age is shown on the x-axis. The children's sex is indicated by the different markers and lines as shown in the legend. 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.

For both quantile regression models (Model 1  $n=443$ , Model 2  $n=442$ ), the regression coefficients for the intercept (which represents male Controls 7.48-years-old with a BMI of  $15.5 \text{ kg/m}^2$ ) fell within the IQR for the Controls' HCC, thus providing a validation of the regression models. Table 2 shows the standardized regression coefficients for significantly associated variables in Models 1 and 2 for the 5<sup>th</sup>, 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup>, and 95<sup>th</sup> percentiles of HCC. Models 1 and 2 unstandardized regression coefficients and

OLS data, and Model 2 example quantile regression graphs are presented in the Supplementary Materials Tables S1, S2, and Figure S1. Confounding for the upper percentiles due to the extremely high HCC, indicated by wide confidence intervals, was still present in both models.

**Table 2.** Summary of standardized quantile regression coefficients\* and range for significant independent variables for Models 1 and 2 for the combined pediatric cohort.

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

BMI = body mass index; ICS = inhaled corticosteroids

\*Coefficients were standardized using the equation reported in the Supplementary Materials and indicate the change in hair cortisol concentration in ng/g per one standard deviation change of the independent variable.

Significant results are in **bold**.

The quantile regression results were not significant for living in Winnipeg, lifetime duration of ICS use, and ICS type. Additionally, ICS dose did not have significant results with a couple of exceptions. Of note, for children in 5<sup>th</sup> percentile of HCC, using >2.5 µg/kg/d was associated with lower HCC (unstandardized regression coefficient ( $\beta$ )=-0.7 ng/g, CI<sub>95%</sub>=-1.0 to -0.04 ng/g), but for children in the 55<sup>th</sup> percentile, using >2.5 µg/kg/d was associated with higher HCC ( $\beta$ =3.8 ng/g, CI<sub>95%</sub>=0.4-5.6 ng/g).

However, an interpretation of variables with significance at only one or two percentiles is possibly over-interpretation [31].

Variables with significant effects on HCC in both models included age, sex, BMI, and intranasal corticosteroid (INC) use. Table 2 shows that, for the combined cohort, age is the most significant factor affecting HCC. With increasing HCC percentile in both Models, age had a significantly positive or negative effect on HCC depending on if the age was below or above 7.48 years, respectively. For Model 1, only the 5-85<sup>th</sup> percentiles were significant. BMI was the next most influential variable with a positive or negative influence on HCC depending on whether the BMI was above or below 15.5 kg/m<sup>2</sup> (5-50<sup>th</sup> percentiles in Model 1, 5-75<sup>th</sup> percentiles in Model 2). Scatterplots showing the relationship of BMI with HCC and age are shown in the Supplementary Materials Figures S2A and B. Of similar influence as BMI, INC use was negatively associated with HCC in Model 1 (15-65<sup>th</sup> percentiles) and Model 2 (25-75<sup>th</sup> percentiles). Finally, females had a slightly but significantly lower HCC in both models (15-55<sup>th</sup> percentiles in Model 1, 5-25<sup>th</sup> percentiles in Model 2). Comparisons of the unadjusted HCC medians between INC users and non-users and between the sexes are shown in the Supplementary Materials Figures S3 and S4.

#### 4.0 Discussion

This exploratory study is one of only a few, and to our knowledge, the largest that has investigated measuring hair cortisol as a potential biomarker of adrenal suppression. Comparison of median HCC among the three groups of children did not show a significant difference, but the comparison was not adjusted for covariates and both control groups had a smaller sample size, which may require a larger observed effect size for significance. Smit et al. also reported no difference between median HCC concentrations in their study comparing children with mild to moderate asthma to controls [32]. These non-significant results are not surprising given that research suggests HCC represents the average, endogenous cortisol concentration over time and only children with chronically lower cortisol would have significantly lower hair cortisol than the controls. Previous studies comparing basal cortisol results (*i.e.* serum or salivary cortisol) and hypothalamic-pituitary-adrenal (HPA) axis stimulation test

results for ICS-treated children to control groups have found that the results for the ICS-treated children were within the normal reference interval although significantly lower than the controls [33–35]. These results suggest the children's HPA axis is unable to respond to the same degree as the controls in the morning or when stimulated. What is not known from these studies is if the children's average cortisol concentrations over the long term would be significantly different from the controls. However, this is the comparison that is made using HCC.

The current study results also contradict the results of our pilot study and another hair cortisol study in children with asthma, which both reported approximately 50% decrease in HCC for children with asthma [21, 22]. The study by Kamps *et al.* [21] may have been underpowered to detect the inter-individual variation in cortisol (n=10 each group) and they did not document ICS use. Our pilot study used an intra-individual comparison rather than an inter-individual comparison, which may account for the conflicting results. Collectively, the literature suggests the ICS-associated adrenal suppression may not be chronic for many children but rather sub-clinical and only realized when an acute stress is applied to the HPA axis. For this reason, hair cortisol analysis may not be sensitive for detecting adrenal suppression in those children since it represents the chronic cortisol response rather than acute cortisol response. Intra-individual comparison, as in our pilot study, may be more sensitive for detecting potentially clinically significant suppression of adrenal function and determining what percentage decrease is clinically significant would be advantageous.

Although an unadjusted comparison of medians was not significant, what is remarkable and warrants further discussion in this pediatric cohort is the variance in HCC. The variation in HCC of the ICS Treated group may indicate distinct subpopulations within this group. The 5.6% of the ICS Treated group with HCC <2.0 ng/g, indicative of children with chronically low cortisol, compared to none in either of the control groups may represent a population with or at risk of having adrenal insufficiency due to ICS therapy especially since only half had been exposed to systemic corticosteroids. It may be that HCC at a certain cut-off could be analogous to diagnosing those with morning serum cortisol <100

nmol/L as having adrenal insufficiency [14]. This theory is supported by previous studies that confirmed adrenal suppression by performing basal cortisol tests in conjunction with the low-dose adrenocorticotrophic hormone (ACTH) stimulation test. Zollner *et al.* reported 6.1% ( $CI_{95\%}=1.8-10.5\%$ ) of children with asthma using ICS had morning serum cortisol  $<83$  nmol/L [13]. Further, a meta-analysis of 27 asthma studies determined that 6.8% ( $CI_{95\%}=3.8-12.0\%$ ) of people  $\geq 12$ -years-old experienced adrenal insufficiency due to ICS use [36]. The similarity in percentages of adults and children diagnosed with adrenal insufficiency using traditionally accepted methods to the 5.6% of children with extremely low HCC in our study suggests HCC may be a suitable biomarker for detecting adrenal suppression. However, adrenal suppression or insufficiency was not confirmed in our study by a basal serum or saliva cortisol or ACTH stimulation test because it was an exploratory study to evaluate HCC as a potential biomarker of adrenal suppression. To verify this theory, confirming adrenal suppression with basal serum or saliva testing and determining the association between HCC and HPA axis stimulation test results would help ascertain if there was a cutoff for HCC similar to that for morning serum cortisol indicative of probable adrenal insufficiency. Additionally, pharmacogenomic investigations, which are planned for this cohort, may reveal potential genomic predictors of adrenal insufficiency from ICS use.

In contrast, there is a subpopulation of 13% of children with higher than expected HCC represented in all three groups. Many studies have observed individuals with higher than expected HCC, measured by immunoassay and liquid chromatography-mass spectrometry, and in many cases have excluded those subjects with values  $>2-3$  SD, thus excluding approximately 2-12%, including "healthy" controls, from the analysis but without proper explanation [37–41]. Even though they are statistical outliers, excluding these data may introduce selection bias. Future research determining if these individuals have a chronic non-pathologic or pathologic hypercortisolemia would provide an enriched examination of HCC as a potential biomarker of endogenous cortisol concentrations and associated conditions. Our groups did not differ in perceived stress as indicated by the PSS score; however, this does not rule out other forms of stress that may have been different among the groups such as physiological

stress. For example, a significant positive correlation existed between children in this study and "asthma exacerbations" (exacerbations overall,  $\rho=0.114$ ,  $P=0.015$ ; major exacerbations requiring emergency visit or hospitalization,  $\rho=0.175$ ,  $P<0.001$ ), which may represent a significant physiological stress. However, our exacerbation data has important limitations due to recall bias and inclusion of events that affected the child's asthma but may not be a true exacerbation, such as anaphylaxis or a respiratory illness (although they are other forms of physiological stress). Therefore, the results should be interpreted with caution. Other explanations for the high cortisol concentrations observed include a genomic association for this subpopulation or contamination from external corticosteroids that were not reported in the data collection. Finally, EIA interference due to inhaled corticosteroids is unexpected due to our previous work showing low cross-reactivity [29]; however, interference due to heterophile antibodies in the hair samples, for example, has not been reported in the literature but cannot be ruled out, although it would not be expected.

Quantile regression was used to adjust for covariates and revealed that age, sex, BMI, and INC use were significant. The majority of literature regarding the association of sex and BMI with HCC indicates males and those with a higher BMI tended to have higher HCC in both children and adults [42, 43]. Both Models 1 and 2 indicated boys had higher HCC than girls and the scatterplot showed a significant difference around 8-9-years-old. BMI was positively associated with HCC in children when their BMI was greater than  $15.5 \text{ kg/m}^2$ . Regarding children's age, the research is conflicting [42]. Similar to our cohort, two studies reported HCC decreases in young children until approximately 8-10-years-old [37, 44]. However, serum cortisol was reported to increase from 2-18-years-old [45], and one study evaluating reference intervals for HCC reported HCC increased in children 5-10-years-old [46]. Finally, we found INC use had a negative association with HCC. Similarly, a meta-analysis determined that 4.2% ( $\text{CI}_{95\%}=0.5\text{-}28.9\%$ ) of individuals using INC had adrenal insufficiency [36]. However, children using ICS alone compared to ICS and INC did not have significantly different morning salivary cortisol concentrations [47].

Unexpectedly, ICS type did not have a significant effect on HCC. Studies have reported fluticasone was associated with adrenal suppression, insufficiency, or crisis [12, 48–50]. However, the studies did not use equipotent doses [48], or used high doses for comparison, *e.g.*, 500 or 1000 µg/d in adults [49]. Conversely, one study found ICS type was not a significant factor [11], and another found budesonide suppressed adrenal function more than fluticasone in children taking ICS for 6 months [34]. Of those studies measuring serum cortisol concentrations, all groups were within the normal reference interval for the study duration [34, 48, 49]. Therefore, while different ICS types are associated with adrenal suppression when tested by an HPA axis stimulation test, they do not generally result in chronically abnormal serum cortisol concentrations and, thus, may not be detected using HCC.

With respect to dose, several studies have found that children on moderate or high dose ICS experienced adrenal suppression or insufficiency [11, 13, 33–35, 51, 52]. The meta-analysis by Broersen *et al.* revealed the percentage of patients with a positive HPA axis stimulation test increased in a dose-dependent manner [36]. Nonetheless, similar to our results, a recent HCC study did not find any correlation between ICS dosage and HCC [32]. For our cohort, ICS dose was only significantly associated with two percentiles of HCC for children taking >2.5 µg/kg/d and, therefore, not considered a significant association. Notably, however, the high dose negatively affected children in the 5<sup>th</sup> percentile (*i.e.* HCC <2.0 ng/g). As previously discussed, it may be hypothesized that these children belong to the population with low basal cortisol concentrations indicative of adrenal insufficiency and, since half of these children were only exposed to ICS and not systemic corticosteroids, the association with ICS dose for this group may truly be significant, also suggesting HCC may be a valid biomarker. The next step would be to perform studies incorporating tests for adrenal suppression or insufficiency and HCC.

A strength of this study is the large pediatric cohort, which, to our knowledge, is the largest to date. Such a large cohort allowed for determining associations for numerous variables with HCC and potentially identified a population of children at risk of adrenal insufficiency. If confirmed by future research, the ability to identify children with adrenal insufficiency using HCC may indicate HCC is as

sensitive as morning serum cortisol. Further, the non-invasive collection of hair makes it a practical tool for cortisol measurement in children.

Another strength of our research was using quantile regression, which allowed representation of all children in the analysis rather than excluding "outliers" and indicated that ICS type was not significant while ICS dose may affect children in the 5<sup>th</sup> percentile of HCC. Unfortunately, some variables were underrepresented in this cohort to properly determine their relationship with HCC and analyses were still confounded by the extremely high HCC. However, this was limited with quantile regression compared to other regression methods.

The use of oral corticosteroids did not significantly affect HCC in this cohort by univariate quantile regression (data not shown). Yet, because dexamethasone cross-reacts with EIA, systemic dexamethasone may be a confounder. Sixty children received dexamethasone during the 3-month period of the hair sample. Confounding due to cross-reactivity would assume that dexamethasone is incorporated into human hair, which is possible but unconfirmed. The correlation for dexamethasone use and HCC was not significant ( $\rho=0.066$ ,  $P=0.156$ ) in this cohort. Alternatively, confounding may be present due to variability in asthma severity either inter-individually or between the No ICS and ICS Treated groups.

In conclusion, this study suggests that HCC may be a potential biomarker for detecting chronic adrenal suppression due to ICS use in children with asthma since 5.6% of ICS Treated children had HCC  $<2.0$  ng/g compared to none in the control groups. Future research may further support the applicability of these results by determining if a cutoff HCC exists that is indicative of those with adrenal suppression or insufficiency. Research incorporating HCC and either a basal cortisol measurement or an HPA axis stimulation test would beneficially address this question and be the logical next step. Additionally, comparing intra-individual concentrations longitudinally rather than inter-individually may provide the most sensitive means of detecting adrenal suppression.

#### ***Funding disclosure***

This study was funded by The Canadian Institutes of Health Research Drug Safety and Effectiveness Network. The funder was not involved in the study design, data collection, analysis, or interpretation, writing the manuscript, or deciding to submit the article for publication.

### ***Disclosure summary***

The Pharmaceutical Outcomes Programme (POPi) has received financial support for its pharmacogenomics research from the following government-funded agencies in Canada: Canada Foundation for Innovation (CFI), Canadian Institutes of Health Research (CIHR), Genome Canada, Genome British Columbia and the Provincial Health Services Authority, the University of British Columbia, and British Columbia Children's Hospital Research Institute. Dr. Carleton is serving on a data safety monitoring board for AEVI Genomic Medicine in 2017.

The remaining authors have nothing to disclose.

### **Acknowledgements**

We would like to thank all of the participants for their interest and participation in our study. Additionally, we thank Dr. Allan Becker, Department of Pediatrics and Child Health, University of Manitoba, for his contributions to the success of this study. For their assistance with the laboratory work, we thank Kiri Latuskie, Helena Pearce-Reilly, and the Motherisk laboratory technicians. Laura Smy received educational funding support from a University of Toronto Scace Graduate Fellowship, Ontario Graduate Scholarship, and Canadian Pharmacogenomics Network for Drug Safety trainee award.

## References

1. The Global Asthma Report 2014 [package insert]. Auckland: The Global Asthma Network; 2014.
2. Thomas EM. Recent trends in upper respiratory infections, ear infections and asthma among young Canadian children. *Health Rep.* 2010;21:47-52.
3. Loughheed MD, Lemiere C, Ducharme FM et al. Canadian Thoracic Society 2012 Guideline update: diagnosis and management of asthma in preschoolers, children, and adults: executive summary. *Can Respir J.* 2012;19:127-164.
4. <sup>Pr</sup>Pulmicort<sup>®</sup> Turbuhaler<sup>®</sup> product monograph [package insert]. Mississauga, ON: AstraZeneca Canada Inc.; 2015.
5. <sup>Pr</sup>Flovent<sup>®</sup> Product Monograph [package insert]. Mississauga, ON: GlaxoSmithKline; 2014.
6. <sup>Pr</sup>Asmanex<sup>®</sup> Twisthaler<sup>®</sup> product monograph [package insert]. Kirkland, QC: Merck Canada Inc.; 2015.
7. <sup>Pr</sup>Alvesco<sup>®</sup> product monograph [package insert]. Oakville, ON: Takeda Canada Inc.; 2012.
8. <sup>Pr</sup>QVAR<sup>™</sup> Inhalation aerosol product monograph [package insert]. Laval, QC: Valeant Canada LP; 2013.
9. Thomas M, Turner S, Leather D et al. High-dose inhaled corticosteroid use in childhood asthma: an observational study of GP prescribing. *Br J Gen Pract.* 2006;56:788-790.
10. Ahmet A, Kim H, Spier S. Adrenal Suppression: A practical guide to the screening and management of this under-recognized complication of inhaled corticosteroid therapy. *Allergy Asthma Clin Immunol.* 2011;7:1-12. DOI:10.1186/1710-1492-7-13.
11. Smith RW, Downey K, Gordon M et al. Prevalence of hypothalamic-pituitary-adrenal axis suppression in children treated for asthma with inhaled corticosteroids. *Paediatr Child Health.* 2012;17:e34-e39.
12. Todd GRG, Cerini CL, Ross-Russell R et al. Survey of adrenal crisis associated with inhaled corticosteroids in the United Kingdom. *Arch Dis Child.* 2002;87:457-461.
13. Zollner EW, Lombard CJ, Galal U et al. Hypothalamic-pituitary-adrenal axis suppression in asthmatic school children. *Pediatrics.* 2012;130:e1512-9. DOI:10.1542/peds.2012-1147.

14. Charmandari E, Nicolaides NC, Chrousos GP. Adrenal insufficiency. *Lancet*. 2014;383:2152-2167. DOI:10.1016/S0140-6736(13)61684-0.
15. Kintz P, ed. *Analytical and Practical Aspects of Drug Testing in Hair*. Boca Raton, FL: Taylor & Francis Group, LLC; 2007.
16. Sauvé B, Koren G, Walsh G et al. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med*. 2007;30:E183-E191.
17. Short SJ, Stalder T, Marceau K et al. Correspondence between hair cortisol concentrations and 30-day integrated daily salivary and weekly urinary cortisol measures. *Psychoneuroendocrinology*. 2016;71:12-18. DOI:10.1016/j.psyneuen.2016.05.007.
18. van Holland BJ, Frings-Dresen MH, Sluiter JK. Measuring short-term and long-term physiological stress effects by cortisol reactivity in saliva and hair. *Int Arch Occup Environ Health*. 2012;85:849-852. DOI:10.1007/s00420-011-0727-3.
19. Manenschijs L, Koper JW, van den Akker EL et al. A novel tool in the diagnosis and follow-up of (Cyclic) Cushing's syndrome: measurement of long-term cortisol in scalp hair. *J Clin Endocrinol Metab*. 2012;97:E1836-43. DOI:10.1210/jc.2012-1852.
20. Russell E, Koren G, Rieder M et al. Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. *Psychoneuroendocrinology*. 2012;37:589-601. DOI:10.1016/j.psyneuen.2011.09.009.
21. Kamps AW, Molenmaker M, Kemperman R et al. Children with asthma have significantly lower long-term cortisol levels in their scalp hair than healthy children. *Acta Paediatr*. 2014;103:957-961. DOI:10.1111/apa.12685.
22. Smy L, Shaw K, Smith A et al. Hair cortisol as a novel biomarker of HPA suppression by inhaled corticosteroids in children. *Pediatr Res*. 2015;78:44-47. DOI:10.1038/pr.2015.60.
23. Cohen S, Williamson GM. Perceived stress in a probability sample of the United States. In: Spacapan S, Oskamp S, eds. *The Social Psychology of Health*. Newbury Park, CA: Sage, 1988

24. Pereg D, Gow R, Mosseri M et al. Hair cortisol and the risk for acute myocardial infarction in adult men. *Stress*. 2011;14:73-81. DOI:10.3109/10253890.2010.511352.
25. Manenschijs L, Koper JW, Lamberts SW et al. Evaluation of a method to measure long term cortisol levels. *Steroids*. 2011;76:1032-1036. DOI:10.1016/j.steroids.2011.04.005.
26. Russell E, Kirschbaum C, Laudenslager ML et al. Toward standardization of hair cortisol measurement: results of the first international inter-laboratory round robin. *Ther Drug Monit*. 2015;37:71-75. DOI:10.1097/FTD.0000000000000148.
27. Gao W, Stalder T, Foley P et al. Quantitative analysis of steroid hormones in human hair using a column-switching LC-APCI-MS/MS assay. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2013;928:1-8. DOI:10.1016/j.jchromb.2013.03.008.
28. Noppe G, de Rijke YB, Dorst K et al. LC-MS/MS-based method for long-term steroid profiling in human scalp hair. *Clin Endocrinol (Oxf)*. 2015;83:162-166. DOI:10.1111/cen.12781.
29. Smy L, Shaw K, Amstutz U et al. Hair cortisol as a hypothalamic-pituitary-adrenal axis biomarker in pregnant women with asthma: a retrospective observational study. *BMC Pregnancy Childbirth*. 2016;16:176. DOI:10.1186/s12884-016-0962-4.
30. Koenker R, Hallock K. Quantile regression: An introduction. *J Econ Perspect*. 2001;15:143-156.
31. Good PI, Hardin JW. *Common errors in statistics (and how to avoid them)*. 4th ed. Hoboken, NJ: John Wiley & Sons; 2012.
32. Smit MP, van Leer EHG, Noppe G et al. Long-Term Cortisol Concentration in Scalp Hair of Asthmatic Children Using Inhaled Corticosteroids: A Case-Control Study. *Horm Res Pediatr*. 2017;88:231-236. DOI:10.1159/000478526.
33. Bakkeheim E, Mowinckel P, Carlsen KH et al. Reduced basal salivary cortisol in children with asthma and allergic rhinitis. *Acta Paediatrica*. 2010;99:1705-1711. DOI:10.1111/j.1651-2227.2009.01598.x.

34. Kannisto S, Korppi M, Remes K et al. Adrenal suppression, evaluated by a low dose adrenocorticotropin test, and growth in asthmatic children treated with inhaled steroids. *J Clin Endocrinol Metab.* 2000;85:652-657. DOI:10.1210/jcem.85.2.6336.
35. Ninan TK, Reid IW, Carter PE et al. Effects of high doses of inhaled corticosteroids on adrenal function in children with severe persistent asthma. *Thorax.* 1993;48:599-602.
36. Broersen LHA, Pereira AM, Jørgensen JOL et al. Adrenal insufficiency in corticosteroids use: systematic review and meta-analysis. *J Clin Endocrinol Metab.* 2015;100:2171-2180. DOI:10.1210/jc.2015-1218.
37. Dettenborn L, Tietze A, Kirschbaum C et al. The assessment of cortisol in human hair: associations with sociodemographic variables and potential confounders. *Stress.* 2012;15:578-588. DOI:10.3109/10253890.2012.654479.
38. Faresjö Å, Theodorsson E, Chatziarzenis M et al. Higher perceived stress but lower cortisol levels found among young Greek adults living in a stressful social environment in comparison with Swedish young adults. *PLoS ONE.* 2013;8:e73828. DOI:10.1371/journal.pone.0073828.
39. Stalder T, Steudte S, Alexander N et al. Cortisol in hair, body mass index and stress-related measures. *Biol Psychol.* 2012;90:218-223. DOI:10.1016/j.biopsycho.2012.03.010.
40. Steudte S, Kirschbaum C, Gao W et al. Hair cortisol as a biomarker of traumatization in healthy individuals and posttraumatic stress disorder patients. *Biol Psychiatry.* 2013;74:639-646. DOI:10.1016/j.biopsycho.2013.03.011.
41. Wosu AC, Gelaye B, Valdimarsdóttir U et al. Hair cortisol in relation to sociodemographic and lifestyle characteristics in a multiethnic US sample. *Ann Epidemiol.* 2015;25:90-95, 95.e1. DOI:10.1016/j.annepidem.2014.11.022.
42. Gray NA, Dhana A, Van Der Vyver L et al. Determinants of hair cortisol concentration in children: A systematic review. *Psychoneuroendocrinology.* 2017;87:204-214. DOI:10.1016/j.psyneuen.2017.10.022.

43. Stalder T, Steudte-Schmiedgen S, Alexander N et al. Stress-related and basic determinants of hair cortisol in humans: A meta-analysis. *Psychoneuroendocrinology*. 2017;77:261-274. DOI:10.1016/j.psyneuen.2016.12.017.
44. Karlén J, Frostell A, Theodorsson E et al. Maternal influence on child HPA axis: a prospective study of cortisol levels in hair. *Pediatrics*. 2013;132:e1333-e1340. DOI:10.1542/peds.2013-1178.
45. Bailey D, Colantonio D, Kyriakopoulou L et al. Marked biological variance in endocrine and biochemical markers in childhood: establishment of pediatric reference intervals using healthy community children from the CALIPER cohort. *Clin Chem*. 2013;59:1393-1405. DOI:10.1373/clinchem.2013.204222.
46. Noppe G, Van Rossum EFC, Koper JW et al. Validation and reference ranges of hair cortisol measurement in healthy children. *Horm Res Paediatr*. 2014;82:97-102. DOI:10.1159/000362519.
47. Heijtsman SM, de Vries TW, Wolhuis A et al. Salivary cortisol levels in prepubertal children using inhaled corticosteroids with or without concurrent intranasal corticosteroids. *Pediatr Pulmonol*. 2011;46:1055-1061. DOI:10.1002/ppul.21474.
48. Clark DJ, Lipworth BJ. Adrenal suppression with chronic dosing of fluticasone propionate compared with budesonide in adult asthmatic patients. *Thorax*. 1997;52:55-58.
49. Derom E, Louis R, Tiesler C et al. Effects of ciclesonide and fluticasone on cortisol secretion in patients with persistent asthma. *Eur Respir J*. 2009;33:1277-1286. DOI:10.1183/09031936.00079908.
50. Drake AJ, Howells RJ, Shield JPH et al. Symptomatic adrenal insufficiency presenting with hypoglycaemia in asthmatic children with asthma receiving high dose inhaled fluticasone propionate. *BMJ*. 2002;324:1081.
51. Cavkaytar O, Vuralli D, Yilmaz EA et al. Evidence of hypothalamic-pituitary-adrenal axis suppression during moderate-to-high-dose inhaled corticosteroid use. *Eur J Pediatr*. 2015;194:1421-1431. DOI:10.1007/s00431-015-2610-9.
52. Molimard M, Girodet P-O, Pollet C et al. Inhaled Corticosteroids and Adrenal Insufficiency: Prevalence and Clinical Presentation. *Drug Safety*. 2008;31:769-774.

### Highlights

- Hair cortisol concentrations (HCC) were compared among children with and without asthma
- 5.6% of children using inhaled corticosteroids (ICS) had very low HCC indicative of chronically low cortisol
- HCC was significantly associated with age, BMI, sex, and intranasal corticosteroid use, but generally not ICS type and dose

# **Assessment of hair cortisol as a potential biomarker for possible adrenal suppression due to inhaled corticosteroid use in children with asthma: a retrospective observational study**

Laura Smy, Kaitlyn Shaw, Ursula Amstutz, Michelle Staub, Shahnaz Chaudhry, Anne Smith, Bruce Carleton, Gideon Koren

## **Supplementary Materials**

### *Hair sample preparation and cortisol extraction*

After washing the hair twice with isopropanol, cortisol was extracted by incubating 10-25 mg of finely chopped hair in methanol for 16 h at 50°C while rotating at 100 rpm. The methanol was transferred to a clean test tube, evaporated under N<sub>2</sub>, reconstituted with 125 µL of PBS at pH 7.2-7.4, and vortexed for one minute.

### *Regression Models*

Two models were created to assess inhaled corticosteroid (ICS) type and dose effects separately. 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 in µg/kg/d. 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 chemical treatments such as colour or 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<sup>2</sup>. 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  $\mu\text{g/kg/d}$  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  $\mu\text{g/kg/d}$ , 1.01-2.5  $\mu\text{g/kg/d}$ , and >2.5  $\mu\text{g/kg/d}$ . The reference group for Models 1 and 2 is a Control male, living in Toronto, aged 7.48 years with a BMI of  $15.5 \text{ kg/m}^2$  who does not have chemically treated hair and does not use intranasal corticosteroids or ICS. Similar to linear regression, the regression coefficient is significant if it is non-zero and the confidence interval does not include zero.

#### *Standardization of Quantile Regression Coefficients*

The unstandardized regression coefficients for Models 1 and 2 were presented in table form (Supplemental Table 2) and Model 2 was graphed (Supplemental Figure 1). 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 [1, 2]. Therefore, calculation of the standardized coefficients was performed using the equation:

$$\text{Eq. 1)} \quad \beta_{\text{standardized}} = \frac{\beta_{\text{unstandardized}} \times s_x}{s_y}$$

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 ( $s_y$ ) [1, 2].

However, since  $s_y$  is common to all independent variables because it is the standard deviation of the hair cortisol concentrations, this variable was eliminated [3]. This also allows the reported standardized coefficients to be in reference to the change in hair cortisol concentration in original units,  $\text{ng/g}$ , which is more intuitive to interpret rather than in units related to a 1-standard deviation change in hair cortisol [3].

## Supplemental Results

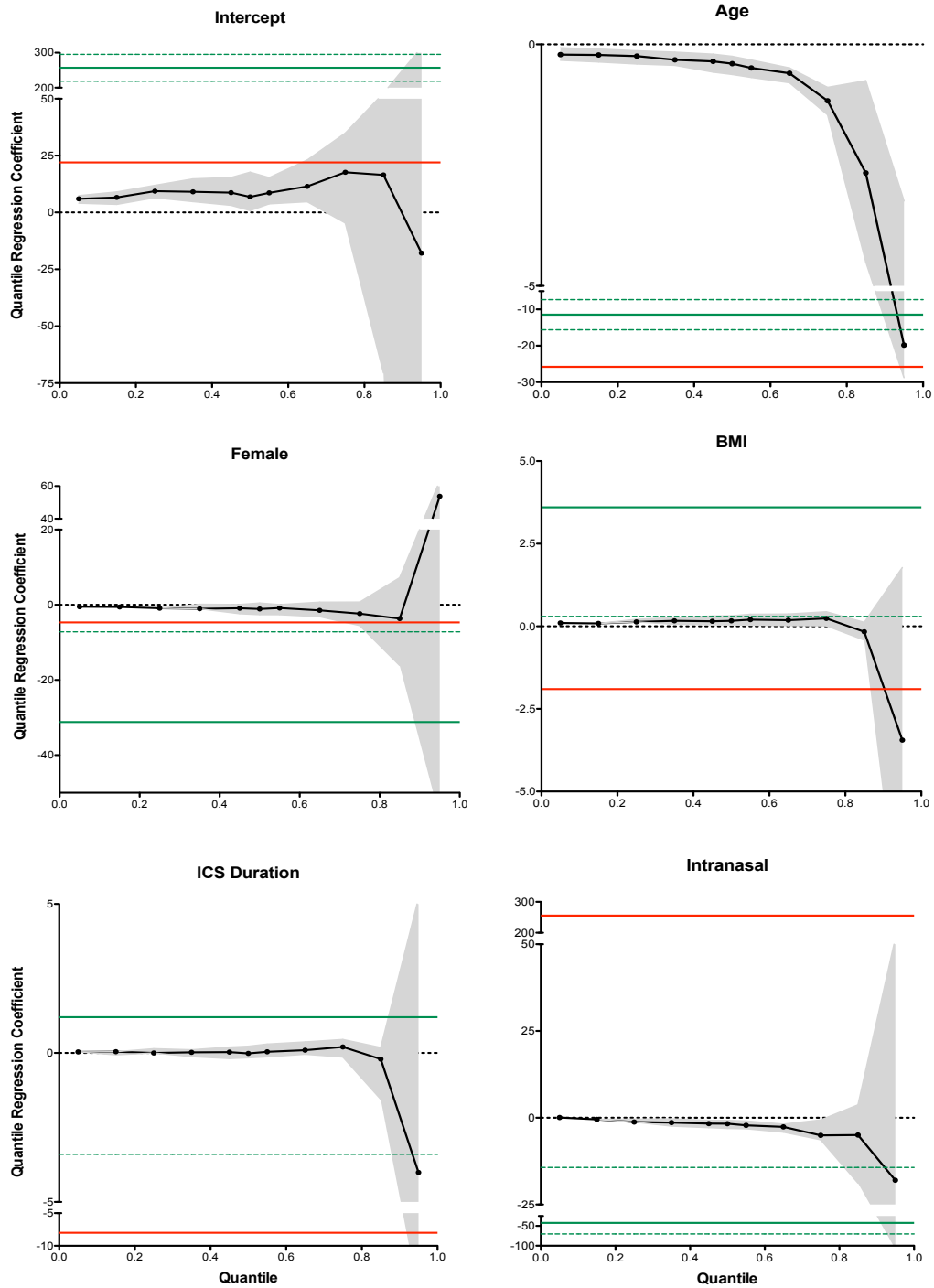


**Figure S1.** Quantile regression graphs for each variable included in Model 2 to evaluate ICS dose-dependent effects on hair cortisol concentrations in the large cohort of children (see next page for continuation of figure and legend).

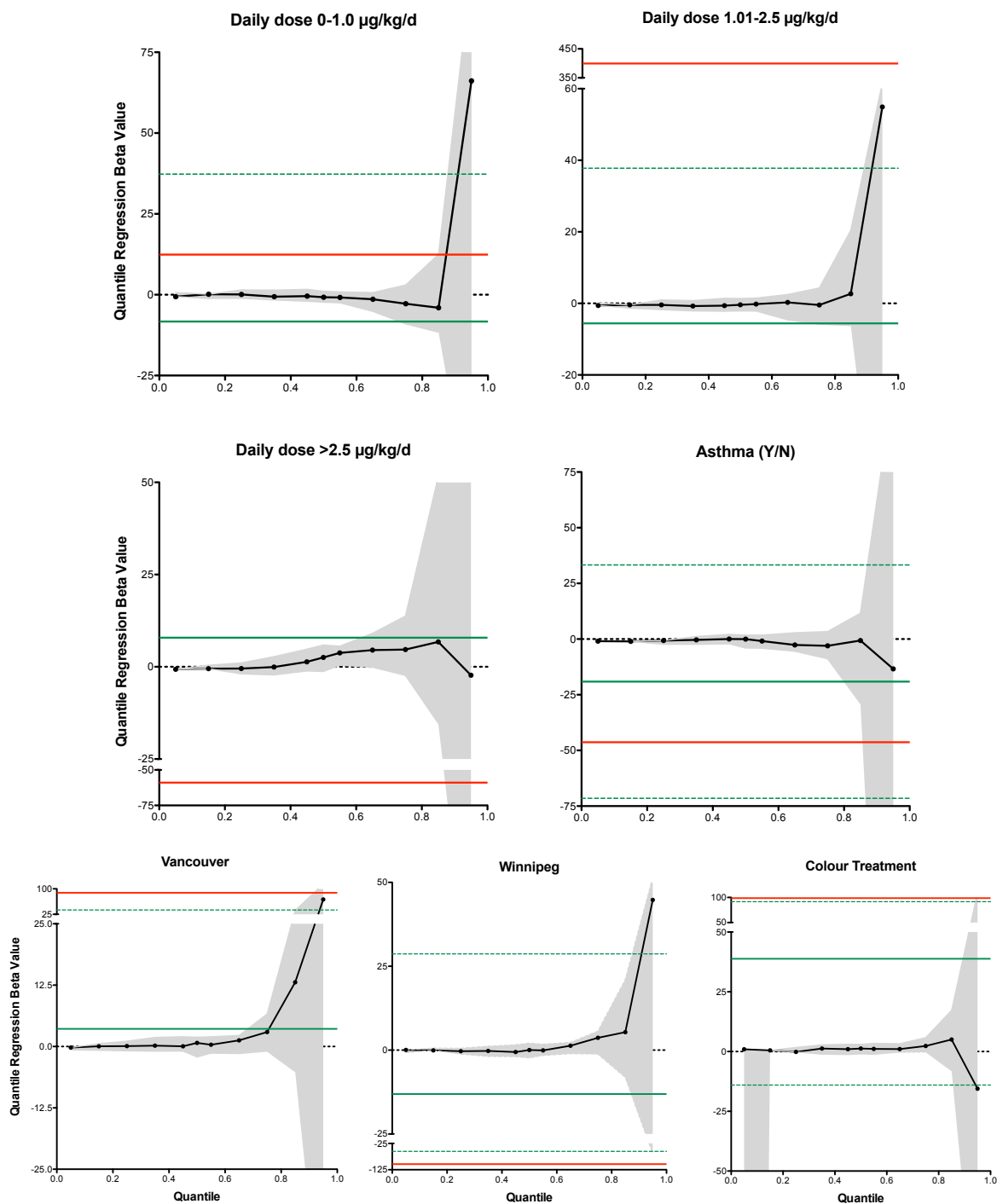


**Figure S1.** Graphs of the quantile regression for each variable included in Model 2 to evaluate ICS dose-dependent effects on hair cortisol concentrations in children. Figure continued from previous page. The unstandardized regression coefficient is plotted on the y-axis and the quantile (or percentile) 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 regression coefficient (beta value) and the grey shaded area indicates the 95% CI. The ordinary least squares (OLS) regression coefficients and 95% CI 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. The comparison among the regression analyses shows that the OLS regression of the original hair cortisol concentrations and rank transformed data do not perform as well as the quantile regression due to the influence of high hair cortisol concentrations as indicated by the significant difference of the beta value (red and green lines) from the quantile regression beta value. Additionally, the confidence intervals for the 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 by this method.

**Table S1.** 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 ICS type.

| Variable                            | Quantile (Percentile)    |                      |                      |                       |                            |                           | Rank OLS†               |
|-------------------------------------|--------------------------|----------------------|----------------------|-----------------------|----------------------------|---------------------------|-------------------------|
|                                     | 0.05                     | 0.25                 | 0.5                  | 0.75                  | 0.95                       | OLS                       |                         |
| Intercept                           | 5.8<br>(0.2, 7.6)        | 9.1<br>(-0.8, 13.1)  | 14.0<br>(5.2, 20.0)  | 13.2<br>(-14.6, 59.0) | -452.8<br>(-850.8, 391.9)  | 54.0<br>(-456.2, 564.3)   | 258.2<br>(219.9, 296.5) |
| Age, years, centred to 7.48 years   | -0.2<br>(-0.3, -0.1)     | -0.3<br>(-0.4, -0.2) | -0.5<br>(-0.7, -0.4) | -1.3<br>(-1.7, -0.9)  | -10.9<br>(-15.5, 5.0)      | -16.6<br>(-70.8, 37.6)    | -12.5<br>(-16.6, -8.4)  |
| Female                              | -0.6<br>(-0.7, 0.01)     | -1.2<br>(-1.6, -0.4) | -1.3<br>(-2.2, -0.3) | -4.1<br>(-6.0, 2.3)   | 8.3<br>(-124.2, 140.3)     | -33.6<br>(-353.8, 286.6)  | -31.5<br>(-55.5, -7.5)  |
| BMI, kg/m², centred to 15.5         | 0.1<br>(0.07, 0.14)      | 0.2<br>(0.1, 0.2)    | 0.1<br>(0.01, 0.3)   | 0.1<br>(-0.2, 0.4)    | -3.6<br>(-5.4, 0.5)        | -4.5<br>(-47.4, 38.4)     | 3.4<br>(0.2, 6.6)       |
| Lifetime duration of ICS use, years | 0.04<br>(-0.05, 0.07)    | 0.00<br>(-0.1, 0.1)  | 0.1<br>(-0.1, 0.2)   | 0.1<br>(-0.4, 0.5)    | -2.6<br>(-20.3, 2.6)       | -12.4<br>(-75.5, 50.7)    | 1.1<br>(-3.7, 5.8)      |
| Intranasal corticosteroid use       | 0.1<br>(-0.3, 0.4)       | -0.9<br>(-1.6, -0.4) | -1.9<br>(-3.1, -0.9) | -3.2<br>(-7.9, 0.1)   | 8.3<br>(-113.9, 83.7)      | 192.6<br>(-176.3, 561.4)  | -40.8<br>(-68.4, -13.1) |
| Living in Vancouver                 | -0.4<br>(-0.6, -0.03)    | 0.03<br>(-0.6, 0.8)  | -0.5<br>(-2.1, 1.6)  | 3.1<br>(-2.8, 11.6)   | 204.1<br>(-23.7, 375.7)    | 65.2<br>(-402.2, 532.7)   | -3.6<br>(-38.7, 31.5)   |
| Living in Winnipeg                  | -0.2<br>(-0.8, 0.2)      | -0.3<br>(-1.2, 0.5)  | -0.9<br>(-3.0, 1.3)  | 0.4<br>(-5.6, 3.6)    | -47.1<br>(-96.9, 89.1)     | -24.3<br>(-596.5, 547.8)  | -24.4<br>(-67.3, 18.6)  |
| Chemically treated hair             | 1.0<br>(-infinity, 1.3)  | 0.4<br>(-0.4, 1.6)   | 1.6<br>(0.01, 3.8)   | 4.7<br>(1.5, 9.0)     | 15.7<br>(-57.4, infinity)  | 58.1<br>(-648.3, 764.4)   | 42.2<br>(-10.8, 95.2)   |
| Has asthma                          | -0.9<br>(-1.8, 0.1)      | -0.6<br>(-2.0, 0.5)  | -0.02<br>(-3.3, 1.7) | -2.2<br>(-6.9, 5.6)   | -40.8<br>(-507.9, 89.8)    | -44.6<br>(-703.1, 613.9)  | -17.2<br>(-66.5, 32.2)  |
| Fluticasone use                     | -0.6<br>(-0.9, 0.2)      | -0.9<br>(-1.5, 0.6)  | -1.1<br>(-2.2, 1.3)  | 2.1<br>(-6.4, 7.9)    | 60.3<br>(-25.7, 402.8)     | 123.3<br>(-364.0, 610.6)  | -4.3<br>(-40.9, 32.2)   |
| Beclomethasone use                  | -0.8<br>(-infinity, 0.9) | -0.2<br>(-1.0, 1.6)  | -0.6<br>(-2.6, 1.7)  | 5.8<br>(-7.1, 106.7)  | 318.7<br>(-90.7, infinity) | 70.3<br>(-711.8, 852.4)   | 5.0<br>(-53.7, 63.6)    |
| Budesonide use                      | -0.4<br>(-0.9, 0.9)      | 0.4<br>(-0.6, 2.5)   | 0.9<br>(-0.1, 2.2)   | 2.0<br>(-5.0, 8.1)    | 101.5<br>(-18.2, 293.9)    | -156.1<br>(-661.5, 973.6) | 24.3<br>(-37.1, 85.6)   |
| Mometasone use                      | 0.7<br>(-infinity, 1.9)  | 0.6<br>(-0.3, 2.5)   | -0.4<br>(-1.7, 2.2)  | 0.5<br>(-7.9, 74.4)   | -4.2<br>(-45.9, infinity)  | 7.2<br>(-1005, 1019)      | 11.5<br>(-64.3, 87.5)   |
| Ciclesonide use                     | -0.5<br>(-0.9, 0.1)      | -0.2<br>(-1.7, 1.3)  | -0.2<br>(-2.5, 1.6)  | -1.1<br>(-16.2, 9.0)  | 52.4<br>(-149.5, 562.1)    | 311.7<br>(-189.2, 812.6)  | -2.7<br>(-40.3, 34.9)   |
| Multiple ICS use                    | 0.2<br>(-0.1, 0.9)       | -0.7<br>(-1.4, 1.1)  | -1.0<br>(-2.2, 2.0)  | -2.6<br>(-14.5, 2.8)  | -60.2<br>(-241.0, 111.1)   | -26.6<br>(-205.7, 152.5)  | 5.2<br>(-8.2, 18.7)     |

OLS = ordinary least squares \*All regression coefficients are unstandardized and reported with the lower and upper limits in parentheses. †Rank OLS regression 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. The 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, are likely due to underrepresentation of that variable in those percentiles.

**Table S2.** 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 ICS dose.

| Variable                                 | Quantile (Percentile)        |                             |                             |                             |                               |                           | Rank OLS†                      |
|------------------------------------------|------------------------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------|---------------------------|--------------------------------|
|                                          | 0.05                         | 0.25                        | 0.5                         | 0.75                        | 0.95                          | OLS                       |                                |
| Intercept                                | <b>6.0</b><br>(4.0, 7.4)     | <b>9.4</b><br>(6.4, 11.9)   | <b>6.9</b><br>(0.8, 17.7)   | 17.7<br>(-4.6, 34.9)        | -17.9<br>(-437.6, 326.9)      | 22.0<br>(-486.5, 530.6)   | <b>257.0</b><br>(218.7, 295.3) |
| Age, years, centred to 7.48 years        | -0.2<br>(-0.3, -0.1)         | -0.2<br>(-0.4, -0.1)        | -0.4<br>(-0.6, -0.2)        | -1.2<br>(-1.7, -0.9)        | <b>-19.8</b><br>(-28.8, -3.2) | -25.8<br>(-80.4, 28.9)    | <b>-11.5</b><br>(-15.6, -7.3)  |
| Female                                   | -0.5<br>(-0.8, -0.01)        | -0.9<br>(-1.4, -0.3)        | -1.1<br>(-2.6, 0.5)         | -2.4<br>(-5.5, 0.7)         | 53.7<br>(-56.5, 188.0)        | -4.7<br>(-323.6, 314.2)   | <b>-31.2</b><br>(-55.2, -7.2)  |
| BMI, kg/m <sup>2</sup> , centred to 15.5 | <b>0.1</b><br>(0.05, 0.13)   | <b>0.1</b><br>(0.07, 0.2)   | <b>0.2</b><br>(0.04, 0.3)   | <b>0.2</b><br>(0.01, 0.4)   | -3.4<br>(-9.9, 1.8)           | -1.9<br>(-44.6, 40.8)     | <b>3.6</b><br>(0.3, 6.8)       |
| Lifetime duration of ICS use, years      | 0.04<br>(-0.04, 0.08)        | 0.00<br>(-0.06, 0.1)        | -0.01<br>(-0.2, 0.2)        | 0.2<br>(-0.1, 0.5)          | -4.0<br>(-17.4, 12.0)         | -8.0<br>(-69.7, 53.7)     | 1.2<br>(-3.4, 5.9)             |
| Intranasal corticosteroid use            | 0.1<br>(-0.5, 0.2)           | <b>-1.2</b><br>(-1.7, -0.4) | <b>-1.7</b><br>(-3.1, -0.8) | <b>-5.1</b><br>(-6.3, -0.6) | -18.0<br>(-108.6, 116.7)      | 255.7<br>(-114.6, 626.1)  | <b>-42.3</b><br>(-70.2, -14.3) |
| Living in Vancouver                      | -0.2<br>(-0.7, 0.1)          | 0.1<br>(-0.9, 1.1)          | 0.7<br>(-2.2, 1.9)          | 2.9<br>(-0.9, 6.6)          | 68.6<br>(-6.0, 293.6)         | 88.2<br>(-367.9, 544.3)   | 3.6<br>(-30.8, 37.9)           |
| Living in Winnipeg                       | 0.07<br>(-0.8, 0.6)          | -0.3<br>(-1.2, 0.6)         | 0.03<br>(-2.3, 2.1)         | 3.7<br>(-1.2, 5.7)          | 44.8<br>(-63.6, 158.0)        | -103.7<br>(-658.7, 451.3) | -13.1<br>(-54.9, 28.7)         |
| Chemically treated hair                  | 1.0<br>(-infinity, 1.3)      | -0.2<br>(-0.8, 1.7)         | 1.2<br>(-1.0, 3.0)          | 2.3<br>(-0.03, 5.8)         | -15.5<br>(-121.2, 179.8)      | 98.6<br>(-601.4, 798.6)   | 38.8<br>(-14.0, 91.5)          |
| Has asthma                               | -1.0<br>(-1.6, 0.2)          | -0.7<br>(-2.3, 0.5)         | -0.02<br>(-4.0, 1.8)        | -3.0<br>(-8.8, 3.4)         | -13.4<br>(-313.7, 126.6)      | -46.3<br>(-741.8, 649.2)  | -19.1<br>(-71.5, 33.3)         |
| Average daily dose >0-1.0 µg/kg/d        | -0.6<br>(-1.0, 0.6)          | 0.1<br>(-1.1, 1.5)          | -0.8<br>(-2.3, 1.1)         | -2.8<br>(-9.0, 3.0)         | 66.1<br>(-68.7, 249.6)        | 12.4<br>(-592.4, 617.2)   | -8.3<br>(-53.8, 37.3)          |
| Average daily dose 1.01-2.5 µg/kg/d      | -0.6<br>(-1.3, 0.1)          | -0.4<br>(-1.7, 0.9)         | -0.4<br>(-2.1, 1.4)         | -0.4<br>(-5.8, 4.3)         | 54.9<br>(-78.4, 221.8)        | 399.6<br>(-177.2, 976.5)  | -5.6<br>(-49.1, 37.8)          |
| Average daily dose >2.5 µg/kg/d          | <b>-0.7</b><br>(-1.0, -0.04) | -0.5<br>(-1.9, 1.1)         | 2.5<br>(-1.3, 5.9)          | 4.7<br>(-2.3, 13.7)         | -2.3<br>(-215.7, 152.3)       | -58.9<br>(-674.2, 556.3)  | 7.9<br>(-38.4, 54.3)           |

OLS = ordinary least squares \*All regression coefficients are unstandardized and reported with the lower and upper limits in parentheses. †Rank OLS regression 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. The confidence limit for the 5<sup>th</sup> percentile for chemical treatment that includes infinity is likely due to underrepresentation of that variable in that percentile.

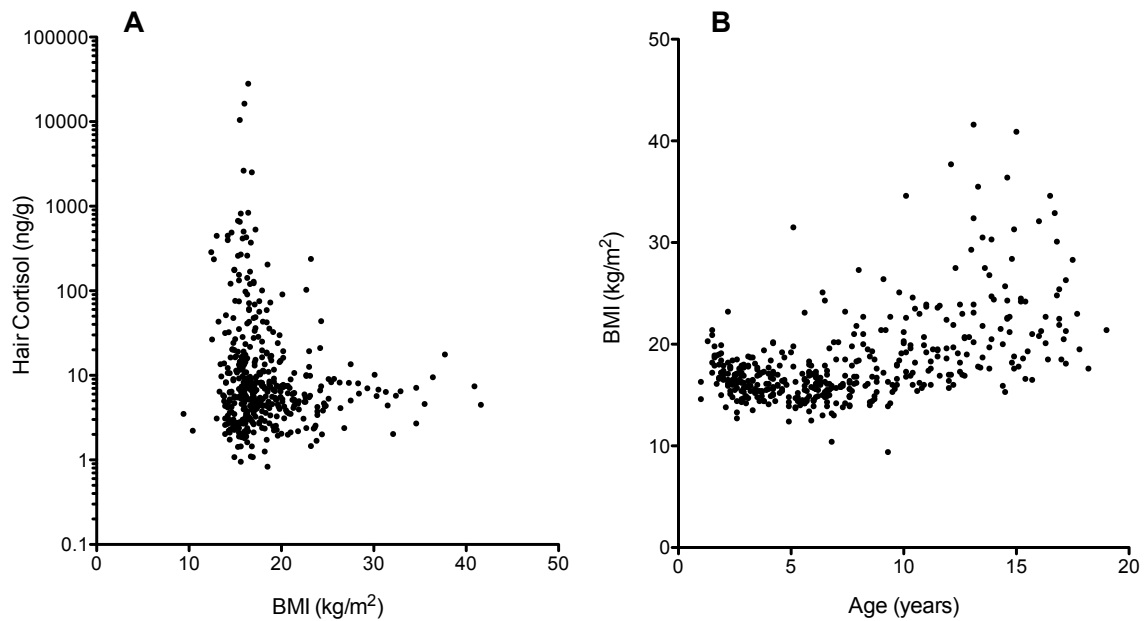


**Figure S2.** Scatterplots of BMI versus hair cortisol concentration and age for the cohort of children. A) Hair cortisol concentration on the y-axis with a logarithmic scale versus BMI shows a complex relationship of hair cortisol concentration with BMI. On one hand, hair cortisol increases greatly within a narrow change in BMI (*e.g.* BMI  $\approx$  15-17 kg/m<sup>2</sup>), but on the other hand, hair cortisol also increases slightly as BMI increases. B) BMI versus age showing there is generally an increase in BMI with age except in very young children.

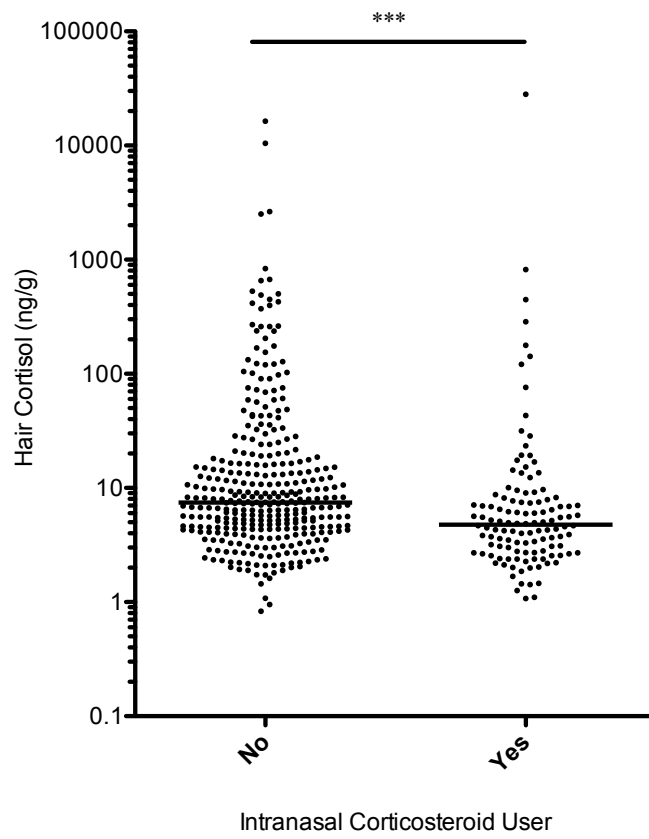


**Figure S3.** Comparison of median hair cortisol concentration between intranasal corticosteroid users and non-users for the pediatric cohort. The hair cortisol concentration, shown on the y-axis with a logarithmic scale, was significantly lower for the children who use intranasal corticosteroids ( $U=14348$ ,  $P<0.0001$ ).

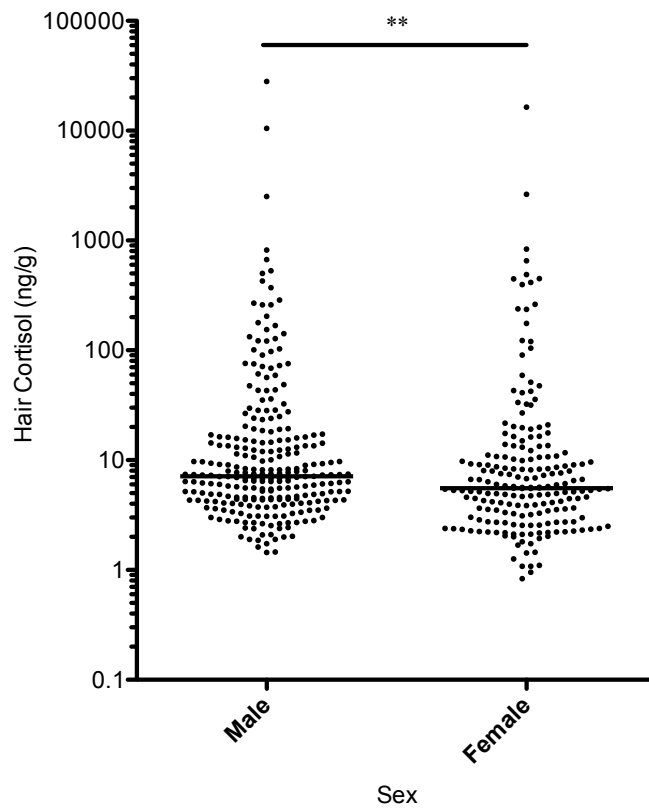


**Figure S4.** Comparison of median hair cortisol concentrations between the sexes in the pediatric cohort. Hair cortisol is shown on the y-axis with a logarithmic scale. Females had a significantly lower hair cortisol concentration than males ( $U=21716$ ,  $P=0.003$ ).

### Supplemental References

1. Rethemeyer K. PAD 705 Handout: Standardized coefficients [Rockefeller College University at Albany website]. January 29, 2004. Available at: [www.albany.edu/faculty/krethema/PAD705/SupportMat/StandardizedCeof.pdf](http://www.albany.edu/faculty/krethema/PAD705/SupportMat/StandardizedCeof.pdf). Accessed April 20, 2016.
2. Williams R. Standardized coefficients [University of Notre Dame website]. 2004. Available at: [www3.nd.edu/~rwilliam/stats1/x92.pdf](http://www3.nd.edu/~rwilliam/stats1/x92.pdf). Accessed April 20, 2016.
3. Williams R. Standardized Coefficients in Logistic Regression [University of Notre Dame website]. February 19, 2016. Available at: [www3.nd.edu/~rwilliam/stats3/L04.pdf](http://www3.nd.edu/~rwilliam/stats3/L04.pdf). Accessed April 20, 2016.