Associations of hair cortisol concentrations with cardio-metabolic risk factors in childhood

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ABSTRACT

Context Biological stress is related to cardiovascular disease in adults. The associations of stress with cardiovascular and metabolic diseases may originate in childhood.

Objective To examine the associations of hair cortisol concentrations at 6 years with cardio-metabolic risk factors at 6 and 10 years.

Design, Setting and participants Cortisol concentrations were measured in hair of 6-year-old children (n = 2,598) participating in the Generation R Study, a population-based prospective cohort study in Rotterdam, the Netherlands.

Main Outcome Measures Blood pressure, heart rate, concentrations of insulin, glucose, lipids and C-reactive protein in blood at 6 and 10 years.

Results Higher hair cortisol concentrations at 6 years were associated with higher systolic blood pressure at 10 years (difference 0.17 standard deviation score (SDS) (95% Confidence Interval (CI) 0.03, 0.31)). The association attenuated into non-significance after adjustment for childhood BMI at 6 years. Higher hair cortisol concentrations at 6 years were associated with an increase in total and LDL cholesterol between 6 and 10 years but not with those measurements at 6 or 10 years. Hair cortisol concentrations were not associated with other cardio-metabolic risk factors at 6 or 10 years.

Conclusions Hair cortisol concentrations were not independent of BMI associated with cardio-metabolic risk factors at 6 or 10 years. The associations of biological stress with cardio-metabolic risk factors may develop at later ages.

Key words: hair cortisol; child; cardio-metabolic risk; blood pressure, heart rate; lipids; cholesterol; insulin; glucose; C-reactive protein
INTRODUCTION

Stress is associated with cardio-metabolic disease in adults.\textsuperscript{1,2} Results from a study among 136,637 subjects showed that stress-related disorders were robustly associated with multiple types of cardiovascular disease, such as hypertensive diseases and heart failure.\textsuperscript{3} Similarly, a prospective cohort study, among 10,308 men and women, reported that those with chronic work stress were twice as likely to develop metabolic syndrome.\textsuperscript{4} It has also been suggested that long-term exposure to elevated cortisol concentrations may lead to long-term physiological alterations compromising the anatomy and function of the cardiovascular and metabolic systems.\textsuperscript{5,6} Cortisol concentrations measured in saliva, serum and urine are subject to situational and intra-individual fluctuations.\textsuperscript{7} Hair cortisol concentrations reflect long-term cumulative cortisol concentrations and are therefore a useful biomarker of long-term systemic cortisol exposure, which is mainly determined by hypothalamic-pituitary-adrenal (HPA)-axis activity.\textsuperscript{7,8} A recent review among 11 cross-sectional studies, shows positive associations of hair cortisol with adverse cardio-metabolic outcomes including higher systolic blood pressure, diabetes, metabolic syndrome and adiposity.\textsuperscript{1} In addition, hair cortisol concentrations have been shown to be associated with an increased risk of having cardiovascular diseases, such as coronary heart disease, stroke, and peripheral arterial disease, in elderly.\textsuperscript{9} The associations of chronic stress with adverse cardio-metabolic outcomes may originate in early life. It is well known that adverse exposures in early life are associated with cardiovascular risk factors development from childhood onwards.\textsuperscript{10,12} Also, cardio-metabolic risk factors tend to track from childhood into adulthood.\textsuperscript{13-16} We previously reported associations of hair cortisol concentrations with childhood body mass index (BMI) and fat mass distribution at the ages of 6 and 10 years.\textsuperscript{17,18} Thus far, studies in children did not report associations between hair cortisol concentrations and cardio-metabolic risk factors.\textsuperscript{19-21} These previous studies had small sample sizes and, mostly, a cross-sectional design.

We hypothesized that chronic exposure to higher cortisol concentrations is associated with an adverse cardio-metabolic risk profile already in school-age children, and thereby predispose individuals to later-life cardiovascular disease. We examined, in a population-based prospective cohort study among 2,598 children, the associations of hair cortisol concentrations at 6 years with
blood pressure, heart rate, lipid profile, glucose metabolism, and C-reactive protein concentrations at 6 and 10 years, and explored the potential mediating role of childhood BMI.

METHODS

Study Design
This study was embedded in the Generation R Study, a population-based prospective cohort study from early pregnancy onwards in Rotterdam, the Netherlands.22 Written informed consent was provided for all children. The Medical Ethics Committee of Erasmus MC approved the study (MEC 198.782/2001/31). This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline. In total, 2,984 children had information on hair cortisol concentrations at 6 years. Twins (N = 58) and children without any measurement of cardio-metabolic risk factors at 6 and 10 years (N = 23) were excluded. Since the highest values of hair cortisol concentrations may be incorrect due to external factors such as glucocorticoid use, we excluded the extreme values of cortisol (N= 305) using Tukey’s definition of outliers (Q1-1.5*IQR and Q3+1.5*IQR).23 There were no substantial differences in the cardio-metabolic risk factors between the population of analysis and the excluded outliers (data not shown). The population for analysis consisted of 2,598 children. The same selection procedure was followed for the cortisone analyses (N= 2,605). The flowchart of participants is given in Supplemental Figure 1.24

Hair Cortisol Concentration Measurements
As described previously, in children aged 6 years, a hair strand of approximately 100 hairs was cut from the posterior vertex using small surgical scissors, as close to the scalp as possible.25 Details on collection, sample preparation, extraction and analysis using the liquid chromatography tandem mass spectrometry (LC-MS/MS) method are provided in the Supplemental Methods.24 To reduce variability and account for right skewedness of the distribution, cortisol and cortisone concentrations outliers defined by Tukey’s definition of outliers (Q1-1.5*IQR and Q3+1.5*IQR) were excluded, after
which values were either divided in quintiles, or natural log transformed and further standardized by the interquartile range (IQR) to ease the interpretation of effect sizes.\textsuperscript{23}

**Cardio-metabolic risk factors**

Outcome assessments were performed at ages 6 and 10 years.\textsuperscript{22} Blood pressure and heart rate were measured at the right brachial artery four times with one minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus (Paramus NJ).\textsuperscript{26} We calculated the mean value for systolic and diastolic blood pressure and heart rate using the last three measurements of each participant. Thirty-minutes fasting venous blood samples were collected to measure serum concentrations of insulin, total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, C-reactive protein at 6 and 10 years, and glucose only at 10 years.\textsuperscript{22} Because the blood samples were collected at different time points during the day, it was not possible to have fasting samples. Participants were asked to stop eating and drinking thirty minutes before the blood draw. Unfortunately we do not have information on the exact time between last meal and sample, nor on the nutrient composition of the used meals. Glucose, total cholesterol, HDL-cholesterol, triglycerides and C-reactive protein concentrations were measured using the c702 module on the Cobas 8000 analyzer. Insulin was measured with electrochemiluminescence immunoassay (ECLIA) on the e601 module (Roche, Almere, The Netherlands).\textsuperscript{27} Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula.\textsuperscript{28,29} We defined children with clustering of cardio-metabolic risk factors being at risk for metabolic syndrome phenotype, in line with other studies.\textsuperscript{30,31} Clustering of cardio-metabolic risk factors was defined as having three or more out of the following four adverse risk factors: android fat mass percentage above the seventy-fifth percentile; systolic or diastolic blood pressure above the seventy-fifth percentile; HDL cholesterol below the twenty-fifth percentile or triglycerides above the seventy-fifth percentile; and insulin above the seventy-fifth percentile of our study population. We measured total body fat mass and fat mass in the abdomen (android fat mass) using a DXA scanner (iDXA, GE140 Lunar, 2008, Madison, WI, USA, enCORE software v.12.6), according to standard procedures.\textsuperscript{32} We calculated android fat mass percentage as android fat mass divided by total body fat mass. For the clustering of cardio-metabolic risk factors at 10 years we also
had visceral fat mass obtained by MRI scans available, as described previously. Because the
distribution of insulin and triglycerides concentrations was skewed, we used their natural logged
values. Since C-reactive protein was not normally distributed and transformation did not yield an
acceptable distribution, we categorized C-reactive protein concentrations into < 3 mg/l (normal levels)
or ≥ 3 mg/l (high levels) in line with previous studies. To enable comparison of effect sizes of
different measures, we constructed SDS (\(\text{observed value} - \text{mean}/\text{SD}\)) for all variables.

**Covariates**

Information on child sex was obtained from midwife/obstetric records. Maternal height was assessed
at the first visit. Information about maternal weight just before pregnancy was obtained by
questionnaire. Maternal pre-pregnancy BMI (kg/m\(^2\)) was calculated. Information on maternal
education, family income, child ethnicity and television watching time was obtained by
questionnaires. Hair color was partially coded through parent report and was completed by two raters
using photographs made at the research center. We calculated BMI (kg/m\(^2\)) at 6 years from height and
weight, both measured without shoes and heavy clothing. Parents completed a questionnaire about
their child on factors which can potentially influence hair cortisol concentrations, such as hair
washing frequency, time since last wash, hair product use and use of and administration route of
glucocorticoid medications at the age of six years. We tested whether birth weight was a confounder
in the associations of hair cortisol concentrations and cardio-metabolic risk factors but birth weight
did not change the effect estimates > 10% and thus was not included in the final confounder model.

**Statistical analysis**

First, we examined differences in subject characteristics between hair cortisol concentration quintiles
with analysis of variance tests for continuous variables and chi-squared tests for categorical variables.
For non-response analyses, we compared participants and non-participants using chi-squared tests,
Student \(t\) tests and Mann-Whitney tests. Second, we used linear regression models to assess the
associations of hair cortisol concentrations at 6 years in quintiles with cardio-metabolic risk factors at
6 and 10 years, and the change in cardio-metabolic risk factor SD scores between these ages (systolic
blood pressure, diastolic blood pressure, heart rate, total cholesterol, HDL and LDL-cholesterol, triglycerides, insulin, glucose). Third, we used logistic regression models to assess the associations of hair cortisol concentrations at 6 years in quintiles with the odds of increased C-reactive protein concentrations (≥ 3 mg/L) and the odds of having clustered cardio-metabolic risk factors at 6 and 10 years. Only cases with complete data on cardio-metabolic outcomes were used for the analyses with clustered cardio-metabolic risk factors. Tests for trend across quintiles were performed by analyzing cortisol quintiles as a continuous variable. Fourth, we performed linear regression models to assess the associations of continuous hair cortisol concentrations (the natural log transformed hair cortisol measures further standardized with the IQR) with all cardio-metabolic outcomes. The basic models were adjusted for child sex, age at cortisol measurement and age at assessment of cardio-metabolic outcomes. The confounder models were additionally adjusted for maternal pre-pregnancy BMI, maternal education, family income, child ethnicity, hair color and average duration of television watching per day. We performed an additional model to assess whether any significant association in the confounder model was explained by childhood BMI. We visualized potential covariates by drawing a directed acyclic graph (DAG) and included the covariates in the models that were associated with exposure and outcome at 6 years and changed the effect estimates > 10% (Supplemental Figure 2). We tested if there was an interaction of cortisol with sex by adding an interaction term to the basic model. After taking multiple testing into account, the interaction was only significant for insulin and triglyceride concentrations at 6 years (p-value < 0.01). For these associations, we performed sex-stratified analyses. As a sensitivity analysis, we only included children without any glucocorticoid use in the three months prior to the hair sample collection (N = 2,296). Also, we repeated all analyses for cortisone, the less active form of cortisol (N = 2,605). Considering three groups of outcomes (blood pressure and heart rate, lipids and glucose metabolism), multiple testing adjustment would lead to a p-value cutoff of < 0.017. We depicted both significance levels (0.05 and 0.017) in the tables and figures. Missing data of covariates were multiple-imputed using a Markov chain Monte Carlo approach. Five imputed datasets were created and analyzed together. All statistical analyses were performed using the Statistical package of Social Sciences.
RESULTS

Subject characteristics

As compared to children in the lower cortisol quintiles, children in the upper cortisol quintiles more often had a mother who had a higher pre-pregnancy BMI, was lower educated and had a lower family income. Also, these children more often had a non-European ethnicity, a higher BMI and systolic blood pressure at 6 and 10 years, a brown or black hair color and a higher average duration of television watching at age 6 years (Tables 1 and 2). Non-response analyses showed that, compared to mothers of participants, mothers of non-participants more often had a higher BMI, a lower family income and lower education. Non-participants more often were boys, had a non-European ethnicity, a higher BMI, brown or dark hair, watched more television and used more often glucocorticoids in the 3 months prior to hair sampling (Supplemental Table 2).

Cardiovascular risk factors

Results from the basic models showed that, as compared to the lowest quintile of hair cortisol concentrations at 6 years, children in the highest quintile had a higher systolic and diastolic blood pressure and heart rate at 6 years (Supplemental Table 2). When we adjusted these models for potential confounders, these associations attenuated into non-significance (Table 3). As compared to the lowest quintile of hair cortisol concentrations at 6 years, children in the highest quintiles had a higher systolic blood pressure at 10 years, in the basic models (Supplemental Table 2). This association remained significant after adjustment for confounders (difference 0.15 SDS (95% CI 0.00, 0.29) and 0.17 SDS (95% CI 0.03, 0.31) for the fourth and fifth quintile, respectively) but attenuated into non-significance after additional adjustment for childhood BMI at 6 years (Table 3 and Supplemental Table 3). Associations for continuous cortisol measures showed similar results (Supplemental Table 4). The tests for trend across the quintiles were not significant.
Metabolic risk factors

Hair cortisol concentrations were not associated with lipid and glucose metabolism biomarkers in the basic and main models (Supplemental Table 5 and Table 4, respectively). In the sex-stratified analyses higher hair cortisol concentrations were associated with lower triglycerides and insulin concentrations among boys only at 6 years (differences -0.11 SDS (95% CI -0.21, -0.01) and -0.09 SDS (95% CI -0.18, 0.00), respectively) (Supplemental Table 6). As compared to the lowest quintile of hair cortisol concentrations at 6 years, children in the highest quintile had a higher increase in total cholesterol and LDL cholesterol concentrations from 6 to 10 years (differences 0.19 SDS (95% CI 0.05, 0.34) and 0.15 SDS (95% CI 0.00, 0.29), respectively), but no difference in change of other metabolic risk factors (Table 4). The association for change in total cholesterol was independent of childhood BMI at 6 years (Supplemental Table 3). The associations for continuous cortisol measures showed similar results (Supplemental Table 4).

Increased C-reactive protein concentrations and clustering of cardiovascular risk factors

Results from the basic models showed that, as compared to the lowest quintile of hair cortisol concentrations at 6 years, children in the highest quintile, had a higher risk of increased C-reactive protein at 6 years (Odds Ratio (OR): 1.76 (95% CI : 1.08, 2.86)) and a higher risk of increased C-reactive protein (OR: 2.23 (95% CI : 1.05, 4.70)) and cardio-metabolic clustering (OR 1.73 (95% CI 1.01, 2.97)) at 10 years (Supplemental Table 7). When we further adjusted the models for potential confounders, these associations attenuated into non-significance (Table 5). The associations for continuous cortisol measures showed similar results (Supplemental Table 8).

Sensitivity analyses

In the confounder models, excluding children with all types of glucocorticoid use in the three months prior to hair sample collection (N = 173), we observed similar but slightly stronger results for systolic blood pressure at 10 years (differences 0.20 SDS (95% CI 0.05, 0.34) (Supplemental Table 9). When we further adjusted the model for childhood BMI, the association attenuated into non-
significance (Supplemental Table 10). In these analyses, results from the confounder model showed that children in the highest quintile of hair cortisol concentrations at 6 years, compared to those in the lowest quintile, had a higher risk of increased C-reactive protein at 6 years (OR: 1.83 (95% CI: 1.06, 3.13)) and 10 years (OR: 2.53 (95% CI: 1.11, 5.77)) (Supplemental Table 11), independent of childhood BMI at 6 years (Supplemental Table 10). Hair cortisone concentrations at 6 years were not associated with any of the cardio-metabolic outcomes at 6 or 10 years (results not shown).

DISCUSSION

In this population-based prospective cohort study among 2,598 children, we observed that hair cortisol concentrations at 6 years were not consistently associated with cardio-metabolic risk factors at 6 and 10 years. The association of higher hair cortisol concentrations at 6 years with higher systolic blood pressure at 10 years was explained by childhood BMI.

Interpretation of main findings

A meta-analysis in 2,832 adults from 11 studies showed that higher hair cortisol concentrations were associated with higher systolic blood pressure, but not with diastolic blood pressure. Also, a review including twenty studies investigating the relationships between various cortisol measures and cardio-metabolic parameters in adults reported that 3 out of 6 studies found positive associations between cortisol measures and systolic blood pressure and reported inconclusive results for the other outcomes. Previous studies in children did not find associations between hair cortisol concentrations and blood pressure, heart rate, lipids, C-reactive protein or glucose metabolism. These studies in children had smaller sample sizes and most of them had a cross-sectional design. Results of studies into the associations of salivary, serum or urinary cortisol with cardio-metabolic risk factors in childhood were not consistent.

In the current study, we observed that higher hair cortisol concentrations at 6 years were associated with a higher systolic blood pressure at 10 years. This finding is in line with the findings of three cross-sectional studies that showed a positive association between serum cortisol concentrations
and systolic blood pressure in children but not, or less clearly, with diastolic blood pressure.\textsuperscript{40-42}

However, we did not find an association between hair cortisol concentrations at 6 years and systolic blood pressure at 6 years. Thus, it may be that higher cortisol concentrations lead to increased systolic blood pressure later in childhood, which is known to track into adulthood.\textsuperscript{13} Additional adjustment of the association between hair cortisol concentrations at 6 years and systolic blood pressure at 10 years for childhood BMI at 6 years resulted in attenuation of this association. This is in contrast with the findings of the three cross-sectional studies mentioned above, which showed that the association remained after adjustment for BMI or total body fat mass.\textsuperscript{40-42} Childhood BMI can be either an intermediate or a confounder in the association of hair cortisol concentration at 6 years and systolic blood pressure at 10 years. We know from previous studies in our cohort that higher hair cortisol concentrations are associated with higher childhood BMI at 6 and 10 years.\textsuperscript{17,18} A bidirectional association between cortisol and adiposity may be present which should be further explored in future studies.\textsuperscript{43} Future studies are also needed to obtain further insight into the role of BMI in the association of hair cortisol concentrations with blood pressure.

We observed an association between higher hair cortisol concentrations and the increase in total cholesterol and LDL concentrations between 6 and 10 years, but not with any of the lipid concentrations at 6 or 10 years. Studies that used different types of samples to measure cortisol did not find an association with lipid concentrations in children.\textsuperscript{20,21,38,39,42} Studies in adults are not consistent about the association between cortisol and lipids, but most provide evidence for a positive association between cortisol and total cholesterol and LDL.\textsuperscript{44-48} It may be that the association between higher hair cortisol and higher total cholesterol and LDL concentrations becomes more apparent at later ages.

In sex-stratified analyses, we observed that higher hair cortisol concentrations were associated with lower triglyceride and insulin concentrations among boys at 6 years, independent of childhood BMI, and higher concentrations of triglycerides and insulin among girls at 6 years. These findings were only significant among boys and not among girls, which may be explained by a higher variability in hair cortisol concentrations among boys. In our study and similarly to previous studies, hair cortisol concentrations were significantly higher among boys than girls.\textsuperscript{36,49} It has been
hypothesized that sex differences in reactivity to psychological stress might contribute to the sex differences in morbidity and mortality rates of cardiovascular diseases. However, studies in adults did not report differences in the associations between hair cortisol concentrations and cardio-metabolic risk factors after stratification on sex.\textsuperscript{36,37,50,51} The sex specific associations of cortisol concentrations with cardiovascular risk factors and disease need further study.

The metabolic syndrome shares many characteristics of Cushing’s Syndrome, caused by the endogenous overproduction of cortisol, such as impaired glucose tolerance, dyslipidemia, abdominal fat distribution and hypertension.\textsuperscript{52} Therefore, it has been suggested that altered activity of the hypothalamus-pituitary-adrenal (HPA) axis leading to the hypersecretion of glucocorticoids may play an important role in the development of metabolic syndrome.\textsuperscript{52-55} However, we did not find clear evidence for an association of higher cortisol concentrations and characteristics of the metabolic syndrome in childhood.

\textbf{Strengths and limitations}

One of the strengths of this study was the prospective data collection from early pregnancy onwards. We had a large sample size and detailed measurements of hair cortisol concentrations and childhood cardio-metabolic risk factors. A limitation of our study is the lack of hair cortisol measurements at the age of 10 years. Therefore, we do not know how cortisol concentrations develop over time. In order to prevent contamination of data caused by hair cortisol outliers we excluded cortisol values using Tukey’s definition of outliers.\textsuperscript{23} Excluding these values would have affected the effect estimates if cardio-metabolic risk factors were different for the excluded children and the population of analysis. However, there were no substantial differences in the characteristics of these groups. Also, the hair cortisol concentration values in the population of analysis, were all within the LC-MS/MS based reference interval for children aged 6, provided by a recent study that aimed to establish age-adjusted reference intervals for hair cortisol in children.\textsuperscript{56} We used non-fasting venous blood samples to measure the serum concentrations of the cardio-metabolic risk factors. The blood samples were collected at different time points during the day, depending on the time of the study visit. Since glucose and insulin concentrations change easily during the day and in response to carbohydrate
intake, this may have caused non-differential misclassification. We think the effect of this potential misclassification will be minor. A previous study reported that insulin resistance or sensitivity in semi-fasted blood samples are moderately correlated with fasting values.\textsuperscript{57} Also, studies reported that non-fasting lipid concentrations can predict increased risks of cardiovascular events later in life.\textsuperscript{58,59} Overall, results should be interpreted with caution and this study should be replicated using fasting samples. Even though the analyses were adjusted for a large number of potential confounding factors, residual cofounding may still be a concern, as in any observational study. Due to the observational design of the study, we cannot establish causality of the observed associations.

\textbf{CONCLUSION}

Our results suggest that hair cortisol concentrations at 6 years are not consistently associated with cardio-metabolic risk factors at 6 and 10 years. The association between hair cortisol concentrations and systolic blood pressure was explained by childhood BMI. The associations of stress with cardio-metabolic risk factors may develop at later ages.
Abbreviations
BMI = Body Mass Index
CI = Confidence Interval
CRP = C-reactive protein
DBP = diastolic blood pressure
HDL = high-density lipoprotein
HPA = hypothalamic-pituitary-adrenocortical
IQR = interquartile range
LDL = low-density lipoprotein
OR = Odds Ratio
SBP = systolic blood pressure
SDS = standard deviation score

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Data availability
The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.
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### Table 1. Family and birth characteristics (N= 2,598)

| Family characteristics | Hair Cortisol Concentrations |
|-------------------------|------------------------------|
|                         | Total group a                 | Quintile 1 a | Quintile 2 a | Quintile 3 a | Quintile 4 a | Quintile 5 a |
|                         | pg/mg (N= 2,598)              | pg/mg (N= 519) | pg/mg (N= 520) | pg/mg (N= 520) | pg/mg (N= 520) |
| Pre-pregnancy BMI, median (95% range), kg/m² | 22.6 (18.2, 35.1) | 22.1 (18.8, 33.9) | 22.7 (18.1, 34.6) | 22.6 (18.1, 35.1) | 22.3 (18.1, 35.9) | 23.0 (17.5, 36.2) | 0.039 |
| Maternal education (%)  | 327 (15.8) | 39 (8.8) | 51 (12.3) | 58 (14.1) | 90 (22.1) | 89 (22.6) | < 0.001 |
| Primary school          | 98 (4.5) | 8 (1.7) | 15 (3.4) | 22 (5.0) | 34 (7.9) | 19 (4.6) | < 0.001 |
| Secondary school        | 815 (37.2) | 151 (32.7) | 150 (34.0) | 167 (38.2) | 169 (39.1) | 178 (42.8) | 178 (42.8) |
| High education          | 1,275 (58.3) | 303 (65.6) | 276 (62.6) | 248 (56.8) | 229 (53.0) | 219 (52.6) | 219 (52.6) |
| Family income (%)       | 78 (4.5) | 39 (8.8) | 51 (12.3) | 58 (14.1) | 90 (22.1) | 89 (22.6) | < 0.001 |
| Low ( < €1600 per month ) | 327 (15.8) | 39 (8.8) | 51 (12.3) | 58 (14.1) | 90 (22.1) | 89 (22.6) | < 0.001 |
| Medium (€1600-4000 per month) | 981 (47.4) | 202 (45.5) | 186 (44.7) | 212 (51.7) | 197 (48.3) | 184 (46.8) | 184 (46.8) |
| High ( > €4000 per month ) | 763 (36.8) | 203 (45.7) | 179 (43.0) | 140 (34.1) | 121 (29.7) | 120 (30.5) | 120 (30.5) |

### Birth characteristics

| Sex, N (%) | 0.054 |
|------------|-------|
| Boys       | 1,237 (47.6) | 223 (43.0) | 248 (47.7) | 247 (47.5) | 247 (47.5) | 272 (52.4) |
| Girls      | 1,361 (52.4) | 296 (57.0) | 272 (52.3) | 273 (52.5) | 273 (52.5) | 247 (47.6) |

| Ethnicity (%) | < 0.001 |
|---------------|---------|
| Boys          | 1,237 (47.6) | 223 (43.0) | 248 (47.7) | 247 (47.5) | 247 (47.5) | 272 (52.4) |
| Girls         | 1,361 (52.4) | 296 (57.0) | 272 (52.3) | 273 (52.5) | 273 (52.5) | 247 (47.6) |
|                  | European | Non-European |
|------------------|----------|--------------|
|                  | 1,644 (65.0) | 885 (35.0)  |
|                  | 419 (82.2)   | 91 (17.8)   |
|                  | 355 (70.9)   | 146 (29.1)  |
|                  | 302 (59.2)   | 208 (40.8)  |
|                  | 283 (55.6)   | 226 (44.4)  |
|                  | 285 (57.1)   | 214 (42.9)  |

*Values are means (standard deviation), medians (95% range) or numbers of subjects (valid %).

b P-values for differences in subject characteristics between cortisol quintiles were tested using one-way ANOVA (Analysis of Variance) tests for continuous variables and chi-square tests for categorical variables.

Abbreviations: BMI: body mass index, N: number.
Table 2. Child characteristics (N= 2,598)

| Hair Cortisol Concentrations | Total group * | Quintile 1 * | Quintile 2 * | Quintile 3 * | Quintile 4 * | Quintile 5 a | P-value b |
|-----------------------------|---------------|--------------|--------------|--------------|--------------|--------------|-----------|
|                             | 0.131-6.764   | 0.131-0.744  | 0.745-1.173  | 1.174-1.831  | 1.832-2.925  | 2.926-6.764 pg/mg |           |
|                             | (N= 2,598)    | (N = 519)    | (N = 520)    | (N = 520)    | (N = 520)    | (N=519)      |           |

Child characteristics at 6 years

Age at measurements, median (95% range), years
5.9 (5.7, 8.1) 5.9 (5.6, 8.1) 5.9 (5.7, 8.1) 5.9 (5.7, 8.2) 5.9 (5.7, 8.2) 5.9 (5.7, 8.1) 0.013

Body mass index, median (95% range), kg/m²
15.8 (13.6, 21.2) 15.7 (13.7, 19.0) 15.7 (13.6, 21.0) 16.0 (13.7, 20.9) 15.9 (13.7, 21.5) 16.1 (13.6, 22.4) < 0.001

Hair cortisol concentrations, median (95% range), pg/mg
1.46 (0.33, 5.62) 0.56 (0.23, 0.74) 0.96 (0.75, 1.17) 1.46 (1.18, 1.81) 2.28 (1.86, 2.88) 3.98 (2.98, 6.60) < 0.001

Hair cortisone concentrations, median (95% range), pg/mg
7.50 (2.63, 29.00) 4.65 (2.00, 7.4) 6.19 (2.95, 10.66) 8.25 (3.31, 11.52) 16.00 (3.65, 44.58) < 0.001

Systolic blood pressure, mean (SD), mmHg
102.6 (8.4) 101.7 (8.1) 102.1 (8.2) 103.1 (8.2) 103.0 (8.2) 103.2 (9.0) 0.007

Diastolic blood pressure, mean (SD), mmHg
60.5 (6.7) 60.1 (6.4) 60.1 (6.6) 60.4 (7.1) 60.4 (6.6) 61.2 (7.0) 0.072

Heart rate, mean (SD), beats/minute
82.7 (9.8) 82.0 (9.0) 83.6 (10.1) 83.1 (10.4) 82.4 (9.6) 82.7 (9.9) 0.091

Insulin, median (95% range), pmol/L
116.90 (18.58, 110.70 (24.60, 130.30 (15.27, 93.49 (13.30, 128.60 (18.26, 115.30 (19.53, 116.90 (18.58, 405.28) 401.89) 411.94) 388.15) 422.35) 455.04) 0.014

Total-cholesterol, mean (SD), mmol/L
4.25 (0.65) 4.27 (0.63) 4.28 (0.62) 4.28 (0.66) 4.23 (0.63) 4.22 (0.68) 0.571

HDL-cholesterol, mean (SD), mmol/L
1.38 (0.32) 1.38 (0.33) 1.37 (0.32) 1.39 (0.33) 1.35 (0.30) 1.39 (0.30) 0.640

LDL-cholesterol, mean (SD), mmol/L
2.37 (0.56) 2.37 (0.55) 2.41 (0.54) 2.39 (0.59) 2.37 (0.54) 2.35 (0.58) 0.690

Triglycerides, median (95% range), mmol/L
0.99 (0.41, 2.35) 0.97 (0.41, 2.64) 0.97 (0.40, 2.35) 1.02 (0.42, 2.14) 0.99 (0.41, 2.37) 1.00 (0.38, 2.30) 0.973
| C-reactive protein, N (%)                  |        |
|------------------------------------------|--------|
| < 3 mg/L                                 | 1,591 (89.2) | 334 (92.0) | 313 (90.2) | 301 (85.8) | 319 (91.1) | 324 (86.9) |
| ≥ 3 mg/L                                 | 193 (10.8)  | 29 (8.0)   | 34 (9.8)   | 50 (14.2)  | 31 (8.9)   | 49 (13.1)  |

| Prevalence cardio-metabolic clustering, N (%) | 218 (10.8)  | 34 (8.4)   | 44 (11.2)  | 47 (11.6)  | 45 (11.1)  | 48 (11.7)  | 0.529 |

| Glucocorticoid use in the 3 months prior to hair sample collection, N (%) |        |
|-----------------------------------------------------------------------|--------|
| No                                                                    | 2,296 (93.0) | 452 (92.2) | 469 (94.6) | 460 (92.4) | 455 (93.0) | 460 (92.7) | 0.622 |
| Yes                                                                   | 173 (7.0)  | 38 (7.8)   | 27 (5.4)   | 38 (7.6)   | 34 (7.0)   | 36 (7.3)   |

| Hair color, N (%)                                                      |        |
|-----------------------------------------------------------------------|--------|
| Red                                                                   | 78 (3.0)  | 20 (3.9)   | 16 (3.1)   | 20 (3.9)   | 11 (2.1)   | 11 (2.1)   | < 0.001 |
| Blond                                                                 | 1,381 (53.2) | 376 (72.4) | 281 (54.0) | 241 (46.4) | 246 (47.4) | 237 (45.7) |
| Brown                                                                  | 857 (33.0) | 111 (21.4) | 175 (33.7) | 207 (39.9) | 180 (34.7) | 184 (35.5) |
| Black                                                                  | 280 (10.8) | 12 (2.3)   | 48 (9.2)   | 51 (9.8)   | 82 (15.8)  | 87 (16.8)  |

| Television watching time, N (%)                                       |        |
|-----------------------------------------------------------------------|--------|
| < 2 hours per day                                                     | 1,631 (81.7) | 381 (88.6) | 338 (83.5) | 318 (79.5) | 303 (78.1) | 291 (78.0) | < 0.001 |
| ≥ 2 hours per day                                                     | 365 (18.3)  | 49 (11.4)  | 67 (16.5)  | 82 (20.5)  | 85 (21.9)  | 82 (22.0)  |

**Child characteristics at 10 years**

| Age at measurements, median (95% range), years                      | 9.7 (9.3, 10.6) | 9.7 (9.3, 10.7) | 9.7 (9.3, 10.4) | 9.7 (9.4, 10.6) | 9.8 (9.2, 10.7) | 9.7 (9.3, 11.0) | 0.155 |
| Body mass index, median (95% range), kg/m²                           | 16.9 (14.0, 24.8) | 16.5 (13.9, 22.4) | 16.7 (14.2, 23.9) | 17.0 (14.0, 23.5) | 17.0 (14.1, 25.4) | 17.2 (13.8, 26.6) | < 0.001 |
| Systolic blood pressure, mean (SD), mmHg                             | 103.2 (8.1)  | 102.4 (7.8) | 102.7 (7.4) | 103.0 (8.3) | 103.9 (8.4) | 104.2 (8.4) | 0.005 |
| Diastolic blood pressure, mean (SD), mmHg                            | 58.6 (6.6)   | 58.4 (6.8) | 58.8 (6.3) | 58.4 (6.7) | 58.5 (6.8) | 59.0 (6.6) | 0.698 |
| Heart rate, mean (SD), beats/minute                                  | 74.3 (10.1)  | 74.3 (10.0) | 74.3 (9.8) | 74.2 (10.4) | 74.8 (10.6) | 74.2 (10.0) | 0.928 |
| Insulin, median (95% range), pmol/L                                  | 193.2 (33.37) | 177.10 (37.21) | 189.50 (30.72) | 201.40 (37.03) | 202.00 (28.19) | 199.55 (30.81) | 0.341 |
|                          | 709.27) | 613.68) | 618.40) | 698.83) | 718.82) | 777.55) |
|--------------------------|---------|---------|---------|---------|---------|---------|
| Glucose, mean (SD), mmol/L | 5.38 (0.92) | 5.42 (0.91) | 5.30 (0.84) | 5.47 (0.99) | 5.35 (0.92) | 5.38 (0.94) |
| Total-cholesterol, mean (SD), mmol/L | 4.30 (0.65) | 4.28 (0.64) | 4.32 (0.64) | 4.29 (0.68) | 4.28 (0.63) | 4.34 (0.66) |
| HDL-cholesterol, mean (SD), mmol/L | 1.47 (0.33) | 1.48 (0.33) | 1.49 (0.35) | 1.46 (0.31) | 1.46 (0.32) | 1.47 (0.33) |
| LDL-cholesterol, mean (SD), mmol/L | 2.33 (0.57) | 2.31 (0.57) | 2.32 (0.56) | 2.34 (0.59) | 2.33 (0.53) | 2.36 (0.58) |
| Triglycerides, median (95% range), mmol/L | 0.93 (0.42, 2.56) | 0.95 (0.39, 2.63) | 0.93 (0.43, 2.84) | 0.93 (0.37, 2.43) | 0.95 (0.45, 2.38) | 0.93 (0.44, 2.65) |

C-reactive protein, N (%)<sup>a</sup>

- < 3 mg/L: 1,303 (93.9) | 272 (96.1) | 266 (94.7) | 254 (94.8) | 267 (92.1) | 244 (91.7) |
- ≥ 3 mg/L: 85 (3.3) | 11 (3.9) | 15 (5.3) | 14 (5.2) | 23 (7.9) | 22 (8.3) |

Prevalence cardio-metabolic clustering, N (%)<sup>b</sup>

- 172 (13.3) | 26 (10.0) | 35 (13.4) | 40 (15.7) | 31 (11.7) | 40 (15.7) | 0.219

<sup>a</sup> Values are means (standard deviation), medians (95% range) or numbers of subjects (valid %).

<sup>b</sup> P-values for differences in subject characteristics between cortisol quintiles were tested using one-way ANOVA (Analysis of Variance) tests for continuous variables and chi-square tests for categorical variables.

<sup>c</sup> pg/mg = picogram per milligram

<sup>d</sup> Clustering of cardio-metabolic risk factors was defined as having three or more out of the following four adverse risk factors: android fat mass percentage above the seventy-fifth percentile; systolic or diastolic blood pressure above the seventy-fifth percentile; HDL cholesterol below the twenty-fifth percentile or triglycerides above the seventy-fifth percentile; and insulin above the seventy-fifth percentile of our study population. We used android fat mass as a percentage of total body fat mass as proxy for waist circumference since this was not available. For the clustering of cardio-metabolic risk factors at 10 years we also had visceral fat mass obtained by MRI scans available, as described previously.<sup>22</sup> (N = 2,022 at 6 years and N = 1,298 at 10 years)
Table 3. Association of hair cortisol quintiles at 6 years with blood pressure and heart rate at 6 years and 10 years and with the change between 6 and 10 years, confounder models

| Hair cortisol quintiles at 6 years | Systolic blood pressure \(^a\) (N = 2,466) | Diastolic blood pressure \(^a\) (N = 2,466) | Heart rate \(^b\) (N = 2,465) |
|----------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------|
| Q1                               | Reference                                 | Reference                                 | Reference                     |
| Q2                               | 0.02 (-0.11, 0.14)                        | -0.02 (-0.14, 0.11)                       | 0.17 (0.05, 0.29)**           |
| Q3                               | 0.11 (-0.02, 0.23)                        | -0.01 (-0.13, 0.11)                       | 0.09 (-0.03, 0.21)           |
| Q4                               | 0.09 (-0.04, 0.22)                        | -0.02 (-0.14, 0.10)                       | 0.01 (-0.11, 0.12)           |
| Q5                               | 0.09 (-0.04, 0.21)                        | 0.09 (-0.03, 0.22)                        | 0.05 (-0.07, 0.17)           |

| Hair cortisol quintiles at 6 years | Systolic blood pressure \(^b\) (N = 1,938) | Diastolic blood pressure \(^b\) (N = 1,938) | Heart rate \(^b\) (N = 1,891) |
|----------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------|
| Q1                               | Reference                                 | Reference                                 | Reference                     |
| Q2                               | 0.03 (-0.11, 0.17)                        | 0.04 (-0.10, 0.19)                       | -0.01 (-0.15, 0.14)          |
| Q3                               | 0.04 (-0.11, 0.18)                        | -0.04 (-0.19, 0.11)                      | -0.04 (-0.19, 0.11)          |
| Q4                               | **0.15 (0.00, 0.29)**\(^*\)               | -0.04 (-0.19, 0.11)                      | 0.01 (-0.13, 0.16)           |
| Q5                               | 0.17 (0.03, 0.31)\(^*\)                   | 0.05 (-0.11, 0.19)                       | -0.01 (-0.16, 0.13)          |

| Hair cortisol quintiles at 6 years | Change in systolic blood pressure \(^c\) (N = 1,829) | Change in diastolic blood pressure \(^c\) (N = 1,828) | Change in heart rate \(^c\) (N =1,789) |
|----------------------------------|-------------------------------------------|-------------------------------------------|-------------------------------|
| Q1                               | Reference                                 | Reference                                 | Reference                     |
| Q2                               | 0.02 (-0.12, 0.16)                        | 0.14 (-0.02, 0.30)                       | -0.08 (-0.22, 0.07)          |
| Q3                               | -0.06 (-0.20, 0.08)                       | 0.01 (-0.15, 0.18)                       | -0.13 (-0.27, 0.02)          |
| Q4                               | 0.06 (-0.09, 0.20)                        | -0.00 (-0.17, 0.16)                      | 0.02 (-0.13, 0.16)           |
| Q5                               | 0.05 (-0.10, 0.19)                        | -0.05 (-0.22, 0.11)                      | -0.07 (-0.21, 0.07)          |

\(^a\) Values are linear regression coefficients (95% confidence interval) and reflect the change in blood pressure and heart rate at 6 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

\(^b\) Values are linear regression coefficients (95% confidence interval) and reflect the change in blood pressure and heart rate at 10 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

\(^c\) Values are linear regression coefficients (95% confidence interval) and reflect the change in blood pressure and heart rate between 6 and 10 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

Confounder models are adjusted for child sex, child age at cortisol assessment, child age at assessment of cardio-metabolic outcomes, maternal pre-pregnancy BMI, maternal education, family income, child ethnicity, television watching time and hair color.

\(\*p< 0.05, **p< 0.017\)
Table 4. Association of hair cortisol quintiles at 6 years with lipids, insulin and glucose at 6 years and 10 years and with the change between 6 and 10 years, confounder models

| Hair cortisol quintiles at 6 years | Total cholesterol \(^a\) | HDL \(^a\) | LDL \(^a\) | Triglycerides \(^a\) | Insulin \(^a\) |
|----------------------------------|--------------------------|-----------|-------------|---------------------|-------------|
| Q1 (N = 1,781)                   | Reference                | Reference | Reference   | Reference           | Reference   |
| Q2 (-0.14, 0.16)                 | 0.01 (-0.14, 0.11)       | 0.08 (-0.06, 0.23) | -0.01 (-0.16, 0.14) | 0.10 (-0.05, 0.24) |
| Q3 (-0.15, 0.15)                 | 0.00 (-0.15, 0.14)       | 0.01 (-0.13, 0.16) | 0.02 (-0.13, 0.16) | -0.10 (-0.25, 0.04) |
| Q4 (-0.14, 0.04)                 | -0.01 (-0.14, 0.16)      | 0.01 (-0.13, 0.17) | 0.02 (-0.13, 0.17) | 0.13 (-0.02, 0.27) |
| Q5 (-0.25, 0.05)                 | -0.04 (-0.19, 0.10)      | -0.03 (-0.18, 0.12) | -0.04 (-0.19, 0.11) |           |

Metabolic risk factors at 6 years

| Hair cortisol quintiles at 6 years | Total cholesterol \(^b\) | HDL \(^b\) | LDL \(^b\) | Triglycerides \(^b\) | Insulin \(^b\) | Glucose \(^b\) |
|----------------------------------|--------------------------|-----------|-------------|---------------------|-------------|-------------|
| Q1 (N = 1,388)                   | Reference                | Reference | Reference   | Reference           | Reference   | Reference   |
| Q2 (-0.11, 0.22)                 | 0.05 (-0.11, 0.20)       | 0.02 (-0.14, 0.18) | 0.01 (-0.15, 0.18) | -0.03 (-0.20, 0.14) | -0.12 (-0.28, 0.05) |
| Q3 (-0.19, 0.13)                 | -0.03 (-0.19, 0.13)      | 0.05 (-0.11, 0.21) | -0.07 (-0.24, 0.09) | 0.08 (-0.09, 0.26) | 0.06 (-0.11, 0.22) |
| Q4 (-0.16, 0.15)                 | -0.01 (-0.16, 0.15)      | 0.02 (-0.14, 0.18) | -0.06 (-0.23, 0.10) | 0.05 (-0.12, 0.22) | -0.05 (-0.21, 0.12) |
| Q5 (-0.27, 0.05)                 | 0.11 (-0.06, 0.27)       | -0.05 (-0.22, 0.12) | 0.03 (-0.15, 0.20) | -0.02 (-0.18, 0.15) |           |

Metabolic risk factors change between 6 and 10 years

| Hair cortisol quintiles at 6 years | Change in total cholesterol \(^c\) | Change in HDL \(^c\) | Change in LDL \(^c\) | Change in Triglycerides \(^c\) | Change in insulin \(^c\) |
|----------------------------------|--------------------------|-----------|-------------|---------------------|-------------|
| Q1 (N = 1,051)                   | Reference                | Reference | Reference   | Reference           | Reference   |
| Q2 (-0.05, 0.23)                 | 0.09 (-0.05, 0.23)       | -0.01 (-0.15, 0.13) | 0.08 (-0.14, 0.30) | -0.14 (-0.39, 0.10) |
| Q3 (-0.05, 0.25)                 | -0.10 (-0.05, 0.25)      | 0.06 (-0.08, 0.20) | -0.01 (-0.24, 0.21) | 0.15 (-0.10, 0.40) |
| Q4 (-0.05, 0.26)                 | 0.12 (-0.03, 0.26)       | 0.06 (-0.09, 0.20) | -0.10 (-0.32, 0.13) | -0.08 (-0.33, 0.17) |
| Q5 (0.34)**                      | 0.15 (0.00, 0.29)**      | 0.04 (-0.18, 0.27) | 0.08 (-0.16, 0.33) |           |

\(^a\) Values are linear regression coefficients (95% confidence interval) and reflect the change in lipids and insulin concentrations at 6 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

\(^b\) Values are linear regression coefficients (95% confidence interval) and reflect the change in lipids, insulin and glucose concentrations at 10 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

\(^c\) Values are linear regression coefficients (95% confidence interval) and reflect the change in the delta of lipids and insulin concentrations between 6 and 10 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

Confounder models are adjusted for child sex, child age at cortisol assessment, child age at assessment of cardio-metabolic outcomes, maternal pre-pregnancy BMI, maternal education, family income, child ethnicity, television watching time and hair color. *p< 0.05, **p< 0.017
Table 5. Association of hair cortisol quintiles at 6 years with risk of increased C-reactive protein and risk of cardio-metabolic clustering at 6 and 10 years, confounder models

| Hair cortisol quintiles at 6 years | Risk of C-reactive protein ≥ 3 mg/l \(^a\) (N = 1,784) | Odds Ratio (95% CI) for outcomes at 6 years | Risk of cardio-metabolic clustering \(^b\) (N = 2,022) |
|-----------------------------------|---------------------------------------------------|-------------------------------------------|---------------------------------------------------|
| Q1                                | Reference                                         | Reference                                 | Reference                                         |
| Q2                                | 1.19 (0.70, 2.01)                                 | 1.39 (0.86, 2.27)                         |
| Q3                                | 1.69 (1.04, 2.77)*                                 | 1.32 (0.81, 2.14)                         |
| Q4                                | 0.98 (0.57, 1.68)                                 | 1.21 (0.74, 1.98)                         |
| Q5                                | 1.54 (0.94, 2.54)                                 | 1.29 (0.79, 2.11)                         |

| Hair cortisol quintiles at 6 years | Risk of C-reactive protein ≥ 3 mg/l \(^a\) (N = 1,389) | Odds Ratio (95% CI) for outcomes at 10 years | Risk of cardio-metabolic clustering \(^b\) (N = 1,299) |
|-----------------------------------|----------------------------------------------------|----------------------------------------------|--------------------------------------------------|
| Q1                                | Reference                                           | Reference                                     | Reference                                       |
| Q2                                | 1.34 (0.59, 3.02)                                  | 1.34 (0.75, 2.39)                           |
| Q3                                | 1.14 (0.50, 2.61)                                  | 1.50 (0.86, 2.64)                           |
| Q4                                | 1.76 (0.82, 3.79)                                  | 1.00 (0.55, 1.82)                           |
| Q5                                | 1.91 (0.88, 4.13)                                  | 1.32 (0.74, 2.35)                           |

\(^a\) Values are odds ratios (95% confidence interval) and represent the risk of childhood high C-reactive protein concentrations (≥ 3 mg/l) at 6 and 10 years for the cortisol quintiles compared to the first quintile.

\(^b\) Values are odds ratios (95% confidence interval) and reflect the odds of cardio-metabolic clustering at 6 and 10 years defined as having three or more out of the following four adverse risk factors: android fat mass percentage above the seventy-fifth percentile; systolic or diastolic blood pressure above the seventy-fifth percentile; HDL cholesterol below the twenty-fifth percentile or triglycerides above the seventy-fifth percentile; and insulin above the seventy-fifth percentile of our study population for the cortisol quintiles compared to the first quintile.

Confounder models are adjusted for child sex, child age at cortisol assessment, child age at assessment of cardio-metabolic outcomes, maternal pre-pregnancy BMI, maternal education, family income, child ethnicity, television watching time and hair color.

*\(p< 0.05\), **\(p< 0.017\)