Differential susceptibility to parenting: Exploring new approaches

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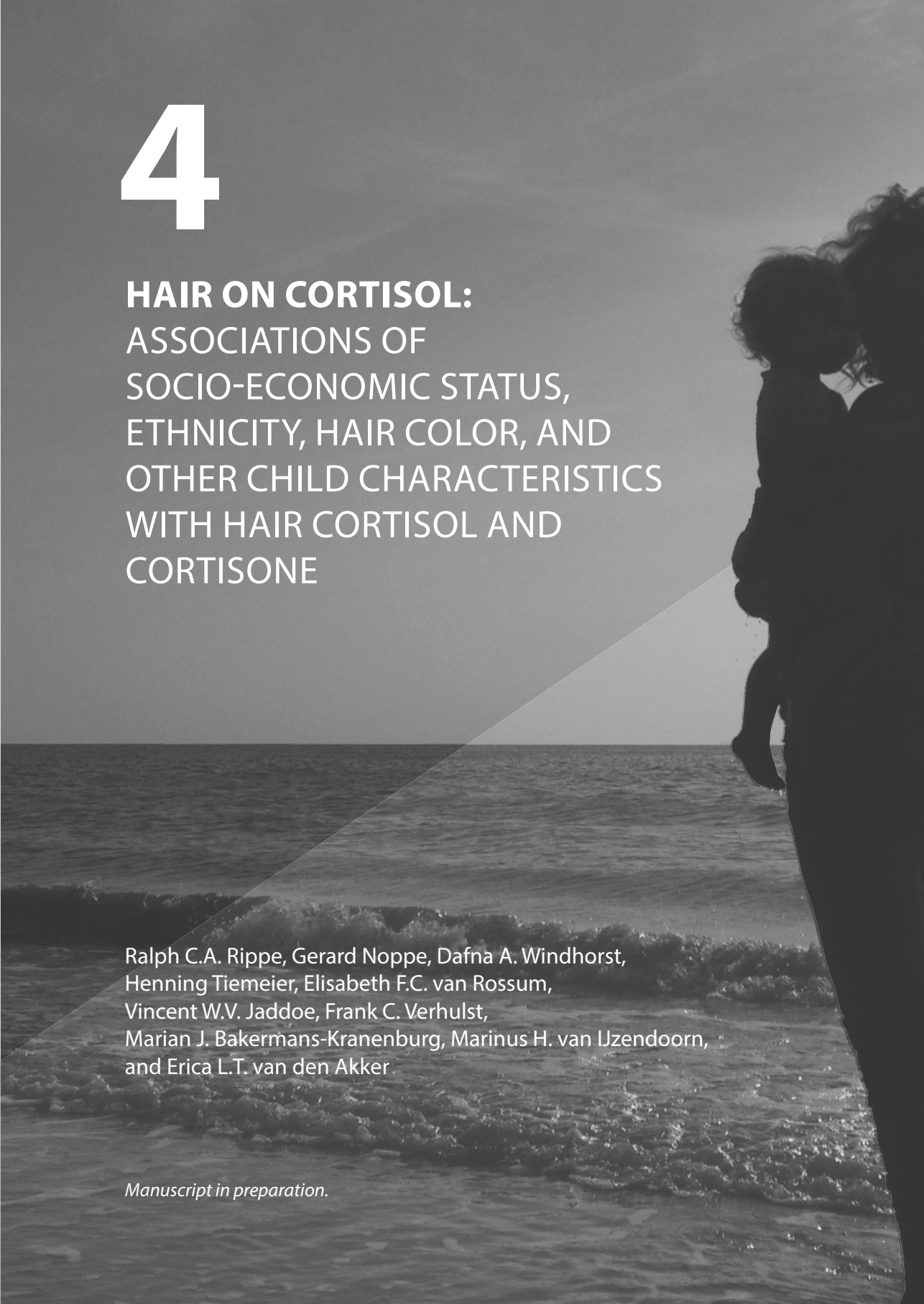
HAIR ON CORTISOL: ASSOCIATIONS OF SOCIO-ECONOMIC STATUS, ETHNICITY, HAIR COLOR, AND OTHER CHILD CHARACTERISTICS WITH HAIR CORTISOL AND CORTISONE



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ABSTRACT

The aim of this study was to examine the association of socio-economic status (SES) and ethnicity with hair cortisol and cortisone and to identify potential child and family characteristics that can assist in choosing confounders to take into account in analyses involving hair cortisol and hair cortisone concentrations.

Hair samples were collected in 2,484 6-year-old children from the Generation R Study, a prospective cohort study in Rotterdam, the Netherlands. Measurements for cortisol and cortisone were used as the outcome in regression analyses. Predictors were SES, ethnicity, hair color and child characteristics such as birthweight, gestational age at birth, BMI, disease, allergy, and medication use.

Lower family income and a larger number of children to be supported by this income were associated with higher hair cortisol and cortisone levels. Furthermore darker hair color and ethnicity (Dutch and more general North European descent) were related to lower levels. Increased amount of sun (in hours) in the month of hair collection was related to lower levels of cortisone only. Boys were found to show higher levels of cortisol and cortisone. Higher BMI was related to higher levels. More recent washing was related to lower levels.

The background characteristics gestational age at birth, birth weight, age, medication use, hair washing frequency, educational level of the mother, marital status of the parents, disease and allergy were not associated with cortisol or cortisone levels.

Our results serve as a starting point for the choice of confounders in studies of substantive predictors or outcomes. Gender, BMI, income, the number of persons in a household, ethnicity, hair color and information on last time of hair washing are strongly suggested to take into account.

Keywords: hair, cortisol, cortisone, generation R, confounder, SES, ethnicity

INTRODUCTION

Hair cortisol is increasingly used as a biological marker of chronic stress and is regarded a promising and nonintrusive method of detecting individual differences in long-term reactions to stressful life experiences. Scalp hair is easy to collect, cheap to store and it is straightforward to obtain cortisol and cortisone concentrations. In the current study we examined the influence of socio-economic status (SES) and ethnicity on hair cortisol levels in 2,484 6-year-old children, and we tested hair characteristics as potential confounders. We expect that key socio-demographic risk indicators such as lower SES and ethnic minority status are associated with higher hair cortisol levels, even after taking into account potential confounders including hair color, sun exposure and hair treatment.

Cortisol is an important biomarker in studies on stressful life experiences. Cortisol is a steroid hormone (glucocorticoid) that shows increased secretion on top of basal secretion in conditions of physical, biological or psychosocial stress. This hormone is important in the suppression the immune system and several anti-inflammatory responses, and it has essential regulatory functions in the metabolism of fat, protein, and carbohydrate. In addition, cortisol plays also a pivotal role in the brain with respect to cognition and mental health (De Kloet, Joëls, & Holsboer, 2005). The formation of glucocorticoids in the adrenal glands is part of the Hypothalamic-Pituitary-Adrenal (HPA) axis, which plays a major role in the general stress response (De Kloet et al., 2005). Cortisone is metabolized from cortisol in the peripheral tissues by the enzyme 11-beta-steroid dehydrogenase type 2. Cortisol has much greater glucocorticoid activity than cortisone, thus, cortisone can be considered an inactive metabolite of cortisol. Reverse metabolism from cortisone to cortisol also occurs continuously by 11-beta-steroid dehydrogenase type 1, but due to lower activity this process is slower. Cortisol levels in the human body follow a diurnal pattern. In general levels peak in the early morning and reach the lowest level three to five hours after sleep onset (Gunnar & Donzella, 2002; Gunnar & Vazquez, 2001; Lupien, McEwen, Gunnar, & Heim, 2009). In response to stressors (either psychological or physical), cortisol levels become elevated (Miller, Chen, & Zhou, 2007).

Thus cortisol is a biomarker of stress and therefore often used in studies in the field of psychiatry, psychology and behaviour. Cortisol levels seem to be gender-specific (Kirschbaum, Klauer, Filipp, & Hellhammer, 1995; Kirschbaum, Wüst, & Hellhammer, 1992), with males having higher cortisol levels due to daily stressors. This holds for non-human primates as well (Laudenslager, Jorgensen & Fairbanks, 2012). Recently, we also showed in adult humans that hair cortisol levels are also significantly higher in men than in women (Staufenbiel, Penninx, De Rijke, Van den Akker, & Van Rossum, 2015). Mårin et al. (1992) showed a relation between elevated cortisol levels and increased body fat as measured by the Body Mass Index (BMI). Several other factors have been shown to influence cortisol levels: biological factors, psychosocial factors (including maternal depression, childhood adversity), and demographic factors such as SES and ethnicity. Similar patterns have been observed in children as well (Noppe et al., 2014, Vanaelst et al., 2012). Hair cortisol and cortisone are increasingly used as a biological marker of chronic stress (Staufenbiel, Penninx, Spijker, Elzinga, & Van Rossum, 2013; Vanaelst et al., 2013, 2014), and, besides clinical metabolic information, it is regarded a promising and suitable method of detecting individual differences in children's long-term reactions to stressful life experiences (Groeneveld et al., 2013).

Among the biological factors, higher cortisol levels have been related to sleep deprivation (Leproult, Copinschi, Buxton, & Van Cauter, 1997) and augmented caffeine use (Lovallo, Farag, Vincent, Thomas & Wilson, 2006). Exercise (both aerobic and anaerobic) has been found to be related to increased levels of cortisol (Kindermann et al., 1982; Skoluda, Dettenborn, Stalder, & Kirschbaum, 2012). A psychosocial factor such as novelty seeking behavior may also be indicated by hair cortisol levels (Laudenslager, Jorgensen, Grzywa, & Fairbanks, 2011). Futhermore, long term effects of (mild) adversities related to SES on cortisol secretion have been observed as well (e.g., Bosch et al., 2012; Halldórsson, Kunst, Köhler & Mackenbach, 2000; Jansen et al., 2009; Lupien et al., 2009; Van Hooijdonk, Droomers, Deerenberg, Mackenbach, & Kunst, 2008). Ethnic minority status may be often related to lower SES and thus to higher cortisol levels but may also imply difficulties to capture commensurable corticosteroid concentration due to differences in pigmentation.

Cortisol extraction from saliva or blood (serum) reflect only cortisol levels at the time of sampling, potentially influenced by daily fluctuations. These fluctuations can be addressed by repeated sampling, but are rather invasive, especially in young participants (<6 years old). Urinary measurements of cortisol are usually collections representing cortisol secretion of the past 24h-48h. Storage of such samples is subject to very specific (and expensive) demands. In contrast, concentrations of cortisol and cortisone extracted from hair samples may reflect accumulated concentrations of cortisol levels up to several months, thus representing a summation of both basal levels and stress-related responses (Manenschijn, Koper, Lamberts, & Van Rossum, 2011; Manenschijn et al., 2012; Noppe et al., 2014).

As discussed above, in small samples it might be difficult to differentiate associations of ethnic variability with cortisol from associations of SES (DeSantis et al., 2007; Lupien, King, Meaney, & McEwen, 2000, 2001; Lupien et al., 2009; Vaghri et al., 2013). Several of the factors discussed above were assessed by Dettenborn, Tietze, Kirschbaum, and Stalder, (2012) in relation to hair cortisol in a relatively small sample (n = 360) aging between 1 and 91 years, using an earlier method of cortisol extraction. Family characteristics were not taken into account. The aim of this paper is to explore the influence of socio-economic and other demographic variables on hair cortisol levels in a large, multi-ethnic sample of homogeneous age. Furthermore, the influence of various characteristics of hair such as hair color, use of hair products, hair washing, and amount of sun light (in hours) in the month of visit on hair cortisol levels will be examined, in order to present a set of potential confounders to be taken into account in substantive studies on hair cortisol and cortisone. In the current study we examined the influence of SES and other family characteristics, as well as individual characteristics and ethnicity on hair cortisol levels in 2,484 6-year-old children, and we tested hair characteristics as potential confounders. Our hypothesis is that lower SES and ethnic minority status are associated with higher hair cortisol levels, even after taking into account potential confounders including hair color, sun exposure and hair treatment.

METHODS

Study sample

The current investigation was embedded in the Generation R Study, a prospective cohort investigating development from fetal life into young adulthood in Rotterdam, the Netherlands (Jaddoe et al., 2012; Kruithof et al., 2014; Tiemeier et al., 2012). Written informed consent was obtained from parents of all participants. The study has been approved by the Medical Ethical Committee of the Erasmus MC, Rotterdam.

The selected sample (n = 2,484) was a mixed-ethnicity subsample of the full population-based cohort, for which measures of hair cortisol and cortisone were available. A comparison of characteristics in the full cohort and the selected subsample, and for males and females within the selected sample, is shown in Table 1.

In total 6,690 children visited the Generation R research center at age 6. Hair samples for cortisol assessment were collected during this lab visit. However, hair collection did not start immediately at onset of this research wave. In the current wave 3,570 were asked to participate in hair sample collection, and 3,034 children (85%) responded positively. Cortisol measurement was successful in 98.4%, cortisone measurement was successful in 96.7%. For 2,897 (95.5%), both cortisol and cortisone concentrations could be quantified. Participants with oral use of corticosteroid drugs and participants for whom this information was absent (n = 365 in total) were excluded due to the risk that endogenous cortisol production might be (partly) suppressed. Moreover, 48 twins were excluded, because only individuals with a low birth weight due to other factors than twin-ship yield accurate measurements without over- or under-correction through covariates (Poulter, Chang, MacGregor, Snieder, & Spector, 1999). Siblings (n = 28) were not excluded for optimal power. Sample size for the current study was thus 2,484 participants.

Hair cortisol and cortisone extraction

Hair samples of approximately 100 strands were cut from the posterior vertex using small surgical scissors, as close to the scalp as possible. Hair locks were then taped to a piece of paper with the scalp end marked, and stored in an envelope at room temperature until further analyses. Parents were requested to fill out a questionnaire for their child on hair washing frequency, time since last wash, hair product use, and use of glucocorticoids. Cortisol and cortisone were measured as described previously, with the exception that hair samples were minced by hand using small surgical scissors, instead of using 1cm segments (Noppe, De Rijke, Dorst, Van den Akker, & Van Rossum, 2015).

Briefly, the proximal 3 cm of hair samples were weighed using an electrical scale and minced. Hair samples were then washed in LC-grade isopropanol for 2 minutes at room temperature, and left to dry for at least 2 days. Deuterium labeled cortisol and cortisone were added prior to extraction. Extraction was performed using LC-grade methanol (MeOH), for 18 hours at 25°C, in a gently shaking water basin. The extract was then transferred to a glass tube, centrifuged at 4300G, and evaporated to dryness at 37°C under a constant flow of N_2 (Noppe et al., 2015).

After reconstitution in 1mL 2% LC-grade MeOH, the extract was loaded on an off-line solid phase extraction plate (HLB Oasis 96-well SPE plate, Waters Chromatography), washed with 1mL 30% LC-grade MeOH, and eluted twice in 300µL 100% LC-grade MeOH. The extract was then evaporated to dryness at 50°C under a constant flow of N_2 and stored at 4°C until further analysis. Prior to analysis, the samples were reconstituted in 100µL eluens, vortexed, and analysed using liquid chromatography tandem mass spectrometry (LC-MS/MS) (Xevo TQS, Waters Chromatography). The obtained concentration measurements were highly skewed, hence the values were log-transformed before use in the analyses, analogous to Gerritsen et al. (2010) and Tordjman et al. (1997).

Predictors

Child characteristics. Age, BMI, data on the use of hair products, hair washing frequency, last time hair was washed, last time a hair product was used and oral medication use were collected at the time of hair sampling. Gender, gestational age at birth, birth weight, parity and ethnicity were collected at baseline. Ethnicity was self-reported by parents at study inclusion, and the following ethnicities were represented in our sample: Dutch, Indonesian, Cape Verdian, Moroccan, Dutch Antilles, Surinamese, Turkish, African, American (western and non-western), Asian (western and non-western), European (general) and Oceania. The full cohort and the study sample differed in the proportion of individuals of Dutch national origin (11% more in the study sample), Dutch Antilles, Surinamese-Creole and Surinamese-unspecified, North-African and sub-Saharan (all fewer in study sample). The latter differences in percentages equal 3% at most, with low absolute prevalences of these ethnicities in both the cohort and the study sample. Due to low prevalences of some ethnicities, ethnicity of the child was classified into the categories 'Dutch', 'Western', and 'Non-Western'.

In order to correct for seasonal effects in human cortisol and cortisone levels (Maes et al., 1997; Persson et al., 2008) and potential seasonal hair growth (Randall & Ebling, 1991), the number of sun hours (obtained from the KNMI, using www.zonurencalculator.nl) in the month of hair sampling were collected and used as a covariate in the analyses. Prevalence of disease and allergy were constructed analogous to the Framingham Heart Study (Preis et al., 2009), from questionnaire scores that were completed at the visit. These scores were highly skewed. Therefore, they were normalized through square root transformation and standardized before analysis.

Hair color was partially coded through parent report and was completed by two raters using front desk photographs. A four category coding was used (red, blond, brown, black). For reference, a set of 50 subjects who were not included in the analysis sample were coded as well, for comparison to parent-reported hair color. To assess multi-rater agreement, the Krippendorff Alpha (Hayes & Krippendorff, 2007) was used to check conformity across all comparisons, and to avoid different statistics for two- and three-rater comparisons. Comparisons were made between the two independent coders and between rating of the coders and hair color as reported by the parents. Inter-coder reliability was high for the two coders (0.79) and somewhat lower with parent report (0.69).

Family characteristics. For socio-economic status, the following variables were available: marital status of the mother (in a relationship, not in relationship), educational level of the mother and family income. Educational level was coded on a 6-point rating scale ranging from 1 (no education finished) to 6 (higher phase 2: higher academic education, PhD). Income was defined as the total net month income in Euro of the household on an 11-point scale, ranging from 1 (<800) to 11 (>5600). Marital status was dichotomized into 'married, registered partnership or living together' versus 'no partner'. Characteristics of the children and families from the full cohort and the selected study sample are listed in Table 1.

Statistical Analyses

Using linear regression models, univariate and multivariate coefficients were estimated for all cases with hair cortisol data, and missing data were imputed, outliers winsorized (n = 2,484). Sensitivity analyses were done with complete cases only without outliers, and for complete cases only with outliers winsorized (n = 1,070).

Missing variables (gestational age at birth (0.8%), gestational age at intake (10.7%), birthweight at intake (0.3%), parity (3.9%), hair color (0.8%), sun hours (25%), disease (5.7%) and allergy (4.2%) were imputed by multivariate imputation. Chained equations were used in the mice package in R, applying imputation models for each individual variable, with 25 random sets and a maximum of 25 iterations per imputation loop. Values lying outside the 3*IQR range were winsorized (Cooper, Gulen, and Schill (2008, p. 1632). This was applied to gestational age at intake, age, weight, BMI, hair cortisol (log10) and hair cortisone (log10). Collinearity checks were performed in the regression analyses and yielded no indication of problematic associations among predictors. The analyses were performed in R (3.1.2), using packages: foreign, stats, Hmisc, irr and mice.

RESULTS

Associations with cortisol

Univariate associations with cortisol levels were found for gender, age, birthweight, BMI, marital status, educational level of the mother, family income, ethnicity, hair product use, hair color and disease (Table 2, left column). Boys had higher cortisol levels, higher age was related to higher cortisol levels, higher weight was related to lower cortisol levels, but higher BMI was associated with higher cortisol levels. Lower family income, lower maternal educational level and being single were associated with higher cortisol levels. Darker hair color, the use of hair products and having more disease symptoms were related to higher cortisol levels. Being of Dutch national origin was related to lower cortisol levels.

Table 1 | Cohort and study sample characteristics

Characteristic	Generation R	Selected subsample	Sign.	Females	Males	Sign.
Sample size	9901	2484		1285	1199	
Gender (% females)	48.6	51.7	**			
Age (years (sd)	6.2 (0.5)	6.2 (0.7)	-	6.2 (0.6)	6.2 (0.7)	-
Gestational age at birth (w (sd))	39.7 (2.0)	39.9 (1.7)	***	39.9 (1.7)	39.9 (1.8)	-
Birthweight (g (sd))	3385.3 (586.7)	3434.5 (548.4)	***	3369.2 (522.0)	3504.6 (567.4)	***
BMI (kg/m^2 (sd))	16.2 (1.9)	16.2 (1.9)	-	16.3 (2.1)	16.2 (1.8)	-
Parity (avg sibs (sd))	0.7 (0.9)	0.6 (0.8)	-	0.6 (0.8)	0.7 (0.9)	-
Ethnicity (% Dutch-Caucasian)	61.7	64.3	*	63.3	65.3	-
Marital status parent (% married)	85.9	87.2	***	86.6	87.8	-
Education level mother (median)	3.7	3.7	-	3.7	3.7	-
Income (median)	7.1	7.2	-	7.1	7.2	-
Number of children in household (m, (sd))	2.2 (0.8)	2.2 (0.8)	-	2.2 (0.8)	2.2 (0.7)	-
Hair washing frequency (%)						
"<1 a week"	18.4	18.6	-	18.3	18.8	-
"1-2 times a week"	48.7	48.1	-	53.2	42.7	***
"3-4 times a week"	25	25.3	-	22.8	27.9	***
">4 times a week "	7.9	8.0	-	5.6	10.6	***
When last hair washed (%)						
"<24 hours ago"	35.1	35.8	-	33.4	38.2	*
"24-48 hours ago"	29.3	28.7	-	29.8	27.3	-
">48 hours ago"	35.6	35.5	-	36.7	34.1	-
Hair product used (%)						
"No"	71.5	72.2	-	76.9	66.7	***
"Yes"	28.5	27.8	-	22.6	33.0	***
Hair color (%)						
"Red"	3.0	3.2	***	1.1	1.0	-
"Blond"	34.9	36.3	***	11.8	12.1	-
"Brown"	48.9	48.2	***	15.9	15.7	-
"Black"	13.1	12.3	***	4.5	3.5	-
Sample size	9901	2484		1285	1199	
Medication use (% no)	91.6	95.0	***	95.6	94.3	**
Sun in month of sampling (h (sd))	152.7 (83.7)	156.4 (82.8)	-	157.4 (82.1)	155.4 (83.4)	-
Disease score (m (sd))	0.0 (1.0)	-0.03 (1.0)	-	0.03 (1.0)	-0.09 (0.9)	**
Allergy score (m (sd))	0.0 (1.0)	-0.08 (0.8)	***	-0.1 (0.7)	-0.01 (0.9)	***
Cortisol in pg/mg (log10 (sd))	5.21 (16.9)	4.49 (15.3)	-	4.4 (15.6)	4.58 (15.1)	-
Cortisone in pg/mg (log10 (sd))	10.2 (8.4)	10.4 (8.6)	-	9.9 (8.6)	10.9 (8.5)	**

* = significant at 0.05, ** = significant at 0.01, *** = significant at 0.001

Table 2 | Regression results for Cortisol (pg/mg, log10)

	Imputed,winsorized univariate, *n* = 2484			Imputed,winsorized (R^2 = 0.078), *n* = 2484			Complete, winsorized (R^2=0.066) , *n* = 1070			Complete, outliers removed (R^2=0.057), *n* = 949		
Variable	**Coefficient**	**SE**	**ES**	**Coefficient**	**SE**	**ES**	**Coefficient**	**SE**	**ES**	**Coefficient**	**SE**	**ES**
Gender	**-0.039***	**0.018**	**-0.087**	**-0.158****	**0.061**	**-0.127**	**-0.159***	**0.066**	**-0.195**	**-0.179****	**0.067**	**-0.231**
Age child	**0.050 ****	**0.001**	**0.148**	-0.001	0.002	-0.018	-0.001	0.003	-0.011	-0.005	0.006	-0.028
Gestational age at birth	-0.010	0.006	-0.073	-0.005	0.008	-0.082	-0.006	0.009	-0.048	0.003	0.010	-0.028
Birthweight	**0.000 ***	**0.000**	**-0.100**	0.000	0.000	0.012	0.000	0.000	0.003	0.000	0.000	-0.023
BMI	**0.025*****	**0.005**	**0.218**	**0.024*****	**0.006**	**0.171**	**0.022****	**0.008**	**0.078**	**0.027****	**0.009**	**0.088**
Parity	0.021	0.011	0.080	0.005	0.019	0.014	0.006	0.021	0.011	0.021	0.022	0.036
Ethnicity	**0.182*****	**0.019**	**0.396**	**0.065***	**0.030**	**0.122**	0.061	0.033	0.081	0.060	0.034	0.091
Marital status of mother	**0.143*****	**0.029**	**0.213**	0.065	0.030	0.144	0.034	0.053	0.013	0.043	0.055	0.024
Educational level of mother	**-0.040*****	**0.009**	**-0.202**	0.010	0.013	0.092	0.028	0.014	0.052	**0.032***	**0.015**	**0.064**
Family income	**-0.028*****	**0.003**	**-0.357**	**-0.014****	**0.006**	**-0.125**	**-0.016****	**0.006**	**-0.087**	**-0.015***	**0.006**	**-0.081**
No. of children in household	0.008	0.013	0.028	**0.036***	**0.014**	**0.030**	**0.039****	**0.015**	**0.076**	0.025	0.016	0.050
Hair washing frequency	0.005	0.011	0.019	-0.021	0.020	-0.055	-0.018	0.021	-0.050	-0.018	0.022	-0.038
Last hair wash	0.015	0.011	0.056	**0.064*****	**0.015**	**0.231**	**0.062*****	**0.016**	**0.116**	**0.046****	**0.017**	**0.104**
Use of hair product	**0.048***	**0.020**	**0.096**	0.015	0.033	0.025	0.031	0.036	0.031	0.031	0.038	0.047
Hair color	**0.136*****	**0.012**	**0.449**	**0.076*****	**0.019**	**0.206**	**0.090*****	**0.021**	**0.146**	**0.064****	**0.021**	**0.099**
Medication use	-0.014	0.131	-0.004	-0.112	0.154	-0.040	-0.220	0.193	-0.034	-0.202	0.206	-0.036
Sunhours	0.000	0.000	-0.069	0.000	0.000	0.015	0.000	0.000	0.005	0.000	0.000	0.040
Total disease	**0.022***	**0.010**	**0.095**	0.020	0.011	0.072	0.015	0.012	0.028	0.017	0.012	0.040
Total allergy	0.014	0.012	0.054	0.024	0.013	0.095	0.014	0.014	0.030	0.019	0.015	0.045
Gender x wash frequency				**0.052***	**0.026**	**0.120**	0.053	0.029	0.085	0.050	0.030	0.084
Gender x product use				-0.054	0.049	-0.063	-0.050	0.054	-0.714	-0.034	0.056	-0.469

* = significant at 0.05. ** = significant at 0.01. *** = significant at 0.001. ES = Cohen's d. Left column: univariate associations, all others columns multivariate associations, controlling for all other characteristics.

Controlling for all variables in the model simultaneously, consistent significant associations ($p < 0.05$) were found, in the same directions as described above, between cortisol and gender, BMI, family income, number of children to be supported from this income, and hair color. Dutch ethnicity was related to lower cortisol levels, and a similar association was present for a more broad classification: being of North European descent was related to lower cortisol levels. Recent hair washing (<24h) was associated with lower cortisol levels. We also found a significant interaction between gender and wash frequency (for females higher wash frequency was related to lower cortisol; for males this association was absent), see Table 2. The characteristics age, gestational age at birth, birthweight, educational level of the mother, marital status of the mother, hair washing frequency, medication use, the number of sun hours in the month of hair sampling, disease, and allergy were not associated with cortisol levels when the other covariates were included in the model. Sensitivity analyses on complete cases without outliers and with outliers winsorized did not show significantly different results.

Associations with cortisone

Univariate associations with cortisone levels were found for gender, BMI, educational level of the mother, family income, ethnicity, hair product use, and hair color (Table 3, left column). Boys had higher cortisone levels, higher BMI was related to higher cortisone levels, the mother being single, lower educational level of the mother and lower family income were also related to higher cortisone. Being of Dutch origin was related to lower cortisone levels. The use of hair products and darker hair color were associated with higher cortisone levels.

Controlling for all variables in the model, consistent significant associations, in the same directions as described above, were found between cortisone and gender, BMI, family income, number of children to be supported from this income, last time hair wash, hair color and sun hours (less sun hours related to higher cortisone). Dutch ethnicity and being of North European descent was related to lower cortisone levels. The interaction between gender and wash frequency showed that for females higher wash frequency was related to lower cortisol and for males this association is absent, see Table 3. The number of sun hours was not associated with cortisone levels in the imputed data. Characteristics such as age, gestational age at birth, birthweight, marital status of the mother, educational level of the mother, hair washing frequency, medication use, disease, and allergy were not associated with cortisone levels. Sensitivity analyses on complete cases without outliers and with outliers winsorized did not show significantly different results.

Table 3 | Regression results for Cortisone (pg/mg, log10)

	Imputed, winsorized univariate, n = 2484			Imputed, winsorized (R^2=0.045), n = 2484			Complete, winsorized (R^2=0.053), n = 1070			Complete, outliers removed (R^2=0.058), n = 949		
Variable	**Coefficient**	**SE**	**ES**	**Coefficient**	**SE**	**ES**	**Coefficient**	**SE**	**ES**	**Coefficient**	**SE**	**ES**
Gender	**-0.053*** **	**0.011**	**-0.186**	**-0.108****	**0.038**	**-0.110**	**-0.102***	**0.042**	**-0.158**	**-0.102***	**0.042**	**-0.153**
Age child	0.000	0.001	0.022	0.000	0.001	-0.051	-0.001	0.002	-0.044	-0.001	0.002	-0.008
Gestational age at birth	-0.003	0.004	-0.037	-0.005	0.005	-0.089	-0.002	0.006	-0.027	-0.002	0.006	-0.016
Birthweight	0.000	0.000	-0.006	0.000	0.000	0.032	0.000	0.000	-0.001	0.000	0.000	-0.024
BMI	**0.009****	**0.003**	**0.118**	**0.009*****	**0.004**	**0.096**	**0.013***	**0.005**	**0.074**	**0.013***	**0.005**	**0.092**
Parity	0.016	0.007	0.093	-0.001	0.012	-0.003	0.000	0.012	0.001	0.005	0.015	0.014
Ethnicity	**0.059*****	**0.012**	**0.200**	**0.011***	**0.019**	**0.038**	0.010	0.021	0.028	0.010	0.021	0.020
Marital status of mother	0.011	0.019	0.027	-0.034	0.025	0.109	-0.032	0.033	-0.031	-0.032	0.033	-0.009
Educational level of mother	**-0.015****	**0.005**	**-0.124**	0.005	0.008	-0.093	0.016	0.009	0.038	0.016	0.009	0.041
Family income	**-0.011*****	**0.002**	**-0.227**	**-0.012****	**0.003**	**-0.167**	**-0.014*****	**0.004**	**-0.121**	**-0.014*****	**0.004**	**-0.120**
No. of children in household	0.013	0.008	0.072	**0.019***	**0.009**	**0.031**	**0.022***	**0.010**	**0.062**	**0.022***	**0.010**	**0.056**
Hair wash freq	0.000	0.007	-0.001	-0.009	0.012	-0.001	-0.012	0.014	-0.023	-0.012	0.014	-0.021
Last hair wash	0.010	0.007	0.063	**0.028*****	**0.009**	**0.174**	**0.031****	**0.010**	**0.105**	**0.031****	**0.010**	**0.102**
Use of hair product	**-0.061*****	**0.013**	**-0.194**	-0.063	0.021	-0.119	-0.048	0.023	-0.057	-0.048	0.023	-0.047
Hair color	**0.033*****	**0.008**	**0.168**	**0.025*****	**0.012**	**0.103**	**0.035****	**0.013**	**0.089**	**0.035****	**0.013**	**0.081**
Medication use	-0.015	0.082	-0.007	0.061	0.097	0.037	-0.033	0.122	-0.005	-0.032	0.122	-0.009
Sunhours	0.000	0.000	0.076	0.000	0.000	0.152	**0.000***	**0.000**	**0.086**	**0.000***	**0.000**	**0.097**
Total disease	0.006	0.007	0.040	0.005	0.007	0.007	0.000	0.008	-0.015	0.000	0.008	-0.001
Total allergy	0.000	0.007	-0.002	0.002	0.008	0.015	-0.004	0.009	0.001	-0.004	0.009	0.002
Gender x wash frequency				**0.019***	**0.017**	**0.052**	0.017	0.018	0.031	0.017	0.018	0.025
Gender x product use				0.004	0.031	-0.034	0.000	0.034	-0.385	0.000	0.034	-0.246

*= significant at 0.05, ** = significant at 0.01, *** = significant at 0.001. Left column: univariate associations, all others columns multivariate associations, controlling for all other characteristics.

DISCUSSION

In this large cohort study, we identified a number of child and family characteristics influencing hair cortisol and cortisone levels. Most importantly, we found that lower SES and belonging to an ethnic minority each independently predicted higher hair cortisol levels, after co-varying a set of potentially confounding variables. We speculate that this association might be due to more stressful life circumstances related to lower SES and minority status, even in a relatively egalitarian society such as the Netherlands.

Hair color is an important potential confounder to account for in substantive analyses, since different pigmentations independently of ethnicity influence cortisol and cortisone levels. Furthermore, both cortisol and cortisone concentrations were found to be higher for males than for females. Other potential confounders were BMI and hair washing, the latter seemed only associated with cortisol and cortisone levels in females. It should be noted that we observed similar associations for cortisol and cortisone, but the strength of the associations with cortisone was lower for all variables. The questionnaire Confounders in the Measurement of Corticosteroids in Hair (CoMCoH) with the potential confounders to be taken into account in analyses using hair cortisol or hair cortisone is provided in Appendix 1.

Because of the large sample the power of our statistical analyses was substantial. Therefore our results pertaining to variables that were not found to be related to hair cortisol or cortisone might be replicable in similar samples. Gestational age at birth, birthweight, use of hair products, use of medication, number of sun hours in the month of sampling, disease and allergy were not identified as a potential confounders affecting cortisol and cortisone levels. Marital status and educational level of the mother were not significantly related to cortisol levels either, after controlling for the other variables. Although previous studies found age-dependent patterns of cortisol, after co-varying other sources of information no effect of age was found here. It should be noted however that the age range in our sample was small, compared to Noppe et al. (2014) who documented significant changes in hair cortisol and cortisone with age.

Cortisol and cortisone levels showed similar associations, albeit with lower coefficients for cortisone. Cortisol is (80-90%) bound to cortisol binding globulin (CBG) and 5-10% is bound to albumin. Only the free fraction (3-10%) is the bio-active hormone. Cortisone also binds with high affinity to CBG. Since it is not completely clear whether only the free fraction of cortisol is built in the hair shaft we may speculate that also CBG levels may influence the correlation between cortisol and cortisone. The difference may be due to the fact that cortisone is built later in the corticosteroid chain, and is not solely related to cortisol levels. Even though cortisone is considered to be more stable over time, its construction may thus be influenced by other (biological) factors.

Some limitations apply to the current study. First, the variance accounted for in the models presented here is relatively modest, ranging from 7.8% for cortisol to 4.5% for cortisone. A similarly modest proportion of explained variance is encountered in Vanaelst et al. (2014). From a purely statistical point of view this can be considered a disadvantage. However, given that our main goal is identification of potential confounders to be considered in other, substantive analyses, low

explained variance here means more residual variance to be explained by genetic information and other, substantive predictors. Second, a limiting factor of using hair as a matrix to measure cortisol over an extended period of time is the time frame in which cortisol levels can be reliably measured, because over time cortisol levels may decrease in parts of the hair samples that are most distal from the scalp (Gao et al., 2013; Kirschbaum, Tietze, Skoluda, & Dettenborn, 2009). The current data capture a timeframe of 3 months, and in the literature there is agreement that the wash-out decline occurs only after a time span of 3–6 months (corresponding to 3–6 cm of hair).

In summary, hair samples are easy to collect and since hair grows back, it facilitates repeated measurements. Once sampled, it is cheap to store, and provides easy access to concentrations of cortisol and cortisone. Hence hair sample extraction can be considered a viable alternative to urinary or saliva extraction. Based on our results, research involving hair cortisol and hair cortisone concentrations should at least take into account the potentially confounding role of gender, BMI, ethnicity, family income, the number of children in the household, time since last hair washing and hair color (see Questionnaire "CoMCoH", Appendix 1). Our study suggests that belonging to low SES and ethnic minority families is associated with higher levels of hair cortisol and cortisone, possibly due to elevated stress the children from such families generally experience.

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Appendix 1 | Risk factors to be assessed in analyses involving hair cortisol concentration and hair cortisone concentration measurements

Confounders in the Measurement of Corticosteroids in Hair (CoMCoH)

		To include when using	
	Potential confounder	**Hair cortisol**	**Hair cortisone**
a	Gender	X	X
b	BMI	X	X
c	Last time of hair washing	X	X
d1	Family income	X	X
d2	Number of children to be supported from income	X	X
e	Hair color	X	X
f	Ethnicity	X	X
g	Sun hours		X
h	Gender * Washing frequency	X	X

Cortisol: log10(cortisol in pg/mg)
Cortisone: log10(cortisone in pg/mg)
Gender: Male, Female
BMI: in kg/m^2, age-corrected if applicable
Last time of hair washing: "<24 hours ago"
"24-48 hours ago"
">48 hours ago"
Family income: ratio scale, preferably, otherwise 8 or more ordinal classes
Number of children to be supported: counted # of children (including non-siblings, incidentally)
Hair color: Red, Blonde, Brown, Black
Ethnicity: at least:
"Dutch"
"Non-Dutch European"
"Non-European "
Sun hours: as reported by National Weather Statistics (or equivalent)
Washing frequency: "<once a week"
"1-2 times a week"
"3-4 times a week "
">4 times a week "