Early-life Stress and Childhood Cardio-metabolic Health

Florianne Olga Lucia Vehmeijer

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1

General introduction

GENERAL INTRODUCTION

Early-life stress and cardio-metabolic health

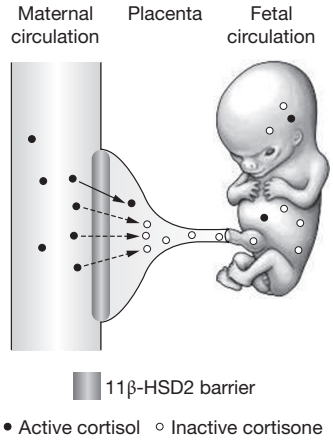
Stress is associated with cardio-metabolic disease in adults.[1, 2] Stress-related disorders lead to an increased risk of multiple types of cardiovascular disease, such as hypertensive diseases, adiposity and heart failure.[3] Also, chronic work stress is associated with development of metabolic syndrome.[4] This seems important since the prevalence of obesity and related cardio-metabolic risk factors, such as hypertension and diabetes, has increased dramatically over the past decades in children and adults.[5-8] Long-term exposure to elevated concentrations of cortisol, also known as the most important stress hormone, has a deleterious effect on the function of cardiovascular and metabolic systems.[1, 2, 9] Exposure to chronic stress in utero and childhood may affect cardio-metabolic health across the life course. Adverse exposures in fetal life and infancy lead to structural and functional developmental adaptations, which predispose individuals to cardio-metabolic diseases in later life.[10, 11] This *Developmental Origins of Health and Disease* concept has been widely accepted and led to a novel approach to identify early life causes of cardio-metabolic diseases.[11, 12]

The main hypothesis for this thesis is that the associations of chronic stress with adverse cardio-metabolic outcomes originate in early life. In 1993, Edwards and colleagues hypothesized that increased early-life exposure to glucocorticoids might be an important biological mechanism linking adverse exposures in early-life to cardiovascular outcomes.[13] Their hypothesis was supported by the finding that in rats decreased concentrations or activity of the enzyme 11 β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which acts as a placental barrier to maternal glucocorticoids, is associated with low birth weight (**Figure 1**).[13, 14] Also, increased exposure of the fetus to exogenous glucocorticoids leads to low birth weight and subsequent hypertension in the offspring.[13] The hypothalamic-pituitary-adrenal (HPA) axis, controlling the secretion of glucocorticoids, is developed during fetal life and might be susceptible to prenatal programming influence with long term cardiovascular consequences. Thus, the lifetime risk of cardiovascular disease may be partly determined by increased exposure to stress in fetal life or childhood. These associations may already be reflected by differences in cardiovascular risk factors in childhood, which tend to track into adulthood.[15-17]

Psychological and physiological stress

Stress can be defined as a threatened homeostasis and refers to the body's response to try to adapt to a perturbation, first mentioned in 1936 by Hans Selye.[9, 18] The source of the stress, the stressor, may be actual or perceived and psychological or physiological.[19] Stress increases the activity of the hypothalamic-pituitary-adrenal (HPA)-axis.[9] In response to stress, the hypothalamus releases corticotrophin releasing hormone (CRH)

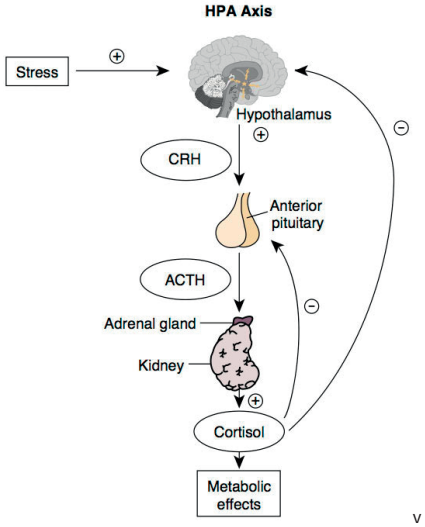
Figure 1. In the normal situation, placental 11β-HSD2 acts as a protective barrier to glucocorticoids because it rapidly inactivates active physiological glucocorticoids (e.g. cortisol) to inert 11-keto forms (e.g. cortisone). Placental glucocorticoid inactivation ensures that high maternal glucocorticoid concentrations are largely excluded from the fetus. Used from Seckl et al.[14]



into the anterior pituitary, causing the release of adrenocorticotropic hormone (ACTH) into the blood flow. ACTH stimulates the generation of glucocorticoids (e.g. cortisol) in the cortex of the adrenal gland, which are released into the blood circulation (**Figure 2**).[20] An increase of HPA-axis activity occurs within seconds after the onset of the stressor, which is followed by an increase in cortisol in several minutes.[21] The adaptive response to stress is critical for the survival of the individual. However, when the stressor becomes chronic, so does the increased HPA activity with increased cortisol concentrations as result. This can have detrimental effects on growth, development and metabolism.[9, 21, 22] Elevated cortisol concentrations, if prolonged, induces insulin resistance, glycolysis and gluconeogenesis which will result in impaired glucose tolerance. Additionally, long-term exposure to elevated cortisol concentrations tend to increase appetite, with a special preference for high-caloric foods. Moreover, cortisol stimulates accumulation of visceral fat. Altogether, chronic cortisol exposure leads to a redistribution of body fat causing truncal obesity, a precursor of the metabolic syndrome. This is a complex of interrelated risk factors including obesity, elevated blood pressure, lipids and glucose. These clinical symptoms are also seen in patients with Cushing's syndrome, caused by the endogenous overproduction of cortisol. [23] Dysregulation of the HPA axis has been associated with cardiovascular disease, insulin resistance and type 2 diabetes in adults.[24 25]

In the studies performed as part of this thesis we focused on the associations of maternal psychological stress in pregnancy and of childhood cortisol concentrations with maternal and childhood adiposity and cardio-metabolic related outcomes. Also, we assessed the role of DNA methylation changes, which might be the result of adverse stressors, in development of childhood body mass index.

Figure 2. In response to stress, the hypothalamus releases corticotrophin releasing hormone (CRH) into the anterior pituitary, causing the release of adrenocorticotropic hormone (ACTH) into the blood flow. ACTH stimulates the generation of glucocorticoids (e.g. cortisol) in the cortex of the adrenal gland, which are released into the blood circulation. Adapted from www.embryology.med.unsw.edu.au.



Maternal psychological distress during pregnancy

Maternal psychological distress during pregnancy could contribute to a suboptimal fetal environment. Psychological distress during pregnancy can be caused by life events, daily hassles, depressive feelings or feelings of anxiety. Pregnancy might be a critical period for distress because of all physical, emotional and social challenges. Psychological distress increases maternal cortisol concentrations, which in turn may lead to fetal exposure to increased concentrations of active glucocorticoids.[14, 26]

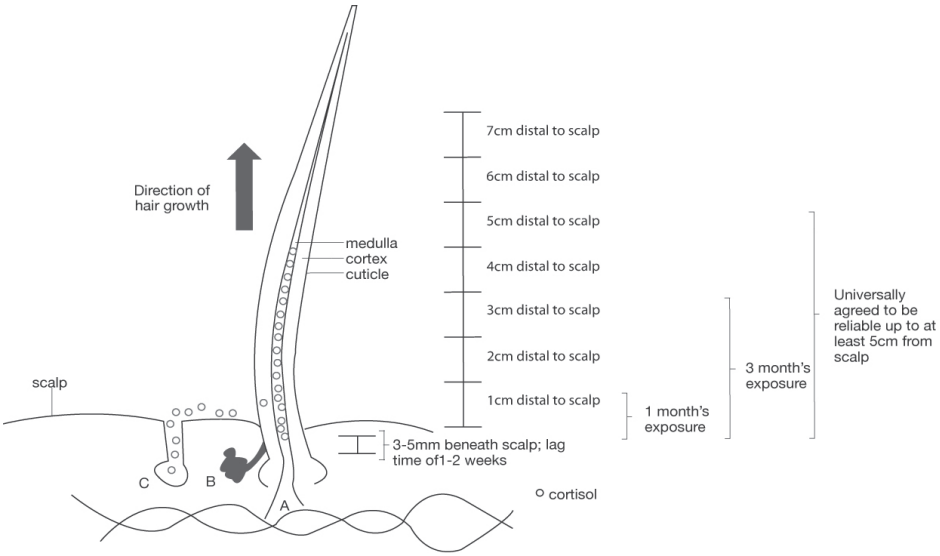
Previous studies showed that psychological distress during pregnancy was associated with adverse maternal and offspring outcomes such as increased risk of pre-eclampsia and impaired fetal growth[27, 28], measured with questionnaires. In this thesis we assessed the associations of psychological distress in pregnancy with gestational weight gain, childhood general and organ fat and cardiovascular risk factors.

Childhood hair cortisol concentrations

Childhood is often conceptualized as a stress-free time. However, chronic exposure to stressful situations is not uncommon.[19, 29, 30] Children are vulnerable to stressors and stress may have an important influence on their psychological and physical health.[29] The physiological stress response can be assessed by measuring cortisol concentrations. Circulating cortisol can be measured through saliva, blood or urine. These traditional measures are not suitable to measure chronic stress since they provide a measurement at a single point in time and show variability depending on factors such

as circadian rhythm, daily variation and acute stressors.[31] Since a few years, cortisol concentrations can be measured in hair. This method is now used as a biomarker of chronic stress.[31, 32] Scalp hair grows approximately 1cm per month, which provides the possibility to measure average cortisol concentrations of a selected period of weeks to months (**Figure 3**).[32] Since hair cortisol concentration is a proxy for the concentration of chronic stress, it may be used to study associations of chronic stress with obesity and cardio-metabolic risk factors. Thus far, studies in children had modest sample sizes, used a cross-sectional design and did not show consistent results.[33, 34 35-37] In this thesis we assessed the associations of hair cortisol concentrations with general and organ fat and cardiovascular risk factors in childhood.

Figure 3. Proposed mechanisms for incorporation of cortisol into hair via blood (A), sebum (B), and sweat (C). Used from Russell et al.[32]

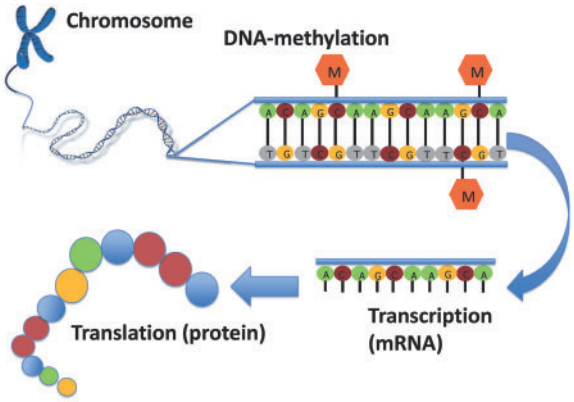


DNA methylation

DNA methylation is one of the potential mechanisms underlying the associations of adverse stressors in fetal and early postnatal life with cardio-metabolic outcomes in later life. DNA methylation is an epigenetic mechanism, which refers to mitotically heritable changes in DNA structure that do not involve changes to the underlying DNA-sequence, but can influence its function.[38] DNA methylation is the most studied epigenetic mechanism in large populations. DNA methylation occurs by the addition of a methyl-group to positions at the DNA where a cytosine is located next to a guanine, a cytosine-phosphate-guanine (CpG) site (**Figure 4**). DNA methylation is required for normal human development and is influenced by genetic and environmental factors and

stochastic events.[39 40] For example, a large study among almost 7,000 mother-child pairs found a robust association of maternal smoking in pregnancy with differential methylation in cord blood with persistence into later childhood.[40] In this thesis, we assessed the associations of haemoglobin concentrations with DNA methylation changes and of DNA methylation with childhood adiposity. Altered maternal haemoglobin is associated with adverse perinatal outcomes such as preterm delivery and intrauterine growth restriction, and thereby a potential physical stressor for the developing fetus.[41]

Figure 4. Schematic representation of DNA methylation



The figure shows a double DNA strand on the top left, with CpG sites which are methylated by the addition of a methyl group (M). DNA is transcribed into messenger RNA (mRNA). DNA methylation can influence transcription either positively or negatively, depending on the location of the methylated site. After transcription, mRNA is translated into proteins. Used from Felix JF et al.[42]

Hypotesis

The main hypothesis for this thesis was that the associations of chronic stress with adverse cardio-metabolic outcomes originate in early life. Both maternal perceived stress during pregnancy and childhood cortisol concentrations may affect maternal, fetal and childhood health.

General aim of this thesis

With this thesis we aimed to extend current knowledge on the associations of early-life stressors with cardio-metabolic outcomes.

The specific aims of this thesis can be summarized as follows:

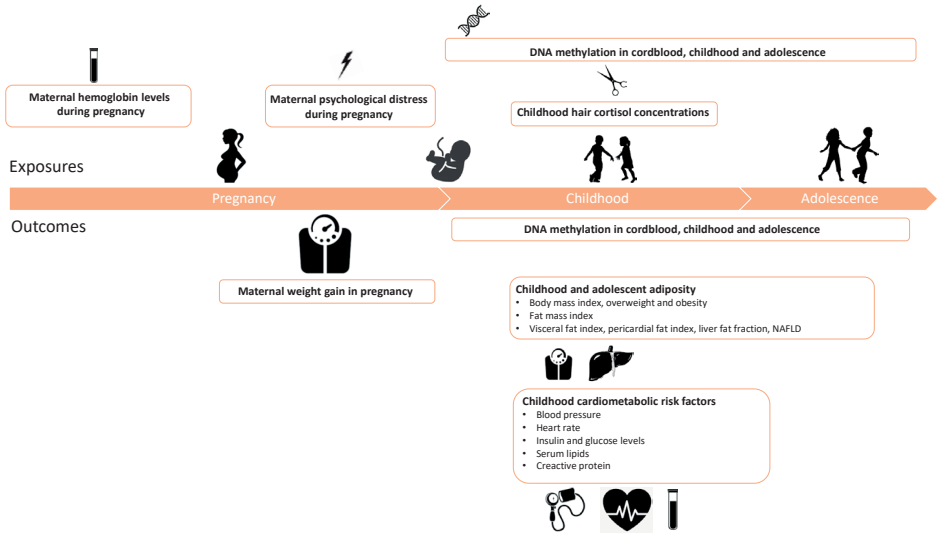
1. To assess the associations of maternal psychological stress during pregnancy with maternal and childhood cardio-metabolic outcomes
2. To assess the associations of hair cortisol concentrations in children with cardio-metabolic outcomes

3. To assess the association of haemoglobin concentrations with DNA methylation and of DNA methylation with BMI from birth to adolescence

General design

The studies presented in this thesis (**Figure 5**) were embedded in the Generation R Study, a population-based prospective cohort study and in the Pregnancy & Childhood Epigenetics Consortium (PACE).

Figure 5. Overview of the exposures and outcomes used in this thesis



The Generation R Study

The Generation R Study is a population-based prospective cohort study in Rotterdam, the Netherlands, following pregnant women and their children from fetal life onwards ("http://www.generationr.nl/").[43] In total, 9,778 pregnant women with a delivery date from April 2002 until January 2006 were enrolled in the study.[43] Enrolment was aimed to be in the first trimester, but was allowed until birth of the child. Response at baseline was 61%, and general follow-up rates until the age of 10 years were around 80%. Women visited the research centre in early (<18 weeks of gestation), mid- (18-25 weeks of gestation) and late- (> 25 weeks of gestation) pregnancy, and postnatal follow-up of mothers and children occurred at approximately six, ten and fourteen years after pregnancy. Data collection for the current thesis included questionnaires during pregnancy and postpartum to obtain information on pre-pregnancy weight, lifestyle factors and symptoms of psychological stress.[44] Detailed mother and child examinations were performed at all trimesters during pregnancy and follow-up assessments at 6 and 10 years.[43] Total

body fat was measured by dual-energy X-ray absorptiometry at 6 and 10 years and organ adiposity measures were obtained from magnetic resonance imaging at 10 years.[43] Biological samples were taken including cord- and childhood blood samples for DNA methylation, cardio-metabolic measures, and scalp hair to measure cortisol concentrations.[45]

Pregnancy And Childhood Epigenetics (PACE) Consortium

We conducted epigenome-wide association studies (EWAS) with partners collaborating in the Pregnancy And Childhood Epigenetics (PACE) Consortium.[38] The consortium brings together researchers and studies from different countries with samples and DNA methylation data in pregnant women, newborns and/or children. Joint analyses in large sample sizes leads to strongly increased power to detect associations and a decreased risk of false-positive associations. Also, a number of studies have measured DNA methylation at multiple time points from birth through childhood and/or in adolescence, which enables investigation into the persistence of differential DNA methylation signals over time.[38]

Outline of this thesis

The objectives of the studies in this thesis are presented in various chapters. **Chapter 2** focuses on the associations of maternal psychological distress with maternal and child cardio-metabolic outcomes. ***Chapter 2.1*** focuses on the influence of maternal psychological distress during pregnancy on fetal outcomes and child cardio-metabolic, respiratory, atopic and neurodevelopment-related health outcomes in a narrative review. In ***Chapter 2.2*** we examined the associations of maternal psychological distress and weight gain in pregnancy. The associations of maternal psychological distress during pregnancy with childhood general and organ fat measures are described in ***Chapter 2.3***. The associations of maternal psychological distress during pregnancy and childhood cardio-metabolic risk factors are assessed in ***Chapter 2.4***. **Chapter 3** focuses on the associations of hair cortisol concentrations with child cardio-metabolic outcomes. In ***Chapter 3.1*** we examined the associations of hair cortisol concentrations in children with childhood general and organ fat measures. The association of hair cortisol concentrations with childhood cardio-metabolic risk factors is discussed in ***Chapter 3.2***. In ***Chapter 4.1*** we examined the association between maternal haemoglobin concentrations in pregnancy and child DNA methylation. Lastly, in ***Chapter 4.2*** we examined whether DNA methylation in cord blood and whole blood in childhood and adolescence was associated with BMI in the age range from 2 to 18 years.

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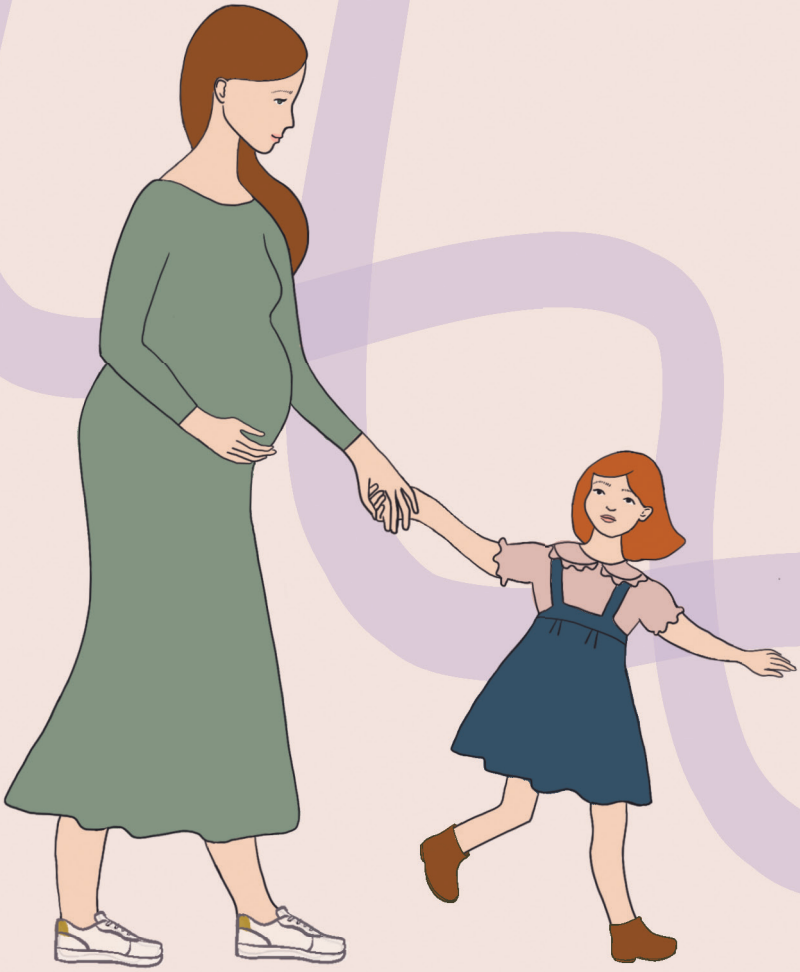

2

Maternal Psychological Distress

2.1

Maternal psychological distress during pregnancy and childhood health outcomes: a narrative review

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ABSTRACT

Maternal psychological distress is common in pregnancy and may influence the risk of adverse outcomes in children. Psychological distress may cause a suboptimal intrauterine environment leading to growth and developmental adaptations of the fetus and child. In this narrative review, we examined the influence of maternal psychological distress during pregnancy on fetal outcomes and child cardio-metabolic, respiratory, atopic and neurodevelopmental-related health outcomes. We discussed these findings from an epidemiological and life course perspective and provided recommendations for future studies. The literature in the field of maternal psychological distress and child health outcomes is extensive and shows that exposure to stress during pregnancy is associated with multiple adverse child health outcomes. Because maternal psychological distress is an important and potential modifiable factor during pregnancy, it should be a target for prevention strategies in order to optimize fetal and child health. Future studies should use innovative designs and strategies in order to address the issue of causality.

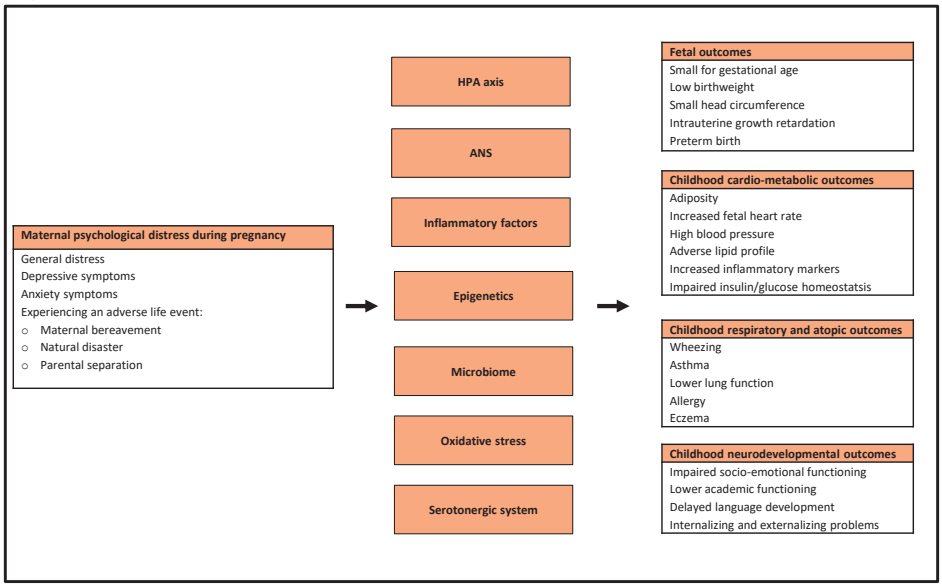
INTRODUCTION

Although pregnancy should be a period of happiness and excitement, 10-20% of pregnant women experience psychological distress.[1-5] Psychological distress can be described as general stress, depressive symptoms, anxiety or experiencing an adverse life event.[6-8] Poverty, low socioeconomic status and single status are some of the risk factors for psychological distress during pregnancy.[9] Studies suggest that maternal psychological distress during pregnancy can have adverse consequences on the development of their child.[10-17] Maternal psychological distress during pregnancy may lead to fetal and postnatal adaptations through intrauterine mechanisms.[18-20]

Experiencing psychological distress by the mother could contribute to a suboptimal fetal environment and may co-occur or interact with adverse lifestyle factors including smoking, alcohol and under- or overnutrition.[14, 21-25] The developing fetus adapts to suboptimal conditions during critical periods of pregnancy by structural and functional changes in cells, organs and tissues.[10] These developmental alterations may have long-term consequences and affect health throughout life. Optimizing maternal and pregnancy health is thus of great importance to improve wellbeing of their children throughout life.

In this narrative review, we provide an update of the findings from recent observational studies and meta-analyses focused on the associations of maternal psychological distress during pregnancy including i.e. general stress, depressive symptoms, anxiety or experiencing an adverse life event with fetal outcomes and cardio-metabolic, respiratory, allergy and neurodevelopmental outcomes throughout childhood, **Figure 1.** These health outcomes are known risk factors for noncommunicable diseases (NCDs) in later life.[26-31] We searched for relevant articles per outcome in the online databases Google Scholar and PubMed using MeSH (Medical Subject Headings) terms of the National Library of Medicine (NLM). We also discuss potential mechanisms underlying the observed associations, causality and challenges for future research. Insight in maternal psychological distress during pregnancy and adverse child outcomes is of great importance, as maternal mental health could be a modifiable target for prevention to improve both maternal and offspring wellbeing.

Figure 1. Conceptual framework of exposures, mechanisms and outcomes in the maternal psychological distress during pregnancy and fetal/child outcomes associations



Abbreviations: ANS = Autonomic Nervous System, HPA axis = Hypothalamic Pituitary Adrenal axis.

FETAL AND BIRTH OUTCOMES

Maternal psychological distress has repeatedly been related to fetal growth using ultrasound measures.[32] In an explorative study of 91 pregnant women maternal depressive symptoms during pregnancy were associated with smaller fetal head circumference in the third trimester, but anxiety symptoms were not.[33] In a case-control study, it was shown that maternal depressive and anxiety symptoms in pregnancy were related to intrauterine growth restriction of the child.[34] Further, in a study that used ultrasound measures in pregnancy, no differences in ultrasound measures at 20 and 34 weeks of gestation (including abdominal circumference, head circumference and femur length) were found, but this study did show that children exposed to maternal depression and/or anxiety had a lower mean birth weight.[35] Another case-control study examined fetal growth rates by using estimated fetal weight at 18-20 weeks and birth weight, and reported a lower estimated fetal weight in mid-gestation and slower fetal growth rate during the second half of pregnancy in fetuses exposed to maternal depressive symptoms.[36] Likewise, in the Generation R study, a prospective population-based multi-ethnic cohort study, ultrasound measures during pregnancy were collected repeatedly and growth trajectories were used in a very large sample of pregnant women. In this study, maternal psychological distress during pregnancy was associated with decreased fetal growth.[37, 38] However, studies are not always consistent. Some studies reported no

association of prenatal maternal psychological distress with fetal growth or birth outcomes. For example, in a prospective observational study prenatal maternal depression was associated with preterm birth but not with birth weight.[39] Interestingly, a meta-analyses on this topic showed that women with depression during pregnancy are at increased risk for children born preterm and born with a low birth weight, but the results should be interpreted with caution because of different magnitudes of effect, ethnicities and socioeconomic status per study.[40] Further, another meta-analysis of 15 studies on maternal anxiety during pregnancy and birth outcomes found that maternal anxiety is associated with an 1.5 increased risk of low birth weight as well as an increased risk of 1.8 of preterm birth.[41] Thus, the current literature suggests that exposure to maternal psychological stress affect fetal growth and birth outcomes, but remains inconsistent, see **Table S1**.

CHILD OUTCOMES

Cardio-metabolic outcomes

Cardio-metabolic health of children might be affected by maternal psychological distress during pregnancy. Maternal psychological distress in pregnancy has been associated with an increased risk of child's adiposity.[42] A potential pathway might be that maternal psychological distress during pregnancy is associated with low birth weight of children and subsequent catch-up growth during infancy. High levels of birth weight and catch-up growth are both strong risk factors for overweight and obesity in later life.[43] In a study among 65,212 mother-child pairs, 10-13-year old children exposed to prenatal stress, defined by being born to mothers who were bereaved by death of a close family member, had an increased risk of overweight.[44] Additionally, a prospective cohort study among 838 mothers and their children showed that depression of the mother during pregnancy was associated with adiposity but a lower body mass index (BMI) at age 3 years.[45] Several studies found that women exposed to an adverse life event, either maternal bereavement or a natural disaster, had a significantly increased risk of children with adiposity.[46-48] Furthermore, parental separation before childbirth has been associated with an increased risk of overweight and obesity at the age of 9-11 years.[49] However, large population studies did not find consistent associations between prenatal maternal stress and adiposity measurements in childhood.[50-53] For example, in the Generation R study, we observed no association between prenatal maternal depression and child BMI at several measurements between age 3 months to age 4 years.[50] Furthermore, in a large study among 37,764 Danish women and child pairs, self-reported stress, depressive or anxiety symptoms at around 30 weeks of gestation were not associated with childhood overweight at 7 years of age.[51] Interestingly, two large prospective cohort studies found

no consistent associations between antenatal maternal depression and child growth, but suggested child sex-specific effects of prenatal maternal stress.[52, 54]

Moreover, an association between maternal psychopathology during pregnancy and increased fetal heart rate has been reported.[55, 56] However, this association, along with other cardiovascular outcome factors in childhood, was not found in two large prospective cohort studies.[57, 58] Although fetal exposure to increased glucocorticoid levels have been linked to adult hypertension, studies do not find an association between maternal psychological distress and child hypertension.[20, 58-60] However, one of these studies observed a positive, non-significant association between prenatal stress and higher systolic and diastolic blood pressure in children.[60] Thus, it might be that these altered blood pressures precede hypertension in later life.

Further, both low-grade chronic inflammation and insulin resistance are known to precede type 2 diabetes.[61] A relation between maternal psychological distress and increased inflammatory markers during pregnancy, such as C-reactive protein (CRP) and interleukins, was shown.[62-64] Animal studies suggest that prenatal maternal psychological stress has lasting effects on the immune system of offspring, but little is known about this effect on human offspring.[65] Only one study reported increased interleukin-4 (IL-4) levels in children exposed to prenatal maternal anxiety.[66] Studies on prenatal maternal psychological distress in relation to child glucose metabolism are also scarce. Both in young adults and adolescents at the average age of 13.5 years, prenatal psychosocial stress exposure was positively associated with insulin secretion and resistance.[67, 68] On the contrary, in 5-6 year old children no associations of prenatal stress and glucose, C-peptide or insulin resistance were found, potentially because the relation was examined early in life.[69] Further, young adults exposed to maternal prenatal stress had higher very low density lipoprotein and lower high density lipoproteins, suggesting differences in fat storage processes.[67] To our knowledge no studies on the association between prenatal stress and lipid profile in childhood have been performed.

Most reviewed studies report that prenatal maternal distress is associated with increased risk for adverse cardio-metabolic child outcomes, see **Table S2**. However, results are often inconclusive and need further investigation.

Respiratory and atopic outcomes

Prenatal exposure to psychological distress has been linked to respiratory and atopic outcomes in children. A meta-analysis found that children whose mothers experienced psychological distress during pregnancy had higher risks of childhood wheezing, asthma, or other respiratory morbidity.[70] Thereafter, a large number of cohort studies have been published with more detailed data.[71] Some studies assessed maternal anxiety and depression symptoms during pregnancy separately,[72-81] while others examined overall psychological distress of the pregnant women, which also included hostility, somatic

problems, or poor self-esteem among others besides anxiety and depression.[75, 82-87] Mixed results were observed when maternal anxiety or depression symptoms during pregnancy were examined separately in relation to child wheezing and asthma. However, overall psychological distress during pregnancy was consistently associated with wheezing and asthma. Other studies examined maternal psychological distress during pregnancy as bereavement or adverse life events, self-reported perceived stress, psychological job strain, or community violence with respiratory outcomes and showed an association of these exposures with an increased risk of child wheezing, asthma or lower lung function.[73, 76, 78-80, 87-103]

Maternal psychological distress during pregnancy may also influence the risk of allergy and eczema in children.[93] Birth cohort studies observed no associations of maternal psychological distress during pregnancy with childhood allergic sensitization (measured with skin prick tests) or physician-diagnosed food allergy.[93, 104] However, maternal psychological distress during pregnancy were associated with an increased risk of physician-diagnosed inhalant allergy in children.[104] These results were independent of maternal psychiatric symptoms after delivery, and of paternal psychiatric symptoms during pregnancy and after delivery. Other birth cohort studies used immunoglobulin E (IgE) levels to identify allergic sensitization.[83, 93, 105-108] Some studies showed that maternal psychological distress during pregnancy was associated with increased umbilical cord blood IgE levels or serum allergen-specific IgE levels, [105-108] while other studies observed no associations.[83, 93] One birth cohort study combined data on food-specific serum IgE levels and on questionnaire obtained food allergy, and observed no association of maternal psychological distress during pregnancy with food allergy.[83, 109] Furthermore, previous birth cohort studies have reported inconsistent results on the association of children of mothers with psychological distress during pregnancy with eczema.[93, 94, 105, 106, 110-112] However, most studies showed that children who were prenatally exposed to maternal psychological distress had an up to 4-fold increased risk of eczema, compared with those unexposed. [94, 105, 111, 112]

Overall, maternal psychological distress during pregnancy appears to be associated with childhood respiratory and atopic health, see **Table S3 and S4**. However, it is unclear which specific respiratory and atopic outcome might be more affected, at what age, and what the long term effects into adulthood are.

Neurodevelopmental outcomes

A number of studies has focused on maternal psychological distress and neurodevelopmental outcomes from birth onwards. Newborns of depressed mothers show less responsiveness to stimulation.[113, 114] Children exposed to maternal psychological distress during pregnancy are more likely to show negative affectivity,[115, 116] and more excessive crying.[117] Maternal psychological distress is also significantly related to attachment

security. [118] On the cognitive domain, children exposed to psychological distress in pregnancy have less advanced or delayed language development,[119, 120] and lower academic skills.[121] Furthermore, children exposed prenatally to maternal psychological distress display altered stress reactivity in early childhood [122] and increased sleep disturbances.[123] Moreover, children exposed to maternal psychological distress during pregnancy are at increased risk to develop internalizing, such as depressive symptoms and being withdrawn.[124] Finally, externalizing problems, such as aggressive behavior and attention deficit hyperactivity disorder symptoms are also more prominent in children exposed to maternal psychological distress during pregnancy.[125-128] The underlying neurobiology of the reported associations is unclear, but evidence is accumulating. Several studies have shown that prenatal exposure to maternal depressive symptoms is related to differences in volumes and white matter microstructure of the limbic system in preschoolers and children aged 6 and 10 years.[129-132] Additionally, prenatal maternal depression has been shown to alter the functional connectivity of the amygdala in early postnatal life.[131] Further, using an exploratory approach rather than only the limbic system exposure to maternal depressive symptoms in pregnancy has been linked to cortical thinning of the frontal and temporal cortex of the brain in three recent studies.[133-135] However, other studies found no or even positive associations of prenatal maternal psychological distress and child neurodevelopmental outcomes (e.g. school achievement, motor and language development).[136-138] and thus these results must be interpreted with caution.
Finally, overall there is a consensus that maternal psychological distress is associated with developmental problems in children and adolescents, including impaired socio-emotional, cognitive and behavioral functioning, however the underlying neurobiology needs further examination, also see **Table S5**.[139-141]

UNDERLYING MECHANISMS

The pathways through which maternal psychological distress during pregnancy may lead to adverse fetal and child outcomes are various and multifactorial, **Figure 1**.[41] One of the most described underlying mechanism includes fetal exposure to increased cortisol levels due to altered activation of the maternal hypothalamic pituitary adrenal (HPA) axis in response to both physiological and psychological distress.[142 146] Fetal exposure to excess cortisol may lead to altered programming of the fetal HPA axis, which in turn may be associated with adverse birth outcomes and could have long term child health consequences.[19, 142-150] A linear relation was shown between fetal and maternal cortisol concentrations.[151] Cortisol stimulates corticotropin releasing hormone (CRH) secretion and production, resulting in positive maternal and fetal feedback loops and increased cortisol concentrations, potentially further amplifying the effect.[152-154] Subsequently,

the uteroplacental blood flow may be reduced in response to increased secretion of maternal cortisol and catecholamines,[155] which may lead to fetal growth restriction and other adverse child health outcomes.[152, 153] Animal studies on the association between excess catecholamines and fetal or child outcomes are conflicting,[156-158] and human studies are scarce. Only one study reported high maternal catecholamine concentration to be associated with an increased risk of spontaneous preterm birth.[159] However, studies are inconsistent about the relation between maternal psychological distress during pregnancy and maternal cortisol levels which needs further investigation.[160-163] A related mechanism is the functioning of 11ß-hydroxysteroid dehydrogenase type 2 (11ß-HSD-2), a placental enzyme that inactivates 50-90% of maternal cortisol.[164, 165] Reduction of 11ß-HSD-2 levels expose the fetus to higher levels of maternal cortisol. Clinical studies showed reduced placental 11ß-HSD-2 gene expression in pregnancies with intrauterine growth restriction and preterm birth.[166, 167] Also, both clinical and animal studies have shown reduced gene expression and/or activity of placental 11ß-HSD-2 in association with maternal anxiety.[166, 167]

Next to the HPA-axis as an underlying mechanism, the autonomic nervous system (ANS) is another physiological mechanism by which organisms react to stress.[168] The ANS is responsible for regulation of several processes in the body such as respiration, digestion, body temperature and metabolism.[169] In contrast to the role of the HPA-axis, the ANS as a possible mechanism is less extensively examined. However, ANS functioning has been described as a mediator in the association of prenatal maternal psychological distress and child health outcomes. For example, heart rate (HR) and heart rate variability (HRV) are regulated through ANS functioning and have been studied in relation to maternal prenatal distress.[144] Maternal depression and anxiety have been associated with both a higher and a reduced baseline fetal heart rate.[170-173] Results on the ANS are not conclusive and need clarification.

Further, another possible mechanism underlying maternal stress during pregnancy and adverse child outcomes are epigenetic changes.[174] Epigenetic changes are chemical modifications to chromatin that regulate genomic transcription.[175] Studies showed that prenatal psychological distress could lead to changes in DNA methylation which subsequently may have a mediating effect on the association between prenatal maternal psychological distress and child health outcomes.[175-183] Epigenetic changes of DNA methylation of the glucocorticoid receptor gene NR3C1 in cord blood and infant salivary samples are associated with maternal stress and could be responsible for the increase of the sensitivity of the fetal HPA axis.[179, 184]

Another potential mechanism involves inflammatory responses to psychological distress during pregnancy.[185] In animal studies, prenatal maternal stress has been shown to program postnatal immunity.[186] Interestingly, higher circulating levels of pro-inflammatory cytokines in pregnant women with psychological distress or depression have been

reported.[62, 187] Further, elevations of inflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) in maternal serum, are associated with increased risk of fetal outcomes such as prematurity.[188]

Additionally, the potential role of the maternal microbiome as a mechanism through which maternal psychological distress during pregnancy can affect the fetus and children should be considered.[189, 190] The microbiome could be defined as the community of microorganisms living in or on the human body.[191] Studies found that the gut microbiome is essential for early development.[192, 193] The HPA axis and the central nervous system regulatory areas affect the gut microbiota composition, which may influence the pathogenesis of diseases.[189] The bidirectional signaling pathways between the gut and brain, the so-called gut-brain axis, have been suggested to underlie neurodevelopmental and psychiatric disorders.[189, 193] Development of the gut microbiome, begins when the infant encounters the maternal vaginal and fecal bacteria during birth.[194] Studies examining the association of maternal psychological distress during pregnancy and child microbiome are scarce. One recent study found an association between maternal stress and the infant intestinal microbiome.[195]

Recently proposed mechanisms are exposure to oxidative stress and serotonergic system as well. Excessive oxidative stress exposure during human pregnancy has been associated with spontaneous abortion, intrauterine growth restriction (IUGR), preterm delivery and allergic diseases in the offspring.[147, 196-199] Evidence, especially in humans, for the involvement of serotonin and tryptophan in the maternal-fetal stress transfer is limited. Animal studies, however, provide preliminary evidence of a potential role for serotonin and tryptophan in fetal programming.[147] Additionally, meta-analyses have shown that women with antenatal depression treated with selective serotonin reuptake inhibitors (SSRIs), medications that increase serotonin levels by blocking reuptake, have a significantly increased risk for preterm birth and low birth weight, [200, 201] and thus support the hypothesis of involvement of the serotonergic system.

In conclusion, the most described underlying mechanism of the association between maternal psychological distress and adverse outcomes in the offspring includes altered HPA-axis functioning but there is emerging evidence that other mechanisms also play a role.

LIMITATIONS

This review suggests that exposure to maternal psychological distress during pregnancy may be associated with long-term health and developmental outcomes from fetal life onwards. Nevertheless, there are several limitations that have to be considered. One of the difficulties with studies on maternal psychological distress during pregnancy

and child outcomes is that (pregnancy-related) psychological distress is a very broad concept of which no specific scales are available.[202] Maternal psychological distress during pregnancy refers to the mental or emotional strain resulting from adverse circumstances that pregnant women experience. Maternal psychological distress during pregnancy can be chronic or acute. Maternal psychological distress during pregnancy can be categorized in two types; objective stress (e.g. lack of food and water following a natural disaster) and subjective distress (i.e. an individual's response to an event). There are many ways to assess psychological distress ranging from questionnaires, like the perceived stress scale,[203] screening tools of psychopathology including depression and anxiety (e.g. Hospital Anxiety and Depression Scale (HADS), State-Trait Anxiety Inventory (STAI), Edinburgh Postnatal Depression Scale (EPDS), Beck's Depression Inventory (BDI) or the Center for Epidemiological Studies-Depression Scale (CES-D), experiencing adverse life events, and exposure to natural disasters.[204] Existing psychological distress scales differ and are not pregnancy-specific.[202, 205] Moreover, some scales include somatic symptoms experienced by many pregnant women, such as nausea and vomiting, which could potentially lead to overestimation of the number of women with psychological distress.[204]

It is possible to assess a number of biological markers in response to stress, including cortisol, adrenalin or cardiovascular measures such as heart rate, blood pressure.[206] Combining psychological distress with biological stress markers will be useful approaches to define psychological distress during pregnancy. For example, studies report associations between maternal cortisol during pregnancy and adverse child outcomes, but interpretation is difficult as study designs and cortisol assessment methods differ.

Further, because studies had different study designs; sample sizes and different age groups, we should interpret the results with caution. The timing and degree of both exposure and outcome also need to be considered. Often studies have only assessed psychological distress once during pregnancy and no assessment before or after pregnancy are available, which makes interpretation and comparison of findings difficult. Studies are inconsistent with regard to the gestational age at which the effects of psychological distress during pregnancy are most pronounced.[16, 151]

Finally, bias due to confounding can never be excluded in cohort studies. In the general population maternal psychological distress often co-occurs with risky behavior such as alcohol consumption, unhealthy diet and tobacco smoking, but also with poverty and separation.[207-211] Psychological distress during pregnancy contributes to adverse maternal health behavior.[23-25, 212, 213] If psychological distress continues to be present after birth, this risk behavior may still affect the development of the child through, for example, parenting and dietary habits.[214-217] Most reviewed studies take into account available confounders, however residual confounding cannot be ruled out.

RECOMMENDATIONS FOR FUTURE RESEARCH

This review shows that the literature on maternal psychological distress during pregnancy and offspring health outcomes is accumulating. **Table 1** shows that children exposed to maternal psychological distress are more likely to have a lower birthweight and increased risk for asthma, however evidence is insufficient for several cardio-metabolic and neurodevelopmental outcomes, see **Table 1**. Although many of the studies were longitudinal, had a relatively large sample size and took many confounding factors into account, the question whether these associations are causal or are confounded by related genetic and environmental factors remains not fully clear. It is important to know the causal nature of the associations to develop evidence-based guidelines for effective intervention and prevention programs. To address the issue of causality and to move forward in the field, we need innovative designs and strategies. A comparison of the effects of maternal and paternal depressive symptoms during pregnancy could uncover potential causal relationships between intrauterine and extrauterine exposures and offspring health.[218] An intrauterine causal relationship should be detectable by a stronger association of maternal than paternal depressive symptoms during pregnancy with health outcomes in children. An equal association of maternal and paternal depressive symptoms during pregnancy with health outcomes might be explained by residual confounding of unmeasured factors. This approach has been used in a few recent studies,[74, 75, 85, 87, 130, 134] but should be utilized more often. For example, in four studies with wheezing and asthma as an outcome, the investigators reported an association between maternal anxiety symptoms or overall psychological distress during pregnancy and wheezing or asthma, while no association was observed of paternal psychological distress during pregnancy with child respiratory morbidity.[74, 75, 85, 87] However, assortative mating for psychiatric disorders - the tendency for women with psychological disorder to pair with men with psychological disorder and vice versa - is well known and a possible limitation.[219, 220]

Another possibility would be to use Mendelian randomization, a method that uses genetic variants as instrumental variables. These genetic variants are robustly associated with the exposure [221], and generate more reliable evidence regarding the causal relationship of maternal psychological distress during pregnancy and child health outcomes. Mendelian randomization relies on the assumption that any association between the genetic instrument(s) and the health outcome is entirely mediated by the exposure (i.e., vertical pleiotropy).[222, 223] However, the polygenic nature of complex traits, such as psychological distress, increases the probability of existing biological links between exposure-associated variants and the outcome not mediated by the exposure itself (i.e., horizontal pleiotropy).[222, 223]

Table 1. Summary findings of discussed literature

	Maternal psychological distress*
Fetal outcomes	
Low birth weight	++
Small head circumference	+
Intrauterine growth retardation	+
Preterm birth	++
Child cardio-metabolic outcomes	
Adiposity	++
Fetal heart rate	+/-
High blood pressure	+/-
Adverse lipid profile	-
Increased inflammatory markers	+/-
Impaired insulin/growth homeostasis	+/-
Child respiratory and atopic outcomes	
Wheezing	++
Asthma	++
Lower lung function	+
Allergy	+/-
Eczema	+
Child neurodevelopmental outcomes	
Lower academic functioning	+/-
Delayed language development	+/-
Internalizing and externalizing problems	+

**defined as general stress, depression, anxiety or experiencing an adverse life event during pregnancy*
Quality of evidence:
++ Good evidence for an association based on consistent results from multiple studies and meta-analyses
+ Moderate evidence for an association based on multiple studies, but with some inconsistencies
+/- Insufficient evidence for an association based on only a few studies, or with substantial inconsistencies
- No or very few studies on the association

Further, little is known about the role of developmental timing - the idea that certain periods in fetal and child development exist when the child is particularly vulnerable to the impact of maternal psychological distress. A strong correlation between prenatal and postpartum maternal depression exists, which may have different effects on child development.[45, 139, 224] Large longitudinal studies, preferably starting during preconception, with repeated measures of psychological distress and repeated measures of child health and development are needed.

Randomized controlled trials (RCTs) of prenatal stress, which allow total control of the type, severity, and timing of the stressor in utero is unethical. However, RCTs of (non-pharmacological) treatment of prenatal psychological distress may give insight on the causal nature of psychological distress during pregnancy and child health outcomes. A

recent randomized controlled trial showed that weekly exercise sessions during pregnancy reduce the level of depressive symptoms.[225] The next step in such studies should focus on longterm follow-up of child health. Another type of design is studying women who experience natural disasters during pregnancy, as this may approximate the random assignment similar to randomization in animal studies. Several of these studies are ongoing and show an increased risk for adverse child outcomes for women exposed to a natural disaster, for example the Project Ice Storm, Iowa Flood Study and Queensland Flood Study.[226] However, such studies have logistical challenges to face when initiating a study of pregnant women in the immediate aftermath of a natural disaster.

Another interesting design is adopted from prenatal cross-fostering animal studies, which allow the maternally provided prenatal environment and inherited factors to be disentangled. Previously, it has not been possible to study human children whose prenatal environment is provided by a biologically unrelated mother. This is now feasible, because of increased use of in vitro fertilization (IVF) as a mean of conception. Children conceived via IVF can be related to both parents (homologous IVF), the mother only (IVF with sperm donation), the father only (IVF with egg donation) or to neither parent (IVF with embryo donation). With egg and embryo donation, the mother provides the intrauterine environment but is not related to the child.[227] These alternative designs can be useful in addressing the question of causality and mechanisms in the association of psychological distress during pregnancy and offspring health, although sample size might be an issue.[227, 228]

Finally, the combination of human studies together with animal studies could provide more insight in underlying mechanisms. Such studies could focus on a variety of aspects like epigenetic changes. Summarized, to address the issue of causality and to move forward in the field, innovative designs and strategies can be useful. However, these designs all have their own strengths and limitations, which must be kept in mind.

CONCLUSION

In this narrative review, we described that in many studies maternal psychological distress during pregnancy is associated with an increased risk for adverse fetal and child health outcomes. The most described underlying mechanism of these associations is fetal exposure to increased maternal cortisol levels, but there is emerging evidence that other mechanisms involving the maternal microbiome, epigenetics and inflammatory factors may also play a role. More detailed population-based prospective cohort studies, as well as intervention randomized controlled trials and Mendelian randomization studies, are needed to further investigate the causal effect of maternal psychological distress during pregnancy on fetal and child outcomes. Maternal mental health during

pregnancy could be a modifiable target for prevention strategies in order to improve both maternal and child wellbeing.

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SUPPLEMENTARY MATERIAL

Table S1. Summary of literature on maternal psychological distress and fetal outcomes

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Fetal outcomes *	Direction of the observed associations
Diego et al. 2009	USA	Case-control	80	Fetal period Birth	Diagnosis of depression (SCID-D and CES-D)	Fetal growth Prematurity Birth weight	Fetal growth ↓ Risk of prematurity ↑ Birth weight ↓
Ding et al. 2014	-	Meta-analysis	12 studies included (total N > 17,000)	Birth outcomes	Different measures of anxiety #	Prematurity Birth weight	Risk of prematurity ↑ Birth weight ↓
Grote et al. 2009	-	Meta-analysis	29 studies included (total N > 40,000)	Birth outcomes	Different measures of depression ##	Fetal growth Prematurity Birth weight	Fetal growth — Risk of prematurity ↑ Birth weight ↓
Henrichs et al 2010	The Netherlands	Population-based prospective	6,313	Fetal period	Psychological distress, depression, anxiety (BSI) Family stress (FAD)	Fetal growth Fetal head and abdominal growth Birth weight	Only anxiety: fetal growth ↓ Only anxiety: birth weight ↓ Fetal weight gain ↓ Fetal head and abdominal growth ↓
Hompes et al. 2012	Belgium	Prospective	91	Fetal period Birth	Anxiety (HADS and PRAQ), depression (EDS and HADS)	Fetal growth Birth weight PI	Fetal growth ↓ Birth weight — BMI and PI at birth ↓
Maina et al.2008	Italy	Case-control	80	Fetal period Birth	Psychiatric disorder (MINI-Plus), stressful life events, depression and anxiety (HRS)	Fetal growth Birth weight Prematurity	Fetal growth — Risk of prematurity — Birth weight ↓
El Marroun et al. 2012	The Netherlands	Population-based prospective	7,696	Fetal period	Depression (BSI)	Fetal body growth Fetal head growth Prematurity	Fetal body growth ↓ Fetal head growth ↓ Risk of prematurity —

Table S1. Summary of literature on maternal psychological distress and fetal outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Fetal outcomes *	Direction of the observed associations
Lewis et al. 2016	-	Systematic review	9 studies included (total N > 7,000)	Fetal period	Different measures of depression, anxiety and stress	Different measurements of fetal growth	Fetal growth ↓
Uguz et al.2011	Turkey	Case-control	148	Fetal period	Diagnosis ### and level of depression and anxiety (BDI and BAI)	Fetal growth	Fetal growth ↓
Wisner et al. 2009	USA	Prospective	238	Fetal period Birth	Diagnosis of depression (SCID)	Prematurity Birth weight	Risk of prematurity ↑ Birth weight —

* = Outcomes were measured by fetal ultrasonography or were obtained from clinical records.

↑ = positive direction of the observed association

↓ = negative direction of the observed association

— = no association observed

Abbreviations: BAI = Beck Anxiety Inventory, BDI = Beck Depression Inventory, BSI = Brief Symptom Inventory, CES-D = The Centre for Epidemiological Studies-Depression scale, EDS = Edinburgh Depression Scale, FAD = Family Assessment Device, HADS = Hospitality Anxiety and Depression Scale, HRS = Hamilton Rating Score for Depression and Anxiety, MINI-Plus = Mini International Neuropsychiatric Interview Plus , PI = Ponderal Index, PRAQ = Pregnancy-Related Anxiety Questionnaire, SCID-D = Structured Clinical Interview for DSM-IV Axis I Disorders, # = Anxiety symptoms or anxiety disorder assessed in all pregnant women by means of self-reported questionnaire or structures psychiatric interview, ## = consistent with the Diagnostic and Statistical Manual of Mental Disorders (Third Edition) or later criteria, ### = The Structured Clinical Interview for the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition.

Table S2. Summary of literature on maternal psychological distress and child cardio-metabolic outcomes

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed associations
Allister et al.	USA	Case control	20	Fetal period	Depression (BDI)	Fetal heart rate (FHR)	Baseline FHR ↑
Dancause et al. 2012	Canada	Prospective	111	5.5	Objective and subjective stress (PNMS)*	Risk of obesity	Risk of obesity ↑ (objective stress)
Dancause et al. 2013	Canada	Prospective	32	13.5	Objective and subjective stress (PNMS)*	BMI, Body fat percentage, fasting and stimulated glucose and insulin, insulin secretion	BMI ↑ (objective stress) BMI z-score ↑(objective stress) Insulin secretion ↑ (objective stress) No associations with subjective stress
Dancause et al. 2015	USA	Prospective	106	2.5-4	Objective (IF100) and subjective distress (IES-R) **	BMI, SS, TR, SS + TR, SS:TR ratio	BMI — Total adiposity ↑ Central adiposity —
Van Dijk et al. 2012	The Netherlands	Prospective longitudinal	2,939	5	Job strain (JCQ)	BMI, waist-to-height ratio (WHtR), fat mass index (FMI)	BMI — WHtR — FMI —
Van Dijk et al. 2012	The Netherlands	Prospective longitudinal	2,624	5-6	Cumulative stress score of five stress scales #	Heart rate (HR), Pre-ejection period (PEP), Respiratory sinus arrhythmia (RSA), Cardiac Autonomic Balance (CAB)	HR — PEP — RSA — CAB —
Van Dijk et al. 2012	The Netherlands	Prospective longitudinal	2,968	5-7	Cumulative stress score of five stress scales #	Blood pressure (BP) Risk for hypertension ##	Systolic BP ↑ Diastolic BP↑ Mean arterial pressure ↑
Van Dijk et al. 2014	The Netherlands	Prospective longitudinal	1,952	5-6	Cumulative stress score of five stress scales #	Fasting glucose, C-peptide, insulin resistance (HOMA-IR)	Fasting glucose — C-peptide — HOMA-IR —

Table S2. Summary of literature on maternal psychological distress and child cardio-metabolic outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed associations
Ertel et al. 2010	USA	Prospective	838	3	Depression (EPDS)	BMI, SS, TR, SS + TR, SS:TR ratio	BMI z-score ↓ Total adiposity — Central adiposity ↑
Guxens et al. 2013	The Netherlands	Population-based prospective	5,238	0,3-4	Psychological distress (BSI) and family stress (FAD)	Weight, height BMI z-scores Risk of overweight	Weight, height — BMI — Risk of overweight —
Hohwü et al. 2015	Denmark	Prospective	2,876	9-11	Self-reported information on parental separation before child birth	BMI Risk of overweight	BMI↑ Risk of overweight ↑
Ingstrup et al. 2012	Denmark	Prospective	37,764	5-8	Stress (GHQ-60), depression or anxiety (SCL-92)	Risk of overweight	Risk of overweight —
Li et al. 2010	Denmark	Prospective	65,212	7-13	Bereavement one year before birth (questionnaire)	BMI Risk of overweight (different ages)	BMI — (before 10 years) Risk of overweight — (before 10 years) BMI ↑ (after 10 years) Risk of overweight ↑ (after 10 years)
Milgrom et al. 2012	Australia	Systematic review	2 studies	0,5 – 12	Depression (EPDS)	BMI, SS, TR, SS + TR, SS:TR ratio	Inconclusive findings
Monk et al. 2000	USA	Case control	17	Fetal period	Anxiety (STPI)	Fetal heart rate	FHR ↑
O'Connor et al. 2013	USA	Prospective longitudinal	1,209	0,2 – 0,5 (2 and 6 months)	Anxiety (PSWQ STAI, SCI for DSM-IV)	Cell-mediated immune response for interferon (IFN)-γ, Interleukin(IL)-2, and IL-4 responder cell frequencies	No effect at 2 months IFN-γ ↓ (at 6 months) IL-4 ↑ (at 6 months) IL-2 — (at 6 months)

Table S2. Summary of literature on maternal psychological distress and child cardio-metabolic outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed associations
Park et al. 2018	USA	Population-based	4,394	0-3	Depression diagnosis (derived from SPARCS)	Weight and height for age, BMI	Weight and height for age ↓ (boys) BMI — (boys) Weight and height for age — (girls) BMI — (girls)
Taal et al. 2013	The Netherlands	Population-based prospective	4,831	6	Psychological distress (BSI)	Blood pressure (BP), carotid –femoral pulse wave velocity, cardiac structures, fractional shortening	BP — Carotid-femoral pulse wave vel. — Left ventricular mass ↓ Other cardiac structures — Fractional shortening —
Wu et al. 2018	Mexico	Prospective	424	4-6	Composite stress index of four stress scales ***	Weight, height, fat mass, percentage body fat, waist circumference, BMI z-score	BMI ↓ Body fat mass ↓ stronger Percentage body fat ↓ for girls Waist circumference ↓

* = Outcomes were measured from (cord) blood samples, vibroacoustic stimulation, electro cardiograms, impedance cardiograms, echocardiographic measurements, air displacement plethysmography, bioelectrical impedance analysis, automatic oscillometric method for blood pressure, assessment of height, weight, waist circumference and using a Holtain caliper for subscapular and triceps skinfold, questionnaires and clinical records.

↑ = positive direction of the observed association

↓ = negative direction of the observed association

— = no association observed

Abbreviations: BDI = Beck Depression Inventory, BMI = Body Mass Index, EPDS = Edinburgh Postnatal Depression Scale, GHQ-60 = General Health Questionnaire 60, IES-R = Impact of Event Scale-Revised: ** objective stress resulting from a natural disaster (flood) was assessed with questions specific to the flood and subjective stress was assessed using a questionnaire on women's psychological reaction, IF-100 = Iowa Flood 100, PNMS = prenatal maternal stress: * objective PNMS resulting from a natural disaster (storm) was assessed with questions specific to the storm and subjective PNMS was assessed using a validated French version of the Impact of Event Scale Revised, PSWQ = Penn State Worry Questionnaire, SCI = Structured Clinical Inventory for Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV), SCL-92 = Symptoms Checklist-92, SPARCS = Statewide Planning and Research Cooperative System, a statewide reporting system for discharge data, SS = subscapular skinfold thickness, TR = triceps skinfold thickness, SS + TR as a measure of total adiposity, SS:TR ratio as a measure for central adiposity, STAI = State-Trait Anxiety Inventory, STPI = State Trait Personality Inventory , *** = Composite index made of Exposure to Violence questionnaire and The Crisis in Family Systems-Revised survey and EPDS and STAI = State-Trait Anxiety Inventory, # = Maternal cumulative stress score made of STAI and CES-D = Center for Epidemiological Studies Depression Scale and PRAQ = Pregnancy Related Anxieties Questionnaire and PDH = Parenting Daily Hassles and JCQ = Job Content Questionnaire, ## = using guidelines from the Fourth Report on the Diagnosis, Evaluation, and Treatment of High Blood Pressure in Children and Adolescents.

Table S3. Summary of literature on maternal psychological distress and child atopic outcomes

	Country	Study Design	Sample size	Age of children (years)	Maternal exposure and assessment during pregnancy	Child outcomes *	Direction of the observed associations
Chang et al. 2016	Korea	Prospective	973 / 1,531	0.5 -5	Depression (CESD), anxiety (STAI-T), distress (K6)	Atopic dermatitis (eczema)	Eczema ↑
Elbert et al. 2016	The Netherlands	Population-based prospective	5,205	0 - 10	Depression, anxiety, overall stress (BSI)	Eczema Allergic sensitization (for inhalant and food allergens) Allergy (inhalant, food)	Eczema ↑ Allergic sensitization (inhalant, food) — Allergy (inhalant) ↑
Hartwig et al. 2014	Australia	Population-based	1,587	7 and 14	Negative life events	Eczema Allergic rhinitis	Eczema 6y —, eczema 14y ↑ Allergic rhinitis 6 and 14y —
Larsen et al. 2014	Denmark	Population-based	32,270	7	Psychological job strain	Atopic dermatitis (eczema)	Eczema ↑
Lin et al. 2003	Taiwan	Prospective	334	0	Psychosocial stress (IQOLA SF-36)	Total IgE	Total IgE ↑
McGowan et al. 2014	USA	Prospective high risk	516	0 - 5	Stress and/or depression (Q)	Food allergy	Food allergy —
Peters et al. 2012	USA	Prospective	403	0	Stress (CRISYS-R)	Total IgE	Total IgE ↑
Reyes et al. 2011	USA	Population-based	279	0 - 5	Demoralization (PERI-D)	Total IgE, specific IgE	Total IgE — Specific IgE —
Sausental et al. 2009	Germany	Prospective	3,004	0 - 6	Stress-related factors (Q/records)	Eczema	Eczema ↑, until 2y only
Wang et al. 2013	Taiwan	Prospective	11,962	3	Work stress (Q)	Atopic dermatitis (eczema)	Eczema ↑
Wen et al. 2011	Taiwan	Birth study	1,264	2	Mental status (Q)	Atopic dermatitis (eczema)	Eczema ↑

* = Outcomes were measured from (cord) blood samples by immunoCAP, immunoradiometric assay, UniCap IgE assay, or Phadia assay (total IgE, specific IgE for inhalant or food allergens) or skin prick tests (food and inhalant allergens), and by questionnaire, telephone interview, clinical history, clinician (eczema, allergic rhinitis, allergy), or combination of those.

↑ = positive direction of the observed association

↓ = negative direction of the observed association

— = no association observed

Abbreviations: BSI = Brief Symptom Inventory, CESD = Center for Epidemiological Studies-Depression, CRISYS-R = Crisis in Family Systems-Revised, IgE = Immunoglobulin E, IQOLA SF-36 = 'Modified Chinese version of Short Form 36 Health Survey', K6 = Kessler six-question psychological distress scale, PERI-D = Psychiatric Epidemiology Research Instrument – Demoralization, Q = Questionnaire not specified, R-CTS = Revised Conflict Tactics Scale, and STAI-T = State-Trait Anxiety Inventory-Trait.

Table S4. Summary of literature on maternal psychological distress and child respiratory outcomes

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed association
Alton et al. 2013	Canada	Randomized controlled trial	791	3	Distress (SQ)	Wheezing	Risk of wheezing — (all) Risk of wheezing ↑(girls)
Bandoli et al. 2016	USA	Population-based	1,193	0 – 6	Pregnancy-related anxiety, chronic stress (PSS), negative life events	Lifetime wheezing Current wheezing Asthma	Risk of wheezing ↑(pregnancy-related anxiety, negative life events) Risk of current wheezing ↑ (pregnancy-related anxiety) Risk of asthma —
Beijers et al. 2010	The Netherlands	Population-based	174	0-1	Anxiety (STAI), Pregnancy-related anxiety (PRAQ-R), daily hassles (APL), pregnancy-related daily hassles (PES)	Respiratory illness (several phenotypes including asthma)	Respiratory illnesses ↑(PES) Respiratory illnesses — (STAI, PRAQ-R, APL)
Brew et al. 2017[ab]	Sweden	Population-based	360,526	0-5	Distress (medication for, or diagnosis of, an anxiety or depressive disorder)	Asthma	Risk of asthma ↑
Cheng et al. 2015	Singapore	Population-based	1,152	0-1	Anxiety (STAI) and depression (EPDS)	Wheezing	Risk of wheezing ↑(depression) Risk of wheezing — (anxiety)
Chiu et al. 2012	USA	Population-based	653	0-2	Negative life events (NLEs)	Wheezing (≥ 2 episodes)	Risk of wheezing ↑ (non-maternal sensitization ↑, maternal sensitization —)
Cookson et al. 2009	UK	Population-based	5,810	7.5	Anxiety (CCEI)	Asthma	Asthma ↑
Fang et al. 2011	Sweden	Population-based	426,334 493,813	1-4 7-12	Bereavement shortly before and during pregnancy	Asthma	Risk of asthma —
Guxens et al. 2014	The Netherlands	Population-based	4,848	0-4 6	Distress, depression, anxiety (BSI)	Wheezing (0-4) Asthma diagnosis (6)	Risk of late wheezing ↑ Risk of persistent wheezing ↑ Risk of asthma —

Table S4. Summary of literature on maternal psychological distress and child respiratory outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed association
Grizenko et al. 2015	Canada	Case-control	201	6-12	Negative life events, scored on the DSM III and DSM-III-R axis IV scale	Asthma	Risk of asthma ↑
Hartwig et al. 2014	Australia	Population-based	994	6 14	Negative life events	Asthma	Risk of asthma — (6 years) Risk of asthma — ↑ (14 years)
Khashan et al. 2012	Sweden	Population-based	3,200,000	0-18	Bereavement 6 month before or during pregnancy	Asthma	Risk of asthma ↑
Larsen et al. 2014	Denmark	Population-based	32,271	7	Psychological job strain (JCQ)	Asthma	Asthma without atopic dermatitis ↑ Asthma with atopic dermatitis —
Lee et al. 2016	USA	Population-based	765	0-6	Negative life events (NLEs)	Asthma	Risk of Asthma ↑ (boys ↑, girls —)
Lee et al. 2017	USA	Population-based	199	7	Negative life events (NLEs)	FEV1, FVC, FEF25-75, FEV1/FVC	FEV1 ↓ FVC ↓ FEF25-75↓ FEV1/FVC —
Lefevre et al. 2011	France	Case-control	247	0-2	Anxiety (STAI), depression (BDI)	Wheezing (>3 episodes)	Wheezing —
Liu et al. 2015	Denmark	Population-based	750,058	0-3 4-15	Bereavement shortly before or during pregnancy	Asthma	Risk of asthma — ↑ (0-3years) Risk of asthma — (4-15 years)
Magnus et al 2017	Norway	Population-based	63,626	7	Depression and anxiety (SCL-5) Negative life events	Asthma	Risk of asthma ↑
de Marco et al. 2012	Italy	Cross-sectional	3,854	3-14	Stressful life events (SLEP)	Wheezing Asthma	Risk of wheezing ↑ Risk of asthma ↑
O'Connor et al. 2017	USA	Population-based	442	4-7	Depression (EPDS), stress during pregnancy (PSS)	Asthma	Risk of asthma —

Table S4. Summary of literature on maternal psychological distress and child respiratory outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed association
Phelan et al. 2015	USA	Population-based	2,802	0-1	Stress (PHS)	Respiratory illness composite score (consisting of cough/ cold, respiratory infections, asthma)	Respiratory illness ↑
Ramratnam et al. 2017	USA	Population-based	467	0-3	Depression (EPDS), stress during pregnancy (PSS)	Recurrent wheezing	Risk of wheezing —
Reyes et al. 2011	USA	Population-based	279	0-5	Psychological distress/ demoralization (PERID-D)	Wheezing	Overall wheezing ↑ Transient wheezing ↑ Late onset wheezing — Persistent wheezing ↑
Rosa et al. 2016	Mexico	Population-based	417	0-4	Stress (CRISYS)	Ever wheezing Current wheezing	Risk of ever wheezing ↑ (boys ↑, girls —) Risk of current wheezing ↑
Smejda et al. 2018	Poland	Population-based	370	0-1	Stress (PSS), occupational stress (SWCQ), negative life events (SRRS)	Wheezing	Risk of wheezing ↑ (PSS, SRRS)
Trump et al. 2016	Germany	Population-based	443	0-5	Stress (PSQ)	Wheezing	Risk of wheezing ↑
Turcotte-Tremblay et al. 2014	Canada	Population-based	68	0-12	Objective stress (Storm32), subjective stress (IES-R), life events (LES), anxiety (GHQ-28)	Wheezing Asthma	Risk of wheezing and asthma ↑ (girls exposed to subjective stress) No other significant associations
Wood et al. 2011	USA	Birth cohort	515	0-1	Anxiety (PAS), depression (EPDS), stress (PSS)	Wheezing (single (1 episode) and multiple (≥ 2 episodes))	Multiple wheezing ↑ (depression, stress) Single wheezing ↑(stress) Multiple wheezing — (anxiety) Single wheezing — (depression, anxiety)

Table S4. Summary of literature on maternal psychological distress and child respiratory outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed association
Zhou et al. 2017	France	Population-based	1,139	0-5	Depression (CES-D)	Wheezing Asthma	Risk of wheezing — Risk of asthma —
Zijlmans et al. 2017	The Netherlands	Population-based	174	2.5-6	Anxiety (STAI), Pregnancy-related anxiety (PRAQ-R), daily hassles (APL), pregnancy-related daily hassles (PES)	Respiratory illness (several phenotypes including asthma)	Respiratory illnesses ↑ (PRAQ-R, anxiety (STAI)) Respiratory illnesses — (APL, PES)

* = Outcomes were measured by spirometry, questionnaire, telephone interview, clinical history of contact, medication and/or hospitalization, clinical diagnosis (asthma) or a combination of those.

↑ = positive direction of the observed association

↓ = negative direction of the observed association

— = no association observed

Abbreviations: APL = Alledaagse Problemen lijst (Dutch), BDI = Beck Depression Inventory, BSI = Brief Symptom Inventory, CES-D = Center for Epidemiological Studies Depression scale, CRISYS = The Crisis in Family Systems – Revised survey, DSM-III/DSM-III-R = Diagnostic and Statistical Manual of Mental Disorders, EPDS = Edinburgh Post-natal Depression Scale, FAD = The McMaster Family Assessment Device, GHQ-28 = 28-item General Health Questionnaire, GHQ-60 = 60-item General health Questionnaire, IES-R = Impact of Event Scale-Revised, IF100 = Iowa Flood 100, JCQ = Job Content Questionnaire, Karasek's Job Strain Model, LES = Life Experiences Survey, NLEs = negative life events score, PAS = Pregnancy Anxiety Scale, PERI-D = Psychiatric Epidemiology Research Instrument-Demoralization scale, PES = Pregnancy Experience Scale, PHS = Psychosocial Hassles Scale, PNMS = Prenatal Maternal Stress, PSS = Perceived Stress Scale, PSQ = Perceived Stress Questionnaire, SCL = Symptom Check List, SLEP = stressful life events during pregnancy, SRRS = Social Readjustment Rating Scale, SQ = The Symptom Questionnaire (includes anxiety and depression), STAI = State Trait Anxiety Inventory, Storm32 = Objective hardship score, SWCQ = Subjective Work Characteristics Questionnaire.

Table 5 Summary of literature on maternal psychological distress and child neurodevelopmental outcomes

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed associations
Atkinson et al. 2000	—	Meta-analyses	2,064 (35 studies)	1-3	Various depression measures #	Attachment (SS or modified SS or Attachment Q-set)	Attachment security ↑
Van den Berg et al 2004	Belgium	Prospective follow-up	71	8-9	Anxiety (STAI) in second and third trimester	Four composites: ADHD symptoms, externalizing problems, internalizing problems, anxiety (CBCL, TRF, CATRS, STAIC)	ADHD symptoms, externalizing problems, anxiety ↑ (Anxiety in second trimester) No other associations
Davis et al. 2004	USA	Longitudinal	22	0.3	Depression (CES-D) and anxiety (STAI)	Behavioral reactivity (HIBRP)	Negative behavioral reactivity to novelty ↑ (STAI and CES-D)
Deave et al. 2008	UK	Prospective	11,098	1.5	Depression (EPDS)	Development (Modified DDST)	Developmental delay ↑
DiPietro et al 2006	USA	Longitudinal	94	2	Anxiety (POMS, STAI), depression (POMS, CES-D), stress (DSI, PSS, PES)	Development (BSID)	Motor development ↑ (anxiety, general stress, depression) Mental development ↑ (anxiety, depression)
Field et al. 2009/2010	USA	Narrative review	Unknown	0-0.5	Depression (CES-D, SCID)	Responses to different stimuli (faces, voices ea)	Responses to different stimuli ↓
Henrichs et al. 2009	The Netherlands	Population-based prospective	2,997	0.5	Pregnancy-specific anxiety (POQ) and anxiety (BSI)	Temperament (IBQ-R)	Activity and sadness ↑ (POQ and BSI) Fearfulness ↑(POQ) Distress to limitations ↑(BSI)

Table 5 Summary of literature on maternal psychological distress and child neurodevelopmental outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed associations
Laplante et al 2008	Canada	Longitudinal	89	5.5	Objective stress (Storm32), subjective stress (IES-R)	IQ and language ability ##	Full Scale IQs ↓ IQs ↓ Objective stress Language abilities ↓ No associations with subjective stress
Li et al. 2013	Australia	Longitudinal	1,038	10	Negative life events	School achievement (using WALNA)	Reading skills ↓ (in girls) Reading skills, mathematic and writing scores ↑ (in boys)
Luoma et al 2004	Finland	Prospective	165	8-9	Depression (EPDS))	Behavior (CBCL)	Problem level ↑
Niederhofer et al. 2004	Austria	Longitudinal	227	6	Stress (unknown questionnaire)	School marks	School marks ↓
O'Conner et al 2007	UK	Prospective	10,323	0.5, 1.5 and 2.5	Depression (EPDS), anxiety (CCEI)	Sleep problems (parent reports)	Sleep problems ↑ (depression and anxiety)
O'Conner et al 2002	UK	Prospective	7,144	4	Anxiety (CCEI)	Behavioral and emotional problems (SDQ)	Behavioral and emotional problems ↑ (also after adjustment for postnatal anxiety)
Van der Wal et al. 2007	The Netherlands	Longitudinal	4,976	0.3-0.5	Depression (CES-D), pregnancy-specific anxiety (PRAQ), parenting stress (PDH), job strain (WEAQ)	Excessive crying	Excessive crying ↑ (CES-D, PRAQ, PDH, QEAQ)
Whitehouse et al 2010	Australia	Longitudinal	2,601	10	Negative life events	Language ability (PPVT-R)	Language ability —
Yong Ping et al. 2015	USA	Longitudinal	94	2.5	Objective stress (IF100) and subjective stress (IES-R)	Stress reactivity (cortisol levels/ increase after stress situation)	Cortisol increase ↑ (Objective and subjective stress)

Table 5 Summary of literature on maternal psychological distress and child neurodevelopmental outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed associations
Childhood neuroimaging outcomes: using structural and functional magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI)							
Lebel et al. 2016	USA	Prospective	52	2.6 – 5.1	Depression (EPDS)	Cortical thickness and white matter structure (FA, RD, AD, MD)	Cortical thickness in right inferior frontal and middle temporal regions ↓ RD and MD in white matter emanating from the inferior frontal area ↓
El Marroun et al. 2016	The Netherlands	Population-based prospective	654	6-10	Depression (BSI)	Structural neuroimaging data: cortical thickness, surface area, gyrification	Superior frontal cortex in left hemisphere ↓ Caudal middle frontal area in left hemisphere ↑ No other associations
El Marroun et al. 2018	The Netherlands	Population-based prospective	636	6-9	Depression (BSI)	White matter microstructure (FA, MD)	MD in the uncinate fasciculus ↑ FA ↓ and MD ↑ in the cingulum bundle
Qiu et al. 2015	Singapore	Prospective	42	0.5	Depression (EPDS)	Amygdala functional connectivity	Amygdala functional connectivity ↑ with several brain regions
Rifkin-Graboi et al. 2013	Singapore	Prospective	157	0-0.1	Depression (EPDS)	Amygdala volume and microstructure (FA, AD)	FA ↓ and AD ↓ in right amygdala FA ↓ in left amygdala Volume amygdala —
Sandman et al 2015	USA	Prospective longitudinal	81	6-9	Depression (CES-D, BDI)	Cortical thickness	Cortical thickness ↓

Table 5 Summary of literature on maternal psychological distress and child neurodevelopmental outcomes (continued)

	Country	Study Design	Sample size	Age of children (years)	Maternal exposures and assessment during pregnancy	Child outcomes *	Direction of the observed associations
Wen et al. 2017	Singapore	Prospective	235	4.5	Depression (EPDS)	Amygdala volume and white matter microstructure (FA)	Right amygdala volume ↑ (only in girls) No other associations

* = Outcomes were measured from blood samples, questionnaires, parent reports, responses to different stimuli, IQ tests, school marks, MRI or DTI.

↑ = positive direction of the observed association

↓ = negative direction of the observed association

— = no association observed

Abbreviations exposures: BSI = Brief Symptom Inventory, BDI = Beck Depression Inventory, CCEI = Crown-Crisp experiential index, CES-D = Center for Epidemiological Studies Depression Inventory, DSI = Daily Stress Inventory, DSM-III = Diagnostic and Statistical Manual of Mental Disorders, 3rd edition, EPDS = Edinburgh Post-natal Depression Scale, FAD = The McMaster Family Assessment Device, GHQ-60 = 60-item General health Questionnaire, IES-R = Impact of Event Scale-Revised, IF100 = Iowa Flood 100, MMPI-2 = Minnesota Multiphasic Personality Inventory, 2nd edition, Modified DDST = modified Denver Developmental Screening Test, PDH = Parenting Daily Hassles, PES = Pregnancy-specific stress, PNMS = Prenatal Maternal Stress, POMS-D = Profile of Mood States-Depression Scale, POQ = Pregnancy Outcome Questionnaire, PPVT-R = Peabody Picture Vocabulary Test-Revised, PSS = Perceived Stress Scale, PRAQ = Pregnancy Related Anxiety Questionnaire, SADS = Schedule for Affective Disorders and Schizophrenia, SCID = Structured Clinical Interview for DSM-IV, SS = Strange Situation, STAI = State-Trait Anxiety Inventory, Storm32 = Objective hardship score, WEAQ = Work Experience and Appreciation Questionnaire based on the Job Content Instrument of Karasek et al, # BDI, POMS-D, DSM-II, CESD-D, EPDS, SADS, MMPI-2
Abbreviations outcomes: AD = Axial Diffusivity, BSID = Bayley Scales of Infant Development , CATRS = Conners Abbreviated Teacher Rating Scale, CBCL = Child Behavior Checklist, FA = Fractional anisotropy, HIBRP = Harvard Infant Behavioral Reactivity Protocol, IBQ-R = Infant Behavior Questionnaire-Revised, MD = mean diffusivity, RD = radial diffusivity, SDQ = Strengths and Difficulties Questionnaire, STAIC = State Trait Anxiety Scale for Children, TRF = Teacher's Report Form, WALNA = Western Australian Literacy and Numeracy Assessment, ## =Wechsler Preschool and Primary Scale of Intelligence-Revised (IQ) and Peabody Picture Vocabulary Test-Revised

2.2

Psychological distress and weight gain in pregnancy: a population-based study

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ABSTRACT

Background: Psychological distress and inappropriate or excessive weight gain are common in pregnancy and are associated with adverse maternal and offspring outcomes. Psychological wellbeing and weight status of women during pregnancy might be interrelated. We aimed to examine whether psychological distress during pregnancy is associated with gestational weight gain.

Methods: In a population-based cohort of 3,393 pregnant women, information about psychological distress, depressive and anxiety symptoms was assessed at 20 weeks of gestation using the Brief Symptom Inventory questionnaire. Weight was repeatedly measured during pregnancy and obtained by questionnaire before and after pregnancy. Linear regression and multinomial logistic regression models were used. Weight gain in the second half of pregnancy, total weight gain and the risks of inadequate and excessive total weight gain were the main outcome measures.

Results: In total, 7.0% of all women experienced psychological distress. Only women with anxiety symptoms had, independently of potential confounders, a lower risk of excessive weight gain (Odds ratio (OR): 0.61 (95% CI 0.48, 0.91)).

INTRODUCTION

Psychological distress is generally defined as general stress, depressive symptoms, anxiety or experiencing an adverse life event.[1, 2] In western countries, 5-20% of pregnant women experience psychological distress.[2-4] Also, more than 60% of pregnant women have either inadequate or excessive weight gain.[5, 6] Pregnancy is a critical period for psychological distress and weight gain, since both are associated with increased risks of adverse maternal and offspring outcomes.[7-11] Previously, we have reported that, compared with low or recommended weight gain, excessive weight gain was associated with a higher risk of gestational hypertension, cesarean delivery, large size for gestational age infants, and childhood overweight.[12] We have also reported that anxiety and depression during pregnancy were associated with impaired fetal growth.[13] Psychological distress and weight gain in pregnant women may also affect each other.[14-17] Two systematic reviews among, in total, 12 studies have been performed on the association between psychological distress and weight gain in pregnancy. One systematic review showed no association and the second systematic review only reported an association of depression, but not psychological distress and anxiety, with increased gestational weight gain.[7, 9] These reviews compiled studies with a modest sample size, used different definitions of psychological distress, depression and anxiety and did not define cutoffs for psychological distress to consider clinical importance.

We hypothesized that psychological distress during pregnancy is associated with gestational weight gain. We examined in a population-based prospective cohort study among 3,393 pregnant women the associations of psychological distress during pregnancy and gestational weight gain. We also explored whether any association was explained by socio-demographic or lifestyle related variables.

METHODS

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early pregnancy until young adulthood in Rotterdam, the Netherlands.[18, 19] The study was approved by the Medical Ethics Committee of Erasmus Medical Center Rotterdam, The Netherlands (MEC 198.782/2001/31). Pregnant women were enrolled between 2002 and 2006. Written informed consent was obtained from all women in the study. In total, 8,879 mothers (91% of the full cohort) were enrolled during pregnancy of whom information about psychological distress during pregnancy was available in 6,650. We further excluded pregnancies not leading to singleton live births

(N = 101) and women without information on weight gain during pregnancy (N = 3,156). This resulted in a population for analysis of 3,393 mothers (**Supplementary Material 1**).

Psychological distress during pregnancy

The Brief Symptom Inventory (BSI) questionnaire was used to examine psychological distress at approximately 20 weeks of gestation. The BSI is a validated self-report questionnaire consisting of 53 items, describing multidimensional psychopathologic problems and complaints in adults in the preceding 7 days.[20-23] The items are divided in 9 subscales (including anxiety, depression, hostility, phobic anxiety, interpersonal sensitivity, obsessive-compulsiveness, paranoid ideation, psychoticism, somatization). As an indicator of overall psychological distress, we used the Global Severity Index (GSI) that is a total score of the 9 subscales (all 53 items of the BSI). Additionally, we used the depression and anxiety subscales separately. We chose these 2 subscales, because they are widely used as valid proxies for psychological distress during pregnancy.[1, 2] The items were rated on a 5-point unidimensional scale ranging from "0" (not at all) to "4" (extremely). A total score was calculated for each symptom scale by summing the item scores of the scales and dividing the results by the number of items in that scale. Higher scores represented an increased occurrence of symptoms. Psychological symptoms were dichotomized (yes/no) by using the following clinical cutoffs derived from a psychiatric outpatient sample of Dutch non-pregnant women: 0.71 for overall psychological symptoms scale, 0.80 for the depression symptoms scale and 0.71 for the anxiety symptoms scale.[23]

Weight measurements during pregnancy

As enrolment in our study was in pregnancy, we were not able to measure weight before pregnancy. Information on pre-pregnancy weight was obtained by questionnaires at enrollment. Pre-pregnancy body mass index (BMI) in kg/m^2 was calculated using pre-pregnancy weight (kg) as reported by the mother and height (m) measured at enrolment and was categorized into underweight (< 18.5 kg/m^2), normal weight (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obesity (≥ 30 kg/m^2) according to the World Health Organization (WHO) 2000 criteria.[24] Weight measurements during pregnancy were performed in a dedicated research center without shoes and heavy clothing. Since we collected information about psychological distress at 20 weeks, we used weight gain in the second half of pregnancy as main outcome. Weight gain in the second half of pregnancy was calculated subtracting weight measured in mid-pregnancy (median 20.4 weeks (95% range 18.6–24.9 weeks)) from the maximum reported weight in pregnancy. The latest weight before delivery was obtained by questionnaire completed 2 months after delivery, hereafter referred to as maximum reported weight in pregnancy. Total weight gain during pregnancy was calculated by subtracting reported pre-pregnancy weight from the maximum reported weight in pregnancy. Total gestational weight

gain was categorized into inadequate, adequate, and excessive weight gain following the 2009 Institute of Medicine (IOM) weight gain recommendation criteria using both categorized pre-pregnancy BMI and total gestational weight gain.[25-27]

Covariates

Gestational age was established using the last menstrual period or first trimester ultrasound measurement.[28] We obtained information on maternal age, ethnicity, parity, educational level, and marital status by questionnaire at enrolment.[18] Information about folic acid intake, smoking, alcohol consumption and nutritional intake in kcal (kilocalories) was assessed by questionnaires during pregnancy.

Statistical analysis

First, we compared subject characteristics between women with and those without psychological distress using Pearson's chi-square tests, independent sample *t* tests and Mann-Whitney tests. We performed non-response analysis using the same tests to assess differences between women with and without information on weight gain during pregnancy. We compared psychological distress and weight gain characteristics between pre-pregnancy BMI categories. Second, we used linear regression models to assess the associations of overall psychological distress, depression and anxiety with weight gain in the second half of pregnancy and total gestational weight gain. We examined potential interactions of maternal psychological distress with pre-pregnancy BMI and ethnicity in the association with gestational weight gain. We observed a statistically significant interaction of maternal psychological distress and pre-pregnancy BMI and thus performed stratified analyses for the clinical BMI categories according to the WHO 2000 criteria (underweight, normal weight, overweight, obesity). Further, we performed sensitivity analyses among full-term mothers, defined as a gestational age of ≥ 37 weeks at birth, to exclude potential bias by preterm birth. Third, we used multinomial logistic regression models to assess the associations of psychological distress, depression, and anxiety with clinical categories of gestational weight gain according to the IOM criteria (inadequate, adequate and excessive weight gain). For all regression models, the basic models were adjusted for maternal age, whereas the full models were adjusted for potential confounders. We included covariates in the models if they were associated with psychological distress and gestational weight gain or if they changed the effect estimates substantially (> 10%). In order to maintain statistical power and reduce bias related to missing data on covariates (missing data on covariates ranged from 0 to 21%, see **Supplementary Material 2**) we performed multiple imputation using the Markov Chain Monte Carlo method. Exploratory analyses showed the data was not missing completely at random (MCAR) (indicated by the Little's test, p-value < 0.05).[29] Comparison between characteristics of complete cases (participants with no missing data) and participants with at least one missing value showed no large

differences. Considering these results and no likely reason for the data to be missing not at random (MNAR), we proceeded with multiple imputation for which missing at random (MAR) is an assumption.[30] Five new datasets were created and pooled results are presented. No major differences in descriptive statistics were found between the original and imputed datasets. Statistical analyses were performed with the Statistical Package of Social Sciences version 21.0 for Windows (IBM Corp, Armonk, NY, USA).

RESULTS

Subject characteristics

Psychological distress, depression, and anxiety were reported by 7.0%, 7.0% and 8.4% of all pregnant women, respectively. **Table 1** shows the subject characteristics. In total, 20.1% and 45.0% of all women had inadequate and excessive gestational weight gain, respectively. Compared to women without psychological distress, those with psychological distress were more often younger, lower educated, without a partner, of a non-Dutch ethnicity, continued smokers and had a lower nutritional intake, alcohol, and folic acid supplement use (**Table 1**). **Table 2** shows that the prevalence of psychological distress, depression and anxiety varies between BMI categories. Women with a normal pre-pregnancy weight had the lowest prevalence of psychological distress and the highest mean weight gain.

Non-response analyses showed that, as compared to women with missing information on weight gain during pregnancy, those with information on gestational weight gain had a higher maternal age, a lower pre-pregnancy BMI, a partner, a higher intake of alcohol and folic acid and a higher total daily energy intake, and were more often nulliparous, higher educated and Dutch-European (**Supplementary Material 2**).

Table 1. Characteristics of study population (N = 3,393)[a]

	Population for analysis (N= 3,393)	Psychological distress (N = 238)	No psychological distress (N = 3,155)	P-value [b]
Age at intake, mean (SD), years	31.0 (4.7)	28.2 (5.8)	31.3 (4.5)	< 0.001
Pre-pregnancy weight, median (95% range), kg	64.0 (49.0, 97.0)	62.0 (47.0, 104.6)	64.0 (49.9, 96.2)	< 0.01
Pre-pregnancy BMI, median (95% range) kg/m^2	22.3 (18.2, 33.6)	22.7 (17.9, 36.7)	22.3 (18.2, 33.3)	< 0.01
Pre-pregnancy BMI clinical categories, N (%)				< 0.01
Underweight	111 (3.8)	9 (4.4)	102 (3.8)	
Normal weight	2,127 (73.0)	140 (68.6)	1,987 (73.3)	
Overweight	491 (16.8)	40 (19.6)	451 (16.6)	
Obesity	185 (6.3)	15 (7.4)	170 (6.3)	

Table 1. Characteristics of study population (N = 3,393)[a] (continued)

Gestational age at birth, median (95% range), weeks	40.1 (36.3, 42.4)	40.3 (36.1, 42.6)	40.0 (36.3, 42.4)	0.21
Total weight gain, mean (SD), kg	14.9 (5.9)	14.4 (6.7)	15.0 (5.8)	0.17
Gestational weight gain clinical categories (IOM criteria), N (%)				0.21
Inadequate weight gain	586 (20.1)	49 (24.0)	537 (19.8)	
Adequate weight gain	1,018 (34.9)	74 (36.3)	944 (34.8)	
Excessive weight gain	1,310 (45.0)	81 (39.7)	1,229 (45.4)	
Weight gain 2nd half of pregnancy, mean(SD), kg	9.6 (4.6)	9.0 (4.8)	9.6 (4.6)	
Parity, N (%)				0.23
Nulliparous	2,059 (60.7)	144 (60.5)	1,915 (60.7)	
Multiparous	1,334 (39.7)	94 (39.5)	1,240 (39.3)	
Education, N (%)				< 0.001
Primary school	178 (5.3)	31 (13.0)	147 (4.7)	
Secondary school	1,280 (37.7)	138 (58.0)	1,142 (36.2)	
Higher education	1,935 (57.0)	69 (29.0)	1,866 (59.1)	
Marital status, N (%)				< 0.001
Married/living together	3,084 (90.9)	185 (77.7)	2,899 (91.9)	
No partner	309 (9.1)	53 (22.3)	256 (8.1)	
Ethnicity, N (%)				< 0.001
Dutch-European	2,448 (72.1)	92 (38.6)	2,356 (74.6)	
Surinamese	200 (5.9)	28 (11.8)	172 (5.5)	
Turkish	178 (5.2)	46 (19.3)	132 (4.2)	
Moroccan	109 (3.2)	24 (10.1)	85 (2.7)	
Cape Verdian	70 (2.1)	19 (8.0)	52 (1.6)	
Dutch Antilles	69 (2.1)	6 (2.5)	62 (2.0)	
Others	319 (9.4)	23 (9.7)	296 (9.4)	
Alcohol consumption, N (%)				< 0.001
No	1,304 (38.4)	130 (54.6)	1,173 (37.2)	
Yes	2,089 (61.6)	108 (45.4)	1,982 (62.8)	
Smoking habits, N (%)				< 0.001
No	2,584 (76.2)	145 (60.9)	2,439 (77.3)	
During first trimester only	318 (9.3)	23 (9.7)	295 (9.4)	
Continued during pregnancy	491 (14.5)	70 (29.4)	421 (13.3)	
Folic acid supplement use, N (%)				< 0.001
No	627 (18.5)	89 (37.4)	538 (17.1)	
Start during first 10 weeks of pregnancy	1,109 (32.7)	102 (42.9)	1,007 (31.9)	
Preconception use	1,657 (48.8)	47 (19.7)	1,610 (51.0)	
Total daily energy intake, mean (SD), kcal	2,076 (535)	2,015 (529)	2,081 (530)	< 0.01

[a] Values are means (standard deviation) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution, and valid percentages for categorical variables. Missing values in covariates are imputed. Percentages of pre-pregnancy BMI clinical categories and gestational weight gain clinical categories are valid percentages.

[b] P-values for differences in subject characteristics between mothers with psychological distress and mothers without psychological distress were calculated performing independent sample t-tests for normally distributed continuous variables, Mann-Whitney test for not normally distributed continues variables and chi-square tests for categorical variables.

Table 2. Psychological distress and gestational weight gain characteristics by pre-pregnancy BMI category (N = 3,393)[a]

	Pre-pregnancy BMI categories			
	Underweight	**Normal weight**	**Overweight**	**Obesity**
	N = 111	N = 2,127	N = 491	N = 185
Weight gain measurements				
Total gestational weight gain, mean (SD)	14.4 (5.3)	15.4 (5.3)	14.3 (6.4)	11.5 (8.6)
Weight gain in the second half of pregnancy, mean (SD)	9.1 (4.5)	9.8 (4.3)	9.5 (4.8)	8.0 (5.7)
Psychological measurements				
Psychological distress	9 (8.1%)	140 (6.6%)	40 (8.1%)	15 (8.1%)
No psychological distress	102 (91.9%)	1,987 (93.4%)	451 (91.9%)	170 (91.9%)
Depression	10 (9.0%)	134 (6.3%)	41 (8.4%)	10 (5.4%)
No depression	101 (91.0%)	1,992 (93.7%)	450 (91.6%)	174 (94.1%)
Anxiety	8 (7.2%)	173 (8.1%)	50 (10.2%)	16 (8.6%)
No anxiety	103 (92.8%)	1,954 (91.9%)	441 (89.9%)	169 (91.4%)

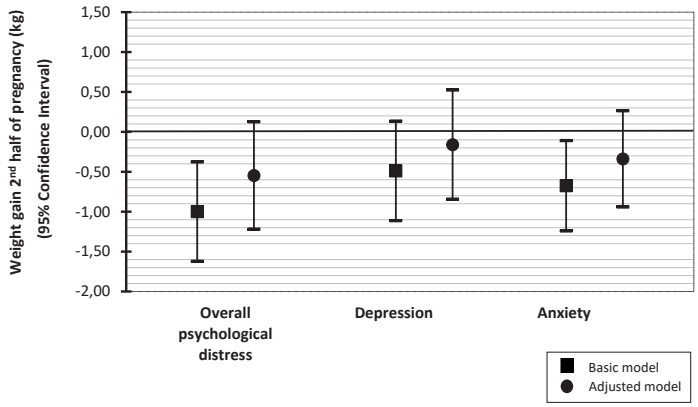
[a] Values are means (standard deviation) for continuous variables with a normal distribution, and valid percentages for categorical variables.

Psychological distress and weight gain in the second half of pregnancy

Figure 1 shows that, in the basic models, overall psychological distress and anxiety were associated with lower weight gain in the second half of pregnancy (differences -1.00 kg (95% Confidence Interval (CI) -1.62, -0.37) and -0.68 kg (95% CI -1.24, -0.11), for overall psychological distress or anxiety, respectively). These associations fully attenuated into non-significance in the adjusted model after taking account for socio-demographic variables such as maternal education and ethnicity. Maternal depression during pregnancy was not associated with weight gain in the second half of pregnancy.

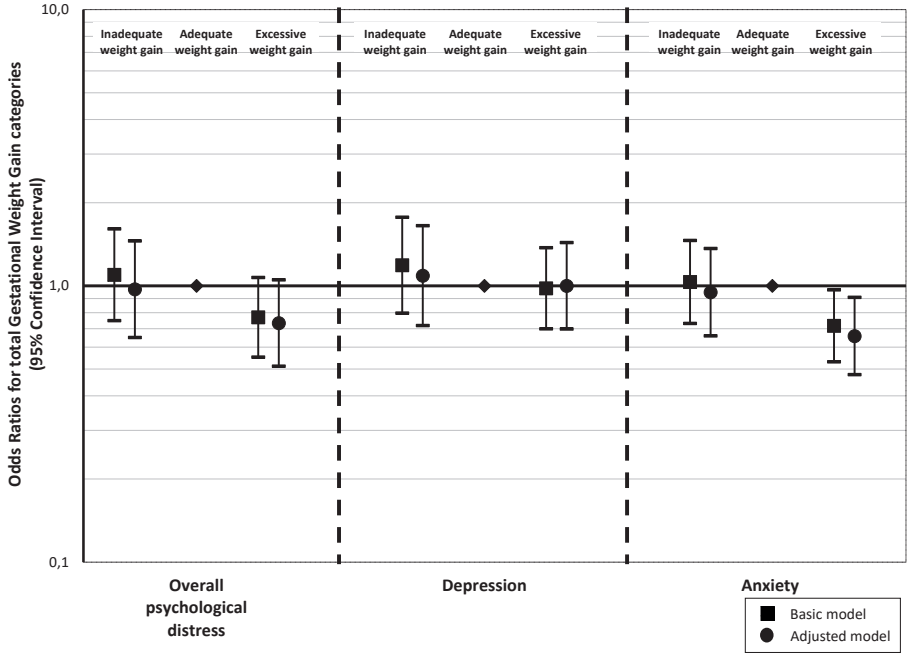
For the associations of maternal psychological distress, depression and anxiety with total gestational weight gain, effect estimates were of the same magnitude and in similar direction as for weight gain in the second half of pregnancy (**Supplementary Material 3)**. The stratified analyses for different BMI categories are shown in **Supplementary Material 4.** Underweight women experiencing overall psychological distress or depression tended to have an increased weight gain from 20 weeks onwards compared with normal, overweight, and obese women with overall psychological distress or depression; however, results were not significant. In obese women experiencing anxiety during pregnancy, weight gain in the second half of pregnancy tended to be lower. Sensitivity analyses among women who had a full term pregnancy only, showed effect estimates of the same size and direction as the main analyses (**Supplementary Material 5)**.

Figure 1. Associations of psychological distress and weight gain in the second half of pregnancy (N = 3263)



Values are linear regression coefficients (95% confidence intervals) and represent the overall change in weight gain in the second half of pregnancy for psychological distress, depression and anxiety compared to no psychological distress, depression or anxiety during pregnancy. The basic model was adjusted for maternal age. The adjusted model was adjusted for maternal age, pre-pregnancy BMI, parity, education, marital status, ethnicity, alcohol intake, smoking, folic acid use and nutritional intake in kcal.

Figure 2. Associations of psychological distress with clinical categories of gestational weight gain (N= 2914)



Odds Ratios (95% Confidence Intervals) represent the risks for the different weight gain categories (inadequate, adequate (reference) and excessive weight gain) according to the 2009 IOM gestational weight gain recommendation categories for women with overall psychological distress, depression and anxiety during pregnancy. The basic model is adjusted for maternal age. The adjusted model is adjusted for maternal age, pre-pregnancy BMI, parity, educational level, marital status, ethnicity, alcohol intake, smoking, folic acid intake and nutritional intake in kcal.

Psychological distress and the risk of inadequate and excessive weight gain in pregnancy

Overall psychological distress and depression were not associated with the risks of inadequate or excessive weight gain (**Figure 2**). Maternal anxiety during pregnancy was, independently of confounders, associated with a lower risk of excessive weight gain (Odds Ratio: 0.61 (95% CI 0.48, 0.91) **(Figure 2).** Similar results were obtained without adjustment for pre-pregnancy BMI.

DISCUSSION

Main findings

In this population-based prospective cohort study, we did not observe consistent associations of overall psychological distress, depression and anxiety with gestational weight gain. Most associations were explained by maternal ethnicity and educational level. Only women with anxiety symptoms had, independently of potential confounders, a lower risk of excessive weight gain.

Interpretation of main findings

We observed that 7.0% to 8.4% of all pregnant women reported psychological distress, depression or anxiety. These percentages are comparable or slightly lower compared to the prevalence reported in previous studies.[2, 3] In our study population 23.1% of women had pre-pregnancy overweight or obesity and 20.1% and 45.0% of women experienced inadequate and excessive weight gain, respectively, which is in line with population figures.[5, 31-34] Although results of some previous studies suggested that psychological distress, depression, or anxiety during pregnancy are associated with gestational weight gain[14-17], one systematic review has reported no association [9]and a second systematic review only reported an association of depression, but not psychological distress and anxiety, with gestational weight gain.[7] Most previous studies did not define cutoffs for psychological distress to consider clinical importance and sample sizes were modest. In our large population-based prospective cohort study, we observed that overall psychological distress and anxiety were associated with a lower gestational weight gain. However, these associations attenuated after adjustment for socio-demographic factors. These findings are in line with a study among 1,605 women in the USA in which the relationship between psychosocial status and adequacy of gestational weight gain was also influenced by socio-demographic factors.[31] In a large study among 13,314 pregnant women in the United Kingdom between 1991 and 1992, the association between antenatal depression and inadequate or excessive gestational weight gain was already non-significant in the unadjusted model.[35] However, this study did not assess the as-

sociation of overall psychological distress and anxiety with gestational weight gain. One previous study in 242 women found that depression during pregnancy was associated with excessive gestational weight gain, only among women with a high pre-pregnancy BMI.[16] We did not find significant differences in the association of psychological distress, depression, or anxiety with weight gain during pregnancy between women of different pre-pregnancy BMI categories. Thus, results from our and some other studies suggest that common socio-demographic factors explain the association between psychological distress and weight gain during pregnancy.

In the present study, we observed a negative association between anxiety and the risk for excessive weight gain, which remained after adjustment. Our finding is not in line with a recent study among 725 women in which lower reported stress was associated with a greater chance of women achieving adequate gestational weight gain.[14] However, some previous studies also suggest that anxiety and depression may be protective of increased weight gain.[17, 36] In the USA-study mentioned above, the association between anxiety and a higher adequacy of weight gain disappeared after adjustment for confounders, among which was physical activity.[31] In our study, the association between anxiety and the lower risk for excessive weight gain remained significant. However, residual confounding by for example physical activity level and sedentary behavior may still be present.

The relationship between psychological distress and weight is complex and might be bidirectional.[7, 37, 38] Since observational studies are not able to clarify the causal directions, mechanistic studies and Mendelian Randomization studies may give further insight in the underlying mechanisms and directions.[39] This is of great importance because both psychological health and weight gain can be targets for preventive strategies in pregnancy. This is shown in a randomized controlled trial in which the effects of a four-session intervention, motivating participants to have a healthy lifestyle during pregnancy, were examined.[40] The study found a reduction of gestational weight gain and levels of anxiety in obese pregnant women after the intervention.

Strengths and Limitations

Strengths of this population-based cohort study were the prospective data collection, the detailed measurements from pregnancy onwards and the large sample size of more than 3000 participants. This study also has limitations. Of all women included during pregnancy, 75% responded to the questionnaire. Only 52% of all women included during pregnancy with information on psychological distress and with singleton live-born children, had information on weight gain during pregnancy. Non-response could have led to selection bias if the associations were different between those included and not included in the analyses. Extrapolating results to all pregnant women should therefore be done with caution. Women with missing information on psychological distress and

weight gain were more often lower educated and of non-European ethnicity. We cannot exclude the possibility that these differences have affected the results. Information on gestational weight gain was self-reported. Self-reported weight tends to be underestimated. We used self-reported weights because the measured weights, weight in the first trimester (median 13.2 weeks, 95% range 9.8–17.5 weeks) and weight in the third trimester (median 30.2 weeks, 95% range 28.5–32.6 weeks), do not comprise the whole pregnancy. However, the correlations between self-reported pre-pregnancy weight and measured weight in the first trimester ($r = 0.96$, $P < 0.001$) as well as the correlation between maximum self-reported weight and measured weight in the third trimester were high ($r = 0.95$, $P < 0.001$). Psychological distress was measured at only one time point during pregnancy, on average at 20 weeks, and refers to the preceding 7 days. Most other studies also have only one assessment point.[41] Therefore, we do not know whether psychological distress symptoms varied in intensity or were persistent throughout pregnancy. Further research is needed to assess the associations of trimester-specific psychological distress on gestational weight gain. To classify psychological symptoms, we have used cutoffs derived from a clinical population of Dutch non-pregnant women, which might not be entirely suitable for our study population. However, cutoffs from a sample of pregnant women are currently not available. In this study, differential misclassification could occur when women with more psychological distress, depression, or anxiety report differently their weight status compared to women without psychological distress, depression or anxiety. This seems unlikely because both pregnant women and data collectors were unaware of the specific research questions under study.[42] Finally, although we used a large number of confounders, residual confounding might still be present.

CONCLUSIONS

Our results do not support the hypothesis that psychological distress, depression and anxiety affect weight gain in pregnant women. Only women with anxiety symptoms had, independently of potential confounders, a lower risk of excessive weight gain. The observed associations of psychological distress with weight gain during pregnancy seem to be largely explained by common socio-demographic factors. Further studies are needed to explore whether psychological distress in pregnancy affects other outcomes in women and their children, such as postpartum weight gain/loss.

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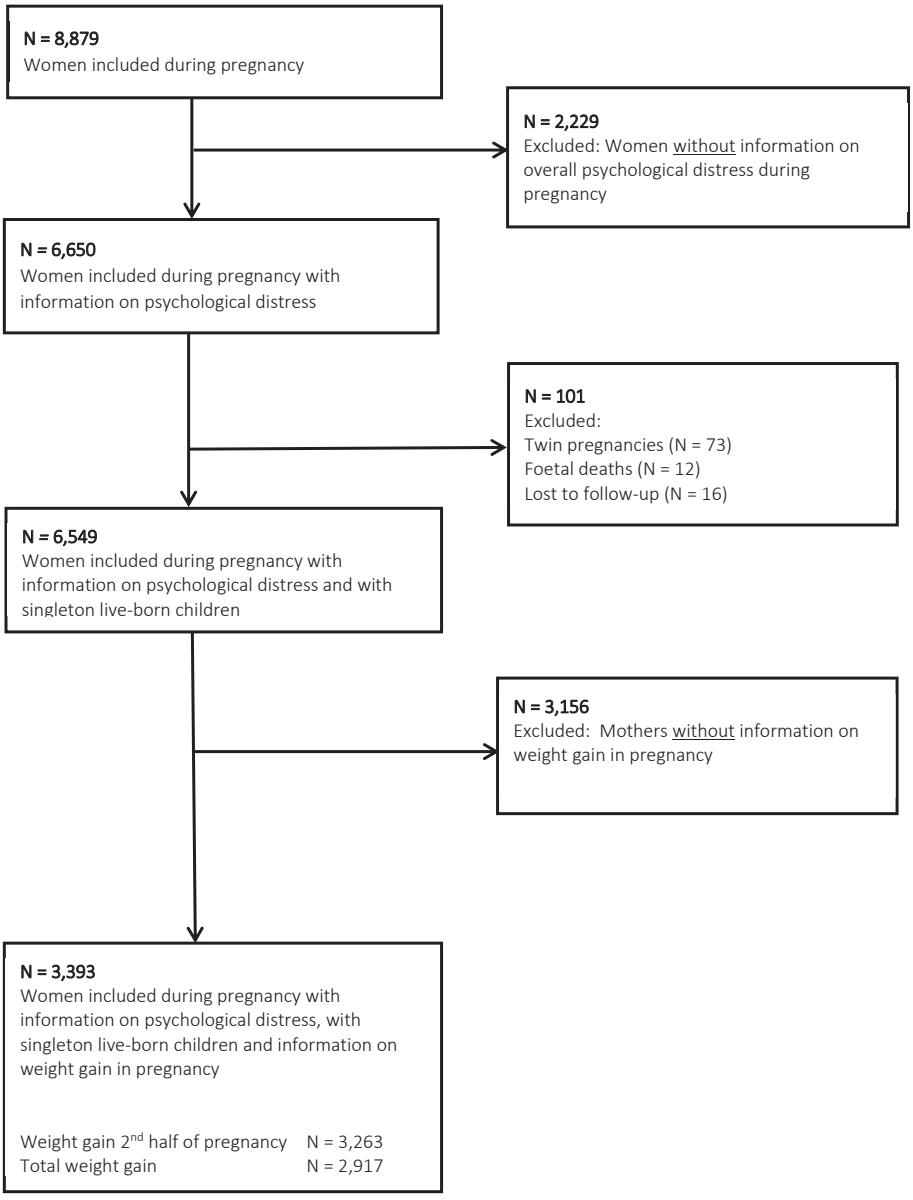
SUPPLEMENTARY MATERIAL

Supplementary Material 1. Flowchart of the study participants



Supplementary Material 2. Characteristics of women with and without information on weight gain in pregnancy (N = 6,549)[a]

	Full group (N=6,549)	Responders (N=3,393)	Non-responders (N=3,156)	P-value[b]
Age at intake, mean (SD), years	30.0 (5.1)	31.0 (4.7)	28.9 (5.4)	< 0.001
Missings, N (%)	1 (0.02)	0 (0.0)	1 (0.03)	
Pre-pregnancy weight, median (95% range), kg	64.0 (48.0, 99.0)	64.0 (49.0, 97.0)	64.0 (48.0, 101.0)	0.38
Missings, N (%)	1,023 (15.6)	476 (14.0)	547 (17.3)	
Height, mean (SD), cm	167.6 (7.4)	168.8 (7.2)	166.4 (7.3)	< 0.001
Missings, N (%)	17 (0.3)	7 (0.2)	10 (0.3)	
Pre-pregnancy BMI, median (95% range), kg/m^2	22.6 (18.0, 34.7)	22.3 (18.2, 33.6)	22.8 (17.7, 35.7)	<0.001
Missings, N (%)	1,030 (15.7)	479 (14.1)	551 (17.5)	
Pre-pregnancy BMI clinical categories, N (%)				< 0.001
Underweight	241 (3.7)	111 (3.3)	130 (4.1)	
Normal weight	3,788 (57.8)	2,127 (62,7)	1,661 (52.6)	
Overweight	1,018 (15.5)	491 (14.5)	527 (16.7)	
Obesity	472 (7.2)	185 (5.5)	287 (9.1)	
Missings	1,030 (15.7)	479 (14.1)	551 (17.5)	
Gestational age at birth, median (95% range), weeks	40.1 (35.9, 42.3)	40.1 (36.3, 42.4)	40.1 (35.4, 42.3)	< 0.001
Missings, N (%)	2 (0.03)	0 (0.0)	2 (0.06)	
Parity, N (%)				< 0.001
Nulliparous	3,786 (57.8)	2,051 (60.4)	1,735 (55.0)	
Multiparous	2,725 (41.6)	1,329 (39.2)	1,396 (44.2)	
Missings, N (%)	38 (0.6)	13 (0.4)	25 (0.8)	
Education, N (%)				< 0.001
Primary school	576 (8.8)	166 (4.9)	410 (13.0)	
Secondary school	2,824 (43.1)	1,237 (36.5)	1,587 (50.3)	
Higher education	2,840 (43.3)	1,914 (56.4)	926 (29.3)	
Missings, N (%)	309 (4.7)	76 (2.2)	233 (7.4)	
Marital status, N (%)				< 0.001
Married/living together	5,388 (82.3)	2,986 (88.0)	2,402 (76.1)	
No partner	829 (12.7)	298 (8.8)	531 (16.8)	
Missings, N (%)	332 (5.1)	109 (3.2)	223 (7.1)	
Ethnicity, N (%)				< 0.001
Dutch-European	3,954 (60.4)	2,441 (71.9)	1,513 (47.9)	
Surinamese	550 (8.4)	197 (5.8)	353 (11.2)	
Turkish	513 (7.8)	174 (5.1)	339 (10.7)	
Moroccan	353 (5.4)	107 (3.2)	246 (7.8)	
Cape Verdian	236 (3.6)	69 (2.0)	167 (5.3)	

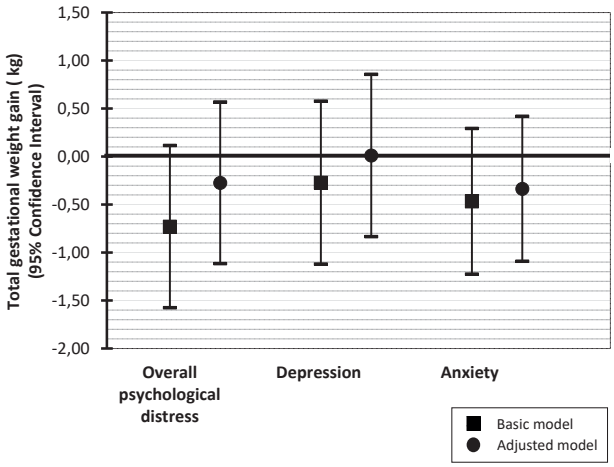
Supplementary Material 2. Characteristics of women with and without information on weight gain in pregnancy (N = 6,549)[a] (continued)

	Full group (N=6,549)	Responders (N=3,393)	Non-responders (N=3,156)	P-value [b]
Dutch Antilles	206 (3.1)	67 (2.0)	139 (4.4)	
Others	592 (9.0)	317 (9.3)	275 (8.7)	
Missings, N (%)	145 (2.2)	21 (0.6)	124 (3.9)	
Alcohol consumption, N (%)				< 0.001
No	2,687 (41.0)	1,177 (34.7)	1,510 (47.8)	
Yes	3,222 (49.2)	1,906 (56.2)	1,316 (41.7)	
Missings, N (%)	640 (9.8)	310 (9.1)	330 (10.5)	
Smoking habits, N (%)				< 0.001
No	4,378 (66.8)	2,375 (70.0)	2,003 (63.5)	
During first trimester only	500 (7.6)	294 (8.7)	206 (6.5)	
Continued during pregnancy	1,115 (17.0)	448 (13.2)	667 (21.1)	
Missings, N (%)	556 (8.5)	276 (8.1)	280 (8.9)	
Folic acid supplement use, N (%)				< 0.001
No	1,298 (19.8)	459 (13.5)	839 (26.6)	
Start during first 10 weeks of pregnancy	1,628 (24.9)	887 (26.1)	741 (23.5)	
Preconception use	2,174 (33.2)	1,366 (40.3)	808 (25.6)	
Missings, N (%)	1,449 (22.1)	681 (20.1)	768 (24.3)	
Total daily energy intake, mean (SD), kcal	2,051 (557)	2,094 (525)	2,001 (589)	< 0.001
Missings, N (%)	1,324 (20.2)	584 (17.2)	740 (23.4)	

[a] Values are means (standard deviation) for continuous variables with a normal distribution, or medians (95% range) for continuous variables with a skewed distribution, and valid percentages for categorical variables. Missing values in covariates are imputed.

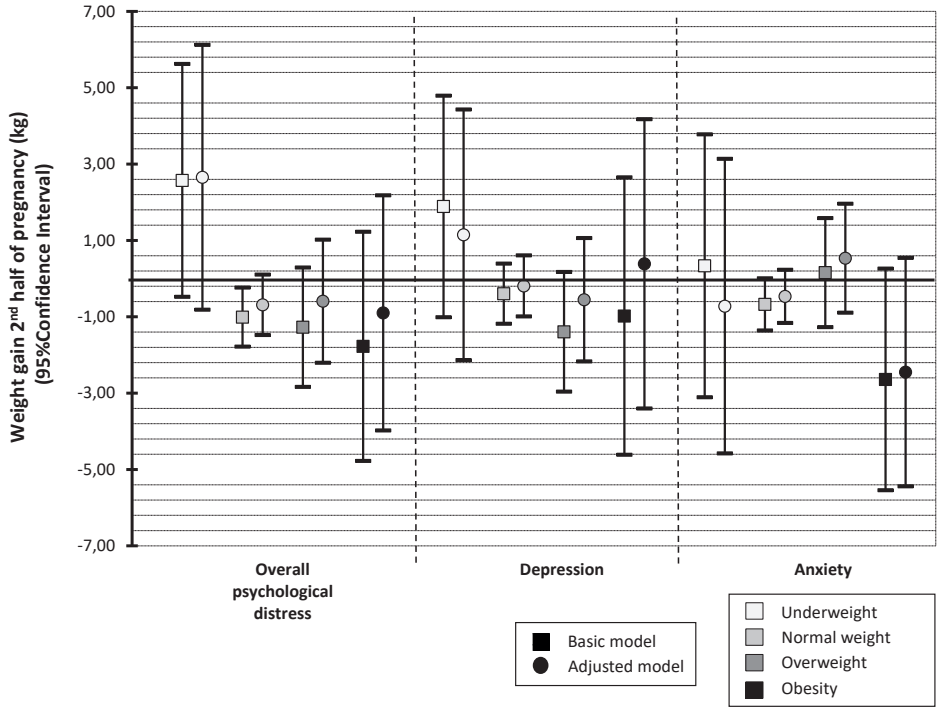
[b] P-values for differences in subject characteristics between responders and non-responders were calculated performing independent sample t-tests for normally distributed continuous variables, Mann-Whitney test for not normally distributed continues variables and chi-square tests for categorical variables.

Supplementary Material 3. Associations of psychological distress with total gestation weight gain (N = 2,917)



Values are linear regression coefficients (95% confidence intervals) and represent the overall change in total gestational weight gain for psychological distress, depression and anxiety compared to no psychological distress, depression or anxiety. The basic model was adjusted for maternal age. The adjusted model was adjusted for maternal age, pre-pregnancy BMI, parity, education, marital status, ethnicity, alcohol intake, smoking, folic acid use and nutritional intake.

Supplementary Material 4. Associations of psychological distress with weight gain in 2nd half of pregnancy for different pre-pregnancy BMI categories (N = 2,784)

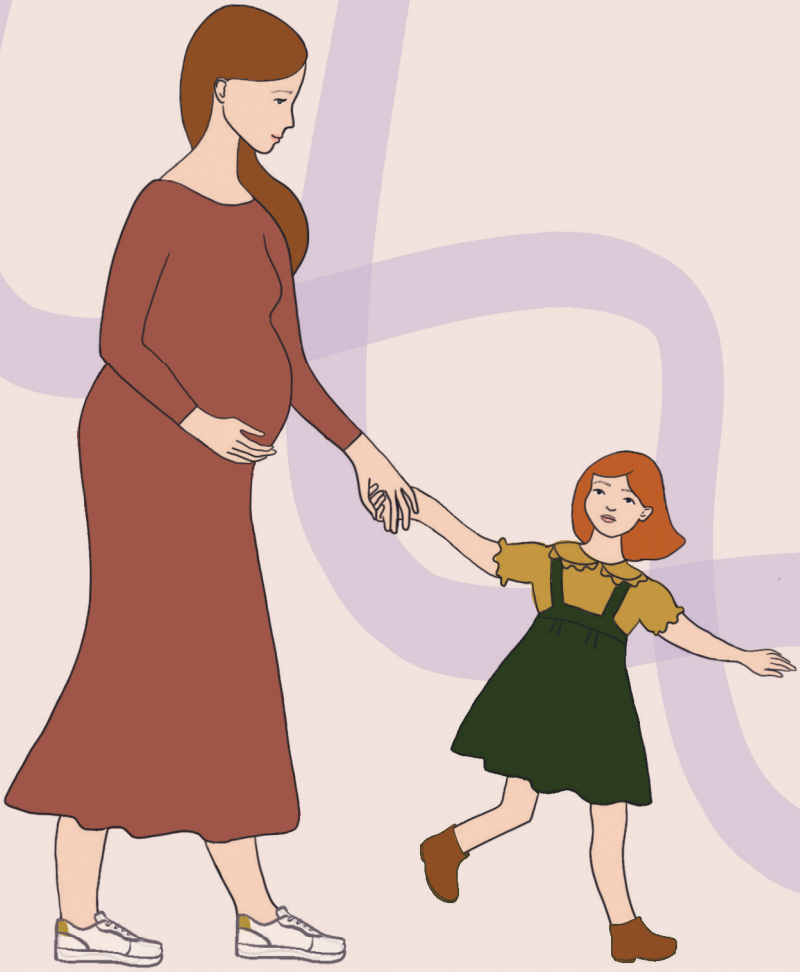


Values are linear regression coefficients (95% confidence intervals) and represent the overall change in the weight gain in the second half of pregnancy for psychological distress, depression and anxiety compared to no psychological distress, depression or anxiety for the different pre-pregnancy BMI groups. The basic model was adjusted for maternal age. The adjusted model was adjusted for maternal age, parity, education, marital status, ethnicity, alcohol intake, smoking, folic acid use and nutritional intake. P-value for pre-pregnancy BMI interaction was < 0.05 for the basic model of overall psychological distress and weight gain in the second half of pregnancy. Pre-pregnancy BMI interaction terms were not significant in the other models.

Supplementary Material 5. Associations of psychological distress stress with weight gain in full term women

	Weight gain 2nd half pregnancy (kg) (N = 3,139)	Total gestational weight gain (kg) (N = 2,813)
Overall psychological distress		
No	Reference	Reference
Yes – basic	**-0.99 (-1.63;-0.35)****	**-0.91 (-1.77;-0.05)***
Yes – adjusted	-0.53 (-1.22;0.16)	-0.39 (-1.24;0.47)
Depression		
No	Reference	Reference
Yes - basic	-0.41 (-1.05;0.23)	-0.14 (-1.00;0.75)
Yes – adjusted	-0.09 (-0.79;0.60)	0.18 (-0.68;1.03)
Anxiety		
No	Reference	Reference
Yes – basic	**-0.75 (-1.32;-0.17)***	-0.66 (-1.43;0.11)
Yes – adjusted	-0.44 (-1.05;0.17)	-0.53 (-1.29;0.23)

Values are linear regression coefficients (95% confidence intervals) and represent the overall change in weight gain in the second half of pregnancy and total gestational weight gain for psychological distress, depression and anxiety compared to no psychological distress, depression or anxiety. The basic model was adjusted for maternal age and gestational age at birth. The adjusted model was adjusted for maternal age, gestational age at birth and pre-pregnancy BMI, parity, education, marital status, ethnicity, alcohol intake, smoking, folic acid use and nutritional intake. *P-value < 0.05. **P-value < 0.01.



2.3

Associations of maternal psychological distress during pregnancy with childhood general and organ fat measures

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ABSTRACT

Background: Psychological distress during pregnancy may influence offspring adiposity. No studies assessed the associations with organ fat measures. We examined the associations of maternal psychological distress, depression and anxiety during pregnancy with child general and organ fat measures.

Methods: In 4,161 mother-offspring pairs, psychological distress was self-reported in pregnancy. We obtained general fat measures including BMI and fat mass index by dual-energy X-ray absorptiometry and organ fat measures (in a subsample of 2,447 children) including subcutaneous, visceral, and pericardial fat indices and liver fat fraction by Magnetic Resonance Imaging at 10 years. Linear and logistic regression models were used.

Results*:* Children of mothers with psychological distress had higher fat mass index (difference 0.14 (95% Confidence Interval (CI) 0.04, 0.24) standard deviation scores (SDS)) and higher risk of obesity (Odds Ratio (OR) 1.73 (95% CI 1.09, 2.74). Maternal anxiety was associated with higher BMI (difference 0.16 (95% CI 0.05, 0.26) SDS), fat mass index (difference 0.19 (95% CI 0.10, 0.28) SDS) and higher risks of overweight and obesity (OR 1.36 (95% CI 1.03, 1.81), 1.78 (95% CI 1.13, 2.81)). Maternal anxiety was associated with higher subcutaneous and visceral fat indices and liver fat fraction (differences 0.16 (95% CI 0.03, 0.29), 0.15 (95% CI 0.01, 0.29), 0.16 (95% CI 0.02, 0.29) SDS). No associations were observed for maternal depression.

Conclusions: Psychological distress and anxiety, but not depression, during pregnancy were associated with higher child general and organ fat measures. A healthy mental state during pregnancy may be important for preventing child adiposity.

INTRODUCTION

Psychological distress is common during pregnancy, affecting 10-20% of pregnant women.[1-4] Psychological distress is mostly defined as perceived stress, depressive symptoms, anxiety or experiencing an adverse life event.[2, 5] Maternal psychological distress during pregnancy is associated with several adverse fetal outcomes such as intra-uterine growth retardation and low birth weight.[6, 7] Psychological distress during pregnancy may lead to developmental adaptations of the fetus, which may have persistent consequences for body composition in later life.[8, 9] One of the most described potentially involved mechanisms includes fetal hypothalamic-pituitary-adrenal (HPA) axis dysregulation in response to increased maternal stress hormones like cortisol.[10, 11] Previous studies showed that an altered fetal HPA axis is associated with an increased risk of adiposity later in life.[12, 13] Although various studies have observed an increased risk of obesity in children exposed to prenatal psychological distress, results are not consistent.[14-20] BMI is easy to obtain, but does not give insight on the body fat distribution. The Framingham Heart Study and the Jackson Heart Study have reported that excess visceral, pericardial and liver fat are related to various cardio-metabolic abnormalities, independently of BMI.[21-24] To date, no studies assessed the association between maternal psychological distress and childhood organ fat measures.

We hypothesized that psychological distress during pregnancy is associated with childhood general and organ fat measures. We examined, in a population-based prospective cohort study among 4,161 mothers and their children, the associations of maternal overall psychological distress, depression and anxiety during pregnancy with offspring BMI, fat mass index measured by dual-energy X-ray absorptiometry (DXA) and subcutaneous fat index, visceral fat index, pericardial fat index and liver fat fraction measured by Magnetic Resonance Imaging (MRI) at 10 years.

METHODS

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early pregnancy until young adulthood onwards in Rotterdam, the Netherlands.[25] The study was approved by the local Medical Ethics Committee of Erasmus MC (MEC 198.782/2001/31). Pregnant women were enrolled between 2002 and 2006. Of all the eligible children in the study area, 61% participated at birth in the study. Written informed consent was obtained for all mothers and children. In total, 8,879 mothers were enrolled during their pregnancy.[26] We excluded pregnancies not leading to singleton live births (N = 246). Of 8,633 mothers and their singleton children, informa-

tion about psychological distress during pregnancy was available in 6,548 mothers. For 2,387 children, no information on any measurement of adiposity at 10 years was available. Thus, the population for analysis consisted of 4,161 mothers and their children **(Figure S1).**

Psychological distress during pregnancy

Information on maternal psychological distress was obtained through questionnaires that were mailed to participants and completed at approximately 20 weeks of gestation. The Brief Symptom Inventory (BSI) is a validated self-report questionnaire consisting of 53 items.[27] These items describe multidimensional psychopathology symptoms that mothers may have experienced in the preceding 7 days. The items are divided in 9 subscales (including anxiety, depression, hostility, phobic anxiety, interpersonal sensitivity, obsessive-compulsiveness, paranoid ideation, psychoticism and somatization).

As an indicator of overall psychological distress, we used the Global Severity Index (GSI), that is a total score of the 9 subscales. Additionally, we used the depression and anxiety subscales separately. We chose these 2 subscales, because they are widely used as valid proxies for psychological distress during pregnancy. [2, 5] Moreover, relating depression and anxiety, separately, with childhood adiposity could uncover different associations, as hypothesized by previous studies. [28]

The items were rated on a 5-point unidimensional scale ranging from '0' (not at all) to '4' (extremely) indicating to what extent the symptom was experienced. A total score was provided for each symptom scale by summing the item scores and dividing the results by the number of reported symptoms. Higher scores represented an increased occurrence of overall psychological symptoms. Then, women were categorized according to the presence of 'clinically' significant psychological symptoms (into "yes" or "no" categories) by using the following cut-offs derived from a psychiatric outpatient sample of Dutch women: 0.71 for the overall psychological symptoms scale; 0.80 for the depression scale and 0.71 for the anxiety scale.[29, 30] In the group of women with symptoms of overall psychological distress (>0.71), depression (>0.80) or anxiety (>0.71), we considered moderate or severe stress, depression or anxiety if below or above the 85th percentile of our study population, respectively.

Measures of adiposity at 10 years

As previously described, children around the age of 10 years were invited to visit our research center at the Erasmus MC-Sophia Children's Hospital to participate in hands-on measurements.[25] We calculated BMI (kg/m^2) at this age from height and weight, both measured without shoes and heavy clothing. We calculated sex- and age- adjusted standard deviation scores (SDS) of childhood BMI based on Dutch reference growth charts (Growth Analyzer 4.0, Dutch Growth Research Foundation).[31] Child BMI catego-

ries (underweight, normal weight, overweight and obesity) were calculated using the International Obesity Task Force cut-offs.[32, 33] We measured total body fat mass using a DXA scanner (iDXA, GE140 Lunar, 2008, Madison, WI, USA, enCORE software v.12.6), according to standard procedures.[34] Previous studies showed that DXA can accurately measure body fat.[35]

Measures of organ fat at 10 years were obtained from MRI scans.[25] MRI has been considered the gold standard for the measurement of intra-abdominal and organ fat deposition because it is an accurate and reproducible technique.[36] Children were scanned using a 3.0 Tesla MRI (Discovery MR 750w, GE Healthcare, Milwaukee, WI, USA) for body fat imaging using standard imaging and positioning protocols, while performing expiration breath-hold maneuvres of maximum 11 seconds duration. They wore light clothing without metal objects while undergoing the body scan.[37] Pericardial fat imaging in short axis orientation was performed using an ECG triggered black-blood prepared thin slice single shot fast spin echo acquisition (BB SSFSE) with multi-breath-hold approach. An axial 3-point Dixon acquisition for fat and water separation (IDEAL IQ) was used for liver fat imaging. This technique also enables the generation of liver fat fraction images.[38] An axial abdominal scan from lower liver to pelvis and a coronal scan centered at the head of the femurs were performed with a 2-point DIXON acquisition (LavaFlex).

The obtained fat scans were subsequently analyzed by the Precision Image Analysis company (PIA, Kirkland, Washington, United States), using the sliceOmatic (TomoVision, Magog, Canada) software package. All extraneous structures and any image artifacts were removed manually.[36] Pericardial fat included both epicardial- and paracardial fat directly attached to the pericardium, ranging from the apex to the left ventricular outflow tract. Total subcutaneous and visceral fat volumes were generated by summing the volumes of the liver, abdominal and if necessary the femoral fat-only scans, encompassing the fat volume ranging from the dome of the liver to the superior part of the femoral head. Fat masses were obtained by multiplying the total volumes by the specific gravity of adipose tissue, 0.9 g/ml. Liver fat fraction was determined by taking four samples of at least 4 cm^2 from the central portion of the hepatic volume. Subsequently, the mean signal intensities were averaged to generate an overall mean liver fat fraction estimation.

To create measures independent of height, we estimated the optimal adjustment by log-log regression analyses and subsequently we divided total and subcutaneous fat mass by $height^4$ (fat mass index and subcutaneous fat index) and visceral and pericardial fat mass by $height^3$ (visceral and pericardial fat indices) (More details given in **Supplemental Methods**).[39, 40]

Covariates

We obtained information on maternal age, pre-pregnancy BMI, ethnicity (European vs non-European), educational level, marital status, smoking habits, alcohol consump-

tion during pregnancy, and folic acid supplement use, by questionnaire at enrollment. Smoking habits, higher alcohol consumption and inadequate folic acid supplement use may be related to childhood adiposity and may be more frequent in pregnant women with psychological distress as these women are more likely to adopt unhealthy behaviors.[41-44] Information on child sex was available from medical records. Information on the average child television watching time was obtained by questionnaires at the age of 10 years filled out by the mother.

Statistical analysis

First, we performed descriptive statistics to gain better understanding on the differences between women with and without psychological distress and between participants and non-participants. Second, we used linear regression models to assess the associations of maternal overall psychological distress, depression and anxiety with offspring adiposity measures at 10 years (BMI, fat mass index, subcutaneous, visceral and pericardial fat indices and liver fat fraction). Third, we used multinomial logistic regression models to assess the associations of maternal overall psychological distress, depression and anxiety with the risk of childhood underweight, overweight or obesity. Fourth, to explore whether the associations differ by severity of stress, we assessed the associations of moderate and severe levels of maternal overall psychological distress, depression and anxiety with childhood adiposity measures at 10 years.

We used a basic model including child sex and age at outcome measurements, and a confounder model, which additionally included all aforementioned covariates. We identified potential covariates based on the graphical criteria for confounding or due to the relation with the outcomes by visualizing a directed acyclic graph (DAG) and included the covariates in the models that changed the effect estimates >10%.[45, 46] **Figure S2** shows a DAG depicting the covariates included in the models. We log-transformed the non-normally distributed childhood DXA and MRI adiposity measures. We constructed SDS [(observed value - mean)/SD] of the sample distribution for DXA and MRI outcomes to enable comparisons of effect sizes. No statistical interactions of maternal psychological distress with maternal ethnicity and child sex were observed in the associations with all childhood adiposity measures. We hypothesized that psychological distress is associated with higher child general and organ fat measures. Since we tested a single hypothesis with several exposures and outcomes, correction for multiple testing seems unnecessary.[47] To enable interpretation of statistical significance level, we presented p-values < 0.05 and p-values < 0.01. In order to maintain statistical power and reduce bias related to missing data on covariates we performed multiple imputation according to Markov Chain Monte Carlo method.[48] The percentage of missing data on covariates ranged from 0 to 21%. Psychological distress and adiposity measures were used as predictor variables only and were not imputed. Covariates were imputed and used as pre-

dictor variables. Five imputed datasets were created and pooled results are presented. No substantial differences in descriptive statistics were found between the original and imputed datasets. All statistical analyses were performed using the Statistical Package of Social Sciences version 24.0 for Windows (IBM SPSS Inc, Chicago, IL, USA).

RESULTS

Participant characteristics

In total, 8.6%, 8.6% and 9.6% of all pregnant women experienced psychological distress, depression and anxiety, respectively. **Table 1** shows the participant characteristics. Women with psychological distress were more often younger, non-European, lower educated, without partner, with higher pre-pregnancy BMI and were more likely to be smokers compared to women without psychological distress. Mothers of children with follow-up data available were slightly older, more often European, higher educated and reported less psychological distress compared to mothers of children without follow-up data available **(Table S1 in Supplementary Material)**.

Table 1. Characteristics of mothers and their children (N= 4,161)[a]

	Total group (N= 4,161)	No psychological distress (N= 3,802)	Psychological distress (N= 359)
Maternal characteristics			
Age at intake, mean (SD), years	30.9 (4.8)	31.2 (4.6)	28.1 (5.8)
Ethnicity, N (%)			
European	2,818 (68.3)	2,712 (71.8)	106 (30.4)
Non-European	1,309 (31.7)	1,066 (28.2)	243 (69.6)
Education, N (%)			
Primary school	256 (6.4)	200 (5.4)	56 (17.2)
Secondary school	1,660 (41.2)	1,459 (39.4)	201 (61.7)
High education	2,115 (52.5)	2,046 (55.2)	69 (21.2)
Marital status, N (%)			
Married/living together	3,565 (89.1)	3,324 (90.8)	241 (71.3)
No partner	434 (10.9)	337 (9.2)	97 (28.7)
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.5 (18.1, 34.2)	22.5 (18.1, 34.0)	23.1 (17.9, 36.1)
Alcohol consumption during pregnancy, N (%)			
Yes	2,254 (59.7)	2,114 (61.1)	140 (44.7)
No	1,519 (40.3)	1,346 (38.9)	173 (55.3)
Smoking during pregnancy, N(%)			

Table 1. Characteristics of mothers and their children (N= 4,161)[a] (continued)

	Total group (N= 4,161)	No psychological distress (N= 3,802)	Psychological distress (N= 359)
Yes	920 (24.1)	784 (22.4)	136 (42.2)
No	2,897 (75.9)	2,711 (77.6)	186 (57.8)
Folic acid supplement use, N (%)			
No	657 (20.0)	547 (18.0)	110 (45.3)
Start during first 10 weeks	1,047 (31.9)	963 (31.7)	84 (34.6)
Preconceptional use	1,581 (48.1)	1,532 (50.4)	49 (20.2)
Child characteristics			
Sex, N (%)			
Boys	2,034 (48.9)	1,839 (48.4)	195 (54.3)
Girls	2,127 (51.1)	1,963 (51.6)	164 (45.7)
Age at visit, mean (SD), years	9.8 (0.3)	9.8 (0.3)	9.8 (0.4)
Height, mean (SD), cm	141.6 (6.6)	141.7 (6.6)	140.6 (7.0)
BMI, median (95% range), kg/m^2	16.9 (14.0, 24.6)	16.9 (14.0, 24.0)	17.8 (13.9, 27.6)
BMI categories, N (%)			
Underweight	292 (7.0)	271 (7.2)	21 (5.9)
Normal weight	3,143 (75.8)	2,915 (76.9)	228 (63.9)
Overweight	573 (13.8)	500 (13.2)	73 (20.4)
Obesity	139 (3.4)	104 (2.7)	35 (9.8)
Total fat mass, median (95% range), g	8,450 (4,466, 21,648)	8,355 (4,466, 20,930)	9,806 (4,408, 27,184)
Subcutaneous fat mass, median (95% range), g	1,305 (599, 5,319)	1,294 (599, 4,974)	1,480 (577, 7,184)
Visceral fat mass, median (95% range), g	367 (164, 969)	366 (164, 950)	370 (161, 1,178)
Pericardial fat mass, median (95% range), g	11 (5, 23)	11 (5, 23)	11 (4, 22)
Liver fat fraction, median (95% range), %	2.0 (1.2, 5.2)	2.0 (1.2, 4.9)	2.1 (1.2, 10.6)
Television watching time at 10 years, N (%)			
< 2 hours/day	2,352 (69.8)	2,228 (71.0)	124 (52.8)
≥ 2 hours/day	1,019 (30.2)	908 (29.0)	111 (47.2)

[a] Values are observed data and represent means (standard deviation), medians (95% range) or numbers of participants (valid %).

Maternal psychological distress and childhood general fat measures

Table 2 shows that, in the basic models, maternal overall psychological distress, depression and anxiety during pregnancy were associated with higher childhood BMI and fat mass index (p-values<0.05). After adjustment for potential confounders, maternal overall psychological distress was associated with higher childhood fat mass index (difference 0.14 (95% Confidence Interval (CI) 0.04,0.24)), and maternal anxiety was associated with higher childhood BMI and fat mass index (differences 0.16 (95% CI 0.05,0.26) SDS, 0.19 (95% CI 0.10, 0.28) SDS, respectively). The associations for maternal depression attenuated and

were no longer significant. When comparing the models with and without each specific covariate, we observed that inclusion of maternal educational level and child television watching time caused the largest change in effect estimates (>45% and >28%, respectively). **Figure 1** shows that in both basic and confounder models, none of the maternal psychological stress scales were associated with the risk of childhood underweight. On the contrary, in the basic models, all maternal stress scales were associated with an increased risk of childhood overweight and obesity (p-values<0.05). After adjustment for confounders, overall psychological distress remained associated with an increased risk of childhood obesity (Odds Ratio (OR) 1.73 (95% CI 1.09, 2.74)), but not overweight. Maternal anxiety remained associated with increased risks of childhood overweight (OR 1.36 (95% CI 1.03, 1.81)) and obesity (OR 1.78 (95% CI 1.13, 2.81)). No significant associations were observed for depression in the confounder model.

When we explored whether the associations differed by severity of maternal psychological distress, depression or anxiety, we observed stronger effect estimates for BMI and fat mass index when mothers had severe rather than moderate stress, depression or anxiety, but the confidence intervals overlapped and thus the differences were not statistically significant (**Figure S3 in Supplementary Material)**.

Table 2. Associations of maternal psychological distress, depression and anxiety scales with childhood general fat measures at 10 years (N= 4,147)

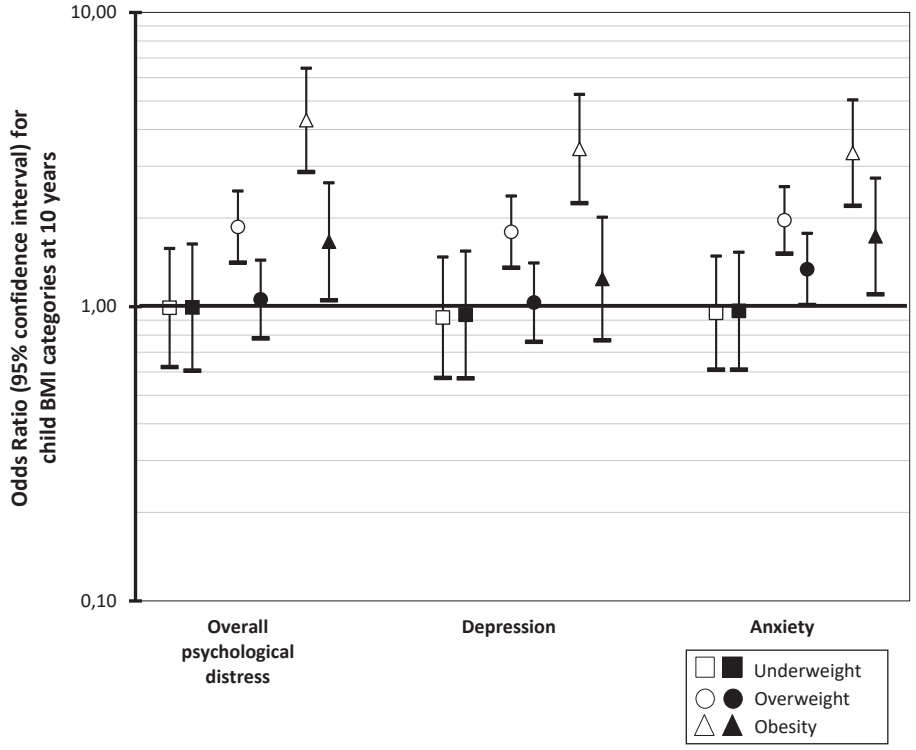
	Measures of general fat at 10 years in SDS			
Maternal psychological distress scales	**Body Mass Index (n=4,147)**		**Fat Mass Index (n=4,097)**	
	Basic Model	**Confounder Model**	**Basic Model**	**Confounder Model**
Overall distress				
No Stress	*Reference*	*Reference*	*Reference*	*Reference*
Stress	0.40 (0.29,0.51)**	0.10 (-0.02,0.21)	0.49 (0.38,0.59)**	0.14 (0.04,0.24)**
Depression				
No Depression	*Reference*	*Reference*	*Reference*	*Reference*
Depression	0.36 (0.25,0.47)**	0.06 (-0.05,0.17)	0.39 (0.28,0.49)**	0.06 (-0.04,0.16)
Anxiety				
No Anxiety	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.37 (0.26,0.48)**	0.16 (0.05,0.26)**	0.43 (0.33,0.53)**	0.19 (0.10,0.28)**

Values are linear regression coefficients (95% confidence interval) and reflect the change in SDS childhood BMI and fat mass index for stress, depression and anxiety, compared to the reference group. Basic models include child´s sex and age (except for sex- and age-adjusted body mass index SDS). Confounder models are additionally adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid use and child TV watching time. *p < 0.05. ** p < 0.01.

Maternal psychological distress and childhood organ fat measures

Table 3 shows that, in the basic models, maternal overall psychological distress, depression and anxiety during pregnancy were associated with higher childhood subcutaneous

Figure 1. Associations of maternal psychological distress, depression and anxiety scales with childhood BMI clinical categories at 10 years (N=4,147)



Values are odds ratios (95% Confidence Intervals) on a logarithmic scale and represent the risk of childhood underweight, overweight and obesity at 10 years for maternal overall psychological distress, depression and anxiety compared to no psychological distress, depression or anxiety. Basic models (□) include child´s sex and age. The confounder models (■) are additionally adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid supplement use and child television watching time.

fat index and liver fat fraction (p-values<0.05). Maternal anxiety was also associated with higher visceral fat index in the offspring (p-values<0.05). We did not observe associations of maternal psychological distress, depression and anxiety with childhood pericardial fat index. After adjustment for potential confounders, only maternal anxiety remained associated with higher childhood subcutaneous fat index (difference 0.16 (95% CI 0.03, 0.29) SDS), visceral fat index (difference 0.15 (95% CI 0.01, 0.29) SDS) and liver fat fraction (difference 0.16 (95% CI 0.02, 0.29) SDS). When comparing the models with and without each specific covariate, we observed that inclusion of maternal educational level and BMI before pregnancy caused the largest change in effect estimates (>60% and >30%, respectively). We observed a tendency towards stronger associations with organ fat measures when mothers had severe rather than moderate stress, depression or anxiety but the differences were not statistically significant (**Figure S4 in Supplementary Material**).

Table 3. Associations of maternal psychological distress, depression and anxiety scales with childhood organ fat measures at 10 years (N = 2,447)

Maternal psychological distress scales	Measures of organ fat at 10 years in SDS							
	Subcutaneous fat index (n=2,141)		Visceral fat index (n=2,141)		Pericardial fat index (n=2,210)		Liver fat fraction (n=2,410)	
	Basic Model	Confounder Model	Basic Model	Confounder Model	Basic Model	Confounder Model	Basic Model	Confounder Model
Overall distress								
No Stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	0.35 (0.20,0.50)**	0.03 (-0.12,0.18)	0.15 (-0.00,0.31)	0.04 (-0.11,0.20)	-0.05 (-0.21,0.11)	-0.08 (-0.24,0.08)	0.23 (0.09,0.37)**	0.09 (-0.06,0.24)
Depression								
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	0.30 (0.15,0.45)**	0.02 (-0.13,0.17)	0.13 (-0.02,0.29)	0.06 (-0.10,0.22)	-0.06 (-0.22,0.10)	-0.07 (-0.24,0.10)	0.22 (0.07,0.36)**	0.10 (-0.05,0.25)
Anxiety								
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.39 (0.25,0.52)**	0.16 (0.03,0.29)*	0.24 (0.10,0.38)**	0.15 (0.01,0.29)*	0.06 (-0.09,0.20)	0.04 (-0.11,0.18)	0.26 (0.13,0.40)**	0.16m (0.02,0.29)*

Values are linear regression coefficients (95% confidence interval) and reflect the change in SDS childhood subcutaneous, visceral, pericardial fat indices and liver fat fraction for overall psychological distress, depression and anxiety, compared to no psychological distress, depression or anxiety. Basic models include child´s sex and age. The confounder models are additionally adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid use and child TV watching time. *p < 0.05. ** p < 0.01.

DISCUSSION

In this population-based prospective cohort study, we observed that maternal psychological distress and anxiety during pregnancy were associated with higher general fat measures and an increased risk of overweight and obesity in the offspring. Maternal anxiety was also associated with higher subcutaneous, visceral and liver fat at 10 years. No associations were observed for maternal depression with childhood general and organ fat measures.

Interpretation of main findings

Psychological distress has been reported by 10-20% of women during pregnancy.[2, 4, 49] We observed a slightly lower but still considerable prevalence of overall psychological distress (8.6%), depression (8.6%) or anxiety (9.6%) in pregnancy. Previous studies examining the association of maternal psychological distress during pregnancy with childhood adiposity mainly focused on BMI. Most studies reported that maternal distress during pregnancy is associated with an increased risk of childhood overweight and obesity.[14, 18, 50, 51] For example, in a study among 65,212 mother-child pairs, 10-13-year old children exposed to prenatal stress, defined by being born to mothers who were bereaved by death of a close family member, had an increased risk of overweight.[14] On the other hand, the same study found no significant association between prenatal maternal stress and the risk of overweight in children younger than 10 years.[14] Likewise, 5-year-old children exposed to maternal psychological distress during pregnancy because of job strain did not have an increased BMI or fat mass index.[52] Also, we previously reported absence of an association between prenatal stress of the mother and offspring BMI in children aged 3 months to 3 years.[15] In the present study, we observed that overall psychological distress and anxiety during pregnancy were associated with an increased risk of childhood obesity and higher child fat mass index, anxiety was additionally associated with higher childhood BMI and an increased risk of child overweight at 10 years. Thus, maternal psychological distress during pregnancy seems not to influence fat mass development in early childhood, but the effects seem to become more apparent at older offspring ages.

Large studies such as the Framingham Heart study and the Jackson Heart Study have reported the important effect of excess ectopic fat deposition on an adverse cardiometabolic risk profile in adults. [21-24] Addressing the influence of maternal psychological distress during pregnancy on organ fat measures in addition to general fat measures gives a more complete understanding of the health risks in children. To our knowledge, the association between maternal distress during pregnancy and childhood organ fat measures has not been studied yet. In the present study, we observed that maternal anxiety during pregnancy was associated with higher subcutaneous fat index, vis-

ceral fat index and liver fat fraction in their 10-year old children. Remarkably, overall psychological distress and depression were not associated with childhood organ fat measures. Thus, maternal anxiety but not overall psychological distress and depression during pregnancy seems to influence organ fat development and ultimately the cardiometabolic health in the offspring.

The influence of maternal psychological distress during pregnancy on general and organ fat measures might be dependent on the levels of stress. Only one previous study investigated a dose-response relation between antenatal depression and childhood adiposity and found no evidence for such a relation.[17] Although we observed a tendency towards stronger associations with childhood adiposity measures when mothers reported severe rather than moderate stress, depression or anxiety, the differences between severe and moderate stress effect estimates were not statistically significant. Only a few women reported severe levels of stress in this study, compromising the statistical power and complicating the detection of significant associations. Thus, the findings may suggest that increasing levels of psychological distress are associated with increased childhood adiposity measures, which emphasizes the need to reduce the severity of psychological distress in pregnant women. There are various pathways through which maternal psychological distress during pregnancy may affect offspring adiposity.[11] Fetal programming of body composition, obesity and metabolic function could be influenced by maternal psychological distress during pregnancy.[53] The most frequently described mechanism includes fetal HPA axis dysregulation due to maternal stress hormones during pregnancy, which subsequently affects fetal and child growth and adiposity.[10, 11, 13] Other mechanisms involving the autonomic nervous system, maternal microbiome, (epi)genetics and inflammatory factors may also play an important (mediating) role.[11] However, from the current observational data, no conclusions can be drawn on the causality of the observed associations. Unmeasured lifestyle-related characteristics might also partly explain the associations. Psychological distress during pregnancy is related to an adverse maternal health behavior such as an unhealthy diet.[54] If antenatal psychological distress continues to be present after birth, this risk behavior may affect the body fat development of the child through, for example, parenting and dietary habits.[55] The consistent associations observed in this study for anxiety with general and organ fat measures and the absence of associations for depression suggest that the mechanisms might be dependent on the specific psychiatric symptom experienced or might be more pronounced with anxiety rather than with depressive symptoms. A tendency for maternal anxiety during pregnancy, and not depression, being associated with offspring outcomes was already observed in the same population as the current study but with fetal growth outcomes.[28]

Our study shows that maternal psychological distress and anxiety during pregnancy are associated with higher child general and organ fat measures, which have impor-

tant adverse cardio-metabolic health consequences. Further studies, using paternal psychological distress as a negative control exposure, are needed to obtain insight into the causality of the observed associations and the underlying biological mechanisms. If these associations are shown to be causal, promoting a healthy mental state during pregnancy is needed for a healthy fat mass development and ultimately to improve cardio-metabolic health of the offspring.

Strengths and limitations

Strengths of this study were the prospective data collection from pregnancy onwards, the large sample size and data available on detailed childhood adiposity measures including organ fat measures assessed by MRI. This study also has limitations. Of all women included during pregnancy with a singleton live-born child, 76% responded to the questionnaire. Only 64% of the children had information on at least one measurement of adiposity at 10 years. Non-response could lead to selection bias if the associations of maternal psychological distress with childhood adiposity measures differ between mothers and children included and excluded in the analyses. As shown in the non-response analyses, mothers of children with and without follow-up data were different regarding the socioeconomic background and frequency of psychological distress. We believe selection bias has little influence on our findings since we adjusted for covariates associated with loss to follow-up such as maternal sociodemographic and lifestyle related factors.[56] Inverse probability weighting techniques were not further applied since previous studies showed no additional effect on the estimates.[56] Another limitation is that we relied on self-report measurements of maternal psychological distress, which may have resulted in underreporting of psychological symptoms and subsequently in an underestimation of observed effects. Maternal psychological distress was measured at only one time point during pregnancy, on average at 20 weeks, and refers to the preceding 7 days. Therefore we do not know whether maternal psychological distress symptoms varied in intensity or were persistent throughout pregnancy. There are no clinical cut-offs to differentiate between moderate and severe levels of psychological distress. We used the 85th percentile, which has been used as cut-off to distinguish risk levels in other fields of research.[57] Future studies should assess the optimal cut-off to classify mothers according to the severity of stress symptoms. The BSI showed good accuracy to identify clinical depression and anxiety in a healthy and outpatient psychiatric population but has not been validated to date in pregnant women.[28] Finally, although we adjusted the analyses for many sociodemographic, lifestyle, and other variables known to influence the associations, residual confounding, for example by maternal nutritional intake, income, chronic conditions and physical activity, might still be present in our study.

CONCLUSION

Our results suggest that maternal overall psychological distress during pregnancy is associated with higher general fat measures and anxiety with higher general and organ fat measures in children aged 10 years. Our findings emphasize the importance of promoting a healthy mental state during pregnancy as it may have long-term consequences on child health.

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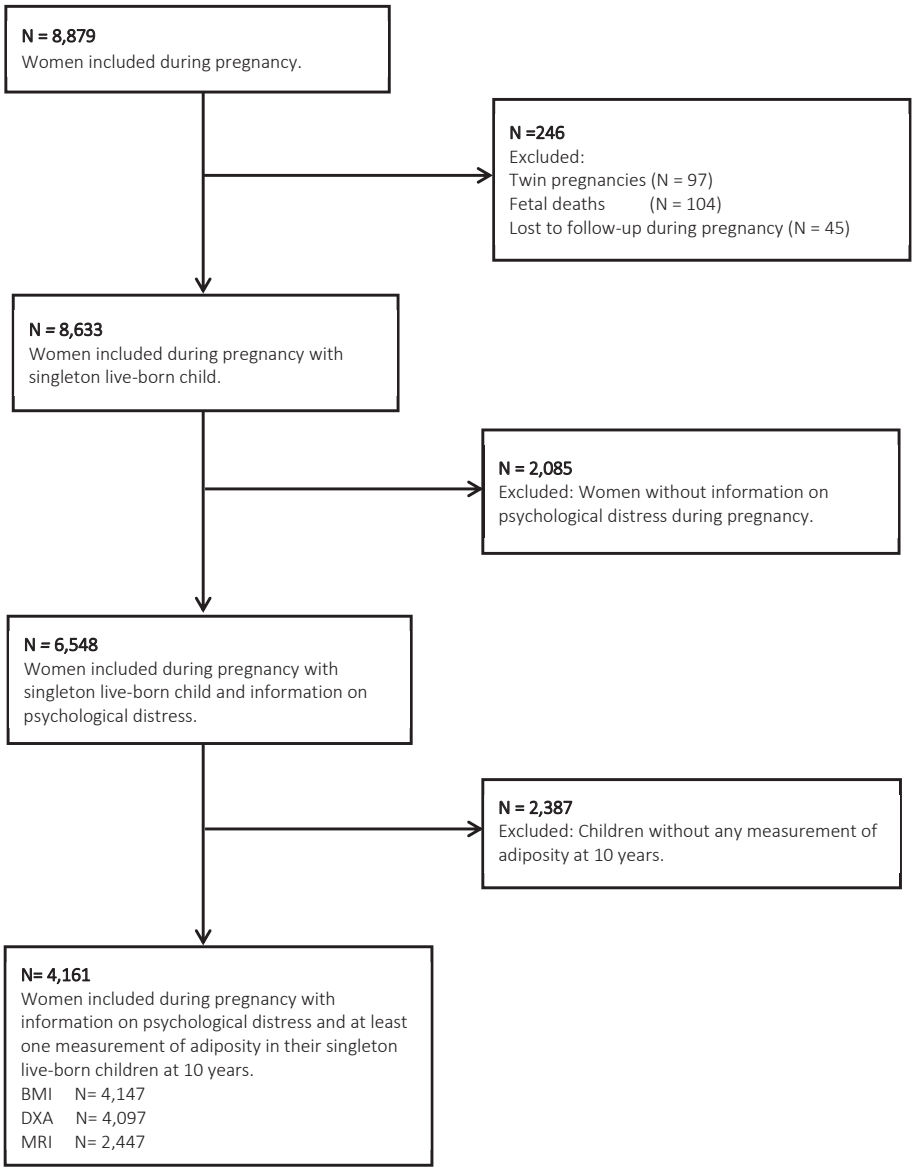
SUPPLEMENTARY MATERIAL

Figure S1. Flowchart of study population



Abbreviations: BMI = Body Mass Index, DXA = Dual-energy X-ray absorptiometry MRI = Magnetic Resonance Imaging

Supplemental Methods: Log-log regression analyses

We estimated the optimal adjustment by log-log regression analyses in order to create measures of child adiposity independent of height at 10 years.[1] Total fat mass, subcutaneous fat mass, visceral fat mass and pericardial fat mass and height were log-transformed, using natural logs. Log-adiposity measures were regressed on log-height. To calculate an index uncorrelated with height, we took the regression slope as the power by which height should be raised. Thus, we divided total fat mass by height^4, subcutaneous fat mass by height^4, visceral fat mass by height^3, and pericardial fat mass by height^3.

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Figure S2. Directed acyclic graph (DAG) for the relationship between maternal psychological distress during pregnancy and child adiposity measures at 10 years depicting the covariates included in the models

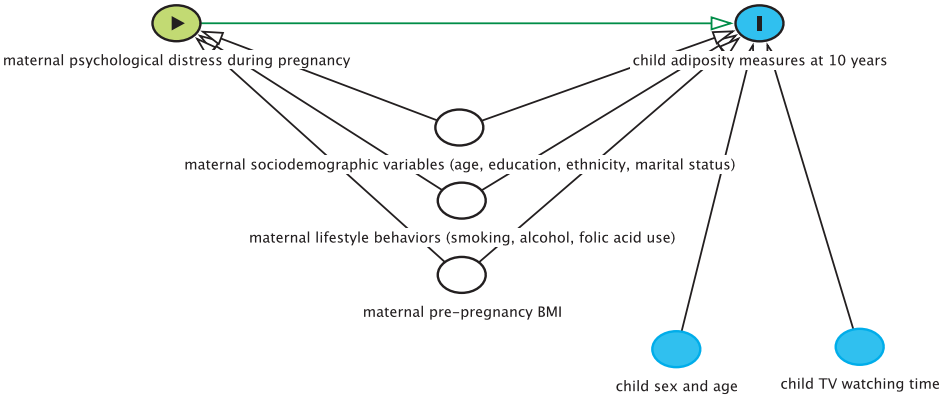
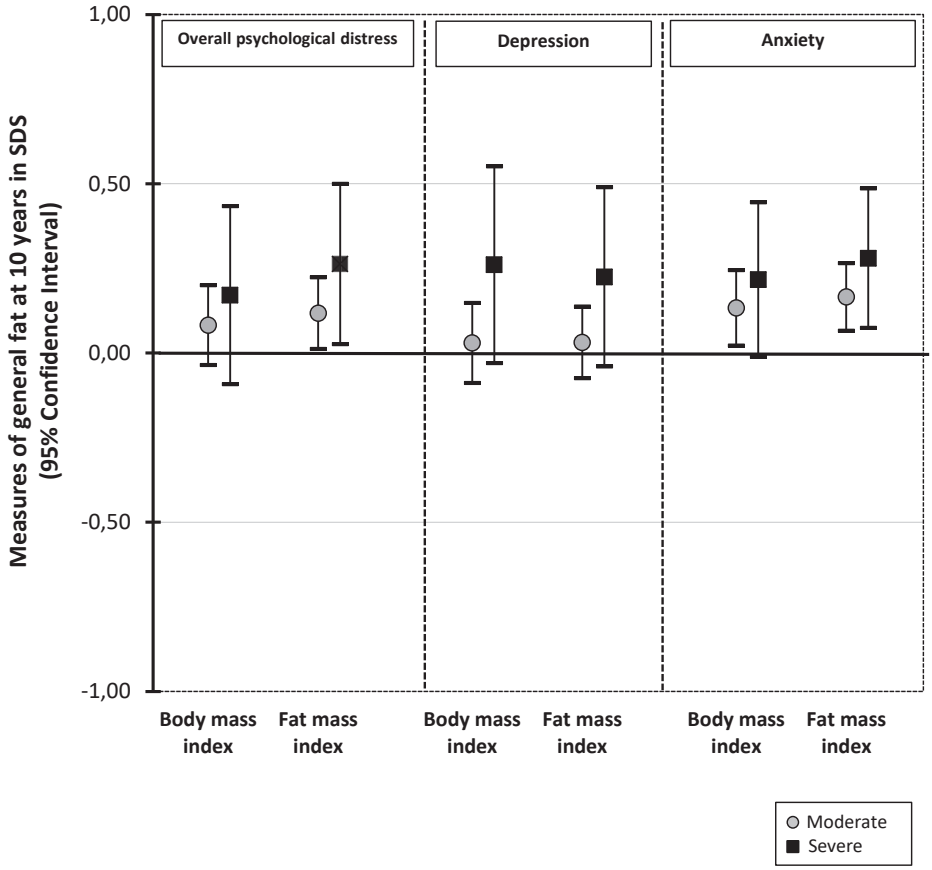


Table S1. Comparison of maternal and child characteristics between mothers and children with and without follow-up data available [a] (N = 6,548)

	With follow-up (N= 4,161)	Without follow-up (N= 2,387)
Maternal characteristics		
Age at intake, mean (SD), years	30.9 (4.8)	28.4 (5.4)
Ethnicity, N (%)		
European	2818 (68.3)	1,136 (49.9)
Non-European	1,309 (31.7)	1,141 (50.1)
Education, N (%)		
Primary school	256 (6.4)	360 (14.5)
Secondary school	1,660 (41.2)	1,164 (52.7)
High education	2,115 (52.5)	725 (32.8)
Marital status, N (%)		
Married/living together	3,565 (89.1)	1,823 (82.2)
No partner	434 (10.9)	395 (17.8)
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.5 (18.1, 34.2)	22.6 (17.7, 35.4)
Alcohol consumption during pregnancy, N (%)		
Yes	2,254 (59.7)	968 (45.3)
No	1,519 (40.3)	1,167 (54.7)
Smoking during pregnancy, N (%)		
Yes	920 (24.1)	694 (31.9)
No	2,897 (75.9)	1,481 (68.1)
Folic acid supplement use, N (%)		
No	657 (20.0)	641 (35.3)
Start during first 10 weeks	1,047 (31.9)	581 (32.0)
Preconceptional use	1,581 (48.1)	593 (32.7)
Overall psychological distress, N (%)		
Yes	359 (8.6)	351 (14.7)
No	3,802 (92.4)	2,036 (85.3)
Child characteristics		
Sex, N (%)		
Boys	2,034 (48.9)	1,226 (51.4)
Girls	2,127 (51.1)	1,161 (48.6)

[a] Values are observed data and represent means (standard deviation), medians (95% range) or numbers of participants (valid %).

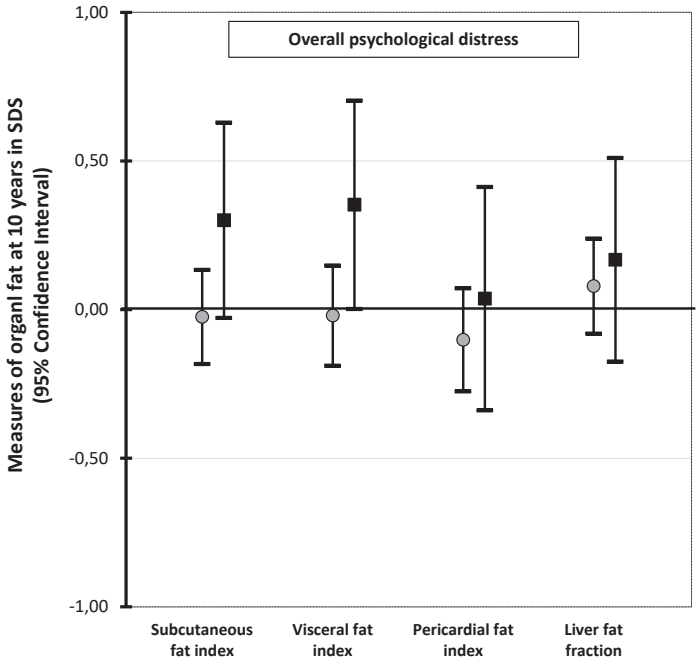
Figure S3. Associations of moderate and severe maternal psychological distress, depression and anxiety with childhood general fat measures at 10 years (N = 4,147)



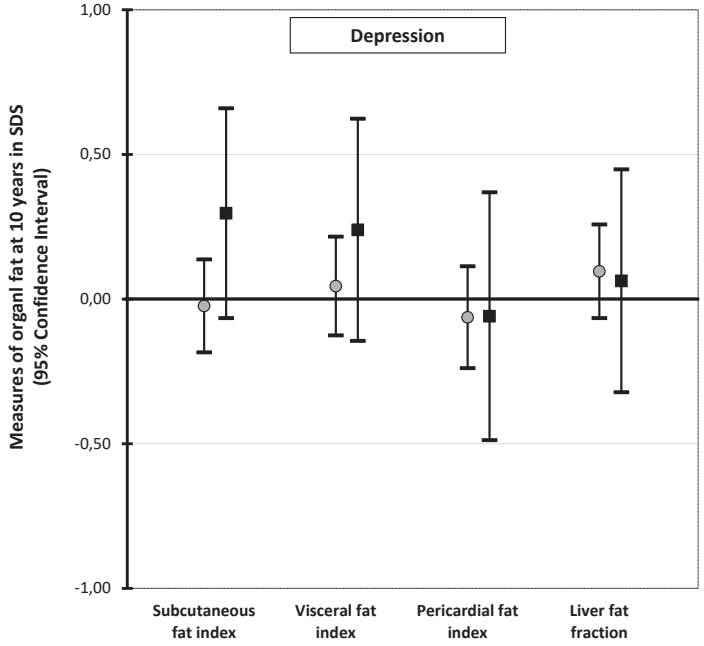
Values are linear regression coefficients (95% confidence intervals) and reflect the change in SDS in childhood BMI and fat mass index for severe and moderate maternal psychological stress, depression or anxiety as compared to no psychological stress, depression or anxiety. Only the confounder models are shown which are adjusted for child sex and age, maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid supplement use and child television watching time.

Figure S4. Associations of moderate and severe maternal psychological distress, depression and anxiety with childhood organ fat measures at 10 years (N = 2,447)

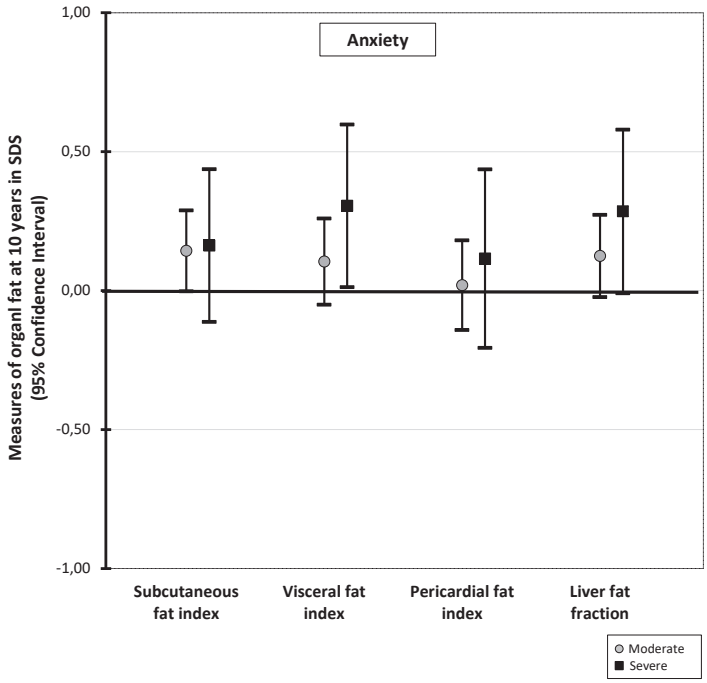
A.



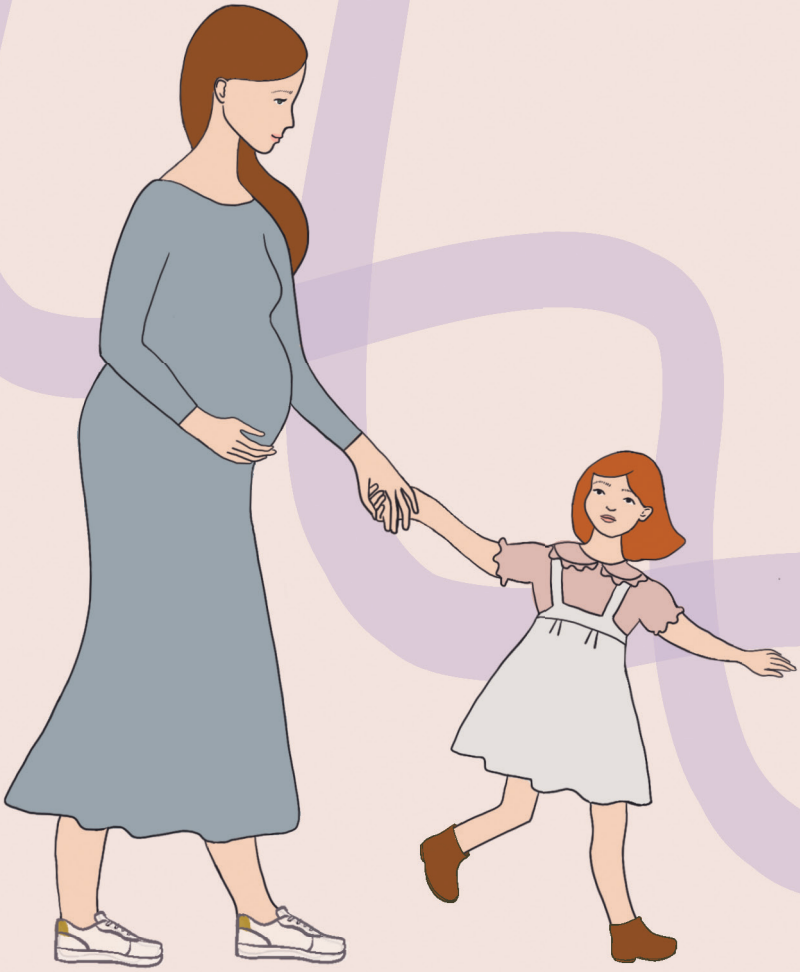
B.



C.



Values are linear regression coefficients (95% confidence intervals) and reflect the change in subcutaneous, visceral, pericardial fat indices and liver fat fraction for moderate and severe maternal psychological stress (A), depression (B) or anxiety (C) as compared to no psychological stress, depression or anxiety. Only the confounder models are shown which are adjusted for child sex and age, maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid supplement use and child television watching time.



2.4

Maternal psychological distress during pregnancy and childhood cardio-metabolic risk factors

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ABSTRACT

Background and Aims: Previous studies suggest that psychological distress during pregnancy may lead to fetal developmental adaptations, which programme cardio-metabolic disease of the offspring. We examined the associations of maternal overall psychological distress, depression and anxiety during pregnancy with cardio-metabolic risk factors in 10-year-old children and explore potential sex-specific differences.

Methods and results: In a population-based prospective cohort study among 4,088 mothers and their children, information about overall psychological distress, including depression and anxiety was obtained through the Brief Symptom Inventory during pregnancy. We measured child blood pressure and heart rate and insulin, glucose, serum lipids and C-reactive protein blood concentrations at 10 years. Analyses were performed in the total group and in boys and girls separately. Psychological distress during pregnancy was associated with higher childhood heart rate among boys only (differences 0.34 (95% Confidence Interval (CI) 0.18, 0.50) standard deviation scores (SDS), 0.22 (95% CI 0.06, 0.38) SDS, 0.33 (95% CI 0.19, 0.48) SDS, for overall psychological distress, depression and anxiety, respectively). Maternal anxiety during pregnancy was associated with higher childhood triglycerides among girls (difference 0.35 (95% CI 0.17, 0.53) SDS). Maternal psychological distress was not associated with childhood blood pressure, cholesterol, insulin, glucose and C-reactive protein concentrations.

Conclusions: Maternal psychological distress may influence their offspring heart rate and triglycerides concentrations. Further studies are needed to replicate these findings and assess the long-term cardio-metabolic consequences of maternal psychological distress.

INTRODUCTION

Pregnancy is a period of great physiological and psychological transformations.[1] Psychological distress has been reported by 10-20% of women during pregnancy.[2] Maternal psychological distress may cause a suboptimal intrauterine environment leading to long-term consequences on growth and health of the offspring.[3,4] More specifically, intrauterine stress exposure may affect offspring cardio-metabolic development via dysregulation of the hypothalamic-pituitary-adrenal axis, increase of inflammatory responses and changes in the balance of the autonomic nervous system.[5-7] In addition, growing evidence suggested sex-specific differences in fetal programming in response to stress, which may result in sex-specific risks for later diseases.[8,9] We have previously reported that maternal psychological distress during pregnancy was not associated with offspring infant heart rate and early-childhood blood pressure.[7,10] Other studies reported inconsistent associations of distress during pregnancy with blood pressure and insulin resistance in children and adolescents.[11-14] To date, no studies have focused on the associations of maternal psychological distress during pregnancy with childhood lipids profile or inflammatory markers. Insight into the associations of maternal distress during pregnancy with childhood cardio-metabolic risk factors may help to develop future preventive strategies. We examined, in a population-based prospective cohort study among 4,088 mothers and their children, the associations of maternal overall psychological distress, depression and anxiety during pregnancy with blood pressure, heart rate, lipids profile, glucose metabolism, and C-reactive protein concentrations in 10-year-old children. We explored whether the associations with cardio-metabolic risk factors differ for boys and girls.

METHODS

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until adulthood in Rotterdam, the Netherlands. The study was approved by the local Medical Ethics Committee of Erasmus MC (MEC 198.782/2001/31). Pregnant women were enrolled between 2002 and 2006. Written informed consent was obtained for all participants. In total, 8,879 mothers were enrolled during prenatal period.[15] We excluded pregnancies not leading to singleton live births (N = 246). Information about psychological distress during pregnancy was available in 6,548 of 8,633 mothers with singleton children. For 2,460 children, no information on any measurement of cardio-metabolic risk factors at 10 years was available. Thus, 4,088 mothers and children had information on psychological distress during pregnancy and at least one measure-

ment of cardio-metabolic risk factors at 10 years. The specific population for analysis for each outcome is shown in the flowchart. (**Figure S1** in Supplementary Material).

Psychological distress during pregnancy

Information on maternal psychological distress was obtained through the Brief Symptom Inventory (BSI) that was mailed to participants and returned at around 20 weeks of gestation. The BSI is a validated self-report questionnaire with 53 items, describing the psychopathologic problems and complaints that mothers may have experienced in the preceding 7 days.[16] These items include a broad spectrum of psychological symptoms, divided in 9 dimensions (anxiety, depression, hostility, phobic anxiety, interpersonal sensitivity, obsessive-compulsiveness, paranoid ideation, psychoticism, somatization). We used the overall psychological distress scale (Global Severity Index) and 2 symptom scales (depression and anxiety) to define psychological distress. We chose these subscales because depression and anxiety are widely used as indicators of psychological distress during pregnancy.[1] To indicate the extent of the symptoms, the items were rated on a 5-point unidimensional scale ranging from '0' (not at all) to '4' (extremely). A total score was provided for each symptom scale by summing the item scores and dividing the results by the number of reported symptoms. Then, the symptoms were dichotomized (into "yes" or "no" categories) by using the following cutoffs derived from a psychiatric outpatient sample of Dutch women: 0.71 for overall psychological symptoms scale; 0.80 for depression scale and 0.71 for anxiety scale.[17, 18]

Cardio-metabolic risk factors at 10 years

As previously described, children around the age of 10 years were invited to visit our research center at Erasmus MC-Sophia Children's Hospital.[19] 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).[20] We calculated the mean value for systolic and diastolic blood pressure and heart rate using the last three measurements of each participant. Non-fasting blood samples were collected to determine serum concentrations of glucose, insulin, total cholesterol, high-density lipoprotein (HDL)-cholesterol and triglycerides. Glucose, total cholesterol, HDL-cholesterol and triglycerides concentrations were measured using the c702 module on the Cobas 8000 analyzer. Insulin was measured with electrochemiluminescence immunoassay (ECLIA) on the E411 module (Roche, Almere, the Netherlands).[21] Low-density lipoprotein (LDL)–cholesterol was calculated according to the Friedewald formula.[22]

Covariates

We obtained information on maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, smoking habits and alcohol consumption during

pregnancy, and folic acid supplement use, by questionnaire. Information on maternal selective serotonin reuptake inhibitors (SSRIs) use in pregnancy was obtained by questionnaires and prescription records from pharmacies.[23] Information on child sex, gestational age at birth and birth weight were available from medical records. We calculated body mass index (kg/m^2) at 10 years from height and weight, both measured without shoes and heavy clothing.

Statistical analysis

We compared subject characteristics between women with and without psychological distress using Pearson's chi-square tests, independent sample t-tests and Mann-Whitney tests. Similar statistical tests were performed to compare characteristics between participants and non-participants. We used linear and logistic regression models to assess the associations of maternal overall psychological distress, depression and anxiety with childhood cardio-metabolic risk factors. We included covariates in the models if they were associated with maternal psychological distress and childhood cardio-metabolic risk factors in our study and if they changed the effect estimates substantially (>10%) for at least one outcome. Thus, all models were adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking, folic acid and selective serotonin reuptake inhibitors use during pregnancy. Child body mass index at 10 years might be in the causal pathway of the associations of maternal overall psychological distress with childhood cardio-metabolic risk factors. We assessed whether these associations were independent of child body mass index, by additionally adjusting our models for this covariate. The distributions of insulin and triglycerides were skewed and natural logged transformed. Since C-reactive protein was not normally distributed and the log-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.[24] To enable comparison of effect sizes of different outcome measures, we constructed standard deviation scores (SDS) ((observed value – mean) / SD). Analyses were performed for the total group and for boys and girls, separately. We found statistically significant sex interactions for the associations of maternal psychological distress with child heart rate and diastolic blood pressure. We did not observe statistical interactions for maternal ethnicity, child's gestational age at birth, birth weight and body mass index at 10 years. To enable interpretation of statistical significance level, we presented p-values<0.05 and p-values<0.01. Missing data in covariates (ranging from 0 to 21%) were multiple-imputed using 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 version 24.0 for Windows (SPSS IBM, Chicago, IL, USA).

RESULTS

Subject characteristics

Participants characteristics are presented in **Table 1**. Of all pregnant women, 8.5%, 8.6% and 9.5% experienced overall psychological distress, depression and anxiety, respectively. Women with psychological distress during pregnancy were more often younger, non-European, lower educated, without partner and were more likely to be smokers compared to women without psychological distress (p-values<0.05). Non-response analyses showed that mothers of children with follow-up data available were slightly older, more often European, higher educated and reported less clinical psychological distress during pregnancy compared to mothers of children without follow-up data available (p-values<0.05) (**Table S1** in **Supplementary Material**).

Table 1. Characteristics of mothers and their children[1]

Maternal characteristics	Total group (N= 4,088)	Overall psychological distress (N= 352)	No overall psychological distress (N= 3,736)	P-value[2]
Age at intake, mean (SD), years	30.9 (4.8)	28.1 (5.8)	31.2 (4.6)	< 0.001
Ethnicity, N(%)				< 0.001
European	2,767 (68.2)	104 (30.4)	2,663 (71.7)	
Non-European	1,288 (31.8)	238 (69.6)	1,050 (28.3)	
Education, N(%)				< 0.001
Primary school	255 (6.4)	56 (17.6)	199 (5.5)	
Secondary school	1,628 (41.1)	195 (61.1)	1,433 (39.4)	
High education	2,076 (52.4)	68 (21.3)	2,008 (55.2)	
Marital status, N(%)				< 0.001
Married/living together	3,502 (89.2)	236 (71.3)	3,266 (90.8)	
No partner	425 (10.8)	95 (28.7)	330 (9.2)	
Pre-pregnancy body mass index, median (95% range) kg/m^2	22.6 (18.1, 34.3)	23.2 (17.9, 36.1)	22.5 (18.1, 34.0)	< 0.05
Alcohol consumption, N (%)				< 0.001
Yes	2,219 (59.9)	137 (44.6)	2,082 (61.3)	
No	1,486 (40.1)	170 (55.4)	1,316 (38.7)	
Smoking, N (%)				< 0.001
Yes	901 (24.0)	132 (41.9)	769 (22.4)	
No	2,847 (76.0)	183 (58.1)	2,664 (77.6)	
Folic acid supplement use, N (%)				< 0.001
No	650 (20.1)	108 (44.8)	542 (18.2)	
Start during first 10 weeks	1,030 (31.9)	84 (34.9)	946 (31.7)	
Preconceptional use	1,546 (47.9)	49 (20.3)	1,497 (50.2)	

Table 1. Characteristics of mothers and their children[1] (continued)

Exposed to SSRIs, N (%)				< 0.001
Yes	43 (1.1)	12 (3.7)	31 (0.9)	
No	3,823 (98.9)	314 (96.3)	3,509 (99.1)	
Child characteristics				
Sex, N (%)				0.06
Boys	1,987 (48.6)	188 (53.4)	1,799 (48.2)	
Girls	2,101 (51.4)	164 (46.6)	1,937 (51.8)	
Gestational age at birth, N (%)				< 0.05
Preterm (< 37 weeks)	178 (4.4)	23 (6.5)	155 (4.1)	
Term (≥ 37 weeks)	3,910 (95.6)	329 (93.5)	3,581 (95.9)	
Birth weight[3], N (%)				< 0.05
Small for gestational age	405 (9.9)	48 (13.7)	357 (9.6)	
Appropriate for gestational age	3,270 (80.1)	277 (78.9)	2,993 (80.2)	
Large for gestational age	409 (10.0)	26 (7.4)	383 (10.3)	
Age at visit, mean (SD), years	9.8 (0.3)	9.8 (0.4)	9.8 (0.3)	< 0.05
Body mass index, median (95% range), kg/m^2	16.9 (14.0, 24.5)	17.8 (13.9, 27.7)	16.9 (14.0, 24.0)	< 0.001
Systolic blood pressure, mean (SD), mmHg	103.1 (8.0)	104.8 (8.9)	102.9 (7.9)	< 0.001
Diastolic blood pressure, mean (SD), mmHg	58.5 (6.4)	59.7 (7.0)	58.4 (6.4)	< 0.001
Heart rate, mean (SD), beats/minute	73.5 (10.0)	76.7 (10.7)	73.2 (9.9)	< 0.001
Insulin, median (95% range), pmol/L	172.9 (35.2, 642.6)	206.8 (40.7, 824.6)	170.2 (34.6, 637.5)	< 0.05
Glucose, mean (SD), mmol/L	5.2 (0.9)	5.2 (0.9)	5.2 (0.9)	0.77
Total-cholesterol, mean (SD),mmol/L	4.3 (0.7)	4.3 (0.7)	4.3 (0.7)	0.53
HDL-cholesterol, mean (SD), mmol/L	1.5 (0.3)	1.4 (0.3)	1.5 (0.3)	< 0.05
LDL-cholesterol, mean (SD), mmol/L	2.3 (0.6)	2.3 (0.6)	2.3 (0.6)	0.96
Triglycerides, median (95% range), mmol/L	1.0 (0.4, 2.6)	1.0 (0.4, 3.0)	1.0 (0.4, 2.5)	0.32
C-reactive protein, median (95% range), mg/L	0.3 (0.3, 5.2)	0.3 (0.3, 12.4)	0.3 (0.3, 4.9)	< 0.001

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

[2] P-values for differences in subject characteristics between groups were calculated performing independent sample t-tests for normally distributed continuous variables, Mann-Whitney test for not normally distributed continuous variables and chi-square tests for categorical variables.

[3] Sex- and gestational age-adjusted birth weight SDS were created based on a North-European reference chart. Small and large size for gestational age at birth were defined as sex- and gestational age-adjusted birth weight below the 10th percentile and above the 90th percentile, respectively.

Maternal psychological distress and childhood blood pressure and heart rate

In the unadjusted models, maternal overall psychological distress, depression and anxiety during pregnancy were associated with higher childhood blood pressure in the total group and among boys (p-values<0.05). Maternal overall distress and anxiety were also associated with higher childhood systolic and diastolic blood pressure, respectively among girls (p-values<0.05). All maternal psychological distress scales were associated with higher childhood heart rate among boys and girls (p-values<0.05) (**Table S2** in **Supplementary Material**). After adjustment for potential confounders, no associations were observed between maternal overall psychological distress, depression and anxiety and childhood blood pressure in boys and girls. All maternal psychological distress scales remained associated with higher childhood heart rate only among boys (differences 0.34 (95% Confidence Interval (CI) 0.18,0.50) SDS, 0.22 (95% CI 0.06,0.38) SDS, 0.33 (95% CI 0.19, 0.48) SDS for overall distress, depression and anxiety, respectively) (**Table 2**). After additional adjustment for child body mass index, similar associations of maternal psychological distress scales with childhood blood pressure and heart rate were observed (**Table S3** in **Supplementary Material**).

Maternal psychological distress and childhood lipids profile

In the unadjusted models, no associations were observed of any maternal psychological distress scales with total cholesterol concentrations. Overall psychological distress and depression were associated with lower HDL-cholesterol concentrations among boys, whereas anxiety was associated with lower HDL-cholesterol and higher triglycerides concentrations among girls (p-values<0.05) (**Table S4** in **Supplementary Material**). After adjustment for potential confounders, only maternal anxiety remained associated with higher childhood triglycerides among girls (difference 0.35 (95% CI 0.17, 0.53) SDS) (**Table 3**). Similar associations were observed after further adjustment for body mass index at 10 years (**Table S5** in **Supplementary Material**). No associations were observed of any maternal psychological distress scale with childhood LDL-cholesterol (**Table S6** in **Supplementary Material**).

Maternal psychological distress and childhood glucose metabolism and inflammatory factors

Maternal overall psychological distress, depression and anxiety during pregnancy were associated with higher childhood insulin concentrations in the total group (p-values<0.05). Maternal depression was associated with higher childhood insulin concentrations among boys and girls, whereas anxiety was associated with higher childhood insulin concentrations among girls only (p-values<0.05). No associations were observed for childhood glucose concentrations. All maternal psychological distress scales were associated with an increased risk of high C-reactive protein concentrations among girls

Table 2. Associations of maternal psychological distress scales with childhood blood pressure and heart rate at 10 years for the total group and stratified for boys and girls

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores								
	Systolic blood pressure			Diastolic blood pressure			Heart rate		
	Total group N = 4,011	**Boys** N = 1,945	**Girls** N = 2,066	**Total group** N = 4,011	**Boys** N = 1,946	**Girls** N = 2,065	**Total group** N = 3,954	**Boys** N = 1,918	**Girls** N = 2,036
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	0.09 (-0.03, 0.20)	0.12 (-0.03, 0.28)	0.06 (-0.11, 0.23)	0.07 (-0.04, 0.19)	0.11 (-0.05, 0.27)	0.03 (-0.14, 0.20)	0.23 (0.12, 0.35)**	0.34 (0.18, 0.50)**	0.14 (-0.03, 0.31)
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	0.01 (-0.10, 0.13)	0.02 (-0.14, 0.18)	0.01 (-0.16, 0.18)	0.05 (-0.07, 0.16)	0.06 (-0.10, 0.23)	0.04 (-0.13, 0.20)	0.17 (0.06, 0.29)**	0.22 (0.06, 0.38)**	0.15 (-0.02, 0.32)
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.09 (-0.02, 0.19)	0.14 (-0.01, 0.28)	0.05 (-0.11, 0.20)	0.09 (-0.01, 0.20)	0.07 (-0.08, 0.22)	0.12 (-0.03, 0.27)	0.21 (0.10, 0.31)**	0.33 (0.19, 0.48)**	0.09 (-0.06, 0.25)

Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood blood pressure and heart rate in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group. Models are adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid and selective serotonin reuptake inhibitors use. *p < 0.05. ** p < 0.01.

Table 3. Associations of maternal psychological distress scales with childhood lipids profile at 10 years, total group and stratified for boys and girls

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores								
	Total Cholesterol			HDL Cholesterol			Triglycerides		
	Total group	Boys	Girls	Total group	Boys	Girls	Total group	Boys	Girls
	N = 2,879	N = 1,397	N = 1,482	N = 2,879	N = 1,397	N = 1,482	N = 2,873	N = 1,398	N = 1,475
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	-0.06 (-0.20, 0.08)	-0.05 (-0.24, 0.14)	-0.01 (-0.22, 0.20)	-0.09 (-0.23, 0.05)	-0.19 (-0.39, 0.00)	0.03 (-0.17, 0.24)	0.02 (-0.13, 0.16)	0.01 (-0.19, 0.21)	0.02 (-0.18, 0.22)
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	-0.04 (-0.18, 0.10)	-0.14 (-0.34, 0.06)	0.12 (-0.09, 0.33)	-0.06 (-0.20, 0.09)	-0.17 (-0.38, 0.03)	0.08 (-0.13, 0.28)	0.04 (-0.11, 0.18)	0.02 (-0.19, 0.23)	0.06 (-0.14, 0.26)
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	-0.01 (-0.14, 0.12)	0.03 (-0.15, 0.21)	-0.02 (-0.21, 0.18)	-0.09 (-0.22, 0.05)	-0.02 (-0.21, 0.17)	-0.15 (-0.33, 0.04)	0.17 (0.04, 0.30)*	0.01 (-0.18, 0.20)	0.35 (0.17, 0.53)**

Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood lipids profile in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group. Models are adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid and selective serotonin reuptake inhibitors use. *p < 0.05. ** p < 0.01.

Table 4. Associations of maternal psychological distress scales with childhood glucose metabolism and inflammatory factors at 10 years, total group and stratified for boys and girls

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores[1]						Odds Ratio (95% CI)[2]		
	Insulin			Glucose			C-reactive protein (≥ 3mg/l)		
	Total group	Boys	Girls	Total group	Boys	Girls	Total group	Boys	Girls
	N = 2,878	N = 1,395	N = 1,483	N = 2,878	N = 1,397	N = 1,481	N = 2,882	N = 1,399	N = 1,483
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	0.03 (-0.11, 0.17)	0.06 (-0.13, 0.26)	0.02 (-0.19, 0.23)	-0.00 (-0.14, 0.14)	0.05 (-0.15, 0.24)	-0.08 (-0.29, 0.14)	1.25 (0.76, 2.07)	1.26 (0.57, 2.79)	1.33 (0.68, 2.58)
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	0.08 (-0.07, 0.22)	0.11 (-0.09, 0.31)	0.05 (-0.15, 0.26)	-0.02 (-0.17, 0.12)	0.06 (-0.14, 0.26)	-0.13 (-0.34, 0.08)	1.09 (0.64, 1.85)	0.80 (0.32, 2.01)	1.38 (0.71, 2.69)
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.06 (-0.08, 0.19)	0.05 (-0.14, 0.23)	0.09 (-0.10, 0.28)	0.04 (-0.09, 0.17)	0.13 (-0.05, 0.32)	-0.06 (-0.25, 0.13)	1.15 (0.69, 1.90)	0.77 (0.32, 1.89)	1.54 (0.83, 2.87)

[1] Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood glucose metabolism in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group.

[2] Values are odds ratios (95% confidence intervals) and represent the risk of childhood high C-reactive protein at 10 years for maternal overall distress, depression and anxiety compared to the reference group.

Models are adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid and selective serotonin reuptake inhibitors use. *p < 0.05. ** p < 0.01.

only (p-values<0.05). (**Table S7** in **Supplementary Material**). The associations were no longer significant after adjustment for potential confounders (**Table 4**) and further adjustment for body mass index at 10 years (**Table S8** in **Supplementary Material**).

DISCUSSION

In this population-based prospective cohort study, the associations of maternal psychological distress with childhood cardio-metabolic outcomes are largely explained by socio-economic and family-based factors. Maternal psychological distress, depression and anxiety during pregnancy were, independent of potential confounders, associated with higher childhood heart rate among boys. Maternal anxiety was also associated with higher triglycerides among girls. Maternal psychological distress was not associated with childhood blood pressure, cholesterol, insulin, glucose and C-reactive protein concentrations.

Interpretation of main findings

Maternal psychological distress during pregnancy may lead to fetal developmental adaptations, which programme cardio-metabolic disease of the offspring. [2] Previous studies suggested an association between maternal distress during pregnancy and a higher risk of hypertension, insulin resistance, and type 2 diabetes in adolescence and adulthood, but not in childhood.[10-14, 25] Next to blood pressure, increased heart rate has been recognized as a risk factor for cardiovascular morbidity and mortality.[26] Previous studies reported that maternal stress during pregnancy is associated with higher fetal heart rate.[27, 28] We have previously described a positive association of maternal distress after pregnancy with infant heart rate, but no association was present for distress during pregnancy.[7] This latter study was performed in a subgroup of the current cohort. To our knowledge, no studies on the association between maternal psychological distress during pregnancy and lipids profile or inflammatory markers in childhood have been performed.

In the current study, the associations of maternal psychological distress, depression and anxiety with offspring blood pressure, cholesterol, insulin, glucose, or C-reactive protein concentrations seem to be explained by family based socio-demographic factors. However, independent of these factors, maternal overall psychological distress, depression and anxiety during pregnancy were associated with higher childhood heart rate at 10 years in boys, but not in girls. It has been proposed that fetal sex-specific placental responsiveness to maternal stress may result in increased risk for later diseases in boys. The higher growth rates of male fetuses may increase their vulnerability and subsequently place them at increased risk of adverse outcomes throughout the life

course.[8] In the current study, we also observed that maternal anxiety, but not overall psychological distress and depression during pregnancy, was associated with higher triglycerides among girls. This suggests that the mechanisms relating maternal stress during pregnancy with childhood triglycerides may relate to specific psychological symptoms and be sex-specific. We cannot exclude the possibility of these results being a chance finding. We considered Bonferroni correction for multiple testing too strict since our outcomes are correlated.[29] However, the observed associations remained significant when considering a p-value of 0.017 (0.05/3 groups of outcomes). Altogether, our findings suggest that maternal psychological distress during pregnancy seems to have a small but persistent influence on cardio-metabolic profile during childhood.

We performed a model additionally adjusted for child body mass index, which might be in the causal pathway of the associations. Since the main results were similar with and without adjustment for child body mass index, the observed associations of maternal psychological distress with childhood heart rate and triglycerides concentrations seem to be independent of childhood adiposity. Fetal programming mechanisms might partly explain these associations. Fetal exposure to increased glucocorticoids levels due to adaptations of the maternal hypothalamic–pituitary–adrenal axis is the most well-known mechanism through which maternal psychological distress may influence the offspring cardio-metabolic outcomes.[4, 5] Another mechanism is the programming of the fetal autonomic nervous system, specifically changes in the balance of sympathetic and parasympathetic nervous system, by maternal psychological stress.[7] An elevated sympathetic nervous system activity established in utero may affect fetal and childhood heart rate and subsequently may lead to cardiovascular diseases later in life. Further research is needed to identify the causality, the underlying mechanisms and to allow a better understanding of the sex-specific responses. Although the observed associations are small and without clinical relevance on individual level, the results may be important from a developmental perspective since cardio-metabolic risk factors tend to track into adulthood. Further studies are needed to replicate our findings and to assess the long-term cardio-metabolic consequences of maternal psychological distress.

Strengths and limitations

Strengths of this study were the prospective design, the large sample size and the detailed data available on childhood cardio-metabolic risk factors. This study also has limitations. We used all data available for each specific analysis in order to optimize statistical power. The analyses for childhood lipids profile, glucose metabolism and C-reactive protein may have lower statistical power due to lower sample sizes. Mothers of children with and without follow-up data were different regarding the socioeconomic background and prevalence of psychological distress. We cannot exclude the possibility of selection bias. We relied on a self-report questionnaire of maternal psychological

distress, which might lead to misclassification bias, due to underreporting of psychological symptoms, and subsequently to underestimation of observed effects.[30] The use of non-fasting blood samples of childhood cardio-metabolic profile may have resulted in misclassification and thus may have led to underestimation of the observed associations. However, previous studies in adults have shown that non-fasting blood lipids levels can accurately predict increased risks of cardiovascular events later in life [31, 32] and that semi-fasted insulin resistance is moderately correlated with fasting values.[33] Finally, although we have adjusted for many sociodemographic and lifestyle variables known to influence the associations, residual confounding might still be an issue due to the observational design of the study.

CONCLUSIONS

The associations of maternal psychological distress with childhood cardio-metabolic outcomes are largely explained by socio-economic family factors. Maternal psychological distress may, independently of these factors, influence offspring heart rate and triglycerides concentrations. Promoting a healthy mental state during pregnancy may improve child cardio-metabolic health.

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SUPPLEMENTARY MATERIAL

Figure S1. Flowchart of study population

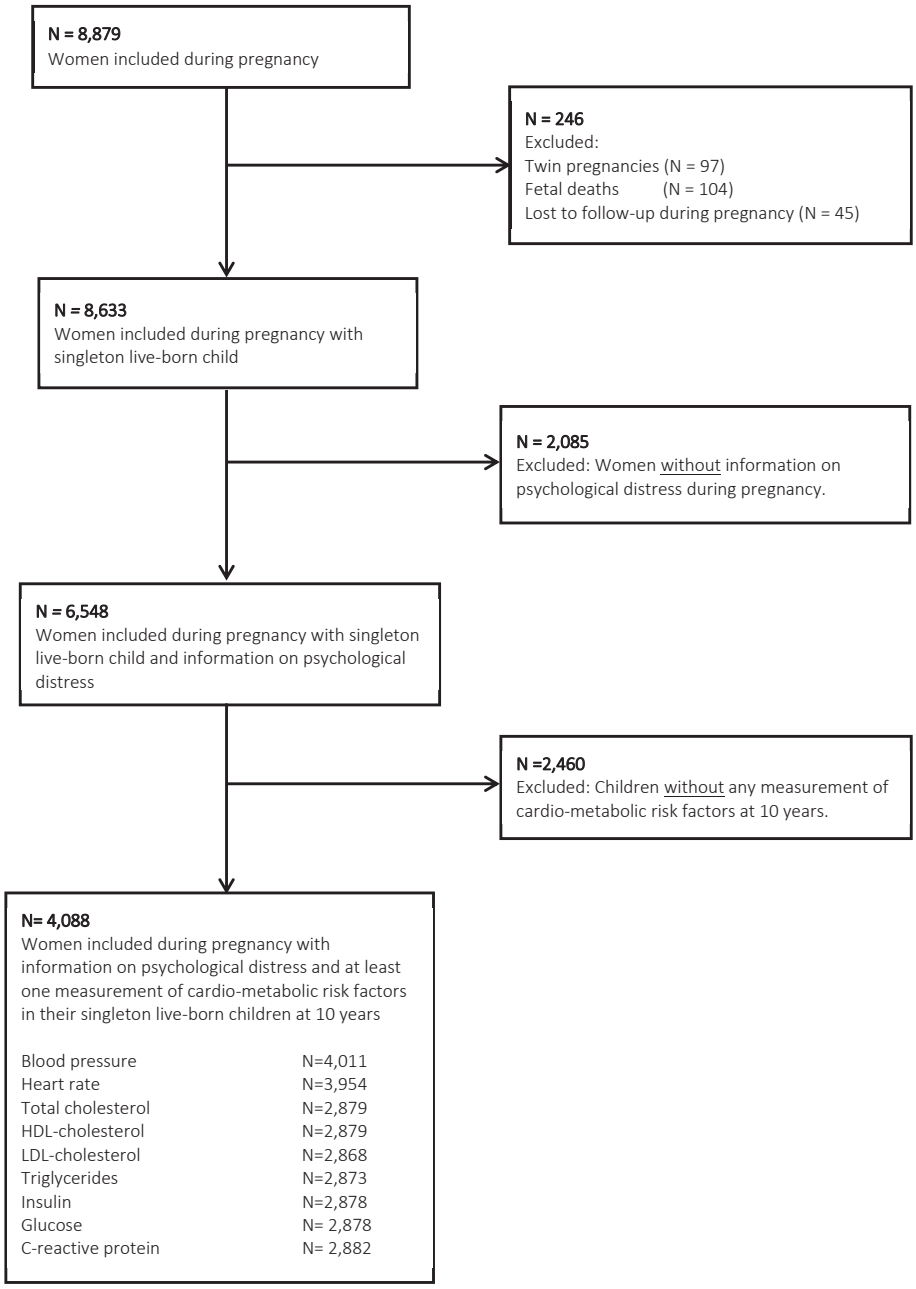


Table S1. Comparison of maternal and child characteristics between mothers and children with and without follow-up data available[1]

	With follow-up (N = 4,088)	Without follow-up (N = 2,460)	P-value[2]
Maternal characteristics			
Age at intake, mean (SD), years	30.9 (4.8)	28.5 (5.4)	< 0.001
Ethnicity, N (%)			< 0.001
European	2,767 (68.2)	1,187 (50.5)	
Non-European	1,288 (31.8)	1,162 (49.5)	
Education, N (%)			< 0.001
Primary school	255 (6.4)	321 (14.1)	
Secondary school	1,628 (41.1)	1,196 (52.4)	
High education	2,076 (52.4)	764 (33.5)	
Marital status, N (%)			< 0.001
Married/living together	3,502 (89.2)	1,886 (82.4)	
No partner	425 (10.8)	404 (17.6)	
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.6 (18.1, 34.3)	22.6 (17.7, 35.1)	< 0.05
Alcohol consumption, N (%)			< 0.001
Yes	2,219 (59.9)	1,003 (45.5)	
No	1,486 (40.1)	1,200 (54.5)	
Smoking, N (%)			< 0.001
Yes	901 (24.0)	713 (31.8)	
No	2,847 (76.0)	1,531 (68.2)	
Folic acid supplement use, N (%)			< 0.001
No	650 (20.1)	648 (34.6)	
Start during first 10 weeks	1,030 (31.9)	598 (31.9)	
Preconceptional use	1,546 (47.9)	628 (33.5)	
Overall psychological distress, N (%)			< 0.001
Yes	352 (8.6)	358 (14.6)	
No	3,736 (91.4)	2,102 (85.4)	
Exposed to SSRIs, N (%)			< 0.001
Yes	43 (1.1)	37 (1.6)	
No	3,823 (98.9)	2,243 (98.4)	
Child characteristics			
Sex, N (%)			< 0.05
Boys	1,987 (48.6)	1,273 (51.7)	
Girls	2,101 (51.4)	1,187 (48.3)	
Gestational age at birth, N (%)			< 0.05
Preterm (< 37 weeks)	178 (4.4)	135 (5.5)	
Term (≥ 37 weeks)	3,910 (95.6)	2,325 (94.5)	
Birth weight[3], N (%)			< 0.05

Table S1. Comparison of maternal and child characteristics between mothers and children with and without follow-up data available[1] (continued)

	With follow-up (N = 4,088)	Without follow-up (N = 2,460)	P-value[2]
Small for gestational age	405 (9.9)	263 (10.8)	
Appropriate for gestational age	3,270 (80.1)	1,941 (79.8)	
Large for gestational age	409 (10.0)	227 (9.3)	
Age at visit, mean (SD), years	9.8 (0.3)	10.0 (0.8)	< 0.001
Body mass index, median (95% range), kg/m^2	16.9 (14.0, 24.5)	17.6 (13.4, 25.1)	< 0.05

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

[2] P-values for differences in subject characteristics between groups were calculated performing independent sample t-tests for normally distributed continuous variables, Mann-Whitney tests for not normally distributed continuous variables and chi-square tests for categorical variables.

[3] Sex- and gestational age-adjusted birth weight SDS were created based on a North-European reference chart. Small and large size for gestational age at birth were defined as sex- and gestational age-adjusted birth weight below the 10th percentile and above the 90th percentile, respectively.

Table S2. Associations of maternal psychological distress scales with childhood blood pressure and heart rate at 10 years, total group and stratified for boys and girls (unadjusted models)

	Difference (95% CI) in standard deviation scores								
Maternal psychological distress scales	**Systolic blood pressure**			**Diastolic blood pressure**			**Heart rate**		
	Total group	**Boys**	**Girls**	**Total group**	**Boys**	**Girls**	**Total group**	**Boys**	**Girls**
	N = 4,011	N = 1,945	N = 2,066	N = 4,011	N = 1,946	N = 2,065	N = 3,954	N = 1,918	N = 2,036
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	0.24 (0.13,0.35)**	0.31 (0.16, 0.46)**	0.18 (0.01, 0.34)*	0.20 (0.09,0.31)**	0.30 (0.15, 0.46)**	0.10 (-0.06, 0.26)	0.35 (0.24,0.46)**	0.47 (0.32, 0.62)**	0.25 (0.09, 0.41)**
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	0.16 (0.05,0.27)**	0.20 (0.05, 0.36)**	0.13 (-0.04, 0.29)	0.16 (0.05,0.27)**	0.24 (0.08, 0.39)**	0.09 (-0.07, 0.25)	0.28 (0.17,0.39)**	0.35 (0.20, 0.50)**	0.24 (0.08, 0.40)**
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.19 (0.08,0.29)**	0.26 (0.12, 0.41)**	0.12 (-0.03, 0.27)	0.18 (0.07,0.28)**	0.21 (0.06, 0.35)**	0.16 (0.01, 0.31)*	0.29 (0.18,0.40)**	0.43 (0.28, 0.57)**	0.17 (0.03, 0.32)*

Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood blood pressure and heart rate in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group. Unadjusted models. *p < 0.05. ** p < 0.01.

Table S3. Associations of maternal psychological distress scales with childhood blood pressure and heart rate at 10 years, total group and stratified for boys and girls (body mass index adjusted models)

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores								
	Systolic blood pressure			Diastolic blood pressure			Heart rate		
	Total group	Boys	Girls	Total group	Boys	Girls	Total group	Boys	Girls
	N = 4,011	N = 1,945	N = 2,066	N = 4,011	N = 1,946	N = 2,065	N = 3,954	N = 1,918	N = 2,036
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	0.06 (-0.05, 0.17)	0.08 (-0.07, 0.23)	0.05 (-0.11, 0.21)	0.07 (-0.05, 0.18)	0.10 (-0.06, 0.26)	0.03 (-0.14, 0.20)	0.23 (0.11, 0.35)**	0.33 (0.17, 0.49)**	0.14 (-0.03, 0.31)
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	-0.01 (-0.12, 0.10)	0.00 (-0.15, 0.15)	-0.01 (-0.17, 0.15)	0.04 (-0.07, 0.16)	0.06 (-0.11, 0.22)	0.03 (-0.14, 0.20)	0.17 (0.06, 0.29)**	0.22 (0.06, 0.37)**	0.15 (-0.02, 0.32)
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.04 (-0.06, 0.14)	0.10 (-0.03, 0.24)	-0.01 (-0.16, 0.13)	0.08 (-0.03, 0.19)	0.06 (-0.09, 0.21)	0.11 (-0.05, 0.26)	0.20 (0.10, 0.31)**	0.33 (0.18, 0.47)**	0.10 (-0.06, 0.25)

Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood blood pressure and heart rate in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group. Models are adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid and selective serotonin reuptake inhibitors use and child body mass index. *p < 0.05. ** p < 0.01.

Table S4. Associations of maternal psychological distress scales with childhood lipids profile at 10 years, total group and stratified for boys and girls (unadjusted models)

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores								
	Total Cholesterol			HDL-Cholesterol			Triglycerides		
	Total group	Boys	Girls	Total group	Boys	Girls	Total group	Boys	Girls
	N = 2,879	N = 1,397	N = 1,482	N = 2,879	N = 1,397	N = 1,482	N = 2,873	N = 1,398	N = 1,475
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	-0.04 (-0.18, 0.09)	-0.00 (-0.19, 0.18)	-0.06 (-0.26, 0.14)	-0.17 (-0.30, -0.03)*	-0.28 (-0.47, -0.10)**	-0.07 (-0.26, 0.13)	0.09 (-0.04, 0.23)	0.13 (-0.06, 0.32)	0.07 (-0.12, 0.26)
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	-0.03 (-0.16, 0.11)	-0.09 (-0.28, 0.09)	0.06 (-0.13, 0.26)	-0.12 (-0.26, 0.01)	-0.25 (-0.44, -0.06)*	-0.01 (-0.20, 0.18)	0.10 (-0.04, 0.23)	0.11 (-0.08, 0.31)	0.09 (-0.10, 0.28)
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.00 (-0.13, 0.13)	0.05 (-0.13, 0.22)	-0.03 (-0.21, 0.16)	-0.14 (-0.27, -0.01)*	-0.11 (-0.29, 0.07)	-0.19 (-0.37,-0.01)*	0.22 (0.09, 0.35)**	0.09 (-0.09, 0.28)	0.37 (0.20, 0.55)**

Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood lipids profile in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group. Unadjusted models.*$p < 0.05$. ** $p < 0.01$.

Table S5. Associations of maternal psychological distress scales with childhood lipids profile at 10 years, total group and stratified for boys and girls (body mass index adjusted models)

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores								
	Total Cholesterol			HDL-Cholesterol			Triglycerides		
	Total group	Boys	Girls	Total group	Boys	Girls	Total group	Boys	Girls
	N = 2,879	N = 1,397	N = 1,482	N = 2,879	N = 1,397	N = 1,482	N = 2,873	N = 1,398	N = 1,475
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	-0.07 (-0.21, 0.08)	-0.06 (-0.25, 0.13)	-0.02 (-0.23, 0.20)	-0.09 (-0.23, 0.05)	-0.19 (-0.38, 0.00)	0.03 (-0.17, 0.23)	0.01 (-0.13, 0.15)	0.00 (-0.20, 0.20)	0.02 (-0.18, 0.22)
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	-0.04 (-0.19, 0.10)	-0.15 (-0.34, 0.05)	0.12 (-0.09, 0.33)	-0.05 (-0.19, 0.09)	-0.17 (-0.37, 0.03)	0.09 (-0.11, 0.28)	0.03 (-0.11, 0.17)	0.01 (-0.19, 0.21)	0.06 (-0.14, 0.26)
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	-0.02 (-0.15, 0.12)	0.03 (-0.15, 0.21)	-0.02 (-0.22, 0.17)	-0.07 (-0.20, 0.06)	-0.02 (-0.20, 0.16)	-0.11 (-0.29, 0.07)	0.16 (0.03, 0.29)*	0.00 (-0.18, 0.19)	0.32 (0.14, 0.50)**

Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood lipids profile in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group. Models are adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid and selective serotonin reuptake inhibitors use and child body mass index. *p < 0.05. ** p < 0.01.

Table S6. Associations of maternal psychological distress scales with childhood LDL-cholesterol at 10 years, total group and stratified for boys and girls

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores								
	Unadjusted models			Adjusted models			Body mass index adjusted models		
	Total group	Boys	Girls	Total group	Boys	Girls	Total group	Boys	Girls
	N = 2,868	N = 1,394	N = 1,474	N = 2,868	N = 1,394	N = 1,474	N = 2,868	N = 1,394	N = 1,474
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	-0.00 (-0.14, 0.13)	0.10 (-0.08, 0.28)	-0.08 (-0.28, 0.11)	-0.02 (-0.17, 0.12)	0.06 (-0.13, 0.25)	-0.06 (-0.27, 0.15)	-0.03 (-0.17, 0.11)	0.05 (-0.14, 0.24)	-0.06 (-0.27, 0.15)
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	-0.01 (-0.14, 0.13)	-0.00 (-0.19, 0.18)	0.02 (-0.18, 0.21)	-0.03 (-0.17, 0.12)	-0.04 (-0.24, 0.15)	0.05 (-0.16, 0.26)	-0.03 (-0.17, 0.12)	-0.05 (-0.24, 0.15)	0.05 (-0.16, 0.26)
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	-0.02 (-0.14, 0.11)	0.08 (-0.09, 0.26)	-0.10 (-0.28, 0.09)	-0.03 (-0.17, 0.10)	0.06 (-0.12, 0.24)	-0.10 (-0.29, 0.10)	-0.04 (-0.18, 0.09)	0.06 (-0.12, 0.24)	-0.11 (-0.30, 0.08)

Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood LDL-cholesterol in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group.

Adjusted models include maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid and selective serotonin reuptake inhibitors use.

Body mass index adjusted models additionally include child body mass index.

*$p < 0.05$. **$p < 0.01$.

Table S7. Associations of maternal psychological distress scales with childhood glucose metabolism and inflammatory factors at 10 years, total group and stratified for boys and girls (unadjusted models)

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores[1]						Odds Ratio (95% CI)[2]		
	Insulin			Glucose			C-reactive protein (≥ 3mg/l)		
	Total group	Boys	Girls	Total group	Boys	Girls	Total group	Boys	Girls
	N = 2,878	N = 1,395	N = 1,483	N = 2,878	N = 1,397	N = 1,481	N = 2,882	N = 1,399	N = 1,483
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	0.15 (0.02, 0.29)*	0.14 (-0.05, 0.32)	0.19 (-0.00, 0.39)	-0.02 (-0.15, 0.11)	0.03 (-0.15,0.22)	-0.09 (-0.28, 0.11)	2.04 (1.28, 3.24)**	1.79 (0.86, 3.72)	2.35 (1.28, 4.30)**
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	0.20 (0.06, 0.33)**	0.19 (0.01, 0.38)**	0.21 (0.02, 0.41)*	-0.04 (-0.17, 0.10)	0.05 (-0.14,0.23)	-0.13 (-0.33, 0.07)	1.77 (1.08, 2.89)*	1.17 (0.49, 2.78)	2.34 (1.28, 4.29)**
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.14 (0.01, 0.27)*	0.11 (-0.07, 0.29)	0.19 (0.01, 0.38)*	0.04 (-0.09, 0.16)	0.12 (-0.05, 0.30)	-0.06 (-0.25, 0.12)	1.62 (1.01, 2.62)*	1.02 (0.43, 2.41)	2.19 (1.22, 3.94)**

[1] Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood glucose metabolism in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group. Unadjusted models.

[2] Values are odds ratios (95% confidence intervals) and represent the risk of childhood high C-reactive protein at 10 years for maternal overall distress, depression and anxiety compared to the reference group. Unadjusted models.

*p < 0.05. ** p < 0.01.

Table S8. Associations of maternal psychological distress scales with childhood glucose metabolism and inflammatory factors at 10 years, total group and stratified for boys and girls (body mass index adjusted models)

Maternal psychological distress scales	Difference (95% CI) in standard deviation scores[1]						Odds Ratio (95% CI)[2]		
	Insulin			Glucos			C-reactive protein (≥ 3mg/l)		
	Total group	Boys	Girls	Total group	Boys	Girls	Total group	Boys	Girls
	N = 2,878	N = 1,395	N = 1,483	N = 2,878	N = 1,397	N = 1,481	N = 2,882	N = 1,399	N = 1,483
Overall distress									
No stress	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Stress	0.03 (-0.11, 0.17)	0.06 (-0.13, 0.25)	0.03 (-0.17, 0.23)	0.00 (-0.14, 0.15)	0.05 (-0.15, 0.25)	-0.07 (-0.28, 0.14)	1.21 (0.72, 2.01)	1.22 (0.55, 2.72)	1.26 (0.63, 2.50)
Depression									
No depression	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Depression	0.07 (-0.07, 0.21)	0.11 (-0.09, 0.30)	0.05 (-0.15, 0.25)	-0.02 (-0.16, 0.13)	0.06 (-0.14, 0.26)	-0.11 (-0.32, 0.09)	1.07 (0.63, 1.83)	0.78 (0.31, 1.99)	1.34 (0.68, 2.65)
Anxiety									
No anxiety	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Anxiety	0.04 (-0.09, 0.17)	0.05 (-0.13, 0.23)	0.05 (-0.13, 0.24)	0.05 (-0.09, 0.18)	0.13 (-0.05, 0.32)	-0.04 (-0.23, 0.15)	1.07 (0.64, 1.78)	0.76 (0.31, 1.87)	1.33 (0.70, 2.54)

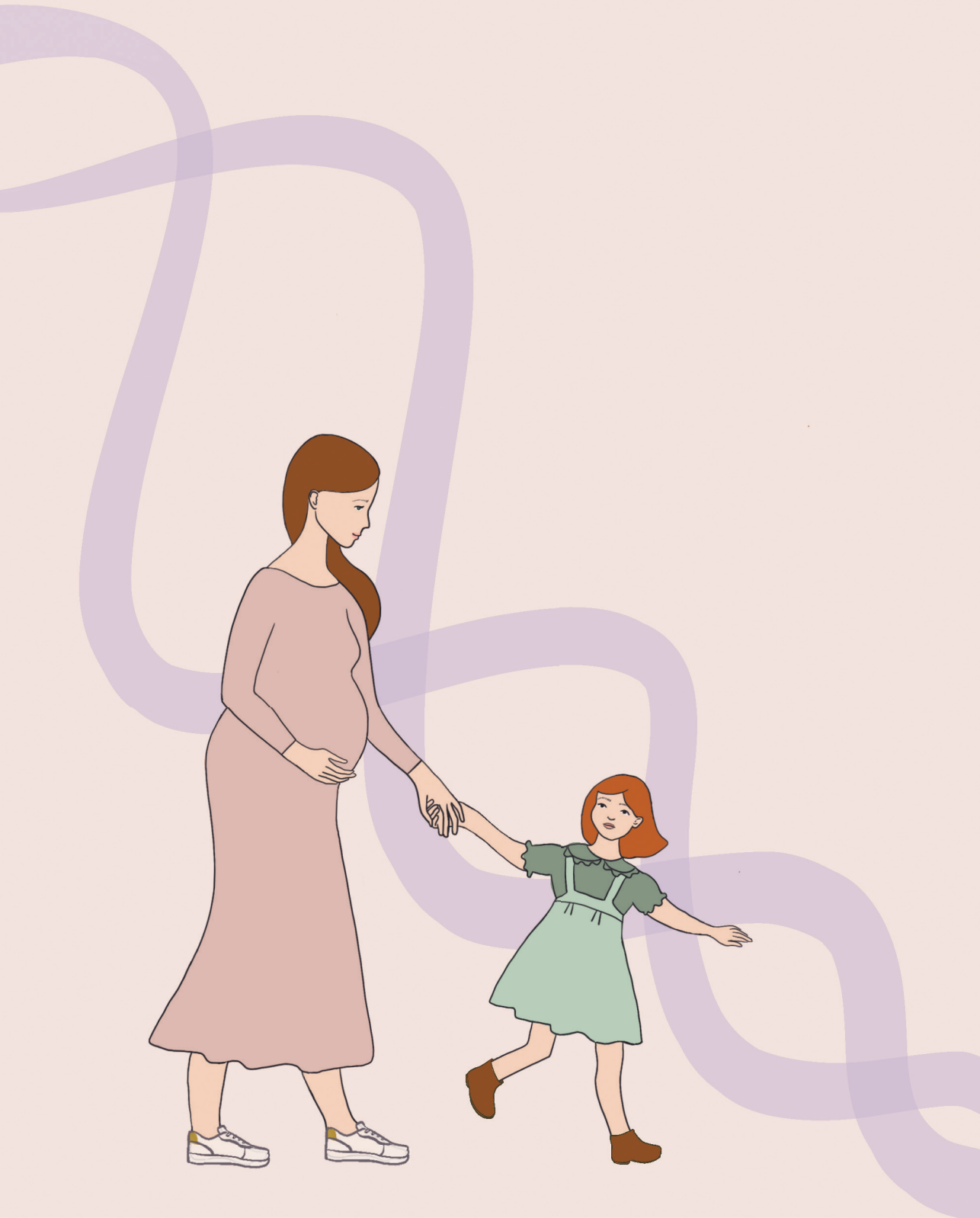
[1] Values are linear regression coefficients (95% confidence intervals) and reflect the change in childhood glucose metabolism in standard deviation scores for maternal overall distress, depression and anxiety, compared to the reference group.

[2] Values are odds ratios (95% confidence intervals) and represent the risk of childhood high C-reactive protein at 10 years for maternal overall distress, depression and anxiety compared to the reference group.

Models are adjusted for maternal age, ethnicity, educational level, marital status, body mass index before pregnancy, alcohol consumption, smoking during pregnancy, folic acid and selective serotonin reuptake inhibitors use and child body mass index. *p < 0.05. ** p < 0.01.

3

Hair Cortisol in Children



3.1

Associations of hair cortisol concentrations with general and organ fat measures in childhood

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ABSTRACT

Context: Stress may lead to an adverse body fat distribution from childhood onwards.

Objective: To examine the associations of hair cortisol concentrations (HCC) at 6 years with general and organ fat measures, risk of overweight and nonalcoholic fatty liver disease (NAFLD) at 10 years and to assess whether these were independent of adiposity measures at 6 years.

Design, Setting and participants: HCC were measured in hair of 6-year old children (N = 2,042) participating in the Generation R Study, a population-based prospective cohort study.

Main Outcome Measures: BMI, fat mass index (FMI) measured by DXA scan, and visceral fat index, pericardial fat index, liver fat fraction measured by MRI and risk of overweight and NAFLD were obtained at 10 years.

Results: The associations of higher HCC at 6 years, with higher BMI, FMI and increased risk of overweight at age 10 years are explained by the relationships observed at 6 years. HCC at 6 years were associated with a higher liver fat fraction (difference 0.11 liver fat fraction standard deviation score (SDS) (95% Confidence Interval (CI) 0.03, 0.18)) and a higher risk of NAFLD at 10 years (OR: 1.95 (95% CI 1.06, 3.56), independent of FMI at 6 years. HCC were not associated with pericardial or visceral fat indices.

Conclusions: Higher HCC at 6 years were associated with higher BMI, FMI, liver fat fraction, and higher risks of overweight and NAFLD at 10 years. Only the associations for liver fat fraction and NAFLD were independent of FMI at 6 years.

INTRODUCTION

Obesity is a major public health problem and is associated with short- and long-term morbidity and mortality.[1] Previous studies suggested that stress is associated with adiposity among adults.[2] Cortisol and cortisone, both glucocorticoids, are objective biomarkers of stress.[3] Long-term dysregulated cortisol secretion can contribute to the development of obesity through insulin resistance of peripheral target tissues and accumulation of visceral fat.[4,5] Unlike the traditional cortisol measures in saliva, serum and urine, hair cortisol concentrations reflect long-term cumulative cortisol concentrations.[3,6,7] Cortisol can be converted into inactive cortisone.[8] The assessment of both glucocorticoids, which are highly correlated, may give more insight into the amount of active and inactive corticosteroids.[9,10] Previous studies reported associations of hair cortisol concentrations with body mass index (BMI), and other adiposity measures in adults.[2,6,11] Thus far, studies in children were of a modest sample size, used a cross-sectional design, did not show consistent results and did not look into the association of cortisol with organ fat measures.[12] We previously reported cross-sectional associations of higher hair cortisol concentrations with higher BMI and fat mass index at 6 years.[13]
Based on these previous results, we hypothesized that chronic exposure to higher cortisol concentrations leads prospectively to an adverse body fat distribution. We examined, in a population-based prospective cohort study among 2,042 children, the associations of hair cortisol concentrations at 6 years with BMI, fat mass index measured by dual-energy X-ray absorptiometry (DXA), and pericardial fat index, visceral fat index, and liver fat fraction measured by magnetic resonance imaging (MRI) and the risks of overweight and nonalcoholic fatty liver disease (NAFLD) at 10 years. We additionally examined whether any association was independent of the previously reported cross sectional associations at 6 years.

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.[14] 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 adiposity at 10 years (N = 648) were excluded. Also, children with extreme values of cortisol (N=236) were excluded using

Tukey's definition of outliers (Q1-1.5*IQR and Q3+1.5*IQR).[15] The population for analysis consisted of 2,042 children. The same selection procedure was followed for the cortisone analyses (N = 2,051). The flowchart of participants is given in **Supplemental Figure 1**.[16]

Hair Cortisol and Cortisone Concentration Measurements

As described previously, hair cortisol and cortisone concentrations were measured in proximal scalp hair.[17] Details on collection, sample preparation, extraction and analysis are provided in the **Supplemental Methods**.[16] 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.[15] The Spearman correlation coefficient between the original variables of hair cortisol and cortisone concentration was 0.63.

General, Visceral, and Organ Fat

Outcome assessments were performed at ages 6 and 10 years.[14] We calculated BMI (kg/m^2) at this age from height and weight, both measured without shoes and heavy clothing. We calculated sex- and age- adjusted standard deviation scores (SDS) of childhood BMI based on Dutch reference growth charts (Growth Analyzer 4.0, Dutch Growth Research Foundation).[18] BMI categories (underweight, normal weight, overweight and obesity) were calculated using the International Obesity Task Force cut-offs.[19,20] We measured total body fat mass using a DXA scanner (iDXA, GE140 Lunar, 2008, Madison, WI, USA, enCORE software v.12.6), according to standard procedures.[21]

Visceral and organ adiposity were obtained from magnetic resonance imaging scans performed at 10 years, as described previously.[14] Briefly, all children underwent imaging using a 3.0-T magnetic resonance imaging scanner (Discovery MR750w; GE Healthcare). Pericardial fat imaging in short axis orientation was performed using an electrocardiogram-triggered black-blood–prepared thin-slice single-shot fast-spin echo acquisition with multi-breath-hold approach. An axial 3-point Dixon acquisition for fat and water separation (IDEAL IQ) was used for liver fat imaging.[22] An axial abdominal scan from lower liver to pelvis and a coronal scan centered at the head of the femurs were performed with a 2-point Dixon acquisition (LavaFlex). The scans were analyzed by the Precision Image Analysis company (PIA, Kirkland, Washington, United States), using the sliceOmatic software package (TomoVision, Magog, Canada). Details on methods and measurements are provided in the **Supplemental Methods**.[16]

To create measures independent of height, we estimated the optimal adjustment by log-log regression analyses and subsequently we divided total fat mass at 10 years by height^4 (fat mass index) and visceral and pericardial fat mass by height^3 (visceral and

pericardial fat indices).[23-25] We log-transformed the non-normally distributed childhood DXA and MRI adiposity measures. We constructed SDS [(observed value - mean)/SD] of the sample distribution for DXA and MRI outcomes to enable comparisons of effect sizes. We used Spearman's rank correlation coefficients to estimate correlations of BMI and fat mass index at 6 years with BMI, fat mass index, pericardial fat mass index, visceral fat mass index and liver fat fraction at 10 years (**Supplemental Table 1**).[16]

Covariates

Information on child sex was obtained from midwife/obstetric records. We collected information on maternal pre-pregnancy BMI and psychological distress during pregnancy by questionnaires. Information on maternal education and marital status, child ethnicity and television watching time was obtained by questionnaires at the age of six years completed by the mother. Hair color was partially coded through parent report and was completed by two raters using front desk photographs at the research center. Parents completed a questionnaire for their child on use and administration route of glucocorticoid medications at the age of six years.

Statistical analysis

First, we examined differences in subject characteristics between hair cortisol concentration quintiles with analysis of variance tests for continuous variables and Chi-square tests for categorical variables. For non-response analyses, we compared participants and non-participants using Chi square 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 with adiposity measures at 10 years (BMI, fat mass index, visceral and pericardial fat indices and liver fat fraction). Third, we used logistic regression models to assess the associations of hair cortisol concentrations at 6 years with the risk of childhood overweight or obesity at 10 years, to which we further refer as overweight. Tests for trends 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 adiposity measures. For NAFLD we only assessed the association with the continuous cortisol measurement since the number of children with NAFLD was too small for some of the cortisol quintiles. Fifth, we examined whether hair cortisol concentrations were associated with change in BMI and fat mass index SD scores between 6 and 10 years. Next, we used conditional regression analyses to assess whether the associations of hair cortisol concentrations at 6 years with adiposity outcomes at 10 years were independent of adiposity measures at 6 years. For these models, we first estimated the standardized residuals from the regression models with the 6 years adiposity measurements as exposures and the 10

years adiposity measurements as outcomes. Subsequently, these residuals were used as outcomes for the associations with hair cortisol concentrations.[26] These residuals should be interpreted as excess in fat measures at 10 years, as would be expected based on the cross sectional analyses at 6 years. Since the organ fat measurements were only available at 10 years, these were conditioned on fat mass index at 6 years based on the strongest correlation (**Supplemental Table 1**).[16] For all continuous and dichotomous adiposity outcomes, we performed sensitivity analyses by adjusting the models focused on the associations of hair cortisol concentrations at 6 years with adiposity outcomes at 10 years for adiposity measures at 6 years. The basic models included child sex and age at cortisol measurement as confounders. The confounder model was additionally adjusted for maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal education and marital status, child ethnicity, hair color and average duration of television watching. We identified potential covariates based on the graphical criteria for confounding by visualizing a directed acyclic graph (DAG) and included the covariates in the models that were associated with exposure and outcome and changed the effect estimates >10% (**Supplemental Figure 2**).[16,27,28] We assessed which covariates had the strongest effects in the associations of continuous hair cortisol concentrations at 6 years with childhood general and organ fat measures at 10 years. We did not observe statistically significant interactions of hair cortisol levels with child ethnicity and sex. As sensitivity analysis, we excluded children with any glucocorticoid use in the three months prior to the hair sample collection (N = 1,805). Also, we repeated all analyses for cortisone (N = 2,051). Because of the correlations between the outcomes (**Supplemental Table1**), we did not perform Bonferroni adjustment.[16,29] However, considering three groups of outcomes (BMI, fat mass index, organ fat measures), multiple testing adjustment would lead to a p-value of < 0.017. We depicted both significance levels (0.05 and 0.017) in the tables and figures. In order to maintain statistical power and reduce bias related to missing data on covariates (**Supplemental Table 2**) we performed multiple imputation according to Markov Chain Monte Carlo method.[16,30] Five imputed datasets were created and pooled results are presented. All statistical analyses were performed using the Statistical Package of Social Sciences (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp).

RESULTS

Subject characteristics

Table 1 shows that as compared to children in the lower cortisol quintiles, children in the upper cortisol quintiles more often had a mother who was younger, lower educated, without a partner and who reported more psychological distress during pregnancy. Also,

these children more often had a lower birth weight, a non-European ethnicity, a brown or black hair color and a higher average duration of television watching at age 6 years. Non-response analyses showed that, compared to participants, non-participants had mothers who were slightly younger, with a higher BMI, who reported more psychological distress during pregnancy and were more often lower educated. Non-participants more often had a higher BMI, a non-European ethnicity, brown or dark hair, and an increased average duration of television watching (**Supplemental Table 2**).[16]

Hair cortisol concentrations and general adiposity measures

As compared to the lowest quintile, children in the highest quintile of hair cortisol concentrations at 6 years, had a higher BMI and fat mass index (differences 0.22 SDS (95% Confidence Interval (CI) 0.09, 0.36) and 0.21 SDS (95% CI 0.09, 0.33), respectively) (**Figures 1A-B**). Tests for trends were significant for BMI and fat mass index (p-value for trend ≤ 0.001). Associations of continuous cortisol concentrations with general adiposity outcomes showed similar results (an IQR increase in the natural log transformed hair cortisol concentrations was associated with a 0.10 (95% CI 0.04 0.26) SDS higher BMI and a 0.09 (95% CI 0.04, 0.15) SDS higher fat mass index (**Supplemental Table 3**).[16] Maternal pre-pregnancy BMI, maternal education and child's sex and child's age were the strongest covariates (**Supplemental Table 4**).[16] Results from basic models were in the same direction and slightly stronger (**Supplemental Table 5)**.[16] Hair cortisol concentrations were not associated with the change in BMI and fat mass index SD scores between 6 and 10 years (**Supplemental Table 6**).[16] Results from the conditional regression analyses showed that the associations of hair cortisol concentrations with BMI and fat mass index residuals were not consistently significant anymore after conditioning the outcomes on adiposity measures at 6 years (**Supplemental Table 7**).[16]

Hair cortisol concentrations and visceral and organ fat measures

Also, as compared to the lowest quintile, children in the highest quintile of hair cortisol concentrations at 6 years had higher liver fat fraction at 10 years (difference 0.26 liver fat fraction SDS (95% CI 0.10, 0.43)). Hair cortisol concentrations were not associated with pericardial or visceral fat indices (**Figures 2A-C**). Test for trends was significant for liver fat fraction (p-value for trend < 0.001). Associations for continuous cortisol measures showed similar results (an IQR increase in the natural log transformed hair cortisol concentration was associated with a 0.15 (95% CI 0.07, 0.22) SDS higher liver fat fraction and a significantly higher risk of NAFLD (Odds Ratio (OR): 2.35 (95% CI 1.31, 4.22)) (**Supplemental Table 2**).[16] Results from basic models were in the same direction and slightly stronger (**Supplemental Table 5)**.[16] The associations of hair cortisol concentrations with liver fat fraction residuals remained significant after conditioning liver fat fraction on fat

Table 1. Subject characteristics (N = 2,042)

		Hair Cortisol Concentrations					
	Total group [a] (N = 2,042)	**Quintile 1 [a] 0.137-0.727 pg/mg (N = 408)**	**Quintile 2 [a] 0.728-1.150 pg/mg (N = 409)**	**Quintile 3 [a] 1.151-1.820 pg/mg (N = 408)**	**Quintile 4 [a] 1.827-2.927 pg/mg (N = 409)**	**Quintile 5 [a] 2.928-6.799 pg/mg (N = 408)**	**P-value [b]**
Family characteristics							
Maternal age, mean (SD), years	31.2 (4.8)	31.8 (4.5)	31.5 (4.7)	30.7 (4.8)	31.0 (4.9)	30.8 (5.1)	0.003
Pre-pregnancy BMI, median (95% range), kg/m²	22.5 (18.2, 35.0)	22.1 (18.8, 34.2)	22.6 (18.4, 34.5)	22.3 (17.8, 35.5)	22.3 (18.3, 33.9)	23.0 (17.7, 36.2)	0.04
Psychological distress during pregnancy, N (%)							0.003
Yes	560 (28.6)	95 (24.1)	95 (24.2)	116 (29.8)	118 (29.8)	136 (35.2)	
No	1,397 (71.4)	299 (75.9)	297 (75.8)	273 (70.2)	278 (70.2)	250 (64.8)	
Maternal educatio, N n (%)							< 0.001
Primary school	68 (3.8)	4 (1.1)	10 (2.8)	15 (4.2)	25 (6.9)	14 (4.0)	
Secondary school	619 (34.3)	115 (31.0)	113 (31.2)	122 (33.8)	131 (36.4)	138 (39.1)	
High education	1,120 (62.0)	2592 (67.9)	239 (66.0)	224 (62.0)	204 (56.7)	201 (56.9)	
Marital status, N (%)							< 0.001
Partner	1,576 (87.4)	342 (92.7)	323 (89.0)	323 (89.2)	293 (82.5)	295 (83.1)	
No partner	228 (12.6)	27 (7.3)	40 (11.0)	39 (10.8)	62 (17.5)	60 (16.9)	
Birth characteristics							
Sex, N (%)							0.09
Boys	970 (47.5)	176 (43.1)	188 (46.0)	194 (47.5)	197 (48.2)	215 (52.7)	
Girls	1,072 (52.5)	232 (56.9)	221 (54.0)	214 (52.5)	212 (51.8)	193 (47.3)	
Birth weight, mean (SD), weeks	3,439 (542)	3,521 (497)	3441 (571)	3,417 (525)	3,409 (556)	3,405 (555)	0.01
Ethnicity (%)							< 0.001
European	1,391 (69.2)	347 (86.3)	302 (74.9)	253 (62.8)	240 (59.4)	249 (62.6)	
Non-European	619 (30.8)	55 (13.7)	101 (25.1)	150 (37.3)	164 (40.6)	149 (37.4)	

Table 1. Subject characteristics (N = 2,042) (continued)

	Hair Cortisol Concentrations						
	Total group [a] (N = 2,042)	**Quintile 1 [a] 0.137-0.727 pg/mg (N = 408)**	**Quintile 2 [a] 0.728-1.150 pg/mg (N = 409)**	**Quintile 3 [a] 1.151-1.820 pg/mg (N = 408)**	**Quintile 4 [a] 1.827-2.927 pg/mg (N = 409)**	**Quintile 5 [a] 2.928-6.799 pg/mg (N = 408)**	**P-value [b]**
Child characteristics at 6 years							
Age at measurements, median (95% range), years	5.9 (5.7, 8.0)	5.9 (5.6, 7.8)	5.9 (5.7, 8.3)	5.9 (5.7, 8.1)	5.9 (5.7, 7.9)	5.9 (5.6, 8.0)	0.15
Body mass index, median (95% range), kg/m²	15.8 (13.6, 20.7)	15.7 (13.7, 18.8)	15.7 (13.6, 20.0)	15.8 (13.6, 20.4)	15.9 (13.7, 21.4)	16.0 (13.4, 22.4)	< 0.001
Hair cortisol concentrations, median (95% range), pg/mg [c]	1.43 (0.32, 5.63)	0.54 (0.23, 0.71)	0.94 (0.73, 1.14)	1.43 (1.16, 1.80)	2.27 (1.84, 2.89)	3.98 (2.99, 6.62)	< 0.001
Hair cortisone concentrations, median (95% range), pg/mg [c]	7.30 (2.64, 29.03)	4.57 (2.02, 8.76)	5.91(2.87, 10.32)	7.93 (3.16, 14.73)	11.77 (3.61, 23.66)	16.27 (3.92, 45.13)	< 0.001
Cortisol / cortisone ratio, median (95% range)	0.17 (0.07, 0.73)	0.11 (0.05, 0.24)	0.16 (0.10, 0.33)	0.18 (0.10, 0.43)	0.19 (0.10, 0.65)	0.24 (0.10, 1.20)	<0.001
Glucocorticoid use in the 3 months prior to hair sample collection, N (%)							0.66
No	1,805 (92.6)	354 (91.9)	369 (93.7)	363 (91.9)	358 (93.7)	361 (91.6)	
Yes	145 (7.4)	31 (8.1)	25 (6.3)	32 (8.1)	24 (6.3)	33 (8.4)	
Hair color, N (%)							< 0.001
Red	63 (3.1)	16 (3.9)	11 (2.7)	17 (4.2)	10 (2.5)	9 (2.2)	
Blond	1,166 (57.1)	310 (76.0)	240 (58.7)	207 (50.7)	204 (50.0)	205 (50.2)	
Brown	620 (30.4)	76 (18.6)	127 (31.1)	149 (36.5)	136 (33.3)	132 (32.4)	
Black	192 (9.4)	6 (1.5)	31 (7.6)	35 (8.6)	58 (14.2)	62 (15.2)	
Television watching time, N (%)							< 0.001
< 2 hours per day	1,381 (83.3)	310 (90.4)	289 (86.5)	270 (80.6)	257 (79.8)	255 (78.9)	
≥ 2 hours per day	276 (16.7)	33 (9.6)	45 (13.5)	65 (19.4)	65 (20.4)	68 (21.1)	
Child characteristics at 10 years							
Age at measurements, median (95% range), years	9.7 (9.3, 10.6)	9.7 (9.3, 10.4)	9.7 (9.3, 10.4)	9.7 (9.4, 10.5)	9.7 (9.2, 10.7)	9.7 (9.3, 11.0)	0.10
Height, mean (SD), cm	141.4 (6.4)	141.8 (5.8)	141.1 (6.4)	140.9 (6.2)	141.4 (6.9)	141.7 (6.7)	0.21
Weight, median (95% range), kg	33.6 (25.4, 54.0)	32.8 (25.4, 49.6)	33.4 (25.1, 51.2)	33.6 (25.2, 50.2)	34.0 (24.9, 56.6)	36.7 (25.4, 57.6)	< 0.001

Table 1. Subject characteristics (N = 2,042) (continued)

		Hair Cortisol Concentrations					
	Total group [a] (N = 2,042)	**Quintile 1 [a] 0.137-0.727 pg/mg (N = 408)**	**Quintile 2 [a] 0.728-1.150 pg/mg (N = 409)**	**Quintile 3 [a] 1.151-1.820 pg/mg (N = 408)**	**Quintile 4 [a] 1.827-2.927 pg/mg (N = 409)**	**Quintile 5 [a] 2.928-6.799 pg/mg (N = 408)**	**P-value [b]**
Body mass index, median (95% range), kg/m^2	16.9 (14.0, 24.8)	16.5 (13.9, 22.2)	16.7 (14.2, 24.0)	16.9 (14.0, 23.5)	17.0 (14.1, 25.4)	17.2 (13.8, 26.6)	< 0.001
Total body fat mass, median (95% range),kg	8.48 (4.46, 21.88)	8.18 (4.59, 18.98)	8.39 (4.44, 20.07)	8.61 (4.45, 20.49)	8.67 (4.31, 24.03)	9.05 (4.62, 24.47)	< 0.001
Pericardial fat mass, median (95% range), g	10.13 (4.48, 22.15)	10.03 (4.60, 20.66)	10.42 (4.62, 20.67)	10.09 (4.45, 22.57)	9.72 (3.95, 21.70)	10.49 (4.29, 24.22)	0.49
Visceral fat mass, median (95% range), kg	0.36 (0.16, 1.01)	0.36 (0.16, 0.91)	0.35 (0.17, 1.06)	0.35 (0.15, 0.98)	0.35 (0.15, 0.99)	0.37 (0.15, 1.19)	0.14
Liver fat fraction, median (95% range), %	1.97 (1.20, 5.04)	1.92 (1.24, 4.45)	1.88 (1.19, 3.63)	1.90 (1.15, 6.16)	2.03 (1.22, 4.23)	2.07 (1.26, 7.91)	<0.001
Nonalcoholic fatty liver disease, N (%)							< 0.001
No	1,327 (97.5)	274 (99.6)	265 (98.9)	263 (94.9)	267 (99.6)	258 (94.5)	
Yes	34 (2.5)	1 (0.4)	3 (1.1)	14 (5.1)	1 (0.4)	15 (5.5)	

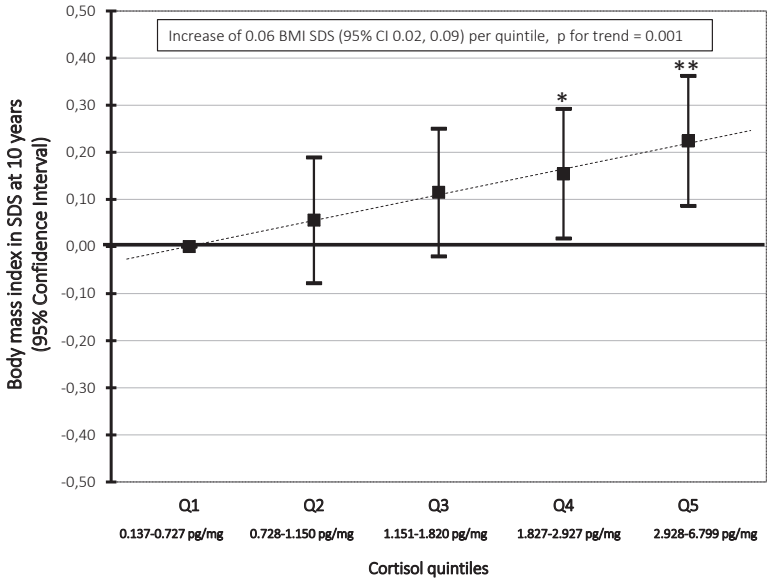
[a] Values are means (standard deviation), medians (95% range) or numbers of subjects (valid %).

[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.

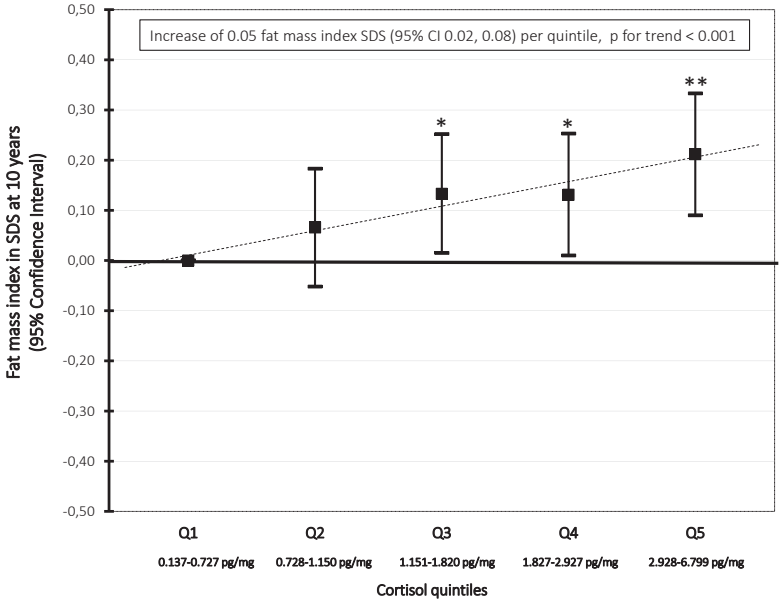
[c] pg/mg = picogram per milligram

Figure 1. Associations of hair cortisol concentrations with general fat measures at 10 years (N = 2,042)

A



B

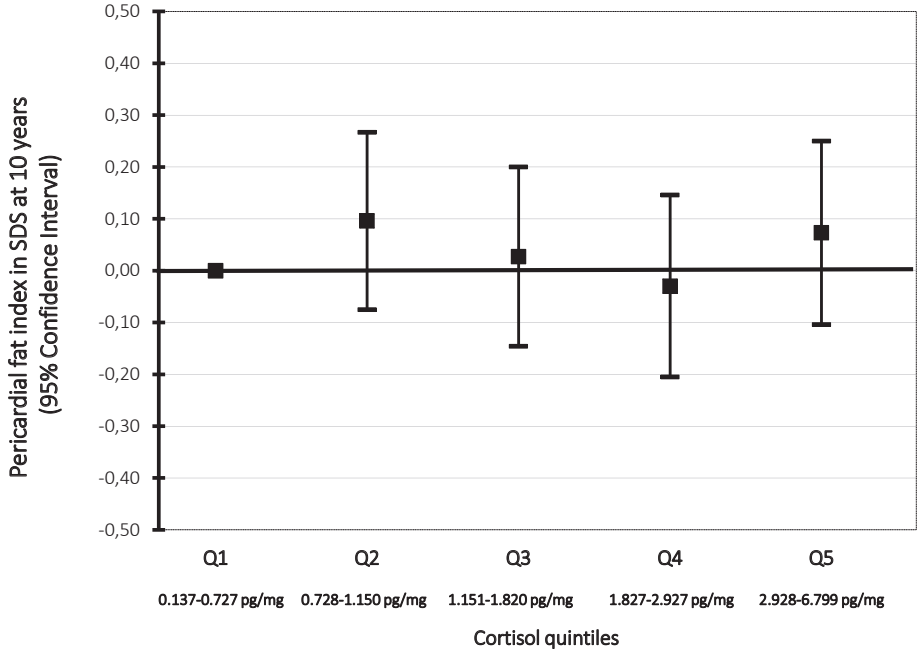


Values are linear regression coefficients (95% confidence interval) and reflect the change in SDS childhood BMI (**A**, N = 2,037) and fat mass index (**B**, N = 2,013) at 10 years for the cortisol quintiles. Models are adjusted for child's sex and age (except for sex- and age adjusted body mass index SDS), maternal pre-pregnancy BMI, psychological distress during pregnancy, maternal educational level and marital status at 6 years, child's ethnicity, hair color and television watching time. Tests for trend were based on multiple linear regression models with hair cortisol concentration quintiles as a continuous variable.

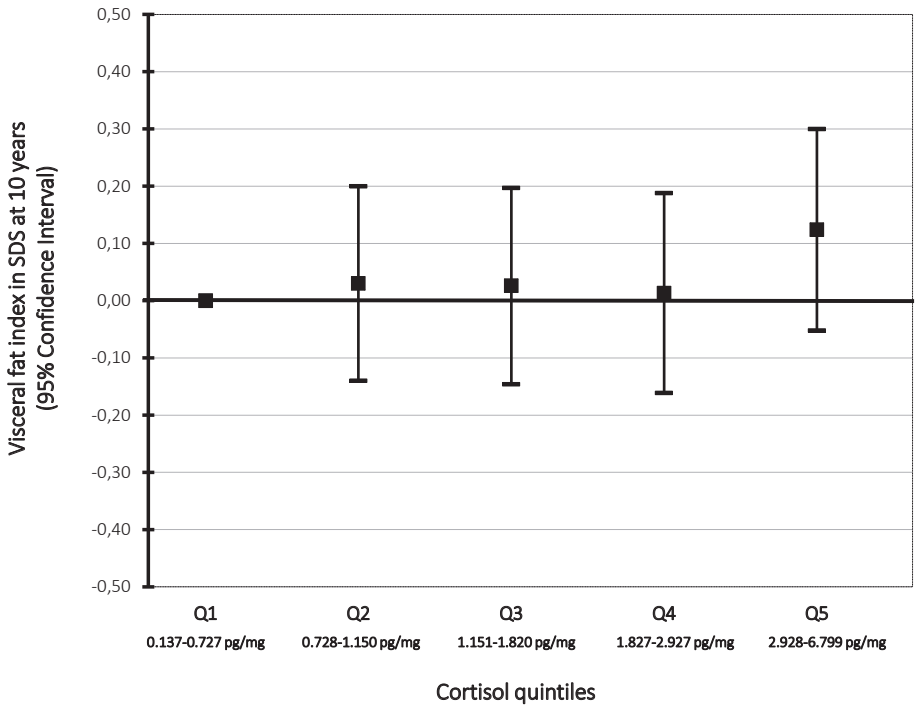
*p< 0.05, **p< 0.017.

Figure 2. Association of hair cortisol quintiles with visceral and organ fat measures at 10 years (N = 1,523)

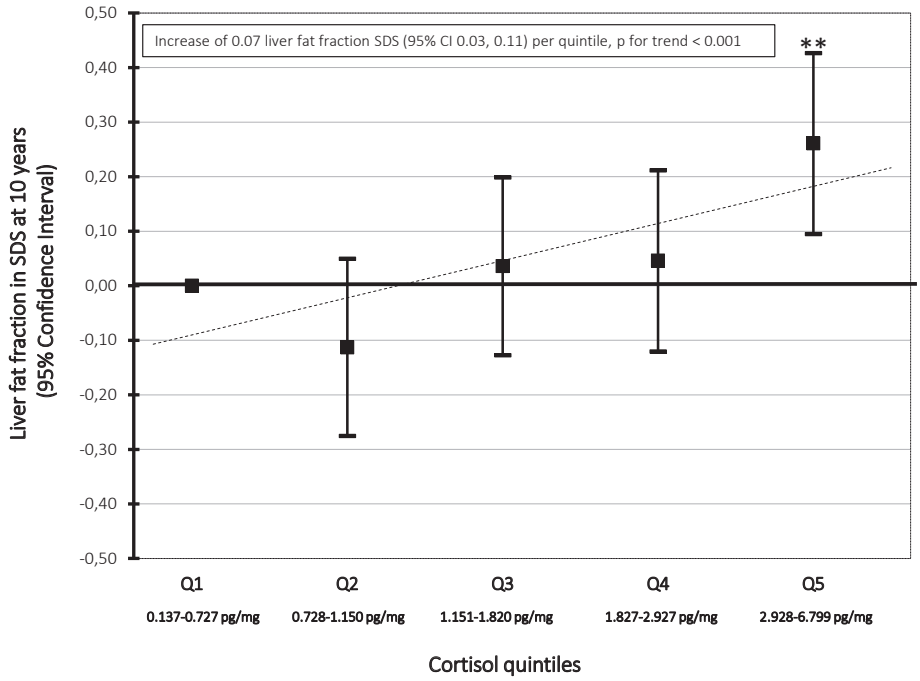
A



B



C



Values are linear regression coefficients (95% confidence interval) and reflect the change in SDS childhood pericardial fat (**A**, N = 1,278**)**, visceral fat (**B**, N = 1,237) indices and liver fat fraction (**C**, N = 1,361) for the cortisol quintiles. Models are adjusted for child's sex and age, maternal pre-pregnancy BMI, psychological distress during pregnancy, maternal educational level and marital status at 6 years, child's ethnicity, hair color and television watching time. Test for trend was based on a multiple linear regression model with hair cortisol concentration quintiles as a continuous variable.
*p< 0.05, **p< 0.017.

mass index at 6 years, suggesting these associations were independent of fat mass index at 6 years (**Supplemental Table 7**).[16]

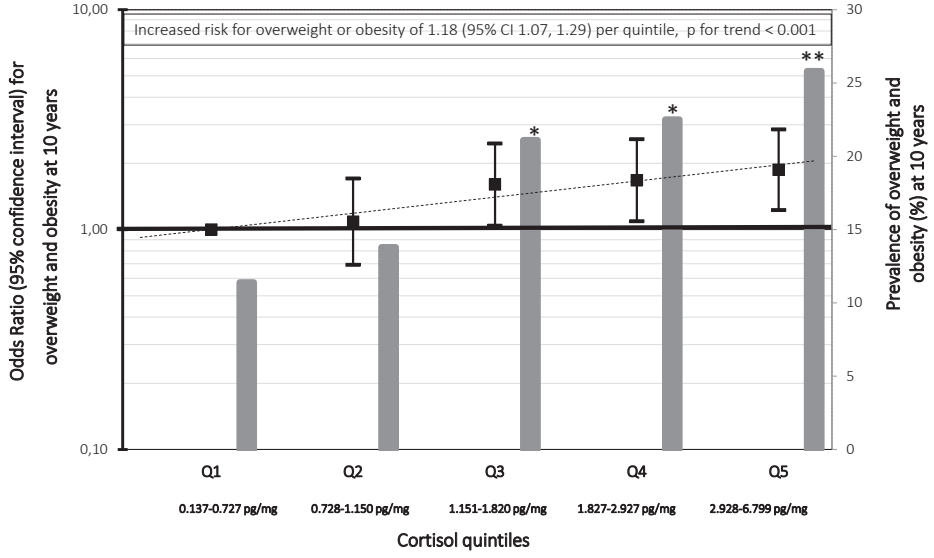
Hair cortisol concentrations and risk of childhood overweight

The prevalence of overweight at 10 year increased from 11.4% in the first quintile to 25.8% in the fifth quintile of hair cortisol concentrations (**Figure 3**). As compared to the lowest quintile, children in the highest quintile of hair cortisol concentrations at 6 years, had a higher risk of overweight (Odds Ratio (OR): 1.87 (95% CI 1.23, 2.86)) at 10 years (**Figure 3**). Test for trend was significant for the risk of overweight (p-value for trend < 0.001). Associations for continuous cortisol measures showed similar results. Results from basic models were in the same direction and slightly stronger (**Supplemental Table 5)**.[16]

Sensitivity analyses

Results from the models excluding children with all types of glucocorticoid use in the three months prior to hair sample collection, were in the same direction and slightly stronger (**Supplemental Table 8**).[16] Higher hair cortisone concentrations were associated with higher BMI and fat mass index, but with a lower pericardial fat index. Higher hair cortisone concentrations were not associated with liver fat fraction, and visceral fat index, or the risk of overweight, although effect estimates were in the same direction as the results for cortisol (**Supplemental Table 9**).[16] The sensitivity analyses, in which we adjusted the main models for adiposity measures at 6 years, showed similar results as the conditional analyses (**Supplemental Table 10)**.[16] Also, these sensitivity analyses suggested that the associations with the risk of NAFLD remained significant, whereas the association with risk of overweight attenuated into non-significance (**Supplemental Table 10)**.[16]

Figure 3. Associations of hair cortisol concentrations with risk of overweight and obesity at 10 years (N = 1,898)



Values on the left y-axis are odds ratios (95% confidence interval) on a logarithmic scale and represent the risk of childhood overweight at 10 years for the cortisol quintiles. Models are adjusted for child's sex and age, maternal pre-pregnancy BMI, psychological distress during pregnancy, maternal educational level and marital status at 6 years, child's ethnicity, hair color and television watching time. Values on the right y-axis are percentages and represent the prevalence (%) of overweight and obesity at 10 years. Test for trend was based on a logistic regression model with cortisol quintiles as a continuous variable.

*p< 0.05, **p< 0.017.

DISCUSSION

In this population-based prospective cohort study among 2,042 children, we observed that higher hair cortisol concentrations at 6 years were associated with higher BMI, fat

mass index, liver fat fraction and increased risk of overweight and NAFLD at age 10. Hair cortisol concentrations were not associated with visceral fat or pericardial fat indices.

Interpretation of main findings

Previous studies reported associations of long-term cortisol concentrations in hair with BMI, and other adiposity measures in adults.[2,6,11] A previous study in our cohort used a cross-sectional design and reported that higher hair cortisol and cortisone concentrations were associated with a higher BMI, fat mass index and increased risk of overweight at age 6 years.[31] We extended this study by examining the prospective associations of hair cortisol and cortisone concentrations at age 6 years with general and organ fat measures measured by MRI at 10 years.

We observed that hair cortisol concentrations at age 6 years were positively associated with childhood BMI, fat mass index and the risk of overweight at 10 years. These findings are in line with the previous study, although we observed somewhat smaller effect sizes.[31] This may partly be explained by a different design and smaller numbers of subjects. Additional conditional analyses and adjustment for BMI at 6 years showed that the associations of hair cortisol concentrations with BMI and risk of overweight were explained by the associations already present at 6 years. Also, we did not observe associations of hair cortisol concentrations with change in BMI or fat mass index SD score between 6 and 10 years, suggesting that the associations already observed at 6 years persist during childhood. A recent review including twelve cohort studies in children reported that a majority of studies showed a positive relationship between hair cortisol concentrations and BMI.[12] Altogether, results from previous studies and our study suggest that higher cortisol concentrations are associated with higher BMI, fat mass index and risk of overweight throughout childhood.

We observed that higher hair cortisol concentrations at 6 years were positively associated with liver fat fraction and a higher risk of NAFLD at 10 years. Additional conditional regression analyses and adjustment for fat mass index at 6 years suggest that these association were independent of fat mass at 6 years. To our knowledge, this study is the first to report associations of hair cortisol concentrations with visceral and organ fat in children. NAFLD is the most common liver disease in western populations, among both children and adults, and closely linked to the development of the metabolic syndrome.[32,33] Our findings are in line with an adult study showing that increased serum cortisol concentration was associated with an increased prevalence of NAFLD.[34] It has been suggested that overactivity of the hypothalamic-pituitary-adrenocortical (HPA) axis and increased glucocorticoids have an important role in the development of NAFLD.[34-36] We did not observe an association of hair cortisol concentrations with visceral adiposity, a well-known consequence of hypercortisolism.[34,37] Pericardial fat is, next to visceral and liver fat, related to adverse cardio-metabolic outcomes in adults which we know

are associated with increased hair cortisol levels in adults.[38,39] We did not observe an association between hair cortisol and pericardial fat in childhood. It may be that the associations between hair cortisol and visceral and pericardial fat become more apparent at older ages.

We performed two sensitivity analyses. The slightly stronger effect estimates after excluding children who used glucocorticoid medication, might be explained by the exclusion of a more heterogeneous group of children with sometimes elevated cortisol concentrations due to exogenous causes. Also, hair cortisone concentrations were associated with childhood adiposity measures, but the effect estimates were weaker than for cortisol. This might be explained by the differences in biological activity.

There are various mechanisms through which cortisol concentrations may affect childhood adiposity. Increased cortisol levels increase appetite, specifically for high-sweet and fatty foods, stimulate adipogenesis, induce insulin resistance and negatively affect brown adipose tissue.[2,40] However, a bidirectional association, where changes in the cortisol metabolism, are consequences of metabolic changes accompanying adiposity, might also be present.[41] Future research should explore the potential of reversed causation and examine underlying mechanisms of these associations. The enzyme 11β -hydroxysteroid dehydrogenase type 1 (11β-HSD1) is expressed in the brain, adipose tissue and the liver and converts cortisone into active cortisol.[42,43] Regeneration of cortisol from inactive cortisone has been found to be increased in adipose tissue in obese individuals.[41,44] The enzyme 11β -hydroxysteroid dehydrogenase type 2 (11β-HSD2) is expressed in the kidneys but also the placenta and fetus highly express this enzyme which converts cortisol into inactive cortisone, protecting the body from mineralocorticoid excess.[44,45] By inactivating the majority of maternal glucocorticoids passing to the fetus, 11β-HSD2 may prevent premature maturation of fetal tissues, decreased birth weight and consequent developmental "programming of later life diseases".[44,46] Adverse circumstances during pregnancy such as maternal psychological distress may induce persistent changes and affect fetal programming of the HPA axis and subsequent body composition and metabolic function.[47-52] Future studies should identify fetal and early-childhood factors that influence cortisol concentrations and thereby lead to developmental adaptations with persistent consequences.

We observed that higher hair cortisol concentrations are associated with an adverse body fat profile, increased liver fat fraction and increased risk of overweight and NAFLD during childhood. These findings seem important, since it is well known that body fat distribution tracks from childhood into adulthood and is associated with cardiovascular disease in later life. [53,54] Future research is needed to obtain further insight into the causality and underlying mechanisms of these associations and to assess whether childhood cortisol concentrations have effect on body fat development throughout adult life.

Strengths and limitations

Strengths of this study were the prospective data collection from early pregnancy onwards, the large sample size, detailed measurements of hair cortisol concentrations and childhood adiposity measures including organ fat measures assessed by MRI. This study also has limitations. Of all children who had information on hair cortisol at 6 years (N = 2,926) only 2,278 had information on at least one measurement of adiposity at 10 years. Selective non-response could lead to selection bias if the associations of hair cortisol concentrations at 6 years with childhood adiposity at 10 years differ between participants and non-participants. This seems unlikely, but cannot be excluded. Another limitation of our study is the lack of hair cortisol measurements at the age of 10 years. Therefore we don't know how cortisol concentrations develop over time and if they partly explain the effects seen at the age of 10 years. Higher cortisol concentrations at both ages could be caused by continued stress but also genetic variation in genes such as *HSD11B1, HSD11B2*, *SPERINA6* or *SPERINA1* may be a cause.[44,55] *HSD11B1* and *HSD11B2,* encode enzymes, 11β-HSD1 and 11β-HSD2, that are involved in the cortisol and cortisone metabolism.[44] Between approximately ages 4 and 7 children undergo an adiposity rebound, resulting in accelerated increase in BMI. An early adiposity rebound is associated with an increased risk of obesity in later life.[56,57] The age at adiposity rebound may be important in the relation between cortisol and adiposity development and this should be addressed in future studies.[58] Detailed information about a large number of potential confounding factors was available in this study. However, residual confounding, for example by maternal and child stress prior to or around the time of hair cortisol examination, might still be present. Also, because of the observational design, no conclusions can be drawn yet on the causality and directionality of the observed associations.

CONCLUSION

Our results suggest that the associations of higher hair cortisol concentrations at age 6 years, with higher BMI, fat mass index and increased risk of overweight at age 10 years are explained by the associations already observed at 6 years. The associations of higher hair cortisol concentrations at 6 years with liver fat fraction and NAFLD at 10 years were independent of fat mass index at 6 years. Future studies are needed to assess the causal pathways underlying these associations, the determinants of early-life cortisol concentrations and the long-term body fat and cardio-metabolic consequences.

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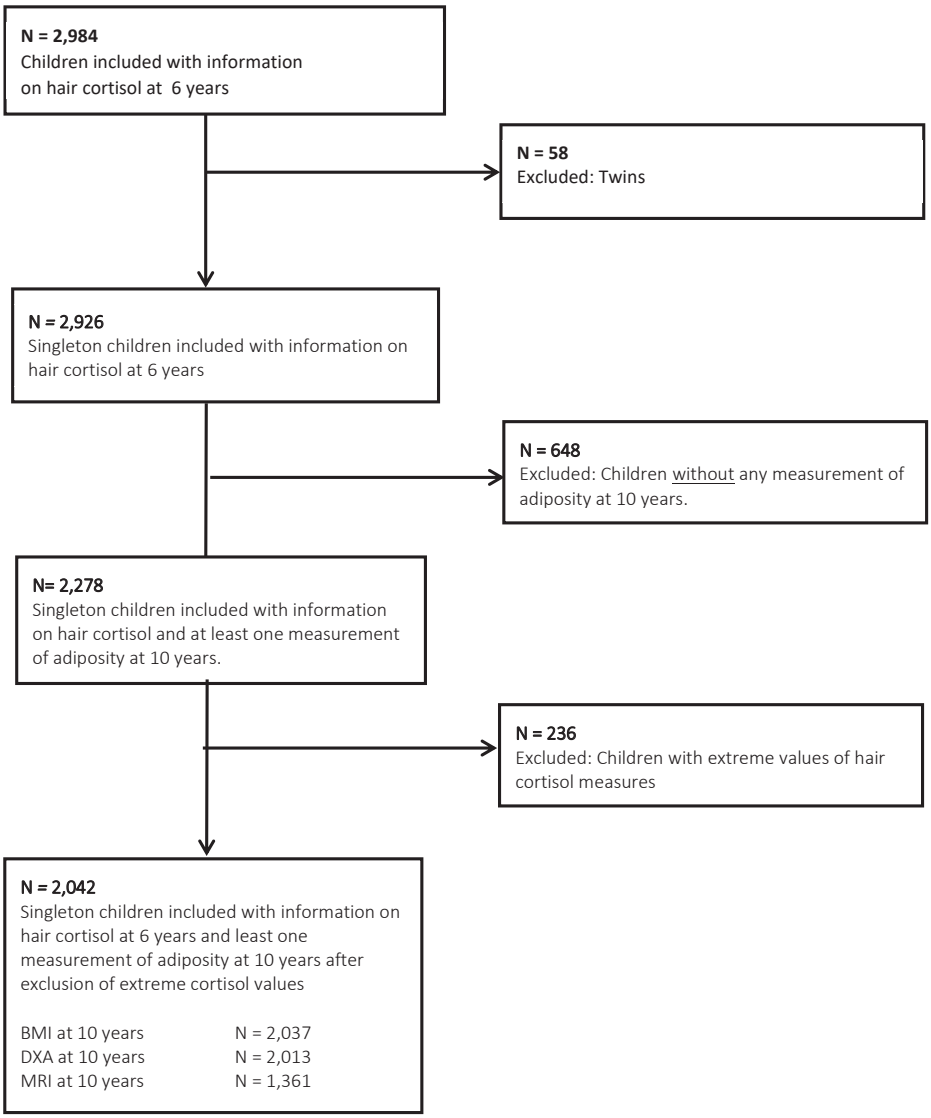
SUPPLEMENTARY MATERIAL

Supplemental Figure 1. Flowchart of study population



Supplemental Methods

Hair Cortisol and Cortisone Concentration Measurements

Hair cortisol and cortisone were measured in proximal scalp hair. A hair strand of approximately 100 hairs was cut from the posterior vertex using small surgical scissors, as close to the scalp as possible.[1] 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. The proximal 3 cm of hair samples were weighed and finely cut. Hair samples were then washed in LC-grade isopropanol for 2 min at room temperature, and left to dry for 2 days. Extraction was performed using LC-grade methanol (MeOH), for 18 h at 25 °C, in the presence of deuterated steroids. Subsequently, the extract was cleaned using solid phase extraction and steroids were quantified on a Xevo TQS liquid chromatography tandem mass spectrometry (LC–MS/MS) (Waters Corporation, Milford, MA, USA).[1]

Visceral and Organ Fat Measurements

Extraneous structures and image artifacts were removed manually.[2] Pericardial fat included both epicardial and paracardial fat directly attached to the pericardium, ranging from the apex to the left ventricular outflow tract. Total visceral fat volume ranged from the dome of the liver to the superior part of the femoral head. Fat mass was obtained by multiplying the total volumes by the specific gravity of adipose tissue, 0.9 g/mL. Liver fat fraction was determined by taking 4 samples of at least 4 cm^2 from the central portion of the hepatic volume. Subsequently, the mean signal intensities were averaged to generate overall mean liver fat fraction estimation. As described previously, nonalcoholic fatty liver disease was defined as liver fat ≥ 5.0%.[3-5]

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Supplemental Figure 2. Directed acyclic graph (DAG) for the relationship between hair cortisol cortisone concentrations at 6 years and child adiposity measures at 10 years depicting the covariates included in the models

Supplemental Table 1. Correlation coefficients between all measures of adiposity at 6 and 10 years (N = 2,042)[a]

	Adiposity measures at 6 years		Adiposity measures at 10 years				
Adiposity measures at 6 years	**Body Mass Index**	**Fat Mass Index**	**Body mass index**	**Fat mass index**	**Pericardial fat index**	**Visceral fat index**	**Liver fat fraction**
Body Mass Index	1	0.70*	0.80*	0.59*	0.25*	0.42*	0.26*
Fat Mass Index	0.70*	1	0.64*	0.81*	0.25*	0.53*	0.33*
Adiposity measures at 10 years	**Body Mass Index**	**Fat Mass Index**	**Body mass index**	**Fat mass index**	**Pericardial fat index**	**Visceral fat index**	**Liver fat fraction**
Body Mass Index	0.80*	0.64*	1	0.81*	0.35*	0.61*	0.38*
Fat Mass Index	0.59*	0.81*	0.81*	1	0.38*	0.71*	0.43*
Pericardial fat Index	0.25*	0.25*	0.35*	0.38*	1	0.49*	0.16*
Visceral fat index	0.42*	0.53*	0.61*	0.71*	0.49*	1	0.41*
Liver fat fraction	0.26*	0.33*	0.38*	0.43*	0.16*	0.41*	1

[a] Values are Spearman correlation coefficients. * P-value < 0.01.

Supplemental Table 2. Comparison of child characteristics between participants and non-participants (N = 2,926)

	Participants [a] (N = 2,042)	Non-participants [a] (N = 884)	P-value [b]
Family characteristics			
Maternal age, mean (SD), years	31.2 (4.8)	29.4 (5.5)	< 0.01
Missings, N (%)	0 (0.0)	0 (0.0)	
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.5 (18.2, 35.0)	22.9 (18.1, 36.9)	0.03
Missings, N (%)	493 (24.1)	246 (27.8)	
Maternal psychological distress during pregnancy, N (%)			< 0.01
Yes	560 (27.4)	362 (41.0)	
No	1,397 (68.4)	481 (54.4)	
Missings, N (%)	85 (4.2)	41 (4.6)	
Maternal education, N (%)			< 0.01
Primary school	68 (3.3)	39 (4.4)	
Secondary school	619 (30.3)	314 (35.5)	
High education	1,120 (54.8)	290 (32.8)	
Missings, N (%)	235 (11.5)	241 (27.3)	
Marital status, N (%)			0.29
With partner (married/living together)	1,576 (77.2)	551 (62.3)	
Without partner	228 (11.2)	103 (11.7)	
Missings, N (%)	238 (11.7)	230 (26.0)	
Birth characteristics			
Sex, N (%)			0.92
Boys	970 (47.5)	444 (50.2)	
Girls	1,072 (52.5)	440 (49.8)	
Missings, N (%)	0 (0.0)	0 (0.0)	
Ethnicity, N (%)			< 0.01
European	1,391 (68.1)	418 (47.3)	
Non-European	619 (30.3)	414 (46.8)	
Missings	32 (1.6)	52 (5.9)	
Child characteristics at 6 years			
Age at visit, median (95% range), years	5.9 (5.7, 8.0)	6.0 (5.7, 8.3)	< 0.01
Missings, N (%)	0 (0.0)	0 (0.0)	
Body mass index, median (95% range), kg/m^2	15.8 (13.6, 20.7)	16.1 (13.4, 22.2)	< 0.01
Missings, N (%)	0 (0.0)	0 (0.0)	
Hair cortisol concentration, median (95% range), pg/mg [c]	1.43 (0.32, 5.63)	2.63 (0.44, 112.56)	< 0.01
Missings, N (%)	0 (0.0)	0 (0.0)	
Hair cortisone concentration, median (95% range), pg/mg [c]	7.30 (2.64, 29.03)	8.72 (2.81, 40.62)	< 0.01
Missings, N (%)	41 (2.0)	45 (5.1)	
Glucocorticoid use in the last 3 months (%)			0.14
No	1,805 (88.4)	749 (84.7)	

Supplemental Table 2. Comparison of child characteristics between participants and non-participants (N = 2,926) (continued)

	Participants [a] (N = 2,042)	Non-participants [a] (N = 884)	P-value [b]
Yes	145 (7.1)	83 (9.4)	
Missings, N (%)	92 (4.5)	52 (5.9)	
Hair color, N (%)			< 0.01
Red	63 (3.1)	24 (2.7)	
Blond	1,166 (57.1)	349 (39.5)	
Brown	620 (30.4)	361 (40.8)	
Black	192 (9.4)	149 (16.9)	
Missings, N (%)	1 (0.0)	1 (0.1)	
Average duration of television watching, N (%)			
< 2 hours per day	1,381 (67.6)	438 (49.5)	<0.01
>= 2 hours per day	276 (13.5)	138 (15.6)	
Missings, N (%)	385 (18.9)	308 (34.8)	

[a] Values are observed data and represent means (standard deviation), medians (95% range) or number of participants (%).
[b] P-values for differences in child characteristics between participants and non-participants were calculated performing Student *t* tests for normally distributed continuous variables, Mann-Whitney test for non-normally distributed continuous variables and Chi-square tests for categorical variables.
[c] pg/mg = picogram per milligram

Supplemental Table 3. Association of continuous hair cortisol concentrations at 6 years with childhood general and organ fat measures at 10 years (N = 2,042)

	Difference (95% CI) in standard deviation scores [a]					Odds Ratio (95% CI) [b]	
	Body mass index (N = 2,037)	Fat mass index (N = 2,013)	Pericardial fat index (N = 1,278)	Visceral fat index (N = 1,237)	Liver fat fraction (N = 1,361)	Risk of overweight and obesity (N = 1,898)	Risk of NAFLD (N = 1,361)
Continuous hair cortisol	**0.10 (0.04, 0.16)****	**0.09 (0.04, 0.15)****	-0.02 (-0.10, 0.06)	0.05 (-0.03, 0.13)	**0.15 (0.07, 0.22)****	**1.41 (1.16, 1.70)****	**2.35 (1.31, 4.22)****

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in childhood body mass, fat mass, pericardial and visceral fat indices and liver fat fraction standard deviation scores for an interquartile range increase in the natural log transformed hair cortisol concentration. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS), maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

[b] Values are odds ratios (95% confidence interval) and represent the risk of childhood overweight and obesity and nonalcoholic fatty liver disease (NAFLD) at 10 years for an interquartile range increase in the natural log transformed hair cortisol concentration. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted BMI categories), maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

*p< 0.05, **p< 0.017.

Supplemental Table 4. Standardized coefficients (betas) for all variables in the associations of continuous hair cortisol concentrations at 6 years with childhood general and organ fat measures at 10 years (N = 2,042)

	Difference (95% CI) in standard deviation scores [a]				
	Body mass index (N = 2,037)	Fat mass index (N = 2,013)	Pericardial fat index (N = 1,278)	Visceral fat index (N = 1,237)	Liver fat fraction (N = 1,361)
Continuous hair cortisol	0.06	0.04	-0.07	-0.00	0.10
Child's sex	NA	0.32	0.04	0.15	0.09
Child's age	NA	0.10	0.07	0.08	0.13
Maternal pre-pregnancy BMI	0.29	0.25	0.14	0.25	0.17
Psychological distress during pregnancy	0.03	0.07	-0.01	0.00	-0.03
Maternal educational level at 6 years - low	0.05	0.07	0.00	0.04	-0.02
Maternal educational level at 6 years - mid	0.08	0.11	0.09	0.09	0.07
Maternal marital status at 6 years	0.07	0.02	-0.05	-0.04	0.02
Child's ethnicity	0.04	0.06	-0.01	-0.05	0.01
Child's hair color - red	0.02	0.02	0.05	-0.02	0.03
Child's hair color - brown	0.03	0.04	-0.03	0.03	0.02
Child's hair color – black	0.02	0.03	-0.04	-0.04	-0.01
Child's television watching time	0.08	0.05	0.01	0.07	0.04

[a] Standardized coefficients (betas) for all variables in the linear regression analyses of continuous hair cortisol (interquartile range increase in the natural log transformed hair cortisol concentrations) at 6 years with general and organ fat measures at 10 years in the original (non-imputed) data. Body mass index is already sex- and age-adjusted therefore results for these variables are not shown (NA).

Supplemental Table 5. Association of hair cortisol quintiles at 6 years with childhood general and organ fat measures at 10 years, basic models (N = 2,042)

	Difference (95% CI) in standard deviation scores [a]					Odds Ratio (95% CI) [b]
Hair cortisol quintiles	**Body mass index (N = 2,037)**	**Fat mass index (N = 2,013)**	**Pericardial fat index (N = 1,278)**	**Visceral fat index (N = 1,237)**	**Liver fat fraction (N = 1,361)**	**Risk of overweight and obesity (N = 1,898)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.12 (-0.02, 0.27)	**0.13 (0.00, 0.26)***	0.09 (-0.09, 0.26)	0.04 (-0.13, 0.21)	-0.10 (-0.26, 0.07)	1.24 (0.81, 1.91)
Q3	**0.23 (0.09, 0.38)****	**0.26 (0.14, 0.39)****	0.02 (-0.15, 0.19)	0.06 (-0.11, 0.23)	0.09 (-0.07, 0.25)	**2.07 (1.38, 3.09)****
Q4	**0.30 (0.16, 0.44)****	**0.29 (0.16, 0.42)****	-0.04 (-0.21, 0.14)	0.05 (-0.12, 0.23)	0.11 (-0.05, 0.28)	**2.24 (1.51, 3.34)****
Q5	**0.41 (0.27, 0.55)****	**0.40 (0.27, 0.53)****	0.10 (-0.08, 0.27)	**0.21 (0.03, 0.38)***	**0.35 (0.18, 0.51)****	**2.69 (1.82, 3.98)****

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in childhood body mass, fat mass, pericardial and visceral fat indices and liver fat fraction standard deviation scores for the cortisol quintiles compared to the first quintile. Basic models are shown and are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS).

[b] Values are odds ratios (95% confidence interval) and represent the risk of childhood overweight and obesity at 10 years for the cortisol quintiles compared to quintile 1. Basic models are shown for sex-and age adjusted BMI categories.

*p< 0.05, **p< 0.017.

Supplemental Table 6. Association of hair cortisol quintiles at 6 years with the change in BMI and fat mass index SD scores between 6 and 10 years (N = 2,037)

	Difference (95% CI) in standard deviation scores	
Hair cortisol quintiles [a]	**Change in BMI SD score between 6 and 10 years (N = 2,037)**	**Change in Fat mass index SD score between 6 and 10 years (N = 1,971)**
Q1	*Reference*	*Reference*
Q2	0.03 (-0.05, 0.11)	0.07 (-0.01, 0.15)
Q3	0.01 (-0.08, 0.09)	0.07 (-0.01, 0.15)
Q4	0.01 (-0.08, 0.09)	0.00 (-0.08, 0.08)
Q5	0.02 (-0.06, 0.11)	0.04 (-0.04, 0.12)
Hair cortisol concentration [b]	0.00 (-0.04, 0.04)	0.00 (-0.04, 0.04)

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in the delta of childhood body mass and fat mass index standard deviation scores between 6 and 10 years for the cortisol quintiles compared to the first quintile. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS), maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

[b] Values are linear regression coefficients (95% confidence interval) and reflect the change in the delta of childhood body mass and fat mass index standard deviation scores between 6 and 10 years for an IQR increase natural log transformed hair cortisol concentrations. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS), maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

Supplemental Table 7. Associations of hair cortisol quintiles at 6 years with childhood general and organ fat measures at 10 years conditional on adiposity measures at 6 years (N = 2,042)

	Difference (95% CI) in standard deviation scores				
Hair cortisol quintiles [a]	**Body mass index (N = 2,044)**	**Fat mass index (N = 2,017)**	**Pericardial fat index (N = 1,280)**	**Visceral fat index (N = 1,234)**	**Liver fat fraction (N = 1,360)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.05 (-0.09, 0.18)	0.13 (-0.01, 0.26)	0.14 (-0.04, 0.31)	0.13 (-0.04, 0.31)	-0.06 (-0.22, 0.12)
Q3	0.02 (-0.11, 0.16)	**0.15 (0.01, 0.28)***	0.05 (-0.13, 0.23)	0.04 (-0.13, 0.22)	0.06 (-0.11, 0.23)
Q4	0.01 (-0.12, 0.15)	0.04 (-0.10, 0.18)	-0.07 (-0.25, 0.11)	-0.04 (-0.22, 0.14)	0.01 (-0.16, 0.18)
Q5	0.06 (-0.08, 0.19)	0.14 (-0.00, 0.28)	0.06 (-0.12, 0.24)	0.07 (-0.11, 0.26)	**0.24 (0.07, 0.41)****
Test for trend [b]	0.01 (-0.02, 0.04)	0.02 (-0.01, 0.05)	-0.01 (-0.05, 0.03)	-0.01 (-0.05, 0.03)	**0.05 (0.01, 0.09)****
Continuous hair cortisol [c]	0.01 (-0.05, 0.07)	0.03 (-0.04, 0.09)	-0.06 (-0.14, 0.03)	-0.01 (-0.09, 0.08)	**0.11 (0.03, 0.19)****

Values are linear regression coefficients (95% confidence interval) and reflect the change in BMI standard deviation scores at 10 years conditional on BMI at 6 years and the change in fat mass, pericardial and visceral fat indices and liver fat fraction standard deviation scores at 10 years conditional on fat mass index at 6 years for the cortisol quintiles compared to the first quintile (a), for one hair cortisol concentration quintile increase (test for trend) (b), for an interquartile range increase in the natural log transformed hair cortisol concentration. For these analyses, we first estimated the standardized residuals form the regression models with the 6 years adiposity measurements as exposures and the corresponding 10 years adiposity measurements as outcomes. Subsequently, these residuals were used as outcomes for the associations with hair cortisol concentrations. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS), maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

*p< 0.05, **p< 0.017.

Supplemental Table 8. Association of hair cortisol quintiles at 6 years with childhood general and organ fat measures at 10 years, excluding children with all types of glucocorticoid use in the 3 months prior to the hair sample collection (N = 1,805)

	Difference (95% CI) in standard deviation scores [a]					Odds Ratio (95% CI) [b]
Hair cortisol quintiles	**Body mass index (N = 1,800)**	**Fat mass index (N = 1,777)**	**Pericardial fat index (N = 1,128)**	**Visceral fat index (N = 1,092)**	**Liver fat fraction (N = 1,203)**	**Risk of overweight and obesity (N = 1,680)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.07 (-0.07, 0.21)	0.09 (-0.03, 0.22)	0.12 (-0.06, 0.30)	0.04 (-0.13, 0.22)	-0.10 (-0.27, 0.07)	1.19 (0.73, 1.93)
Q3	0.12 (-0.02, 0.26)	**0.15 (0.03, 0.28)***	0.04 (-0.14, 0.22)	0.00 (-0.18, 0.18)	0.02 (-0.15, 0.19)	**1.97 (1.25, 3.13)****
Q4	**0.15 (0.01, 0.29)***	**0.15 (0.02, 0.28)***	-0.01 (-0.19, 0.17)	0.03 (-0.15, 0.22)	0.05 (-0.13, 0.22)	**1.90 (1.20, 3.01)****
Q5	**0.25 (0.10, 0.39)****	**0.24 (0.11, 0.37)****	0.08 (-0.11, 0.26)	0.14 (-0.04, 0.33)	**0.30 (0.13, 0.47)****	**2.21 (1.40, 3.48)****

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in childhood body mass, fat mass, pericardial and visceral fat indices and liver fat fraction standard deviation scores for the cortisol quintiles compared to the first quintile after excluding children with all types of glucocorticoid use in the 3 months prior to the hair sample collection (N = 237: 145 children used glucocorticoids prior to hair sample collection and 92 children had missings for glucocorticoid use). Models are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS), maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

[b] Values are odds ratios (95% confidence interval) and represent the risk of childhood overweight and obesity at 10 years for the cortisol quintiles compared to the first quintile after excluding children with all types of glucocorticoid use in the pvast 3 months (N = 237). The model are adjusted for maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

*p< 0.05, **p< 0.017.

Supplemental Table 9. Association of hair cortisone quintiles at 6 years with childhood general and organ fat measures at 10 years (N = 2,051)

	Difference (95% CI) in standard deviation scores [a]					Odds Ratio (95% CI) [b]
Hair cortisol quintiles	**Body mass index (N = 2,044)**	**Fat mass index (N = 2,017)**	**Pericardial fat index (N = 1,280)**	**Visceral fat index (N = 1,234)**	**Liver fat fraction (N = 1,360)**	**Risk of overweight and obesity (N = 1,904)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.06 (-0.08, 0.19)	0.07 (-0.05, 0.19)	-0.12 (-0.29, 0.05)	0.02 (-0.15, 0.18)	-0.01 (-0.16, 0.15)	0.72 (0.48, 1.08)
Q3	0.05 (-0.09, 0.18)	0.08 (-0.04, 0.20)	-0.10 (-0.27, 0.08)	0.05 (-0.12, 0.22)	-0.06 (-0.21, 0.10)	0.96 (0.65, 1.43)
Q4	0.01 (-0.13, 0.15)	0.01 (-0.11, 0.13)	-0.16 (-0.33, 0.02)	-0.06 (-0.23, 0.11)	0.02 (-0.13, 0.18)	0.81 (0.54, 1.21)
Q5	**0.15 (0.02, 0.29)***	**0.18 (0.06, 0.30)****	**-0.21 (-0.38, -0.04)****	0.04 (-0.13, 0.21)	0.12 (-0.03, 0.28)	1.26 (0.85, 1.85)

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in childhood body mass, fat mass, pericardial and visceral fat indices and liver fat fraction standard deviation scores for the cortisone quintiles compared to the first quintile. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS), maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

[b] Values are odds ratios (95% confidence interval) and represent the risk of childhood overweight and obesity at 10 years for the cortisone quintiles compared to the first quintile. The model is adjusted for maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time.

*p< 0.05, **p< 0.017.

Supplemental Table 10. Association of hair cortisol quintiles at 6 years with childhood general and organ fat measures at 10 years, additionally adjusted for adiposity measures at 6 years (N = 2,042)

	Difference (95% CI) in standard deviation scores [a]					Odds Ratio (95% CI) [b]	
Hair cortisol quintiles	**Body mass index (N = 2,044)**	**Fat mass index (N = 2,017)**	**Pericardial fat index (N = 1,280)**	**Visceral fat index (N = 1,234)**	**Liver fat fraction (N = 1,360)**	**Risk of overweight and obesity (N = 1,904)**	**Risk of NAFLD (N = 1,361)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	NA
Q2	0.03 (-0.05, 0.11)	0.07 (-0.01, 0.14)	0.13 (-0.04, 0.29)	0.11 (-0.04, 0.25)	-0.06 (-0.21, 0.10)	1.05 (0.60, 1.83)	NA
Q3	0.02 (-0.06, 0.10)	**0.08 (0.01, 0.16)***	0.05 (-0.12, 0.22)	0.04 (-0.11, 0.18)	0.05 (-0.11, 0.21)	1.32 (0.78, 2.24)	NA
Q4	0.02 (-0.07, 0.10)	0.03 (-0.05, 0.10)	-0.07 (-0.24, 0.10)	-0.03 (-0.18, 0.12)	0.01 (-0.15, 0.17)	1.20 (0.71, 2.05)	NA
Q5	0.05 (-0.04, 0.13)	**0.08 (0.00, 0.16)***	0.06 (-0.12, 0.23)	0.06 (-0.09, 0.21)	**0.22 (0.06, 0.38)****	1.15 (0.67, 1.96)	NA
	Difference (95% CI) in standard deviation scores [c]					**Odds Ratio (95% CI) [d]**	
Continuous hair cortisol	0.01 (-0.03, 0.05)	0.03 (-0.01, 0.06)	-0.05 (-0.13, 0.03)	-0.01 (-0.08, 0.06)	**0.11 (0.03, 0.18)****	1.10 (0.86, 1.40)	**1.95 (1.06, 3.59)***

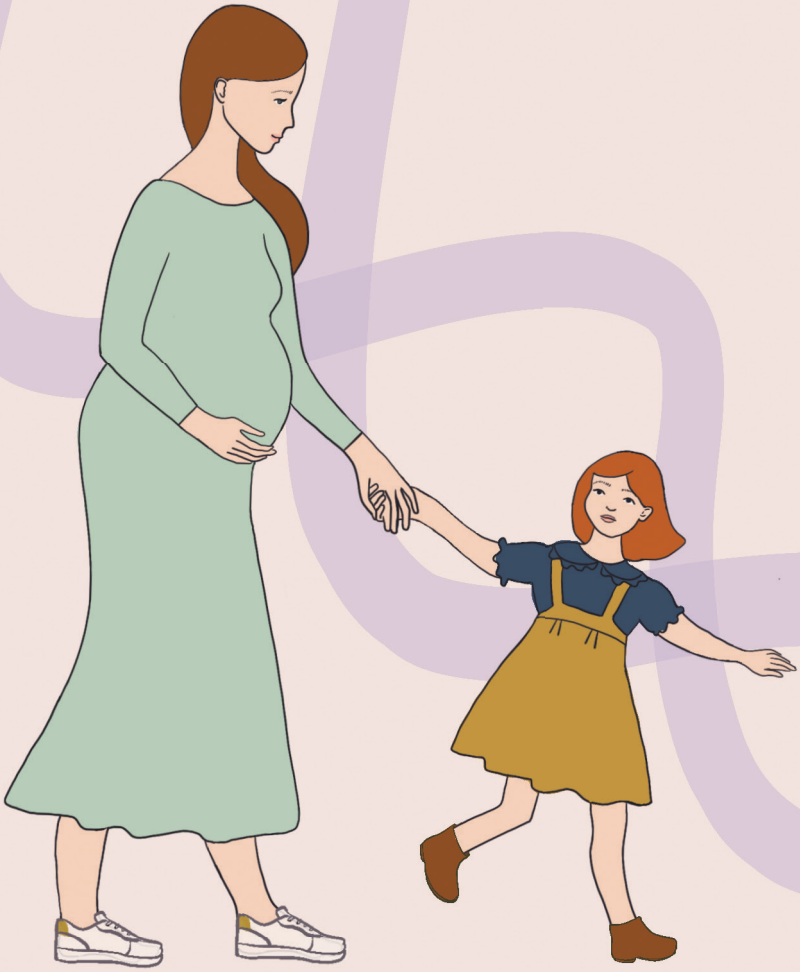
[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in childhood body mass, fat mass, pericardial and visceral fat indices and liver fat fraction standard deviation scores for the cortisone quintiles compared to the first quintile. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS), maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color, television watching time and. The model of BMI at 10 years is additionally adjusted for BMI at 6 years. The models for fat mass index and organ fat measures at 10 years are additionally adjusted for fat mass index at 6 years.

[b] Values are odds ratios (95% confidence interval) and represent the risk of childhood overweight and obesity at 10 years for the cortisone quintiles compared to the first quintile. Models is adjusted for maternal pre-pregnancy BMI, maternal psychological distress during pregnancy, maternal educational level and marital status at 6 years, child ethnicity, hair color, television watching time and is additionally adjusted for BMI at 6 years.

[c] Values are linear regression coefficients (95% confidence interval) and reflect the change in childhood body mass, fat mass, pericardial and visceral fat indices and liver fat fraction standard deviation scores for an interquartile range increase in the natural log transformed hair cortisol concentration. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted body mass index SDS), maternal pre-pregnancy BMI, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time. The model of BMI at 10 years is additionally adjusted for BMI at 6 years. The models for fat mass index and organ fat measures at 10 years are additionally adjusted for fat mass index at 6 years.

[d] Values are odds ratios (95% confidence interval) and represent the risk of childhood overweight and obesity and nonalcoholic fatty liver disease (NAFLD) at 10 years for an interquartile range increase in the natural log transformed hair cortisol concentration. Models are adjusted for child sex and age (except for the model of sex- and age-adjusted BMI categories), maternal pre-pregnancy BMI, maternal educational level and marital status at 6 years, child ethnicity, hair color and television watching time. The model for risk of overweight and obesity at 10 years is additionally adjusted for BMI at 6 years. The model for risk of NAFLD at 10 years is additionally adjusted for fat mass index at 6 years.

*p< 0.05, **p< 0.017.



3.2

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.

INTRODUCTION

Stress is associated with cardio-metabolic disease in adults.[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.[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.[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.[5,6] Cortisol concentrations measured in saliva, serum and urine are subject to situational and intra-individual fluctuations.[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.[7,8] A recent review among 11 cross-sectional studies in adults, shows positive associations of hair cortisol with adverse cardio-metabolic outcomes including higher systolic blood pressure, diabetes, metabolic syndrome and adiposity.[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.[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.[10-12] Also, cardio-metabolic risk factors tend to track from childhood into adulthood.[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.[17,18] Thus far, studies in children did not report associations between hair cortisol concentrations and cardio-metabolic risk factors.[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.[23]

Cardio-metabolic risk factors

Outcome assessments were performed at ages 6 and 10 years.[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).[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.[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).[27] Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald formula.[28,29] We defined children with clustering of cardio-metabolic risk factors being at risk for metabolic syndrome phenotype, in line with other studies.[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.[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.[22] 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.[33,34] To enable comparison of effect sizes of different measures, we constructed SDS ([observed value – mean]/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**).[24] 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.[35] Five imputed datasets were created and analyzed together. All statistical analyses were performed using the Statistical package of Social Sciences version 24.0 for Windows (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, version 24.0. Armonk, NY: IBM Corp).

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**).[24] 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**).[24]

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**).[24] When we adjusted these models for potential confounders, these associations attenuated into non-significance (**Table 3**).[24] 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)**.[24] 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**).[24] Associations for continuous cortisol measures showed similar results (**Supplemental Table 4**).[24] The tests for trend across the quintiles were not significant.

Table 1. Family and birth characteristics (N = 2,598)

		Hair Cortisol Concentrations					
	Total group [a] 0.131-6.764 (N = 2,598)	Quintile 1 [a] 0.131-0.744 pg/mg (N = 519)	Quintile 2 [a] 0.745-1.173 pg/mg (N = 520)	Quintile 3 [a] 1.174-1.831 pg/mg (N = 520)	Quintile 4 [a] 1.832-2.925 pg/mg (N = 520)	Quintile 5 [a] 2.926-6.764 pg/mg (N = 519)	P-value [b]
Family characteristics							
Pre-pregnancy BMI, median (95% range), kg/m^2	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 (%)							< 0.001
Primary school	98 (4.5)	8 (1.7)	15 (3.4)	22 (5.0)	34 (7.9)	19 (4.6)	
Secondary school	815 (37.2)	151 (32.7)	150 (34.0)	167 (38.2)	169 (39.1)	178 (42.8)	
High education	1,275 (58.3)	303 (65.6)	276 (62.6)	248 (56.8)	229 (53.0)	219 (52.6)	
Family income (%)							< 0.001
Low (< €1600 per month)	327 (15.8)	39 (8.8)	51 (12.3)	58 (14.1)	90 (22.1)	89 (22.6)	
Medium (€1600-4000 per month)	981 (47.4)	202 (45.5)	186 (44.7)	212 (51.7)	197 (48.3)	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)	
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
European	1,644 (65.0)	419 (82.2)	355 (70.9)	302 (59.2)	283 (55.6)	285 (57.1)	
Non-European	885 (35.0)	91 (17.8)	146 (29.1)	208 (40.8)	226 (44.4)	214 (42.9)	

[a] 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

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).[24] 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**).[24] 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**).[24] The association for change in total cholesterol was independent of childhood BMI at 6 years (**Supplemental Table 3**).[24] The associations for continuous cortisol measures showed similar results (**Supplemental Table 4**).[24]

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)**.[24] When we further adjusted the models for potential confounders, these associations attenuated into non-significance (**Table 5**).[24] The associations for continuous cortisol measures showed similar results (**Supplemental Table 8**).[24]

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**).[24] When we further adjusted the model for childhood BMI, the association attenuated into non-significance (**Supplemental Table 10**).[24] 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**).[24] Hair cortisone concentrations at 6 years were not associated with any of the cardio-metabolic outcomes at 6 or 10 years (results not shown).

Table 2. Child characteristics (N = 2,598)

		Hair Cortisol Concentrations					
	Total group [a] 0.131-6.764 (N = 2,598)	**Quintile 1 [a] 0.131-0.744 pg/mg (N = 519)**	**Quintile 2 [a] 0.745-1.173 pg/mg (N = 520)**	**Quintile 3 [a] 1.174-1.831 pg/mg (N = 520)**	**Quintile 4 [a] 1.832-2.925 pg/mg (N = 520)**	**Quintile 5 [a] 2.926-6.764 pg/mg (N = 519)**	**P-value [b]**
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/m^2	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 [c]	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 [c]	7.50 (2.63, 29.00)	4.65 (2.00, 8.78)	6.19 (2.95, 10.66)	8.25 (3.31, 14.56)	11.52 (3.60, 23.18)	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, 405.28)	110.70 (24.60, 401.89)	130.30 (15.27, 411.94)	93.49 (13.30, 388.15)	128.60 (18.26, 422.35)	115.30 (19.53, 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 (%)							0.025
< 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 (%) [d]	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 (%)							0.622
No	2,296 (93,0)	452 (92.2)	469 (94.6)	460 (92.4)	455 (93.0)	460 (92.7)	
Yes	173 (7.0)	38 (7.8)	27 (5.4)	38 (7.6)	34 (7.0)	36 (7.3)	
Hair color, N (%)							< 0.001
Red	78 (3.0)	20 (3.9)	16 (3.1)	20 (3.9)	11 (2.1)	11 (2.1)	
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 (%)							< 0.001
< 2 hours per day	1,631 (81.7)	381 (88.6)	338 (83.5)	318 (79.5)	303 (78.1)	291 (78.0)	
≥ 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/m^2	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, 709.27)	177.10 (37.21, 613.68)	189.50 (30.72, 618.40)	201.40 (37.03, 698.83)	202.00 (28.19, 718.82)	199.55 (30.81, 777.55)	0.341
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)	0.227
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)	0.790
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)	0.831
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)	0.835
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)	0.910
C-reactive protein, N (%)							0.140
< 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 (%) [d]	172 (13.3)	26 (10.0)	35 (13.4)	40 (15.7)	31 (11.7)	40 (15.7)	0.219

[a] 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.

[c] pg/mg = picogram per milligram

[d] 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.[22] (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

	Cardiovascular risk factors at 6 years Difference (95% CI) in standard deviation scores		
Hair cortisol quintiles at 6 years	**Systolic blood pressure [a] (N = 2,466)**	**Diastolic blood pressure [a] (N = 2,466)**	**Heart rate [a] (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)
	Cardiovascular risk factors at 10 years Difference (95% CI) in standard deviation scores		
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)
	Cardiovascular risk factors between 6 and 10 years Difference (95% CI) in standard deviation scores		
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

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 in adults, showed that higher hair cortisol concentrations were associated with higher systolic blood pressure, but not with diastolic blood pressure.[36] 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.[37] Previous studies in children did not find associations between hair cortisol concentrations and blood pressure, heart rate, lipids, C-reactive protein or glucose metabolism.[19-21] 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.[38-42] 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.[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.[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.[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.[17,18] A bidirectional association between cortisol and adiposity may be present which should be further explored in future studies.[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.

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

	Metabolic risk factors at 6 years Difference (95% CI) in standard deviation scores				
Hair cortisol quintiles at 6 years	**Total cholesterol [a] (N = 1,781)**	**HDL [a] (N = 1,781)**	**LDL [a] (N = 1,782)**	**Triglycerides [a] (N = 1,773)**	**Insulin [a] (N = 1,766)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.01 (-0.14, 0.16)	-0.04 (-0.19, 0.11)	0.08 (-0.06, 0.23)	-0.01 (-0.16, 0.14)	0.10 (-0.05, 0.24)
Q3	-0.02 (-0.17, 0.13)	0.00 (-0.15, 0.15)	0.01 (-0.13, 0.16)	0.02 (-0.13, 0.16)	-0.10 (-0.25, 0.04)
Q4	-0.08 (-0.23, 0.07)	-0.11 (-0.26, 0.04)	0.01 (-0.14, 0.16)	0.02 (-0.13, 0.17)	0.13 (-0.02, 0.27)
Q5	-0.10 (-0.25, 0.05)	-0.01 (-0.16, 0.14)	-0.04 (-0.19, 0.10)	-0.03 (-0.18, 0.12)	-0.04 (-0.19, 0.11)

	Metabolic risk factors at 6 years Difference (95% CI) in standard deviation scores					
Hair cortisol quintiles at 6 years	**Total cholesterol [b] (N = 1,388)**	**HDL [b] (N = 1,387)**	**LDL [b] (N = 1,377)**	**Triglycerides [b] (N = 1,380)**	**Insulin [b] (N = 1,382)**	**Glucose [b] (N = 1,387)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.05 (-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	0.01 (-0.16, 0.17)	-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	-0.01 (-0.18, 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	0.10 (-0.07, 0.27)	0.04 (-0.12, 0.20)	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 Difference (95% CI) in standard deviation scores				
Hair cortisol quintiles at 6 years	**Change in total cholesterol [c] (N = 1,051)**	**Change in HDL [c] (N = 1,050)**	**Change in LDL [c] (N = 1,043)**	**Change in Triglycerides [c] (N = 1,041)**	**Change in insulin [c] (N = 1,036)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.09 (-0.05, 0.23)	0.03 (-0.11, 0.18)	-0.01 (-0.15, 0.13)	0.08 (-0.14, 0.30)	-0.14 (-0.39, 0.10)
Q3	0.10 (-0.05, 0.25)	0.07 (-0.08, 0.22)	0.06 (-0.08, 0.20)	-0.01 (-0.24, 0.21)	0.15 (-0.10, 0.40)
Q4	0.12 (-0.03, 0.26)	0.13 (-0.02, 0.28)	0.06 (-0.09, 0.20)	-0.10 (-0.32, 0.13)	-0.08 (-0.33, 0.17)
Q5	**0.19 (0.05, 0.34)****	0.02 (-0.13, 0.16)	**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

	Odds Ratio (95% CI) for outcomes at 6 years	
Hair cortisol quintiles at 6 years	**Risk of C-reactive protein ≥ 3 mg/l [a] (N = 1,784)**	**Risk of cardio-metabolic clustering [b] (N = 2,022)**
Q1	*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)
	Odds Ratio (95% CI) for outcomes at 10 years	
Hair cortisol quintiles at 6 years	**Risk of C-reactive protein ≥ 3 mg/l [a] (N = 1,389)**	**Risk of cardio-metabolic clustering [b] (N = 1,299)**
Q1	*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

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.[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.[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.[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.[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.[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.[52-55] However, we did not find clear evidence for an association of higher cortisol concentrations and characteristics of the metabolic syndrome in childhood.

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.[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.[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.[57] Also, studies reported that non-fasting lipid concentrations can predict increased risks of cardiovascular events later in life.[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.

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.

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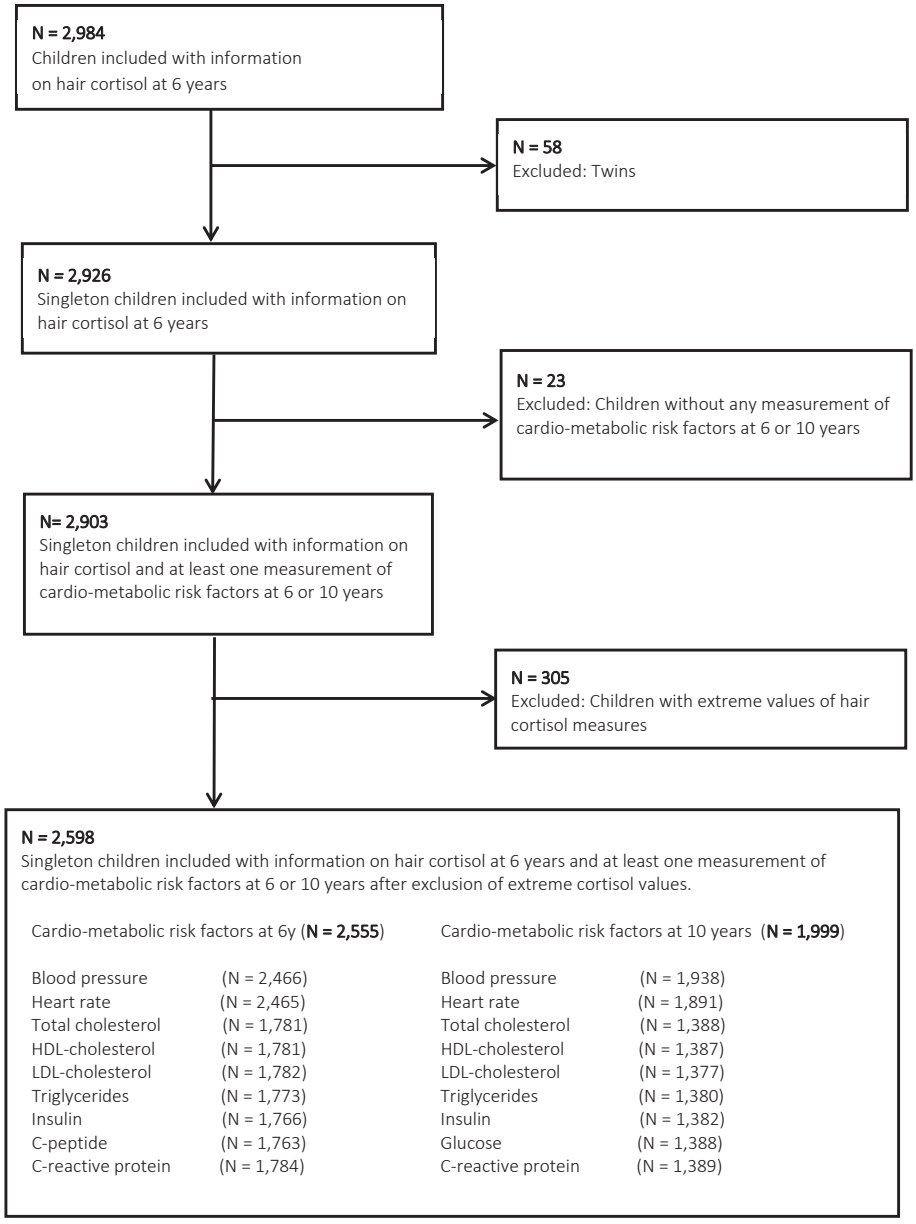
SUPPLEMENTARY MATERIAL

Supplemental Figure 1. Flowchart of study population



Supplemental Methods

Hair cortisol concentration measurements

Hair cortisol and cortisone were measured in proximal scalp hair. A hair strand of approximately 100 hairs was cut from the posterior vertex using small surgical scissors, as close to the scalp as possible.[1] 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. The proximal 3 cm of hair samples were weighed and finely cut. Hair samples were then washed in LC-grade isopropanol for 2 min at room temperature, and left to dry for 2 days. Extraction was performed using LC-grade methanol (MeOH), for 18 h at 25 °C, in the presence of deuterated steroids. Subsequently, the extract was cleaned using solid phase extraction and steroids were quantified on a Xevo TQS liquid chromatography tandem mass spectrometry (LC–MS/MS) (Waters Corporation, Milford, MA, USA).[1]

Reference

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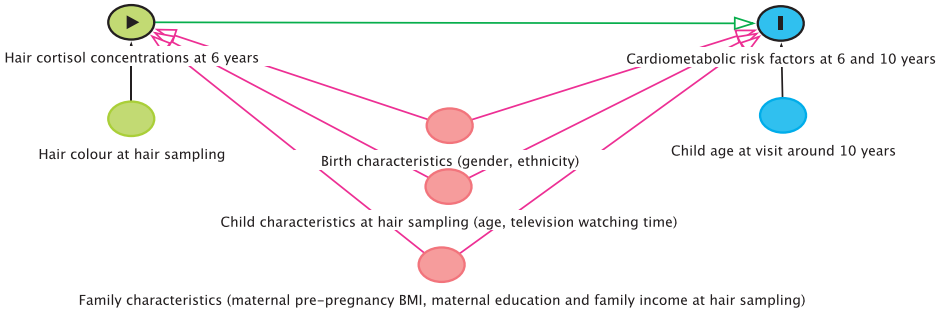
Supplemental Figure 2. Directed acyclic graph (DAG) for the relationship between hair cortisol concentrations at 6 years and child cardio-metabolic risk factors at 6 and 10 years depicting the covariates included in the models



Supplemental Table 1. Comparison of child characteristics between participants and non-participants (N = 2,926)

	Participants [a] (N = 2,598)	Non-participants [a] (N = 328)	P-value [b]
Family characteristics			
Pre-pregnancy body mass index, median (95% range), kg/m^2	22.6 (18.2, 35.1)	24.3 (18.3, 38.2)	< 0.001
Maternal education, N (%)			0.046
Primary school	98 (4.5)	9 (3.4)	
Secondary school	815 (37.2)	118 (45.0)	
High education	1,275 (58.3)	135 (51.5)	
Family income, N (%)			0.028
Low (< €1600 per month)	327 (15.8)	55 (22.3)	
Medium (€1600-4000 per month)	981 (47.4)	113 (45.7)	
High (> €4000 per month)	763 (36.8)	79 (32.0)	
Birth characteristics			
Sex, N (%)			0.030
Boys	1,237 (47.6)	177 (54.0)	
Girls	1,361 (52.4)	151 (46.0)	
Ethnicity, N (%)			< 0.001
European	1,644 (65.0)	165 (52.7)	
Non-European	885 (35.0)	148 (47.3)	
Child characteristics at 6 years			
Age at visit, median (95% range), years	5.9 (5.7, 8.1)	6.2 (5.7, 8.2)	0.217
Body mass index, median (95% range), kg/m^2	15.8 (13.6, 21.2)	16.4 (13.2, 22.6)	0.005
Hair cortisol concentration, median (95% range), pg/mg [c]	1.46 (0.33, 5.62)	14.25 (0.90, 144.37)	< 0.001
Hair cortisone concentration, median (95% range), pg/mg [c]	7.50 (2.63, 29.00)	10.67 (3.89, 61.24)	< 0.001
Cortisol / cortisone ratio, median (95% range)	0.17 (0.07, 0.73)	1.42 (0.11, 14.51)	< 0.001
Glucocorticoid use in the last 3 months, N (%)			< 0.001
No	2,296 (93.0)	258 (82.4)	
Yes	173 (7.0)	55 (17.6)	
Hair color, N (%)			< 0.001
Red	78 (3.0)	9 (2.7)	
Blond	1,381 (53.2)	134 (40.9)	
Brown	857 (33.0)	124 (37.8)	
Black	280 (10.8)	61 (18.6)	
Average duration of television watching, N (%)			
< 2 hours per day	1,631 (81.7)	188 (79.3)	0.371
>= 2 hours per day	365 (18.3)	49 (20.7)	

[a] Values are observed data and represent means (standard deviation), medians (95% range) or numbers of participants (valid %).

[b] P-values for differences in child characteristics between participants and non-participants were calculated performing Student *t* tests for normally distributed continuous variables, Mann-Whitney tests for non-normally distributed continuous variables and chi-square tests for categorical variables.

[c] pg/mg = picogram per milligram

Supplemental Table 2. 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, basic models.

	Cardiovascular risk factors at 6 years Difference (95% CI) in standard deviation scores		
Hair cortisol quintiles at 6 years	**Systolic blood pressure [a] (N = 2,466)**	**Diastolic blood pressure [a] (N = 2,466)**	**Heart rate [a] (N = 2,465)**
Q1	*Reference*	*Reference*	*Reference*
Q2	0.04 (-0.09, 0.16)	0.00 (-0.12, 0.13)	**0.19 (0.07, 0.31)****
Q3	**0.15 (0.02, 0.27)***	0.04 (-0.08, 0.16)	**0.15 (0.03, 0.27)****
Q4	**0.14 (0.01, 0.26)***	0.04 (-0.09, 0.16)	0.08 (-0.04, 0.20)
Q5	**0.16 (0.03, 0.28)****	**0.16 (0.04, 0.28)****	**0.13 (0.01, 0.25)***
	Cardiovascular risk factors at 10 years Difference (95% CI) in standard deviation scores		
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	0.04 (-0.10, 0.18)	0.06 (-0.09, 0.20)	0.01 (-0.13, 0.15)
Q3	0.08 (-0.07, 0.22)	0.01 (-0.14, 0.15)	0.01 (-0.14, 0.15)
Q4	**0.19 (0.05, 0.33)****	0.02 (-0.13, 0.16)	0.07 (-0.08, 0.21)
Q5	**0.23 (0.09, 0.37)****	0.11 (-0.04, 0.25)	0.04 (-0.10, 0.18)
	Cardiovascular risk factors between 6 and 10 years Difference (95% CI) in standard deviation scores		
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, 0.31)	-0.08 (-0.21, 0.06)
Q3	-0.06 (-0.20, 0.08)	0.02 (-0.14, 0.18)	-0.13 (-0.27, 0.01)
Q4	0.06 (-0.08, 0.20)	0.01 (-0.15, 0.17)	0.00 (-0.14, 0.14)
Q5	0.06 (-0.08, 0.20)	-0.04 (-0.20, 0.12)	-0.08 (-0.22, 0.06)

[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 the delta of blood pressure and heart rate between 6 and 10 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

Basic models are adjusted for child sex, child age at cortisol assessment and child age at assessment of cardio-metabolic outcomes.

*p< 0.05, **p< 0.017

Supplemental Table 3. Association of hair cortisol quintiles at 6 years with cardio-metabolic risk factors with significant findings in the confounder models, BMI models.

	Cardio-metabolic risk factors Difference (95% CI) in standard deviation scores		
Hair cortisol quintiles at 6 years	**Systolic blood pressure at 10 years [a] (N = 1,938)**	**Change in total cholesterol between 6 and 10 years [b] (N = 1,051)**	**Change in LDL between 6 and 10 years [b] (N = 1,043)**
Q1	*Reference*	*Reference*	*Reference*
Q2	0.03 (-0.11, 0.16)	0.10 (-0.05, 0.24)	-0.01 (-0.15, 0.13)
Q3	0.01 (-0.13, 0.15)	0.10 (-0.05, 0.24)	0.06 (-0.09, 0.20)
Q4	0.10 (-0.04, 0.24)	0.12 (-0.03, 0.26)	0.05 (-0.09, 0.20)
Q5	0.10 (-0.03, 0.24)	**0.20 (0.05, 0.34)****	0.14 (-0.00, 0.28)

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in systolic blood pressure at 10 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 the delta of total cholesterol and LDL cholesterol concentration between 6 and 10 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

BMI 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, hair color and additionally adjusted for childhood BMI at 6 years.

*p< 0.05, **p< 0.017

Supplemental Table 4. Association of continuous hair cortisol concentrations at 6 years with blood pressure, heart rate, lipids, insulin and glucose concentrations at 6 and 10 years and with the change at 6 and 10 years

	Cardio-metabolic risk factors at 6 years Difference (95% CI) in standard deviation scores							
Continuous hair cortisol at 6 years	**Systolic blood pressure [a] (N = 2,466)**	**Diastolic blood pressure [a] (N = 2,466)**	**Heart rate [a] (N = 2,465)**	**Total cholesterol [a] (N = 1,781)**	**HDL [a] (N = 1,781)**	**LDL [a] (N = 1,782)**	**Triglycerides [a] (N = 1,773)**	**Insulin [a] (N = 1,766)**
Basic model	**0.09 (0.03, 0.15)****	**0.08 (0.03, 0.14)****	0.03 (-0.02, 0.09)	-0.03 (-0.10, 0.03)	-0.02 (-0.08, 0.05)	-0.01 (-0.08, 0.06)	-0.01 (-0.08, 0.05)	-0.01 (-0.08, 0.05)
Confounder model	0.06 (-0.00, 0.11)	0.05 (-0.01, 0.11)	-0.01 (-0.07, 0.04)	-0.04 (-0.11, 0.03)	-0.02 (-0.09, 0.05)	-0.02 (-0.09, 0.05)	-0.02 (-0.08, 0.05)	-0.01 (-0.07, 0.06)

	Cardio-metabolic risk factors at 10 years Difference (95% CI) in standard deviation scores								
Continuous hair cortisol at 6 years	**Systolic blood pressure [b] (N = 1,938)**	**Diastolic blood pressure [b] (N = 1,938)**	**Heart rate [b] (N = 1,891)**	**Total cholesterol [b] (N = 1,388)**	**HDL [b] (N = 1,387)**	**LDL [b] (N = 1,377)**	**Triglycerides [b] (N = 1,380)**	**Insulin [b] (N = 1,382)**	**Glucose [b] (N = 1,387)**
Basic model	**0.12 (0.05, 0.18)****	0.03 (-0.03, 0.10)	0.03 (-0.04, 0.09)	0.05 (-0.03. 0.12)	-0.03 (-0.10, 0.04)	0.06 (-0.01, 0.14)	0.00 (-0.07, 0.08)	0.05 (-0.03, 0.13)	0.00 (-0.08, 0.07)
Confounder model	**0.09 (0.02, 0.15)****	-0.00 (-0.07, 0.07)	-0.00 (-0.07, 0.07)	0.04 (-0.04. 0.12)	0.01 (-0.07, 0.08)	0.06 (-0.02, 0.13)	-0.03 (-0.10, 0.05)	0.03 (-0.05, 0.11)	0.01 (-0.07, 0.08)
BMI model	0.05 (-0.01, 0.11)								

	Cardio-metabolic risk factors between 6 and 10 years Difference (95% CI) in standard deviation scores							
Continuous hair cortisol 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)**	**Change in total cholesterol [c] (N = 1,051)**	**Change in HDL [c] (N = 1,050)**	**Change in LDL [c] (N = 1,043)**	**Change in Triglycerides [c] (N =1,041)**	**Change in insulin [c] (N = 1,036)**
Basic model	0.02 (-0.04, 0.09)	-0.05 (-0.12, 0.03)	-0.01 (-0.08, 0.05)	**0.07 (0.01, 0.14)***	-0.00 (-0.07, 0.07)	0.06 (-0.00, 0.13)	0.03 (-0.07, 0.13)	0.07 (-0.04, 0.18)
Confounder model	0.02 (-0.05, 0.08)	-0.06 (-0.14, 0.02)	-0.01 (-0.07, 0.06)	**0.09 (0.02, 0.15)****	0.03 (-0.04, 0.09)	**0.08 (0.01, 0.14)***	0.00 (-0.10, 0.10)	0.04 (-0.08, 0.15)
BMI model				**0.09 (0.02, 0.16)****		**0.08 (0.01, 0.14)***		

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in childhood cardio-metabolic risk factors at 6 years in standard deviation scores (SDS) for an inter-quartile range increase in the natural log transformed hair cortisol concentration.

[b] Values are linear regression coefficients (95% confidence interval) and reflect the change in childhood cardio-metabolic risk factors at 6 years in standard deviation scores (SDS) for an inter-quartile range increase in the natural log transformed hair cortisol concentration.

[c] Values are linear regression coefficients (95% confidence interval) and reflect the change in the delta of cardio-metabolic risk factors between 6 and 10 years for an IQR increase natural log transformed hair cortisol concentrations.

Basic models are adjusted for child sex, child age at cortisol assessment and child age at assessment of cardio-metabolic outcomes.
Confounder models are additionally adjusted for maternal pre-pregnancy BMI, maternal education, family income, child ethnicity, television watching time and hair color.
BMI models are confounder models additionally adjusted for childhood BMI at 6 years.
*p< 0.05, **p< 0.017

Supplemental Table 5. Association of hair cortisol quintiles at 6 years with lipids, insulin and glucose concentration at 6 years and 10 years and with the change between 6 and 10 years, basic models.

	Metabolic risk factors at 6 years Difference (95% CI) in standard deviation score				
Hair cortisol quintiles at 6 years	**Total cholesterol [a] (N = 1,781)**	**HDL [a] (N = 1,781)**	**LDL [a] (N = 1,782)**	**Triglycerides [a] (N = 1,773)**	**Insulin [a] (N = 1,766)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.02 (-0.13, 0.17)	-0.03 (-0.17, 0.12)	0.08 (-0.06, 0.23)	-0.02 (-0.16, 0.13)	0.09 (-0.05, 0.24)
Q3	0.00 (-0.14, 0.15)	0.01 (-0.13, 0.16)	0.03 (-0.12, 0.17)	0.02 (-0.13, 0.16)	-0.12 (-0.26, 0.03)
Q4	-0.07 (-0.22, 0.08)	-0.10 (-0.24, 0.05)	0.02 (-0.12, 0.17)	0.02 (-0.13, 0.16)	0.11 (-0.04, 0.25)
Q5	-0.08 (-0.23, 0.07)	-0.00 (-0.15, 0.14)	-0.02 (-0.17, 0.12)	-0.03 (-0.17, 0.12)	-0.05 (-0.19, 0.10)

	Metabolic risk factors at 10 years Difference (95% CI) in standard deviation score					
Hair cortisol quintiles at 6 years	**Total cholesterol [b] (N = 1,388)**	**HDL [b] (N = 1,387)**	**LDL [b] (N = 1,377)**	**Triglycerides [b] (N = 1,380)**	**Insulin [b] (N = 1,382)**	**Glucose [b] (N = 1,387)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.06 (-0.10, 0.22)	0.02 (-0.14, 0.18)	0.02 (-0.13, 0.18)	0.03 (-0.13, 0.19)	-0.01 (-0.18, 0.16)	-0.13 (-0.29, 0.03)
Q3	0.02 (-0.14, 0.18)	-0.06 (-0.22, 0.10)	0.06 (-0.10, 0.22)	-0.05 (-0.21, 0.11)	0.10 (-0.07, 0.28)	0.05 (-0.12, 0.21)
Q4	0.01 (-0.16, 0.17)	-0.05 (-0.21, 0.10)	0.04 (-0.11, 0.20)	-0.02 (-0.18, 0.14)	0.08 (-0.09, 0.25)	-0.07 (-0.23, 0.09)
Q5	0.12 (-0.05, 0.28)	-0.03 (-0.19, 0.13)	0.13 (-0.03, 0.29)	0.01 (-0.16, 0.17)	0.08 (-0.09, 0.25)	-0.04 (-0.20, 0.13)

	Metabolic risk factors between 6 and 10 years Difference (95% CI) in standard deviation score				
Hair cortisol quintiles at 6 years	**Change in total cholesterol [c] (N = 1,051)**	**Change in HDL [c] (N = 1,050)**	**Change in LDL [c] (N = 1,043)**	**Change in Triglycerides [c] (N = 1,041)**	**Change in insulin [c] (N = 1,036)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.09 (-0.06, 0.23)	0.00 (-0.14, 0.15)	-0.02 (-0.16, 0.12)	0.12 (-0.10, 0.34)	-0.11 (-0.35, 0.13)
Q3	0.09 (-0.06, 0.23)	0.04 (-0.11, 0.18)	0.05 (-0.09, 0.19)	0.01 (-0.21, 0.24)	0.19 (-0.06, 0.43)

Q4	0.10 (-0.05, 0.24)	0.08 (-0.06, 0.23)	0.03 (-0.11, 0.17)	-0.05 (-0.27, 0.17)	-0.03 (-0.27, 0.22)
Q5	**0.17 (0.03, 0.31)***	-0.04 (-0.18, 0.11)	0.12 (-0.02, 0.26)	0.10 (-0.13, 0.32)	0.14 (-0.10, 0.39)

[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.

Basic models are adjusted for child sex, child age at cortisol assessment and child age at assessment of cardio-metabolic outcomes. *
p< 0.05, **p< 0.017

Supplemental Table 6. Association of continuous hair cortisol concentrations at 6 years with triglycerides and insulin concentrations at 6 years, separately for boys and girls

	Metabolic risk factors at 6 years Difference (95% CI) in standard deviation scores	
Continuous hair cortisol at 6 years	**Triglycerides (N = 1,773)**	**Insulin (N = 1,766)**
Boys – basic model	**-0.11 (-0.20, -0.01)***	**-0.10 (-0.19, -0.01)***
Boys – confounder model	**-0.11 (-0.21, -0.01)***	**-0.09 (-0.18, -0.00)***
Boys – BMI model	**-0.12 (-0.21, -0.02)****	**-0.10 (-0.19, -0.01)***
Girls – basic model	0.08 (-0.01, 0.17)	0.08 (-0.02, 0.17)
Girls – confounder model	0.08 (-0.01, 0.18)	0.09 (-0.02, 0.19)
Girls – BMI model	0.08 (-0.02, 0.17)	0.07 (-0.03, 0.17)

Values are linear regression coefficients (95% confidence interval) and reflect the change in triglycerides and insulin concentration at 6 years in standard deviation scores (SDS) for an interquartile range increase in the natural log transformed hair cortisol concentration for boys and girls seperately.

Basic models are adjusted for child sex, child age at cortisol assessment and child age at assessment of cardio-metabolic outcomes.

Confounder models are additionally adjusted for maternal pre-pregnancy BMI, maternal education, family income, child ethnicity, television watching time and hair color.

BMI models are confounder models additionally adjusted for childhood BMI at 6 years.

*p< 0.05, **p< 0.017

Supplemental Table 7. Association of hair cortisol quintiles at 6 years with risk of increased C-reactive protein concentration and risk of cardio-metabolic clustering at 6 and 10 years, basic models

	Odds Ratio (95% CI) for cardio-metabolic outcomes at 6 years	
Hair cortisol quintiles at 6 years	**Risk of C-reactive protein ≥ 3 mg/l[a] (N = 1,784)**	**Risk of cardio-metabolic clustering[b] (N = 2,022)**
Q1	*Reference*	*Reference*
Q2	1.27 (0.75, 2.13)	1.42 (0.88, 2.30)
Q3	**1.91 (1.17, 3.09)****	1.43 (0.89, 2.29)
Q4	1.13 (0.66, 1.91)	1.37 (0.85, 2.20)
Q5	**1.76 (1.08, 2.86)***	1.53 (0.96, 2.44)
	Odds Ratio (95% CI) for cardio-metabolic outcomes at 10 years	
Hair cortisol quintiles at 6 years	**Risk of C-reactive protein ≥ 3 mg/l[a] (N = 1,389)**	**Risk of cardio-metabolic clustering[b] (N = 1,299)**
Q1	*Reference*	*Reference*
Q2	1.40 (0.63, 3.10)	1.45 (0.84, 2.51)
Q3	1.36 (0.60, 3.05)	**1.72 (1.01, 2.94)***
Q4	2.10 (1.00, 4.40)	1.19 (0.68, 2.09)
Q5	**2.23 (1.05, 4.70)***	**1.73 (1.01, 2.97)***

[a] Values are odds ratios (95% confidence interval) and represent the risk of childhood high C-reactive protein concentration (≥ 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.

Basic models are adjusted for child sex, child age at cortisol assessment and child age at assessment of cardio-metabolic outcomes.

*p< 0.05, **p< 0.017

Supplemental Table 8. Association of continuous hair cortisol concentrations at 6 years with risk of increased C-reactive protein concentration and risk of cardio-metabolic clustering at 6 and 10 years

	Odds Ratio (95% CI) for outcomes at 6 years	
Continuous hair cortisol at 6 years	**Risk of C-reactive protein ≥ 3 mg/l[a] (N = 1,784)**	**Risk of cardio-metabolic clustering[b] (N = 2,022)**
Basic model	**1.27 (1.02, 1.58)***	**1.26 (1.02, 1.55)***
Confounder model	1.18 (0.94, 1.48)	1.15 (0.92, 1.43)
	Odds Ratio (95% CI) for outcomes at 10 years	
Continuous hair cortisol at 6 years	**Risk of C-reactive protein ≥ 3 mg/l[a] (N = 1,389)**	**Risk of cardio-metabolic clustering[b] (N = 1,299)**
Basic model	**1.41 (1.01, 1.95)***	1.23 (0.96, 1.56)
Confounder model	1.27 (0.90, 1.80)	1.07 (0.82, 1.38)

[a] Values are odds ratios (95% confidence interval) and represent the risk of childhood high C-reactive protein concentration (≥ 3 mg/l) at 6 and 10 years for an interquartile range increase in the natural log transformed hair cortisol concentration.

[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 an interquartile range increase in the natural log transformed hair cortisol concentration.

Basic models are adjusted for child sex, child age at cortisol assessment and child age at assessment of cardio-metabolic outcomes.

Confounder models are additionally adjusted for maternal pre-pregnancy BMI, maternal education, family income, child ethnicity, television watching time and hair color.

*p< 0.05, **p< 0.017

Supplemental Table 9. Association of hair cortisol quintiles at 6 years with blood pressure, heart rate, lipids, insulin and glucose concentrations, excluding children with all types of glucocorticoid use in the 3 months prior to the hair sample collection, confounder models

	Cardio-metabolic risk factors at 6 years Difference (95% CI) in standard deviation scores							
Hair cortisol quintiles at 6 years	**Systolic blood pressure [a] (N = 2,180)**	**Diastolic blood pressure [a] (N = 2,180)**	**Heart rate [a] (N = 2,179)**	**Total cholesterol [a] (N = 1,587)**	**HDL [a] (N = 1,587)**	**LDL [a] (N = 1,588)**	**Triglycerides [a] (N = 1,579)**	**Insulin [a] (N = 1,573)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.03 (-0.11, 0.16)	-0.01 (-0.14, 0.12)	**0.17 (0.04, 0.29)****	0.02 (-0.14, 0.17)	-0.05 (-0.21, 0.10)	0.09 (-0.05, 0.24)	0.00 (-0.15, 0.15)	0.10 (-0.05, 0.25)
Q3	0.12 (-0.01, 0.25)	0.01 (-0.12, 0.14)	0.11 (-0.02, 0.23)	0.02 (-0.13, 0.18)	-0.01 (-0.16, 0.15)	0.06 (-0.09, 0.21)	0.03 (-0.13, 0.18)	-0.14 (-0.29, 0.01)
Q4	0.08 (-0.06, 0.21)	-0.02 (-0.14, 0.11)	0.03 (-0.09, 0.16)	-0.07 (-0.22, 0.09)	-0.11 (-0.27, 0.04)	0.02 (-0.13, 0.17)	0.05 (-0.10, 0.21)	0.12 (-0.04, 0.27)
Q5	0.08 (-0.06, 0.21)	0.09 (-0.05, 0.21)	0.08 (-0.05, 0.21)	-0.12 (-0.27, 0.04)	-0.01 (-0.16, 0.15)	-0.06 (-0.21, 0.09)	-0.04 (-0.19, 0.12)	-0.08 (-0.23, 0.08)

	Cardio-metabolic risk factors at 10 years Difference (95% CI) in standard deviation scores								
Hair cortisol quintiles at 6 years	**Systolic blood pressure [b] (N = 1,711)**	**Diastolic blood pressure [b] (N = 1,710)**	**Heart rate [b] (N = 1,669)**	**Total cholesterol [b] (N = 1,228)**	**HDL [b] (N = 1,234)**	**LDL [b] (N = 1,234)**	**Triglycerides [b] (N = 1,233)**	**Insulin [b] (N = 1,225)**	**Glucose [b] (N = 1,228)**
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.04 (-0.10, 0.19)	0.05 (-0.10, 0.20)	-0.01 (-0.15, 0.14)	0.06 (-0.10, 0.23)	0.03 (-0.13, 0.19)	0.03 (-0.13, 0.19)	0.02 (-0.14, 0.19)	-0.02 (-0.19, 0.16)	-0.10 (-0.27, 0.07)
Q3	0.04 (-0.11, 0.19)	-0.05 (-0.20, 0.11)	-0.00 (-0.15, 0.15)	0.02 (-0.15, 0.20)	-0.05 (-0.21, 0.12)	0.08 (-0.09, 0.24)	-0.06 (-0.23, 0.11)	0.06 (-0.12, 0.24)	0.04 (-0.13, 0.21)
Q4	**0.16 (0.01, 0.31)***	-0.02 (-0.17, 0.13)	0.04 (-0.12, 0.19)	0.02 (-0.15, 0.18)	0.00 (-0.16, 0.16)	0.05 (-0.11, 0.21)	-0.06 (-0.23, 0.10)	0.04 (-0.14, 0.21)	-0.06 (-0.23, 0.11)
Q5	**0.20 (0.05, 0.34)****	0.06 (-0.09, 0.22)	0.04 (-0.12, 0.19)	0.08 (-0.10, 0.25)	0.04 (-0.12, 0.21)	0.07 (-0.10, 0.24)	-0.05 (-0.22, 0.12)	0.03 (-0.15, 0.21)	-0.02 (-0.19, 0.16)

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in cardio-metabolic risk factors 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 cardio-metabolic risk factors at 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 and 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

Supplemental Table 10. Association of hair cortisol quintiles at 6 years with cardio-metabolic risk factors with significant findings in the confounder models, excluding children with all types of glucocorticoid use in the 3 months prior to the hair sample collection, BMI models

	Differences (95% CI) in standard deviation scores	Odds Ratio (95% CI)	Odds Ratio (95% CI)
Hair cortisol quintiles at 6 years	**Systolic blood pressure at 10 years [a] (N = 1,711)**	**Risk of C-reactive protein ≥ 3 mg/l at 6 years [b] (N = 1,590)**	**Risk of C-reactive protein ≥ 3 mg/l at 10 years [b] (N = 1,235)**
Q1	*Reference*	*Reference*	*Reference*
Q2	0.04 (-0.10, 0.18)	1.33 (0.75, 2.34)	1.65 (0.68, 4.00)
Q3	0.02 (-0.13, 0.16)	**1.89 (1.10, 3.24)***	1.34 (0.54, 3.34)
Q4	0.12 (-0.03, 0.26)	1.23 (0.69, 2.18)	2.02 (0.86, 4.71)
Q5	0.13 (-0.02, 0.27)	**1.73 (1.00, 2.97)***	2.18 (0.93, 5.09)

[a] Values are linear regression coefficients (95% confidence interval) and reflect the change in systolic blood pressure at 10 years in standard deviation scores (SDS) for the cortisol quintiles compared to the first quintile.

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

BMI 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, hair color and additionally adjusted for childhood BMI at 6 years.

*p< 0.05, **p< 0.017

Supplemental Table 11. Association of hair cortisol quintiles at 6 years with risk of increased C-reactive protein concentration and risk of cardio-metabolic clustering at 6 and 10 years, excluding children with all types of glucocorticoid use in the 3 months prior to the hair sample collection, confounder models

	Odds Ratio (95% CI) for outcomes at 6 years	
Hair cortisol quintiles at 6 years	**Risk of C-reactive protein ≥ 3 mg/l [a] (N = 1,590)**	**Risk of cardio-metabolic clustering [b] (N = 1,797)**
Q1	*Reference*	*Reference*
Q2	1.37 (0.78, 2.42)	1.33 (0.81, 2.17)
Q3	**1.93 (1.13, 3.29)****	1.20 (0.73, 1.97)
Q4	1.26 (0.71, 2.24)	1.19 (0.73, 1.96)
Q5	**1.83 (1.06, 3.13)***	1.17 (0.71, 1.93)
	Odds Ratio (95% CI) for outcomes at 10 years	
Hair cortisol quintiles at 6 years	**Risk of C-reactive protein ≥ 3 mg/l [a] (N = 1,235)**	**Risk of cardio-metabolic clustering [b] (N = 1,150)**
Q1	*Reference*	*Reference*
Q2	1.59 (0.66, 3.80)	1.39 (0.77, 2.50)
Q3	1.38 (0.57, 3.37)	1.55 (0.87, 2.75)
Q4	2.26 (0.99, 5.13)	1.08 (0.59, 1.97)
Q5	**2.53 (1.11, 5.77)***	1.31 (0.73, 2.38)

[a] Values are odds ratios (95% confidence interval) and represent the risk of childhood high C-reactive protein concentration (≥ 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 and 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

4

Early-life DNA methylation

4.1

Maternal haemoglobin concentrations in pregnancy and child DNA methylation: a study in the Pregnancy And Childhood Epigenetics Consortium

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ABSTRACT

Altered maternal haemoglobin levels during pregnancy are associated with pre-clinical and clinical conditions affecting the fetus. Evidence from animal models suggests that these associations may be partially explained by differential DNA methylation in the newborn with possible long-term consequences. To test this in humans, we meta-analyzed the epigenome-wide associations of maternal haemoglobin levels during pregnancy with offspring DNA methylation in 3,967 newborn cord blood and 1,534 children and 1,962 adolescent whole-blood samples derived from 10 cohorts. DNA methylation was measured using Illumina Infinium Methylation 450K or MethylationEPIC arrays covering 450,000 and 850,000 methylation sites, respectively. There was no statistical support for the association of maternal haemoglobin levels with offspring DNA methylation either at individual methylation sites or clustered in regions. For most participants, maternal haemoglobin levels were within the normal range in the current study, whereas adverse perinatal outcomes often arise at the extremes. Thus, this study does not rule out the possibility that associations with offspring DNA methylation might be seen in studies with more extreme maternal haemoglobin levels.

BACKGROUND

Maternal haemoglobin is routinely monitored throughout pregnancy as altered haemoglobin levels have been associated with adverse perinatal outcomes such as preterm delivery and intrauterine growth restriction.[1-6] Low maternal haemoglobin is estimated to affect 38% of all pregnancies worldwide translating to 32 million mothers annually.[7] During pregnancy, maternal haemoglobin levels normally decrease until about 20 weeks of gestation, mainly due to dilution because of an increase in plasma volume. Haemoglobin levels then rise to around 30 weeks of gestation due to increased red blood cell production; thereafter they remain relatively stable.[1] Low maternal haemoglobin levels may relate to insufficient oxygen and/or nutrient delivery to the foetus, whilst high levels may indicate incomplete haemodilution resulting in high blood viscosity which may lead to fetal hypoxia due to impairment of maternal-foetal exchange.[8]

A potential mechanism underlying the associations between maternal haemoglobin levels and adverse perinatal outcomes could include DNA methylation.[9,10] Methylation at cytosine-guanine dinucleotide sites (CpGs) in the DNA is the most widely studied epigenetic modification and its genome-wide pattern is highly determined during intrauterine development, partly due to environmental factors.[11] DNA methylation has been suggested as a mechanism underlying known associations of early-life exposures with later-life health outcomes. While associations of a number of maternal pregnancy characteristics and outcomes, including maternal BMI, maternal smoking, hypertensive disorders of pregnancy, gestational age and child birth weight, with offspring DNA methylation have been explored, it is unknown if maternal haemoglobin levels are associated with offspring DNA methylation.[12-16]

Thus, in this epigenome-wide association study (EWAS), we meta-analysed harmonised cohort-specific associations between maternal haemoglobin level and DNA methylation in the offspring at birth, in childhood and in adolescence, using data from ten studies in the Pregnancy And Childhood Epigenetics (PACE) Consortium.

MATERIAL AND METHODS

Participating cohorts

Ten studies participated in the current meta-analyses. Details of cohort-level characteristics and methods are shown in the **Supplementary Methods**. We included seven cohorts in the meta-analysis of maternal haemoglobin levels and newborn (cord blood) DNA methylation: the Avon Longitudinal Study of Parents and Children (ALSPAC), the Mother-child Cohort on the Prenatal and Early Postnatal Determinants of Child Health and Development (EDEN), the Finnish Gestational Diabetes Study (FinnGeDi), the

Generation R Study (Generation R), the Environment and Childhood Project (INMA), the Prediction and Prevention of Preeclampsia and Intrauterine Growth Restriction Study (PREDO) and the Programming Research in Obesity, Growth Environment and Social Stress Study (PROGRESS).[17-26] Five cohorts participated in the meta-analysis of maternal haemoglobin and childhood (cohort mean age 4 – 7 years) DNA methylation: ALSPAC, EDEN, Generation R, INMA and the Postpartum Outcomes in Women with Gestational Diabetes and Their Offspring Study (POGO) and three cohorts in the meta-analysis of maternal haemoglobin and adolescent (cohort mean age 16 – 17 years) DNA methylation: ALSPAC, the Northern Finland Birth Cohort 1986 (NFBC1986) and the Raine Study.[27-29] All cohorts acquired ethics approval and informed consent from participants.

Maternal haemoglobin level during pregnancy

Where studies had more than one pregnancy maternal haemoglobin levels, the value assessed at the oldest gestational age was used because in previous studies, extreme maternal haemoglobin level at late pregnancy was more often associated with adverse pregnancy outcomes.[30] Measurement methods and units varied between cohorts (see **Supplementary Methods**) so standardised maternal haemoglobin (i.e. each cohort centred and scaled the variable using their own study specific mean and standard deviation = (maternal haemoglobin – mean(maternal haemoglobin)) / SD(maternal haemoglobin)) were used. Observations more than five standard deviations from the mean were excluded as outliers. According to this threshold, one observation was excluded from the Generation R newborn analysis and one from the ALSPAC childhood analysis.

DNA methylation data and quality control

DNA from cord or offspring peripheral blood underwent bisulphite conversion using the EZ-96 DNA Methylation kit (ZymoResearch Corporation, Irvine, USA). DNA methylation was measured either using the Infinium Human Methylation 450K BeadChip or the MethylationEPIC BeadChip platform (Illumina, San Diego, USA). Cohorts performed quality control and normalisation using their own preferred method, indicated in the **Supplementary Methods**. Untransformed beta values representing the level of methylation and ranging from 0 to 1 were used in all analyses. We excluded DNA methylation values below the 25th percentile minus 3 times the interquartile range (IQR) and values above the 75th percentile plus 3 times the IQR.

Cohort-specific statistical analyses

The association of maternal haemoglobin and offspring DNA methylation was analysed using robust linear regression separately for each methylation probe. Robust regression with White's covariance matrix estimator for calculating standard errors were chosen because of possible heteroscedasticity in the DNA methylation beta values.[31] Association analyses

were performed in the following age categories: newborns (cord blood), children (age 4 – 7 years) and adolescents (age 16 – 17 years). Cohort-specific analyses were performed using *rlm* function in *MASS* package for R.[32,33] P-values and standard errors were estimated using *coeftest* function with the function *vcovHC* from *sandwhich* package for White's type of covariance matrix.[34,35] Newborn and childhood initial models were adjusted for gestational week at maternal haemoglobin measurement, child sex, DNA methylation batch and white blood cells estimated with the Bakulski reference panel for newborn samples and with the Houseman reference panel for childhood and adolescent samples provided by the *minfi* package for R.[33,36-38] Main analyses were further adjusted for maternal parity, education, and smoking, gestational age at birth, and child age at time of DNA methylation measurement (in the analyses of child and adolescent DNA methylation). Gestational age at maternal haemoglobin measurement was not available in the Raine Study and only for a subsample in NFBC1986 so this covariate was not included in the adolescent models. One case-control study (FinnGeDi study) was included in the newborn meta-analysis and for this, also a selection factor (control vs. gestational diabetes case) was included to account for the design. Each cohort used their own categorisation for maternal education. Parity was defined as a dichotomous variable (nulliparity/multiparity) and maternal smoking as a three-level categorical variable (never smoked/stopped in early pregnancy/smoked throughout pregnancy). The FinnGeDi study only included non-smokers and therefore did not adjust for smoking. Only six women in PROGRESS reported smoking during pregnancy and were removed from the analysis. Non-smoking vs. smoking environment was included instead in the PROGRESS analysis. For detailed information of all variables see **Supplementary Table 1 and the Supplementary Methods.**

Meta-analyses

Cohort-specific results were meta-analysed with METAL, using inverse-variance weighting.[39] Multiple testing was accounted for using the Bonferroni correction with 0.05/number of analysed CpG sites as P-value cut off for statistical significance.[39] Bonferroni-corrected P-values were considered as primary indicator for statistical significance, but less stringent false discovery rate (FDR) -adjusted P-values with 0.05 as cut off for statistical significance were also reported for comparison.[40] Cross reactive probes as well as probes for which results from only one study were available, sample size was below 20 and those mapped to X or Y chromosomes, were excluded from the meta-analyses and the subsequent analyses.[41,42] Polymorphic CpG sites i.e. sites located near genetic variants were flagged in the results because the adjacent variant might affect the methylation status of the CpG site.[41] The meta-analyses were conducted by two research groups independently and the results were compared.

Differentially methylated regions

Differentially methylated regions were analysed with *comb-p* and *DMRcate*.[43,44] In short, *comb-p* uses methylation probes' P-values to define differentially methylated regions. Regional P-values are calculated first using the Stouffer-Liptak-Kechris correction that accounts for autocorrelation and then adjusted for multiple testing with a one-step Šidák correction. *DMRcate* analysis was performed using the t-statistics from meta-analysis results as input. The program applies Gaussian kernel smoothing for t-statistics using a bandwidth lambda. P-values for regions are calculated based on the Satterthwaite method and corrected using FDR correction. Parameter settings for *DMRcate* and *comb-p* were chosen.[45] Mallik et al. evaluated power, precision, area under precision-recall curve (AuPR), Matthews correlation coefficient, F1 score and type I error rate from four different DMR analysis methods, including DMRcate and comb-p.[45] Settings for best performance were defined as the parameters yielding highest AuPR value and were set for *comb-p* as seed=0.05, dist=750 and for *DMRcate* as lambda=500, C=5. Differentially methylated regions that were identified with both programs, were accepted to be significant. Partial overlap between regions identified by both programs was accepted.

Study heterogeneity

Inter-study heterogeneity (I^2) statistic was used to assess between study heterogeneity of the associations between maternal haemoglobin and offspring DNA methylation. I^2 represents the percentage of total variation across studies due to heterogeneity. I^2 value of 50% or above indicated high heterogeneity.

Sensitivity analyses

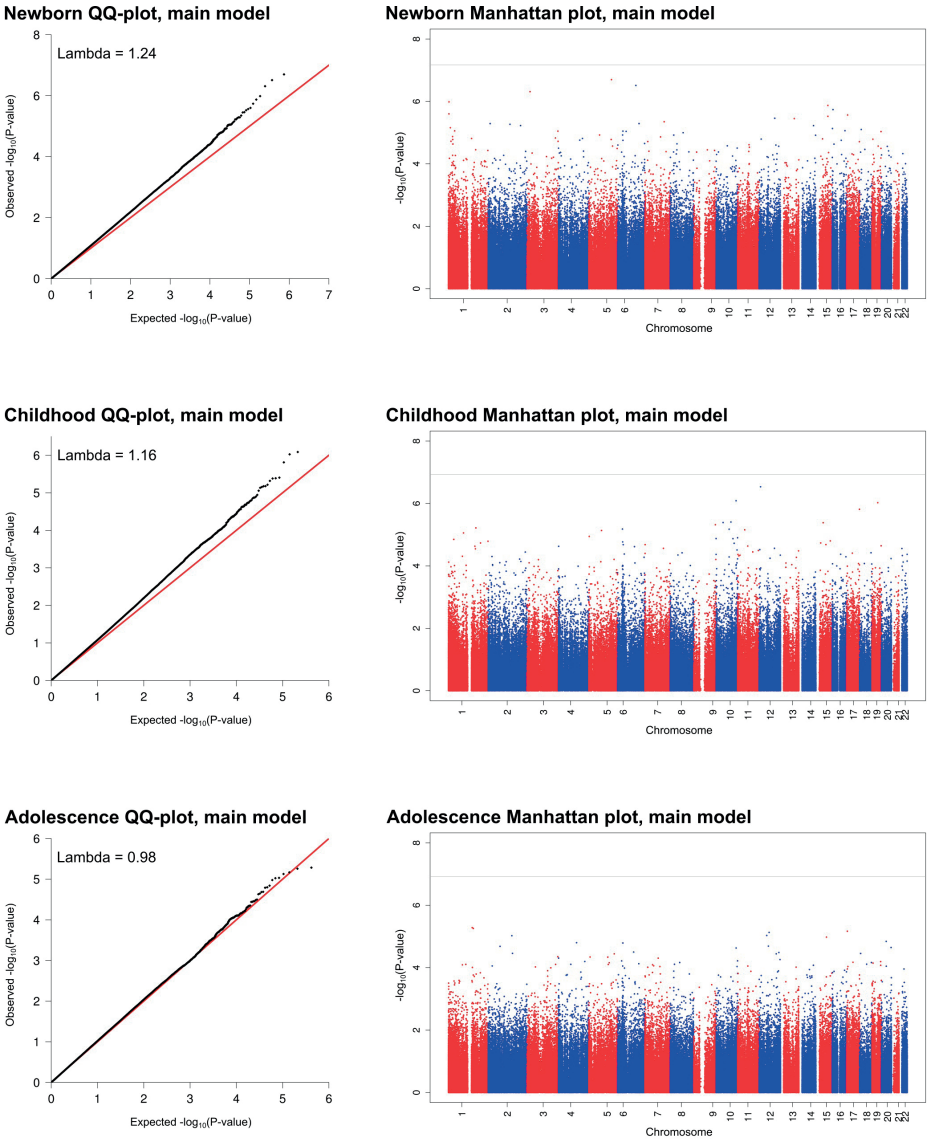
Given the influence of gestational age on maternal haemoglobin levels and the variation in gestational age at which blood for maternal haemoglobin was collected, we also repeated the meta-analyses in two subgroups: those with maternal haemoglobin measured in early pregnancy and those who had it measured in late pregnancy. Of the seven studies contributing to the analysis of newborn DNA methylation, two studies, reflecting 48% (1,893/3,967) of participants, assessed maternal haemoglobin levels at a mean gestational age of 15 weeks or less and five studies, reflecting 52% (2,074/3,967) of participants, assessed maternal haemoglobin levels at a mean gestational age of 27 weeks or more (**Table 1**). For childhood DNA methylation, two of five studies (77% of participants, 1,178/1,534) assessed the levels at 15 weeks or less and three of them (23% participants, 356/1,534) assessed maternal haemoglobin levels at 27 weeks or more. For the childhood DNA methylation analyses, numbers for late pregnancy maternal haemoglobin were too small for subgroup analyses and for the adolescent DNA methylation analyses, only two studies had information on gestational age at maternal haemoglobin measurement, both had a mean maternal haemoglobin measurement at 10 weeks of gestation.

Table 1. Characteristics of the cohorts involved in the meta-analyses

Life-stage	Cohort	N	Females, %	mHb, g/L mean (SD)	GA at mHb, weeks mean (SD)	GA at birth, weeks mean (SD)	Child age at DNAm, years mean (SD)
Newborn Cord blood	ALSPAC	688	52.3	124.5 (9.0)	9.7 (2.4)	39.6 (1.5)	0
	EDEN	123	41.5	119.3 (10.5)	27.2 (1.1)	39.4 (1.5)	0
	FinnGeDi	484	51.4	123.8 (9.6)	36.6 (3.0)	39.9 (1.3)	0
	Generation R	1,205	49.5	124.6 (8.7)	14.9 (3.7)	40.2 (1.5)	0
	INMA	363	49.0	115.1 (9.9)	32.2 (4.3)	39.8 (1.3)	0
	Predo	709	47.7	121 (12.7)	30.3 (7.6)	39.8 (1.6)	0
	PROGRESS	395	45.6	128.2 (9.3)	31.6 (1.0)	38.5 (1.5)	0
Childhood 4 to 7 years	ALSPAC	749	51.3	124.4 (8.9)	9.7 (2.4)	39.6 (1.5)	7.4 (0.1)
	EDEN	121	41.3	119.1 (10.5)	27.2 (1.1)	39.4 (1.5)	5.7 (0.1)
	Generation R	429	53.4	124.2 (8.7)	14.8 (3.7)	40.2 (1.6)	6.0 (0.4)
	INMA	185	48.1	115.0 (10.1)	32.6 (3.7)	39.9 (1.3)	4.4 (0.2)
	POGO	71	49.3	123.8 (11.1)	34.7 (4.9)	38.5 (2.0)	7.6 (3.0)
Adolescence 16 to 17 years	ALSPAC	750	52.4	124.6 (8.8)	9.7 (2.4)	39.6 (1.5)	17.1 (1.0)
	NFBC1986	451	61.9	131.4 (10.2)	10.7 (2.9)	40.1 (1.3)	16.1 (0.4)
	Raine Study	761	49.3	122.8 (9.0)	NA	39.6 (1.7)	17.1 (0.3)

N, sample size; SD, standard deviation from mean; mHb, maternal haemoglobin; GA, gestational age; DNAm, DNA methylation; NA, not available.

Figure 1. Maternal haemoglobin during pregnancy and offspring DNA methylation at birth, childhood and adolescence main models



The fully adjusted main models for newborn and childhood data are adjusted for gestational weeks at maternal haemoglobin measurement, maternal parity, maternal education, maternal smoking, child sex, gestational age at birth, child age at time of DNA methylation measurement, DNA methylation batch and white blood cells estimates. The main model for adolescent data is adjusted for the same variables as the newborn and childhood models except for gestational weeks at maternal haemoglobin measurement. The grey line shows the Bonferroni-corrected significance threshold for multiple testing.

RESULTS

Study characteristics

Total sample sizes were 3,967 for the newborn analyses, 1,534 for childhood analyses and 1,962 for adolescent analyses. Cohort-specific study characteristics are presented in **Table 1**. Detailed information on all characteristics used in the models is shown in **Supplementary Table 1**.

Table 2. Summary of cohort-specific and meta-analysis results for offspring EWAS on maternal haemoglobin during pregnancy

Life-stage	Cohort	N	Initial model [1]			Main model [2]		
			Lambda	Hits	Probe N	Lambda	Hits	Probe N
Newborn Cord blood	ALSPAC	688	0.96	0	468,622	0.96	0	468,622
	EDEN	123	1.68	33	439,306	1.59	21	439,306
	FinnGeDi	484	1.06	0	687,640	1.01	0	687,640
	Generation R	1,205	1.04	0	450,068	1.03	0	450,116
	INMA	363	1.57	0	465,930	1.62	0	465,930
	Predo	709	0.88	0	428,619	0.88	0	428,603
	PROGRES	395	1.44	1	846,258	1.49	2	846,257
	Meta-analysis	**3,967**	**1.24**	**0**	**737,758**	**1.24**	**0**	**738,318**
Childhood 4 to 7 years	ALSPAC	749	1.06	1	471,078	1.07	0	471,078
	EDEN	121	1.83	62	439,306	1.73	47	439,306
	Generation R	429	1.02	0	457,863	1.01	0	457,866
	INMA	185	0.74	0	465,930	0.80	0	465,929
	POGO	50	0.84	0	845,824	0.84	0	845,699
	Meta-analysis	**1,534**	**1.12**	**1**	**424,780**	**1.16**	**1**	**425,188**
Adolescence 16 to 17 years	ALSPAC	750	1.10	0	470,334	1.10	0	470,334
	NFBC1986	451	0.83	0	466,289	1.28	0	466,284
	Raine Study	761	0.82	0	462,927	0.85	0	462,927
	Meta-analysis	**1,962**	**0.98**	**0**	**418,039**	**0.98**	**0**	**418,438**

N, sample size; hits, statistically significant CpG sites after Bonferroni correction; probe N, number of CpG sites analysed.
[1] Initial model for newborn and childhood data is adjusted for gestational week at haemoglobin measurement, child sex, DNA methylation batch, selection factor in the case of randomised controlled trial and white blood cell estimates. Adolescence model is initial model without adjustment for gestational week at maternal haemoglobin measurement.
[2] Main model for newborn and childhood data is initial model adjusted for maternal parity, maternal education, maternal smoking, gestational age at birth and child age at the time of DNA measurement. Adolescence model is main model without adjustment for gestational week at maternal haemoglobin measurement.

Epigenome-wide association studies

Table 2 shows a summary of cohort specific EWAS results. The newborn and childhood models in the individual studies showed minimal inflation of associations with low P-value under the global null hypothesis (meta-analysis lambdas 1.24 and 1.16,

Table 3. CpG sites with the lowest P-values in the meta-analysis of associations between maternal haemoglobin during pregnancy and offspring DNA methylation at birth

CpG site	Chr	Gene	Regression coefficient	SE	P-value	FDR-corrected P-value
cg05470963*	5	*ARHGAP26*	0.0015	0.0003	2.00E-07	0.114
cg18479141	6	*HDAC2*	-0.0022	0.0004	3.08E-07	0.114
cg04181092	3		0.0013	0.0003	4.88E-07	0.120
cg24953596	1	*MEGF6*	-0.0043	0.0009	1.03E-06	0.190
cg04365443	15	*MPI*	-0.0005	0.0001	1.34E-06	0.198
cg00736299*	16	*MGRN1*	0.0027	0.0006	1.83E-06	0.225
cg20169893	1	*PRDM16*	-0.0018	0.0004	2.51E-06	0.238
cg06928695	17	*PITPNM3*	-0.0030	0.0006	2.73E-06	0.238
cg09126014	15	*SCAMP2*	0.0022	0.0005	2.99E-06	0.238
cg23912509	12	*MIR135A2*	0.0015	0.0003	3.47E-06	0.238
cg05454731	13		-0.0040	0.0009	3.55E-06	0.238
cg04140066	7		-0.0033	0.0007	4.47E-06	0.259
cg14801038	6	*TCF21*	-0.0023	0.0005	5.13E-06	0.259
cg15753546*	2		0.0015	0.0003	5.13E-06	0.259
cg02935826	2		0.0022	0.0005	5.40E-06	0.259
cg06522562	2	*FAM117B*	0.0006	0.0001	5.95E-06	0.259
cg08908586	14	*FBLN5*	-0.0010	0.0002	5.96E-06	0.259
cg13305114	1	*VPS13D*	0.0009	0.0002	6.94E-06	0.263
cg05924031	16	*CACNA1H*	0.0026	0.0006	7.38E-06	0.263
cg14500916	18	*LOC101927410*	0.0009	0.0002	7.92E-06	0.263
cg24542758	16		-0.0023	0.0005	8.67E-06	0.263
cg09364660	1	*MYCBP, RP5-864K19.4, RP5-864K19.6, RP5-864K19.7*	0.0007	0.0002	8.82E-06	0.263
cg02662362	6	*HLA-DPB2*	-0.0007	0.0002	8.94E-06	0.263
cg24392197	3	*RN7SL36P, XXYLT1, XXYLT1-AS2*	-0.0032	0.0007	8.97E-06	0.263
cg15520639	6		0.0011	0.0002	9.11E-06	0.263
cg23076906	19	*ZNF444*	-0.0011	0.0002	9.27E-06	0.263
cg10250335	8	*LOC101927040*	0.0057	0.0013	1.01E-05	0.275
cg19681474	5		-0.0019	0.0004	1.19E-05	0.289
cg20757478	6		0.0044	0.0010	1.19E-05	0.289
cg20794351*	8		-0.0039	0.0009	1.20E-05	0.289
cg08008938	14	*ADSSL1*	-0.0017	0.0004	1.21E-05	0.289
cg18878872	1	*MAN1C1*	0.0052	0.0012	1.34E-05	0.295
cg16815082	7		0.0035	0.0008	1.37E-05	0.295
cg09041485	3	*USP13*	-0.0009	0.0002	1.49E-05	0.295
cg21961202	1		-0.0006	0.0001	1.53E-05	0.295
cg04342176	4	*DCLK2*	-0.0007	0.0002	1.54E-05	0.295

Table 3. CpG sites with the lowest P-values in the meta-analysis of associations between maternal haemoglobin during pregnancy and offspring DNA methylation at birth (continued)

CpG site	Chr	Gene	Regression coefficient	SE	P-value	FDR-corrected P-value
cg03927133	15	*ITPKA*	-0.0008	0.0002	1.59E-05	0.295
cg12751042	12	*CDKN1B*	0.0019	0.0004	1.61E-05	0.295
cg03726569	19	*SAFB2*	0.0012	0.0003	1.62E-05	0.295
cg26556719	5	*AC005609.17, PCDHA1 - PCDHA13*	-0.0026	0.0006	1.66E-05	0.295

There are no significant CpG sites after Bonferroni correction (P-value < 6.77E-08). The fully adjusted models for newborn and childhood data were adjusted for gestational weeks at maternal haemoglobin measurement, maternal parity, maternal education, maternal smoking, child sex, gestational age at birth, child age at time of DNA methylation measurement, DNA methylation batch, selection factor in the case of randomised controlled trial and white blood cells estimates. The main model for adolescent data is adjusted for the same variables as the newborn and childhood models except for gestational weeks at maternal haemoglobin measurement. CpG, cytosine-phosphate-guanine; Chr, chromosome; Regression coefficient, difference in offspring DNA methylation beta value per one SD unit increase in maternal haemoglobin; SE, standard error. Polymorphic CpG sites are indicated with an asterisk after the site name.

respectively), whereas in the adolescent analyses there was little evidence of departure from the global null (lambda 0.98, **Table 2, Figure 1 and Supplementary Figure 1**). The number of analysed CpG sites was 738,318 in newborn, 425,188 in childhood and 418,438 in adolescent models. I^2 values were below 50% i.e. they did not indicate high inter-study heterogeneity in 602,276 (81.6%), 371,919 (87.5%) and 347,638 (83.1%) CpG sites in the newborn, childhood, and adolescent models, respectively.

After Bonferroni correction for 738,318 tests (P-value < 6.77E-08), there were no significant associations of maternal haemoglobin levels with offspring DNA methylation at any CpG sites in newborns. The forty CpG sites with the lowest P-values for main model are shown in **Table 3** and for the minimally adjusted model in **Supplementary Table 2**. Similarly, there was no statistical support for associations of maternal haemoglobin levels and DNA methylation in childhood (Bonferroni correction for 425,188 tests, P-value < 1.18E-07) or in adolescence (Bonferroni correction for 418,536 tests, P-value < 1.19E-07). Volcano plots of the meta-analysis results are provided in **Supplementary Figure 2**. The forty CpG sites with the lowest P-values in the childhood and adolescent models are listed in **Supplementary Tables 3 – 6**. CpG sites that were statistically significant in individual cohorts are listed in **Supplementary Table 7**. We also corrected for multiple testing using the less stringent false discovery rate (FDR) threshold by Benjamini and Hochberg and found no statistical support for association (P < 0.05).[40]

To investigate the effect of maternal haemoglobin measurement timing on the associations, we conducted sensitivity analyses by stratifying the newborn studies into those with early (mean maternal haemoglobin level measured before or at gestational week 15) and late (mean maternal haemoglobin level measured after gestational week 27) maternal haemoglobin measurements. Global P-values were not inflated for the early

Table 4. Differentially methylated regions in offspring DNA associated with maternal haemoglobin

Life-stage	Chr	Gene	Start	End	N	P-value
Newborn Cord blood	1	*PLEKHG5*	6,471,656	6,471,754	3	1.80E-02
	3	*MBNL1-AS1, MBNL1*	152,268,820	152,269,011	6	4.75E-02
	3	*XXYLT1*	195,147,697	195,147,779	3	7.68E-03
	6	*LY6G5C*	31,682,957	31,683,502	18	1.41E-09
	7	***HOXA2***	**27,103,615**	**27,103,860**	**7**	**6.58E-03**
	7	*UPP1*	48,090,199	48,090,396	5	2.11E-05
	10	*MIR378C*	130,885,180	130,885,192	2	1.97E-02
	12	*LOC101593348, DIABLO*	122,227,440	122,227,666	8	6.68E-04
	15	*FOXB1*	60,002,198	60,003,114	5	2.46E-07
	16	*TEPP*	57,985,961	57,986,081	3	1.16E-02
	17	*TBC1D3P5*	27,380,401	27,380,510	2	2.87E-02
	19	*RPS9*	54,206,998	54,207,425	4	5.90E-05
Childhood 4 to 7 years	2	*GDF7*	20,670,326	20,671,642	8	1.35E-15
	3	*LRRC15*	194,369,747	194,370,002	5	1.59E-06
	5	*FAM172A*	94,111,781	94,111,996	5	2.69E-02
	6	*PSORS1C3*	31,180,554	31,180,881	14	8.39E-03
	6	*VARS*	31,794,631	31,795,000	11	1.64E-02
	6	*HLA-DQB1*	32,664,553	32,665,387	16	9.01E-08
	6	*TAPBP*	33,312,274	33,312,678	12	3.02E-06
	6	*CRISP2*	49,713,464	49,713,679	7	2.03E-02
	7	*GPR146, C7orf50*	1,055,828	1,056,085	5	3.94E-02
	7	*HOXA-AS3, HOXA6*	27,147,752	27,147,942	6	1.60E-03
	10	*PRXL2A*	80,408,000	80,408,019	3	9.96E-05
	10	*GLRX3*	130,191,038	130,191,586	7	5.11E-08
	11	*PGGHG*	289,773	289,967	3	2.84E-02
	11	*IFITM5*	299,389	300,491	11	6.71E-08
	11	*TNNT3*	1,927,702	1,927,884	5	2.06E-02
	11	*ACY3*	67,650,634	67,650,935	11	3.55E-03
	12	*RIMBP2*	130,633,880	130,634,110	4	4.02E-03
	12	*ADGRD1*	131,132,498	131,132,548	3	1.24E-02
	14	*CDC42BPB*	103,058,561	103,058,653	3	5.11E-03
	17	***C17orf107, CHRNE***	**4,901,378**	**4,901,544**	**2**	**4.66E-02**
	17	*RAB34*	28,718,024	28,718,159	5	2.53E-02
	17	*NBR2*	43,126,117	43,126,364	7	1.64E-02
	17	*SEC14L1*	77,100,119	77,100,301	3	8.71E-03
	18	*SALL3*	78,506,264	78,506,438	3	1.19E-04
	19	*IZUMO1*	48,741,313	48,741,418	3	2.16E-02
	20	*CDH4*	61,773,104	61,773,352	3	4.89E-02
	20	*RTEL1-TNFRSF6B, TNFRSF6B*	63,696,614	63,696,742	3	2.30E-02

Table 4. Differentially methylated regions in offspring DNA associated with maternal haemoglobin (continued)

Life-stage	Chr	Gene	Start	End	N	P-value
Adolescence 16 to 17 years	1	*RNU1-1, RNU1-3, RNVU1-18, RNU1-2, RNU1-4*	143,717,589	143,717,820	2	3.90E-05
	1	*MIR5087*	148,328,899	148,329,313	3	3.52E-04
	3	*CACNA1D*	53,495,988	53,496,221	3	2.05E-02
	3	*COL6A6*	130,649,213	130,649,552	6	5.95E-05
	4	*CTBP1-DT*	1,250,060	1,250,299	7	3.57E-07
	4	*EXOC1L*	55,794,161	55,794,295	3	3.75E-03
	6	*LINC00533*	28,633,491	28,633,743	12	6.11E-03
	7	***HOXA2***	**27,103,615**	**27,103,860**	**7**	**8.72E-03**
	10	*GLRX3*	130,190,896	130,191,293	5	2.37E-04
	11	*KCNQ1*	2,807,294	2,807,549	4	1.50E-03
	15	*LOC100130111*	29,675,827	29,675,992	3	2.22E-02
	15	*TTC23*	99,249,416	99,249,651	5	7.89E-04
	17	***C17orf107, CHRNE***	**4,901,378**	**4,901,544**	**2**	**4.89E-05**
	19	*SMIM24*	3,480,364	3,480,675	5	1.57E-03
	22	*RFPL2*	32,203,523	32,203,662	4	3.44E-02

The fully adjusted models for newborn and childhood data are adjusted for gestational week at haemoglobin measurement, child sex, DNA methylation batch, white blood cell estimates, possible selection factor, gestational age at birth, child age at the time of DNA methylation measurement, maternal smoking, parity and maternal education. The main model for adolescent data is adjusted for the same variables as the newborn and childhood models except for gestational weeks at maternal haemoglobin measurement. The overlapping region in chromosome 7 between the newborn and adolescent model as well as in chromosome 17 between the childhood and the adolescent model are highlighted. Chr, chromosome; N, number of CpG sites; P-value, Sidak-corrected P-value (significant when < 0.05).

gestational age measurements (meta-analysis lambda 0.98) and there was minimal inflation for those with late maternal haemoglobin measurements (meta-analysis lambda 1.24). There was no statistical support for associations of maternal haemoglobin levels with newborn DNA methylation when analyses were conducted separately for early and late maternal haemoglobin measurement (**Supplementary Figure 3**).

Differentially methylated regions

Using *comb-p*, we found 12 differentially methylated regions in the newborn analyses, 27 in childhood and 17 in the adolescence models (**Table 4**).[43] None of the differentially methylated regions overlapped between all of the ages, but there was an overlap of one differentially methylated region annotated to *HOXA2* between the newborn and the adolescent models and a region annotated to *CHRNE* between the childhood and the adolescent models. We did not find any differentially methylated regions using *DMRcate*.[44]

DISCUSSION

In the current study, we analysed associations of maternal haemoglobin levels during pregnancy with offspring DNA methylation at birth, in childhood and in adolescence. We meta-analysed EWAS summary statistics of ten studies comprising 3,967 neonatal, 1,534 childhood and 1,962 adolescent offspring DNA methylation samples and their maternal haemoglobin levels during pregnancy. We did not find statistical support for an association between maternal haemoglobin levels during pregnancy and offspring DNA methylation at any of the three age ranges.

We found some evidence of association between maternal haemoglobin levels and differentially methylated regions in the offspring DNA using *comb-p*.[43] We identified one shared region on chromosome 7 between newborn and adolescent models and one on chromosome 17 between childhood and adolescent models. Of these, one locus is situated in the homeobox A2 (*HOXA2*) gene, which encodes a transcription factor that is important during embryonic development. *HOXA2* located in chromosome 7, has a role in the development of the lower and middle part of the face and middle ear and its deficiency has been associated with ear microtia.[46] *Comb-p* is a flexible tool specifically for meta-analysed EWAS summary statistics as it uses P-values by sliding windows and takes into account the correlation between near-by sites; however, *comb-p* has been shown to produce false positive results, especially if the signal in the original data was weak.[47] As there is no consensus on the best method for analysis of differentially methylated regions with meta-analysis data, we also analysed the results using *DMRcate* which did not support the *comb-p* results.[44] As the differentially methylated regions were identified by one method only, we conclude that the highlighted regions may be artefacts and should be interpreted with caution.

The large sample size covering the newborn, childhood and adolescent age periods was a major strength of the current study. Nearly 80% of the meta-analysed CpG sites show only little or moderate evidence for between study heterogeneity suggesting that the observed effects were reasonably consistent across cohorts. This is another strength, as lower heterogeneity improves the interpretability of the results. However, this study also had some technical limitations. Although the current method for epigenome-wide analysis of methylated CpG sites is arguably the best choice for high-throughput studies, the 450,000 or 850,000 sites analysed by the Illumina Infinium Methylation450K and MethylationEPIC arrays, respectively, account for only 2 to 4 % of the CpG sites in the whole genome. It is possible that DNA methylation at sites not covered on either array could be related to maternal haemoglobin levels.[48]

There is a large and ongoing proliferation of published methods for quality control, processing and analysis of DNA methylation data. The optimal method may vary between cohorts based on technical issues prior to data analysis, such as bisulphite conversion

efficiency, sample distribution on the chip and the chip reading efficiency. In addition, the multitude of methods are often published with insufficient evaluation of how these alter results or compare with other methods. Thus, we allowed each cohort, with their familiarity with how the samples were processed in their study, to assess normalisation method and apply their own correction. This might have influenced the downstream analysis. However, we have previously shown, that there are no large differences between a meta-analysis of cohorts which all used their own preferred normalization method and a meta-analysis of the non-normalized data of those same cohorts.[13] Due to the restrictions in data transfer permissions, we used a meta-analysis of summary statistics of individual studies, which is a standard practice in the PACE Consortium. Thus, the participating cohorts conducted their own EWAS locally and sent the summary statistics to the meta-analysis team, which then conducted the meta-analysis. This may also lessen the effect of differing normalisation as the same normalisation was always used within the cohort. That is, we would expect any true associations to be identified within the individual cohorts, regardless of the normalization method and then to also come up in the meta-analysis.

Although the sample size in the current study was relatively large, it might have been insufficient to detect weak associations that might exist between variation of maternal haemoglobin levels within the normal range and the offspring DNA methylation. Furthermore, maternal haemoglobin levels are routinely monitored during pregnancy and if low haemoglobin was detected, it is likely that measures were taken in an attempt to increase levels by administration of iron supplements. This may have lowered the number of individuals with low maternal haemoglobin level in our analysis. In addition, we have used linear models in the current analyses, while the fact that both high and low maternal haemoglobin levels have been shown to associate with adverse pregnancy outcomes would support a non-linear approach. There were not enough individuals in the cohort-specific strata of low/high maternal haemoglobin levels to make analyses in categories of low, normal and high haemoglobin levels feasible. Future studies in populations with a higher prevalence of high or low maternal haemoglobin levels, such as those living at high altitudes or in low-income countries, respectively, will provide insight into potential associations at more extreme maternal haemoglobin levels.[4, 49]

The mean gestational age at which maternal haemoglobin levels were measured varied substantially between cohorts, from 9.7 to 36.6 weeks. During pregnancy, maternal haemoglobin levels normally decrease due to haemodilution until 20 weeks of gestation and begin to increase at around 30 weeks. We adjusted the models for gestational age at maternal haemoglobin measurement; however, this might not account for inter-cohort differences. To investigate this further, we conducted sensitivity analyses separately for studies that measured maternal haemoglobin levels during early and late pregnancy

in newborn models and found no strong statistical support for associations in either of these strata.

One mechanism by which maternal haemoglobin levels could influence DNA methylation of the offspring is through non-physiological intrauterine hypoxia.[9] Both low and high maternal haemoglobin levels may expose the fetus to hypoxia; low levels via insufficient oxygen availability and high levels via increased blood viscosity.[8] Hypoxia has been shown to increase methylation of approximately half of the CpG sites that would in normoxic conditions become hypomethylated in the placental trophoblasts.[10] Non-physiological hypoxia may affect the developing fetus either in a pre-placental, utero-placental or post-placental manner.[50] From these, only pre-placental hypoxia influences both mother and fetus whereas utero-placental and post-placental hypoxia may not be reflected in the maternal haemoglobin levels. Thus, the maternal haemoglobin levels investigated in the current study may represent only pre-placental hypoxia. Further mechanistic studies are warranted to fully understand the relationship between non-physiological intrauterine hypoxia and offspring DNA methylation.

CONCLUSION

This study is the first to date to ascertain a possible association between maternal haemoglobin levels and DNA methylation in the offspring at three age ranges from birth to adolescence. We did not find evidence to support epigenetic programming by physiological variations of maternal haemoglobin levels during pregnancy.

Detailed acknowledgements and Supplementary Data can be found in the published article online: https://pubmed.ncbi.nlm.nih.gov/33331245/

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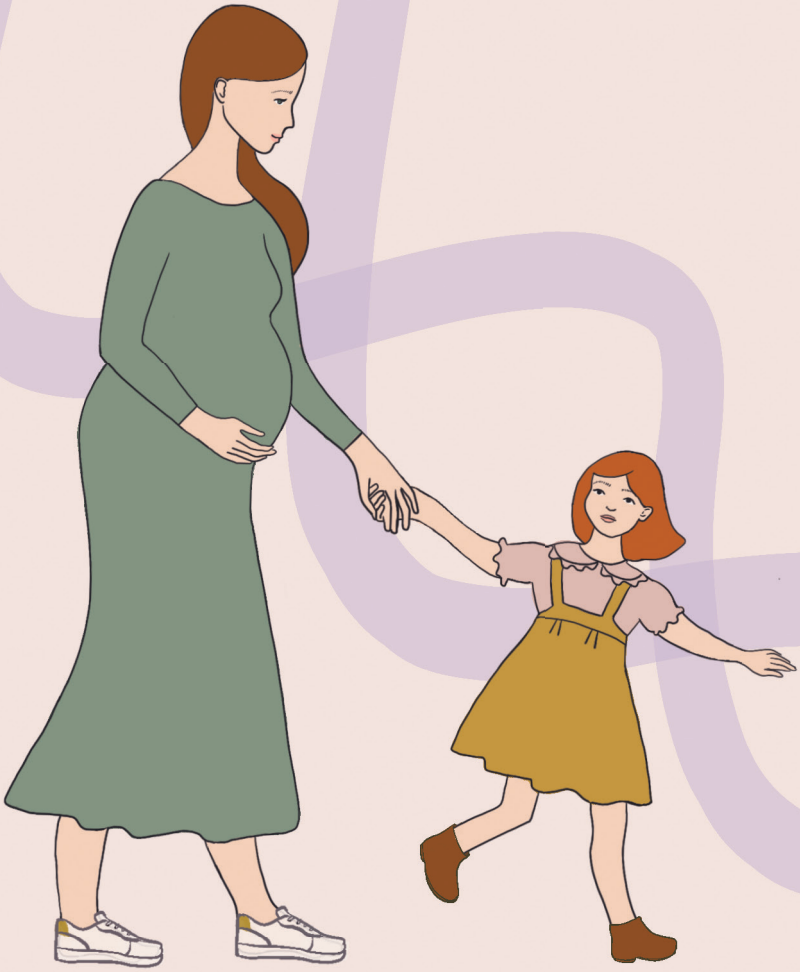

4.2

DNA methylation and body mass index from birth to adolescence: meta-analyses of epigenome-wide association studies

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ABSTRACT

Background: DNA methylation has been shown to be associated with adiposity in adulthood. However, whether similar DNA methylation patterns are associated with childhood and adolescent body mass index (BMI) is largely unknown. More insight into this relationship at younger ages may have implications for future prevention of obesity and its related traits.

Methods: We examined whether DNA methylation in cord blood, and in whole blood in childhood and adolescence was associated with BMI in the age range from 2-18 years using both cross-sectional and longitudinal models. We performed meta-analyses of epigenome-wide association studies including up to 4,133 children from 23 studies. We examined the overlap of findings reported in previous studies in children and adults with those in our analyses and calculated enrichment.

Results: DNA methylation at three CpGs (cg05937453, cg25212453 and cg10040131), each in a different age range, was associated with BMI at Bonferroni significance, $P < 1.06 \times 10^{-7}$, with a 0.96 standard deviation score (SDS) (standard error (SE) 0.17), 0.32 SDS (SE 0.06) and 0.32 BMI SDS (SE 0.06) higher BMI per 10% increase in methylation, respectively. DNA methylation at nine additional CpGs in the cross-sectional childhood model was associated with BMI at False Discovery Rate significance. The strength of the associations of DNA methylation at the 187 CpGs previously identified to be associated with adult BMI, increased with advancing age across childhood and adolescence in our analyses. In addition, correlation coefficients between effect estimates for those CpGs in adults and in children and adolescents also increased. Among the top findings for each age range, we observed increasing enrichment for the CpGs that were previously identified in adults (birth $P_{enrichment} = 1$; childhood $P_{enrichment} = 2.00 \times 10^{-4}$; adolescence $P_{enrichment} = 2.10 \times 10^{-7}$).

Conclusions: There were only minimal associations of DNA methylation with childhood and adolescent BMI. With advancing age of the participants across childhood and adolescence, we observed increasing overlap with altered DNA methylation loci reported in association with adult BMI. These findings may be compatible with the hypothesis that DNA methylation differences are mostly a consequence rather than a cause of obesity.

BACKGROUND

An accumulating body of evidence suggests that exposures in early life are associated with childhood BMI.[1] It is hypothesized that changes in DNA methylation may underlie the associations of early-life exposures with childhood adiposity.[2-4] Thus far, most of the evidence regarding DNA methylation and adiposity stems from adult studies.[5-9] The largest epigenome-wide association study (EWAS) in adults identified cross-sectional associations between DNA methylation at 187 loci and BMI in over 10,000 participants.[5] Previous studies of the associations between epigenome-wide DNA methylation and childhood and adolescent adiposity were small and inconclusive.[10-16] Candidate gene studies in childhood identified associations of DNA methylation in cord and childhood blood with measures of adiposity.[17-24] Epigenome-wide association studies in children and adolescents, with sample sizes ranging from 40 to 700 individuals, identified a limited number of CpGs associated with BMI.[11-13, 15, 25] Although findings of some studies suggest that differences in DNA methylation may precede the development of adiposity, recent studies in adults, using methods such as Mendelian randomization, posit that alterations in DNA methylation are predominantly the consequence of adiposity, rather than the cause.[4, 5, 9, 26, 27] The direction of any causal pathway has not been robustly appraised in children. Obtaining more knowledge on the association between DNA methylation and adiposity already in childhood may have implications for future prevention of obesity and its related traits.

We performed a meta-analysis of epigenome-wide association studies of BMI in up to 4,133 participants from 23 studies. We assessed associations of DNA methylation in cord blood, in childhood and adolescence with BMI in children aged 2-18 years. We also compared effect estimates and examined whether there was enrichment in our data for CpGs previously identified for their association with adolescent and adult adiposity.

METHODS

Participants

We meta-analyzed epigenome-wide associations studies of cord or whole blood methylation with childhood or adolescent body mass index (BMI). We used data from up to 4,133 participants from 23 studies collaborating in the Pregnancy And Childhood Epigenetics (PACE) consortium, LifeCycle Project and NutriProgram Project (**Table S1A-D and Supplementary Methods**)[28, 29]: ALSPAC, BAMSE, CHAMACOS, CHOP Study, CHS, DOMInO trial, GECKO Drenthe cohort, Generation R Study, GOYA study, Healthy Start Study, HELIX, INMA, IOW F1, IOW F2, MoBa1, MoBa2, NEST, NFBC 1986, PIAMA study, PREDO study, Project Viva, Raine and STOPPA (full names in **Supplementary Methods**).

Cohort participants were mainly of European ancestry, but there were also cohorts with (partly) non-European ethnicities (African, Hispanic, and Aboriginals). Most cohorts are prospective birth cohorts. We excluded multiple births, siblings (maximum one child per family), physician-diagnosed syndromic obesity cases, and any type of maternal diabetes (including gestational diabetes). Informed consent was obtained for all participants, and all studies received approval from their local ethics committees (**see Supplementary Methods**).

DNA methylation

DNA methylation was measured in cord blood and whole blood samples, in children and adolescents using the Illumina Infinium® HumanMethylation450 BeadChip assay (Illumina, San Diego, California, USA).[30] Each cohort independently conducted their preferred quality control and normalization method, see **Supplementary Methods** for details. Untransformed normalized beta-values of individual Cytosine-phosphate-Guanine (CpG) sites were used as exposure variables. If multiple measurements of DNA methylation and BMI were available within an age range, we used the oldest age within that range for which BMI and DNA methylation were available at the same time point. Outlying methylation beta values were excluded using the following method: values < (25^{th} percentile – 3*interquartile range (3IQR)) and values > (75^{th} percentile + 3IQR) were removed.[31] DNA methylation is expressed as the proportion of alleles at which the DNA was methylated at a specific site and hence takes values from zero to one.

Childhood BMI

Height and weight were measured in each study using established protocols as described in detail in the **Supplementary Methods**. The primary outcome was BMI, calculated as weight/height^2 in kg/m^2, on a continuous scale measured in three age ranges: 2-5 years (early childhood), 5-10 years (late childhood), and 14-18 years (adolescence). If multiple BMI and DNA methylation measurements were available, we used the measurements at the oldest age within the age range for which BMI and DNA methylation were available at the same time point. BMI values were then transformed into sex- and age-adjusted standard deviation scores (SDS) using LMSGrowth.[32-34] The International Obesity Task Force (IOTF) standard was used to define cut-offs for BMI for underweight, normal weight, overweight, and obesity in children, created with the British1990 growth reference and information of participants on BMI, sex, and age.[35, 36] In secondary analyses we used a binary outcome variable with normal weight children as controls and overweight or obese children as cases. Underweight children were excluded from these secondary analyses. If a study had =<10 participants in one of the (case or control) groups, this study was excluded from the secondary analyses.

Covariates

Covariates included in all models were maternal covariates: maternal age, maternal educational level (cohort definition), maternal smoking status during pregnancy (any smoking versus no smoking), maternal pre-pregnancy or early pregnancy BMI, and parity (multiparous versus nulliparous), and gestational age at birth. For details on cohort-specific collection methods, see **Supplementary Methods**. We estimated white blood cell proportions (B-cells, CD8+ T-cells, CD4+ T-cells, granulocytes, NK-cells, and monocytes) using the reference-based Houseman method with the Reinius reference in the minfi package in R.[37-40] A sensitivity analysis using the cord blood-specific Bakulski reference was performed in the Generation R and ALSPAC Studies.[41] Batch effects were adjusted for using cohort-specific methods, see **Supplementary Methods.** Additional covariates added in the cross-sectional childhood analyses were birth weight and breast feeding. The adolescent analyses were additionally adjusted for adolescent age, sex, own smoking status, and puberty status. Puberty status was categorized into early puberty (if both breast and pubic hair Tanner stages (or comparable classification) were 1,2 or 3 and if girls were pre-menarcheal or boys did not have voice change yet) and late puberty (if either breast or pubic hair Tanner stages (or comparable classification) were 4 or 5 or if girls were post-menarcheal or boys had had their voice change).[42-44] Further details are provided in the study-specific **Supplementary Methods.**

Study-specific analyses

Associations of DNA methylation with childhood or adolescent BMI were performed in individual studies on participants with complete data on all covariates. In studies with more than one ethnic group, each group was analyzed separately. We used robust linear regression models for the continuous outcome of BMI-SDS and generalized linear regression models for the case/control analyses of overweight and obesity versus normal weight, according to a pre-specified analysis plan. EWAS analyses were conducted using DNA methylation at three time points: birth, childhood and adolescence, and BMI data collected at three time-points: early childhood (2-5 years), late childhood (5-10 years), and adolescence (12-18y) (**Table 1**). We categorized the childhood period into early- and late childhood to overcome any age-specific effects and the potential influence of the adiposity rebound on the results.[45] Depending on data availability, cohorts participated in one or more of four analyses: (Analysis A) longitudinal associations of cord blood DNA methylation with early childhood BMI (2-5y; 3,295 children from 13 studies), (Analysis B) longitudinal associations of cord blood DNA methylation with late childhood BMI (5-10y; 4,133 children from 12 studies), (Analysis C) cross-sectional associations of childhood blood DNA methylation with childhood BMI (2-10y; 3,371 children from 11 studies) and (Analysis D) cross-sectional associations of adolescent blood DNA methylation with

adolescent BMI (14-18 y; 2,842 adolescents from 7 studies) (**Table 1**). Participating studies per analysis are shown **Table S1A-D**.

Analyses of cord blood were adjusted for maternal age, educational level, smoking status, pre-pregnancy or early pregnancy BMI, parity, gestational age, batch, and estimated cell type proportions. The cross-sectional analysis in childhood was additionally adjusted for child covariates birth weight and breastfeeding, in contrast the cross-sectional analysis in adolescence was adjusted for the same covariates as analysis C plus adolescent sex, age, smoking and puberty status.

Table 1. Overview of main analyses, secondary analyses and sensitivity analyses

	Main analyses			Secondary analyses	Sensitivity analyses	
Analysis	DNA methylation in blood	BMI SD-scores	N	Binary model (N) Cases = overweight and obesity Controls = normal weight	Europeans only (N)	Without studies > 30% overweight and obesity (N)
			Cord blood analyses			
A	Birth (cord blood)	Early childhood (2-5y)	3,295	Cases = 491 Controls = 2540	2,902	2,989
B	Birth (cord blood)	Late childhood (5-10y)	4,133	Cases = 707 Controls = 3217	3,657	3,489
			Cross-sectional analyses			
C	Childhood (whole blood)	Childhood (2-10y)	3,371	Cases = 644 Controls = 2567	3,026	3,171
D	Adolescence (whole blood)	Adolescence (12-18y)	2,842	Cases = 507 Controls = 2188	NA	NA

Analyses A and B were adjusted for maternal age, educational level, smoking status, pre-pregnancy or early pregnancy BMI, and parity, gestational age, batch and estimated cell type proportions.

Analyses C was adjusted for maternal age, educational level, smoking status, pre-pregnancy or early pregnancy BMI, and parity, gestational age, batch, estimated cell type proportions, birth weight and breastfeeding.

Analyses D was adjusted for maternal age, educational level, smoking status, pre-pregnancy or early pregnancy BMI, and parity, gestational age, batch, estimated cell type proportions, birth weight, breastfeeding, adolescent sex, age, smoking and puberty status.

Meta-analyses

After performing quality control on all studies, we combined results in a fixed-effects inverse variance weighted meta-analysis using METAL.[46, 47] All follow-up analyses were conducted in R.[39] The meta-analyses were done independently by two study groups and the results were compared. After exclusion of probes that were measured in only one study, that mapped to X and Y chromosomes and probes that co-hybridized to alternate sequences (cross-reactive probes) we included 429,959 probes for Analysis A, 429,959 probes for Analysis B, 429,957 probes for Analysis C and 428,967 probes for Analysis D.[48, 49] In the result files of the main meta-analyses, we flagged probes that map to DNA containing a single nucleotide polymorphism (SNP), to repetitive sequence elements,

or DNA harboring an INDEL (**Table S2A-D**).[48, 49] We corrected for multiple testing using both the Bonferroni correction, which gives a significance threshold of $p<1.16x10^{-7}$ (0.05/429,959) and the less stringent false discovery rate (FDR) threshold using the method by Benjamini and Hochberg.[50] EWAS results were summarized as mean (and standard error) differences in BMI-SDS per 10% increase in methylation for each CpG. We created volcano plots to visualize magnitude and direction of effect (reduced or increased methylation) along with the level of statistical significance. We calculated the I^2 statistic to explore heterogeneity across studies. The I^2 estimates the proportion of variation in the meta-analysis results for each CpG site that is due to between-study differences rather than random/sampling variation. Heterogeneity was defined as an I^2 value of >50 and shown graphically in forest plots. We performed leave-one-out analyses, in which we reran the main meta-analysis repeatedly with one of the 23 studies removed each time, to explore if any study influenced individual findings. We enhanced the annotation provided by Illumina using the UCSC Genome Browser. All of the annotations use the human February 2009 (GRCh37/hg19) assembly. We updated the gene names manually in all result files using HUGO gene nomenclature and, in case they were not found there, we used the NCBI gene website on November 5th, 2019.[51-53]

To explore the associations for the extreme upper values of the BMI distribution we performed case/control analyses (overweight and obesity versus normal weight). Underweight children were excluded from these analyses, leading to sample sizes of N = 491 cases and 2,540 controls (Analysis A), N = 707 cases and 3,217 controls (Analysis B), N = 644 cases and 2,567 controls (Analysis C), N = 507 cases and 2,188 controls (Analysis D) (**Table S3A-D**).

To examine whether any of the Bonferroni significant or FDR significant CpGs in our analyses where close to BMI SNPs, we assessed if these CpGs were located within a 4Mb window (+/- 2 Mb) surrounding the 15 genetic loci associated with childhood body mass index.[2, 54] For the FDR-significant CpGs that were flagged because they were potentially influenced by a SNP, we visually inspected density plots in the Generation R Study to see whether these deviated from unimodality (**Supplementary Information, Figure S6**).

To explore DNA methylation patterns in the regions around the significant CpGs, we assessed the associations of all CpGs located within a 10Kb window (+/- 5Kb) surrounding these CpGs with BMI in the relevant models (**Table S4**).

Sensitivity analyses

To explore whether ethnic heterogeneity may have affected our results, we repeated the meta-analyses including studies with participants of European ancestry only (N = 2,902 (excluding three studies for analysis A), N = 3,657 (excluding three studies for analysis B), N = 3,026 (excluding two studies for analysis C)), the largest ethnic subgroup (**Table S5A-C**). Ethnicity was defined using self-reported questionnaires unless specified other-

wise in the study-specific **Supplementary Methods.** We performed additional analyses excluding studies with a high percentage (>30% (percentage calculated after exclusion of underweight children)) of children with overweight and obesity to explore whether any associations found may be driven by more extreme values of BMI (included N = 2,989 (excluding two studies for analysis A), N = 3,489 (excluding four studies for analysis B), N = 3,171 (excluding one study for analysis C) (**Table S6A-C**). We also performed a third, conservative, sensitivity analysis in all age groups, excluding cohorts of non-Europeans, studies with a high percentage (>30%) of children with overweight or obesity, and studies in which the sample was selected on or enriched for any particular exposure or outcome (**Table S7A-D**).

Comparison with previous findings

We explored whether CpG sites associated with childhood, adolescent, or adult BMI in previous studies were associated with BMI in our data. For previous candidate gene studies and smaller EWASs (N<1,000), we performed a look-up of the hits, using a Bonferroni-adjusted P-value cutoff per study, so for each study, the cutoff was 0.05/(N CpGs from that study) (**Table S8**).[7, 15, 17, 18, 20, 21, 23, 24, 55] If the specific CpGs from a study were not available in our dataset, we looked up all CpGs annotated to the relevant genes.[17, 24]
To establish whether the CpG sites associated with BMI in previously reported larger EWASs (N≥1,000) were over-represented among our CpGs with the smallest P-values, we examined the absolute overlap of the top CpGs from literature with the top CpGs in our analyses.[5, 6, 9, 25, 26, 56-59] The latter were defined using two cut-offs: a stringent cut-off of P-value < $1x10^{-5}$ and a more lenient one of P-value <0.05. (**Table S9**). We used a hypergeometric test to calculate enrichment with the phyper function in the R Stats package in R.
We examined the 187 CpGs identified in the largest adult study (N=10,261) to date in more detail in our results.[5] We tested whether the enriched CpGs significantly overlapped between our analyses using chi-square tests. We used Pearson's correlation coefficients to examine the correlations between the effect estimates of these 187 CpGs in adults and those in our analyses.[5] Using Fisher's exact test we calculated whether the correlation coefficients at the various ages were significantly different from each other.

Functional analyses

We explored the potential functional interpretation of the most significantly associated CpGs (p-value < $1x10^{-4}$) in all models using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. We used the missMethyl package, which enabled us to correct for the number of probes per gene on the 450k array, based on the May 5, 2020 version of the GO, and the October 23, 2019 version of the KEGG source databases.[60] To filter out the large, general pathways we set the number of genes

for each gene set between 5 and 2000, respectively. We report nominal P-values < 0.05 and FDR for enrichment (**Table S10**).

RESULTS

Participants

We included 2,842 to 4,133 participants from 23 independent cohorts from the Pregnancy And Childhood Epigenetics (PACE) Consortium.[28] We assessed associations of DNA methylation in cord blood with BMI in early childhood (2-5 years) (N = 3,295, analysis A), DNA methylation in cord blood with BMI in late childhood (5-10 years) (N = 4,133, analysis B), DNA methylation in childhood with BMI in childhood (2-10 years) (N = 3,371, analysis C) and DNA methylation in adolescence with BMI in adolescence (12-18 years) (N = 2,842, analysis D). Details of participants and studies used in the different analyses are presented in **Table 1, Table S1A-D and in the Supplementary Methods**.

Meta-analyses

Main, secondary, and sensitivity analyses are outlined in **Table 1**. Genomic inflation factors (lambdas) for the main meta-analyses ranged between 0.97 and 1.27 (**Supplementary information, Figures 1A-D**). Genomic inflation factors (lambdas) of all cohort-specific analyses are shown in **Table S1A-D**. The main results are shown in **Table 2 and Figure 1**. We did not identify associations at genome-wide significance of DNA methylation in cord blood with BMI in early childhood (**Analysis A, Figure 1A, and Table S2A**). DNA methylation at one CpG, cg05937453 (*SFRP5*), in cord blood was significantly associated with late-childhood BMI (**Analysis B, Figure 1B, and Table S2B**). For each 10% increase in DNA methylation at cg05937453 in cord blood, late-childhood BMI increased 0.96 SD (standard error (SE) 0.17). Cord blood DNA methylation at this CpG was nominally significantly associated with BMI in early-childhood (P-value = 0.004), but DNA methylation in childhood and adolescence was not associated with BMI in the cross-sectional analyses (**Table S11**).

In the cross-sectional analysis (Analysis C), childhood DNA methylation at cg25212453 (*SLC43A2*) was associated with childhood BMI after Bonferroni correction. A 10% increase in DNA methylation at cg25212453, was associated with a 0.32 SD (SE 0.06) increase in childhood BMI (**Figure 1C and Table S2C**). DNA methylation at this CpG at birth and in adolescence was not associated with BMI (**Table S11**). DNA methylation in childhood at nine additional CpGs in or near other genes was associated with childhood BMI using FDR P-value < 0.05 (**Figure 1C and Table S2C**). DNA methylation in adolescence at cg10040131 (*SFXN5*) was associated with adolescent BMI after Bonferroni correction (**Analysis D, Figure 1D and Table 2D**). A 10% increase in DNA methylation at cg10040131

Table 2 CpG sites at which DNA methylation was associated with child or adolescent BMI

CpG	CHR	Location	Coef	SE	P-value	FDR p-value	Nearest gene
Analysis B = Association of cord blood DNA methylation with late childhood BMI (5-10y)							
cg05937453	10	99531765	0.96288	0.16871	1.15×10^{-8}	0.0049	*SFRP5*
Analysis C = Cross-sectional association of whole blood DNA methylation with childhood BMI (2-10y)							
cg25212453	17	1509953	0.31925	0.05978	9.27×10^{-8}	0.02075	*SLC43A2*
cg03500056	16	8814507	0.30577	0.05767	1.15×10^{-7}	0.02075	*ABAT*
cg05281708	3	44690673	0.65856	0.12614	1.78×10^{-7}	0.02075	*ZNF35*
cg15125798	5	122621645	0.49705	0.09548	1.93×10^{-7}	0.02075	-
cg04456029	12	113496126	0.27587	0.05358	2.63×10^{-7}	0.0226	*DTX1*
cg03431111	11	62621406	0.19261	0.03791	3.77×10^{-7}	0.0270	*SNORD30;SNORD22;SNORD29; SNORD31;SNHG1*
cg26889953	15	22915992	0.31743	0.06391	6.81×10^{-7}	0.0304	*CYFIP1*
cg19743522	12	113495566	0.33854	0.0682	6.92×10^{-7}	0.0304	*DTX1*
cg25877069	8	95003236	-0.45126	0.09092	6.94×10^{-7}	0.0304	-
cg13931559	20	33146515	-0.84718	0.17082	7.07×10^{-7}	0.0304	*MAP1LC3A*
Analysis D = Cross-sectional association of whole blood DNA methylation with adolescent BMI (12-18y)							
cg10040131	2	73178866	0.32434	0.0566	1.00×10^{-8}	0.0043	*SFXN5*

Coefficients (coef) and standard errors (SE) are presented per 10% increase in methylation level.
Analyses B was adjusted for maternal age, educational level, smoking status, pre-pregnancy or early pregnancy BMI, parity, gestational age, batch and estimated cell type proportions. Analysis C was additionally adjusted for child covariates birth weight and breastfeeding, whereas Analysis D was adjusted for the same covariates as analysis C plus adolescent sex, age, smoking and puberty status.

was associated with a 0.32 SD (SE 0.06) higher BMI in adolescence. DNA methylation at this CpG in childhood was nominally significantly associated with childhood BMI (P-value = 0.0002). The association of DNA methylation at this CpG in cord blood and BMI in childhood was not significant (**Table S11**).

Associations of DNA methylation with BMI did not show a preferential direction of effect in any of the analyses (Volcano plots, **Supplementary Information, Figures S2A-D**). We observed very little evidence of heterogeneity between studies among the Bonferroni-significantly associated CpG sites, with all $I^2 \leq 50$ (**Table 2A-D and forest plots, Supplementary Information, Figures S3A, B and L**). We found evidence of between-study heterogeneity ($I^2 > 50$) for 3 of the 9 FDR significantly associated CpG sites (**Table 2C and forest plots, Supplementary Information, Figures S3C-K**). The results for the twelve Bonferroni or FDR-significantly associated CpGs were stable after omitting one study at a time (leave-one-out analyses, **Supplementary Information, Figures S4A-L**).

When BMI was dichotomized into normal and overweight/obesity, only one CpG in the cross-sectional model in childhood, cg06991974 (*PRDM16-DT*), showed evidence of association. In the cross-sectional model during childhood, which included 644 children with overweight/obesity and 2,567 normal weight children, DNA methylation at

cg06991974 was associated with an increased risk of overweight/obesity in childhood (Odds Ratio (OR) 3.10 (95% Confidence Interval (CI) 2.08, 4.63) (**Table S3A-D**).
None of the three individual Bonferroni significant CpGs in the three different age ranges nor the 9 FDR significant CpGs were within a 4Mb window surrounding the 15 known genetic loci associated with childhood body mass index.[54]
Four of the 12 FDR significant CpGs contained a single-nucleotide polymorphism (SNP).[48, 49] We found no indication of non-unimodal distribution for any of these CpGs suggesting that methylation measurements at these sites were not markedly affected by SNPs (**Supplementary Information, Figure S6**).
Two of the three Bonferroni-significant CpGs (cg05937453 and cg25212453) had other nearby CpGs within a 10Kb window (+/- 5Kb) measured on the 450K array (**Table S4**). Cg05937453 (model B) was surrounded by 24 other CpGs, of which one was nominally significantly associated with BMI (p-value < 0.05). Both were located in the TSS200 region of *SFRP5* with effect estimates in the same direction. Cg25212453 (model C) was surrounded by 13 other CpGs, of which three were nominally significant (p-values < 0.05). All were located in the gene body of *SLC43A2* with effect estimates in the same direction. Results for Bonferroni- and FDR- significant CpGs are shown in **Table S4**.

Sensitivity Analyses

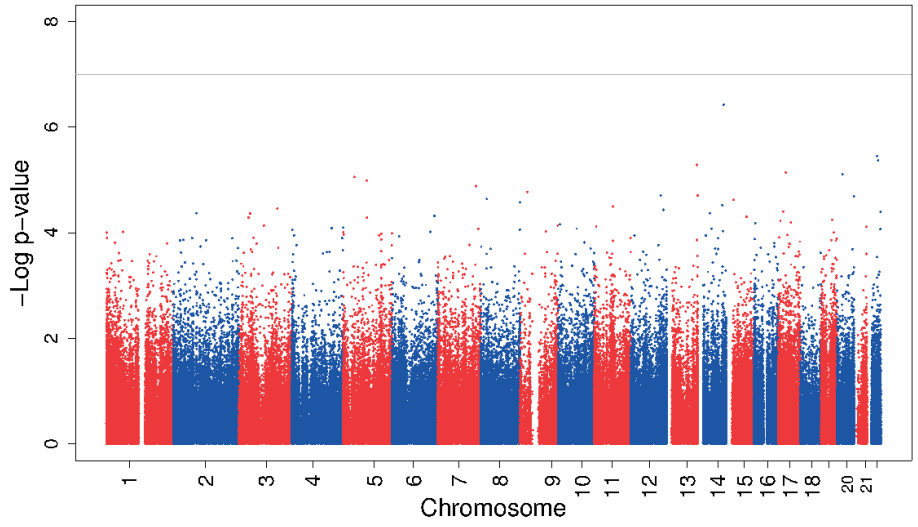
Findings were consistent with the main results when restricted to up to 3,657 participants of European ethnicity (Pearson correlation coefficients of the effect estimates across all CpG sites were 0.86-0.97 and were 0.99 across top CpG sites (p-value < $1x10^{-4}$) for all models (**Table S5A-C**). Similarly, when the studies with a high percentage (>30%) of children with overweight or obesity were excluded, results were also consistent with the main analyses (Pearson correlation coefficients of the effect estimates across all CpG sites were 0.89-0.98 and were 0.99 across top CpG sites (p-value <$1x10^{-4}$) for all models (**Table S6A-C)**. Lastly, when the studies of non-Europeans participants, a high percentage of children with overweight or obesity and studies in which the sample was selected on or enriched for any particular exposure or outcome were all excluded, results remained strongly correlated to those from the main models. Pearson correlation coefficients of the effect estimates across all CpG sites were 0.64-0.97 and 0.95-0.99 across top CpG sites (p-value < 1x10-4) for all models (**Table S7A-D**).

Comparison with previous findings

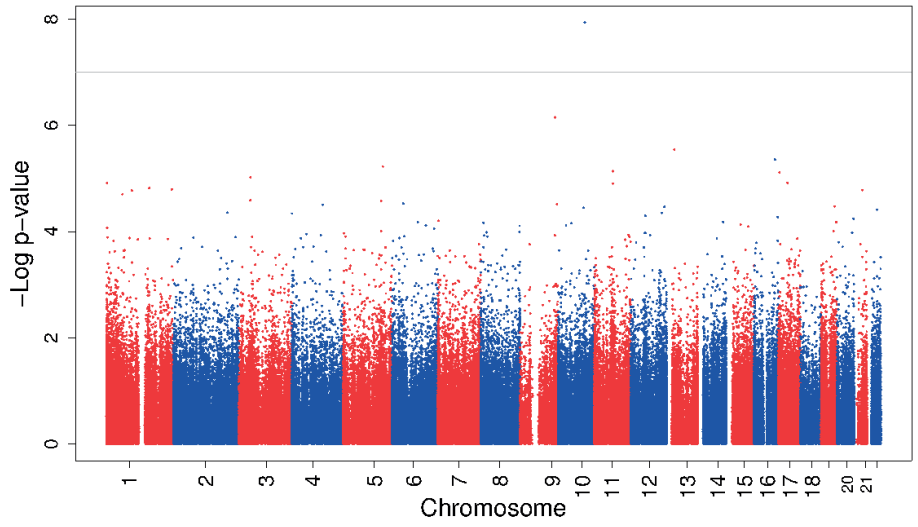
Most CpGs identified to be associated with BMI in previous candidate-gene studies or smaller EWASs (N <1,000) did not replicate in our results (**Table S8**).[7, 15, 17, 18, 20, 21, 23, 24, 55]
When comparing the genome-wide significant findings from the largest BMI EWASs (N > 1,000) in adults to our most significant findings across the four age ranges, we found an increasing overlap with age (**Table 3 and Table S9**).[5, 6, 9, 25, 26, 56-59] We used two cutoffs to

Figure 1. Manhattan plots for the meta-analyses of DNA methylation and childhood or adolescent BMI

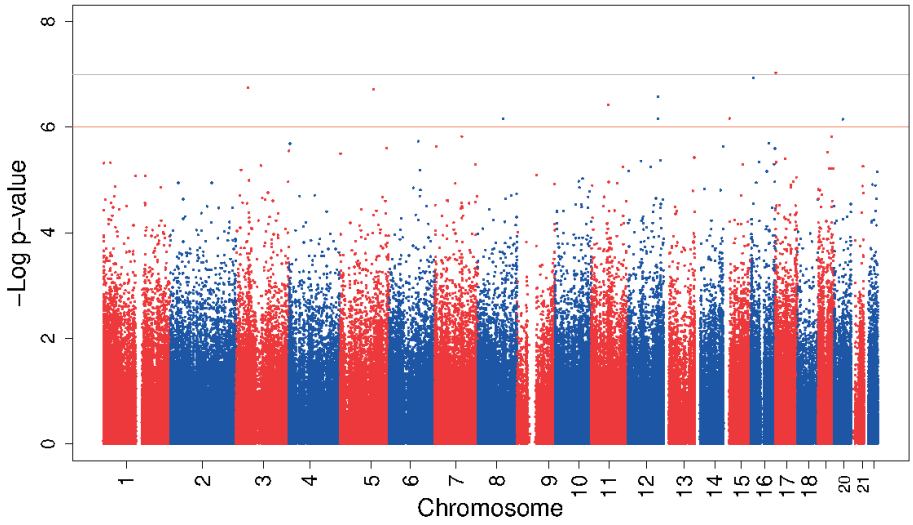
A



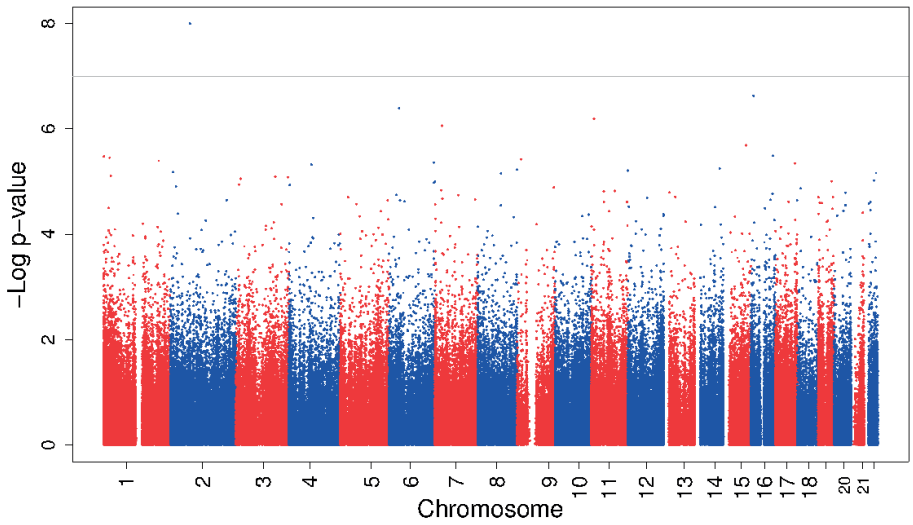
B



C



D



Manhattan plots showing the meta-analysis results for associations of DNA methylation in cord blood with early childhood BMI (Figure 1A) and late childhood BMI (Figure1B), of DNA methylation in whole blood in childhood with childhood BMI (Figure 1C) and of DNA methylation in whole blood in adolescence with adolescent BMI (Figure 1D). The grey line shows the Bonferroni-corrected significance threshold for multiple testing ($p<1.06x10^{-7}$). The orange line shows the FDR-corrected significance threshold for multiple testing.

Table 3. Absolute number of overlapping CpGs and P-values for enrichment of significant CpGs from previous EWASs (N > 1,000) in our data

Previous study (N sites associated with BMI)	Significance level	Analysis A Association of cord blood DNA methylation with early childhood BMI (2-5y)	Analysis B Association of cord blood DNA methylation with late childhood BMI (5-10y)	Analysis C Cross-sectional analysis of whole blood DNA methylation with childhood BMI (2-10y)	Analysis D Cross-sectional analysis of whole blood DNA methylation with adolescent BMI (12-18y)
	$1x10^{-5}$	**N = 7**	**N = 8**	**N = 51**	**N = 26**
	0.05	**N = 22,687**	**N = 20,645**	**N = 37,074**	**N = 25,292**
Ali et al.[56] (3 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$
	0.05	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	1/3 $P_{enrichment} = 0.24$	0 $P_{enrichment} = 1$
Aslibekyan et al.[6] (8 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$
	0.05	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	1/8 $P_{enrichment} = 0.51$	2/8 $P_{enrichment} = 0.08$
Campanella et al.[57] (26 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	**1/26** **$P_{enrichment} = 0.002$**
	0.05	3/26 $P_{enrichment} = 0.16$	1/26 $P_{enrichment} = 0.72$	**6/26** **$P_{enrichment} = 0.02$**	**11/26** **$P_{enrichment} = 1.006 \times 10^{-7}$**
Geurts et al.[58] (310 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	**2/310** **$P_{enrichment} = 0.0006$**	**2/310** **$P_{enrichment} = 0.0002$**
	0.05	12/310 $P_{enrichment} = 0.90$	13/310 $P_{enrichment} = 0.73$	**103/310** **$P_{enrichment} = 3.92 \times 10^{-34}$**	**125/310** **$P_{enrichment} = 6.63 \times 10^{-70}$**
Mendelson et al.[9] (83 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	**2/83** **$P_{enrichment} = 4.67 \times 10^{-5}$**	**3/83** **$P_{enrichment} = 1.81 \times 10^{-8}$**
	0.05	4/83 $P_{enrichment} = 0.64$	**8/83** **$P_{enrichment} = 0.045$**	**28/83** **$P_{enrichment} = 1.36 \times 10^{-10}$**	**45/83** **$P_{enrichment} = 3.02 \times 10^{-33}$**

Sayols-Baixeras et al.[59] (96 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$
	0.05	8/96 $P_{enrichment} = 0.13$	**9/96** **$P_{enrichment} = 0.04$**	**24/96** **$P_{enrichment} = 1.53 \times 10^{-6}$**	**30/96** **$P_{enrichment} = 1.85 \times 10^{-14}$**
Sun et al.[26] *black participants* (36 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	**1/36** **$P_{enrichment} = 0.002$**
	0.05	3/36 $P_{enrichment} = 0.30$	**6/36** **$P_{enrichment} = 0.007$**	**13/36** **$P_{enrichment} = 4.98 \times 10^{-6}$**	**22/36** **$P_{enrichment} = 1.50 \times 10^{-18}$**
Sun et al.[26] *white participants* (349 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$
	0.05	12/349 $P_{enrichment} = 0.959$	22/349 $P_{enrichment} = 0.12$	**86/349** **$P_{enrichment} = 4.13 \times 10^{-19}$**	**116/349** **$P_{enrichment} = 1.75 \times 10^{-54}$**
Wahl et al.[5] (187 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	2/187 $P_{enrichment} = 0.0002$	**3/187** **$P_{enrichment} = 2.10 \times 10^{-7}$**
	0.05	8/187 $P_{enrichment} = 0.77$	11/187 $P_{enrichment} = 0.29$	**61/187** **$P_{enrichment} = 1.97 \times 10^{-20}$**	**77/187** **$P_{enrichment} = 1.68 \times 10^{-44}$**
Wang et al.[25] (54 CpGs)	$1x10^{-5}$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	0 $P_{enrichment} = 1$	**1/54** **$P_{enrichment} = 0.003$**
	0.05	2/54 $P_{enrichment} = 0.79$	4/54 $P_{enrichment} = 0.26$	**23/54** **$P_{enrichment} = 2.49 \times 10^{-11}$**	**33/54** **$P_{enrichment} = 3.98 \times 10^{-27}$**
N CpGs in ≥ 2 adult studies	0.05	9/52 (17.3%)	23/75 (30.7%)	98/347 (28.2%)	163/465 (35.1%)

Two cut-offs were used to select the significant findings in our results: a P-value < $1x10^{-5}$, to identify "suggestive" findings, and a less stringent, nominal P-value < 0.05, to identify any trends. We used a hypergeometric test to calculate enrichment with the phyper function in the R Stats package in R. Results in bold are nominally significant. Of those findings from adult studies that had a nominal p-value (< 0.05) in our models, 17-35% were reported by more than one adult study.

select the most significant findings in our results: a P-value < $1x10^{-5}$, to identify "suggestive" findings, and a less stringent, nominal P-value < 0.05. The number of CpGs that met these criteria are provided in **Table 3**. First, we examined the absolute number of overlapping CpGs between the studies in adults and our findings with a P-value < $1x10^{-5}$ and calculated enrichment. With advancing age across childhood and adolescence, we observed increasing enrichment for the 187 CpGs previously reported to be associated with adult BMI in the largest study to date (N = 10,261).[5] For the two cord blood models, there was no overlap with the adult findings ($P_{enrichment}$ = 1), for the cross-sectional model in childhood 2/187 adult hits overlapped, ($P_{enrichment}$ = 0.0002), and for the cross-sectional model in adolescence 3/187 overlapped ($P_{enrichment}$ = $2.10x10^{-7}$) (**Table 3 and Table S9**). Using the less stringent cut-off (P-value < 0.05) this trend was even clearer. The overlap between the 187 CpGs from the adult EWAS and the CpGs in our data with a P-value <0.05 was 8/187 CpGs ($P_{enrichment}$ = 0.77, Analysis A) for the association of cord blood DNA methylation and early childhood BMI and 11/187 CpGs ($P_{enrichment}$ = 0.30, Analysis B) for the association of cord blood DNA methylation and late childhood BMI. For the cross-sectional model in childhood, the overlap was 61/187 CpGs ($P_{enrichment}$ = $1.97x10^{-20}$, Analysis C), and in adolescence, the overlap was 77/187 CpGs ($P_{enrichment}$ = $1.68x10^{-44}$, Analysis D) (**Table 3 and Table S9**). Twenty-seven CpGs were among the enriched CpGs in both the childhood and the adolescent model. This overlap was not significant (p = 0.88).

Correlation coefficients between the effect estimates of the 187 hits and the effect estimates for those CpGs in the four models increased with age (Analysis A = -0.186 (p = 0.01), Analysis B = -0.013 (p = 0.86), Analysis C = 0.604 (p = $5.31x10^{-20}$) and Analysis D = 0.816 (p = $7.89x10^{-46}$). The difference in correlation coefficients was significant for all comparisons (p's for comparison between correlation coefficients < 0.01) except for the comparison between models A and B (p = 0.09).

Effect sizes of the associations for these 187 adult BMI CpGs in our analyses increased with advancing age of children in our analyses (**Supplementary Information, Figure S5**). We found similar trends for enrichment in other EWASs in adults and adolescents (**Table 3**).[6, 9, 25, 26, 56-59] Of those findings from adult studies that had a nominal p-value (< 0.05) in our models, 17-35% were reported by more than one adult study. Most of these were found in two or three studies, but four, cg06500161, cg19750657, cg12593793, and cg18181703, were reported in six or seven previous analyses.

Functional analyses

A functional enrichment analysis using genes linked to the CpGs with p-values < $1\text{-}x10^{-4}$ in each of the models showed no functional enrichment of Gene Ontology (GO) terms or Kyoto Encyclopedia of Genes and Genomes (KEGG) terms (FDR <0.05) (**Table S10**).

DISCUSSION

In this large meta-analysis of EWASs of childhood and adolescent BMI, we found little evidence of an association between DNA methylation and childhood or adolescent BMI. DNA methylation at three different CpGs, each one in a different age range, was associated with BMI in early life. With advancing age of children in our analyses, we observed increasing enrichment of CpGs previously identified for their relation with adolescent or adult adiposity. In addition, for the 187 CpGs identified in the largest previous study of adult BMI, we found increasing effect sizes and increasing correlations between the adult effect sizes and those in our analyses, with age.

Interpretation of main findings

Childhood obesity is a major public health problem and associated with short- and long-term morbidity and mortality.[61] Although there is some evidence from previous studies that DNA methylation may mediate associations of pregnancy-related exposures with offspring adiposity, only few specific CpG sites have been identified.[4, 27] Thus far, most of the evidence for associations of DNA methylation with adiposity stems from adult studies.

In this study, we found little evidence of an association between DNA methylation and childhood or adolescent BMI. DNA methylation at three CpGs (cg05937453, cg25212453 and cg10040131), each in a different age range, was associated with BMI at Bonferroni significance, $P < 1.06 \times 10^{-7}$. However, we did observe increasing enrichment and increasing point estimates of CpGs previously reported in relation to adult adiposity, with increasing age of the participants in our study. [5, 6, 9, 25, 26, 57-59] Also, correlation coefficients between effect estimates from the adult study and effect estimates in our models increased with age of the participants in our study. After exclusion of invariable probes (N = 114,204) using an adult reference the trend of increasing enrichment of CpGs associated with adult adiposity with advancing age remained. This result suggests that probes reported to be invariable in adults, did not strongly affect the results of the enrichment analyses.[62] These trends were most clearly seen in the cross-sectional analyses in childhood and adolescence, although there was no significant overlap in the enriched CpGs between the two time points. This trend may partly be explained by a difference in study sample size, age range, and covariates between the models. These findings may indicate that over time, exposure to higher “levels” of BMI may lead to differential DNA methylation. DNA methylation has been shown to be responsive to the environment and could also change in response to metabolic changes and the altered adipokine/cytokine environment associated with a higher BMI.[63-65] Methylation differences may be either induced by the altered environment, or result from cellular selection in this altered environment. If differential DNA methylation is the result of exposure to higher BMI, it

may be part of a pathway that links adiposity to metabolic and cardiovascular disease.[5, 7] Several studies have reported that DNA methylation levels at obesity-associated CpG sites were associated with cardio-metabolic factors such as lipids, insulin resistance and blood pressure.[26, 64]

Recent studies, using methods such as Mendelian Randomization, suggested that alterations in DNA methylation are predominantly a consequence of adiposity, rather than a cause.[5, 7, 9, 26] In these studies, Mendelian randomization was used to investigate the potential causal relationships, independent of unmeasured confounders, between DNA methylation and BMI using genetic variants as instrumental variables.[66, 67] Although in our study, we cannot determine whether any of the associations are causal, our results may be compatible with this hypothesis. One alternative explanation for the increasing enrichment of CpGs previously reported in relation to adult and adolescent adiposity with age in our data could be that BMI at different ages does not represent the same biological phenotype. The DNA methylation profile may simply reflect the transition of childhood BMI into a different, more adult-like BMI-phenotype over time. BMI (weight(kg)/height(m^2)) is likely to have a different biological interpretation at different ages and with increase of age the biological phenotype becomes more similar to adult BMI.[68] DNA methylation at specific CpG sites is known to change with age. We did not see any increased enrichment of age-related CpGs identified in previous childhood and adolescent studies with advancing age in our models (all p-values > 0.19), making it unlikely that our results represent a strong effect of age.[69, 70]

We observed only three CpGs at which DNA methylation in three different age ranges was Bonferroni-significantly associated with BMI in childhood or adolescence. Cg05937453, at which DNA methylation in cord blood was associated with late childhood BMI is annotated to Secreted frizzled-relate protein 5 *(SFRP5)*. This gene is part of the *SFRP* family that acts by modulating Wnt signal transduction.[71] The Wnt family and SFRPs have roles in multiple biological processes, including embryonic development, inflammation, and immunity.[72] *SFRP5* is an anti-inflammatory adipokine that may be induced during preadipocyte proliferation, differentiation and maturation.[65, 72] Less is known about the other two CpGs, cg25212453 and cg10040131, and their potential relation to adiposity. In the cross-sectional analyses in childhood, DNA methylation at cg25212453, in the gene body of Solute Carrier Family 43 Member 2 (*SLC43A2)*, was associated with BMI. *SLC43A2* transcripts have been described to be associated with fasting insulin in a whole blood transcriptome-wide association analysis of three cohort studies.[73] DNA methylation at cg10040131, located in the gene body of Sideroflexin 5 *(SFXN5)*, was associated with BMI in adolescence. *SFXN5* has not been described in relation to adiposity or related phenotypes.

Based on histone marks mapped by Roadmap Epigenomics Data Complete Collection extracted from the UCSC Genome Browser, all 3 CpG-sites coincide with a region of weak

transcription in blood, and 2 CpG-sites coincide with a region of weak transcription in adipose tissue, except for cg25212453 (at *SLC43A2)* which coincides with an enhancer in adipose tissue.[74] This overlap with key regulatory elements may indicate that DNA methylation at these CpGs could have regulatory consequences.[75, 76]

Many previous studies that examined the associations between DNA methylation and childhood BMI were not genome-wide, were of modest sample size or used only FDR or less stringent cut-offs for significance.[10-13, 18, 77] Previous candidate-gene studies reported that methylation of CpGs annotated to Proopiomelanocortin (*POMC),* Retinoid X Receptor Alpha *(RXRA)* and Nitric Oxide Synthase 3 (*NOS3 or eNOS)* was associated with BMI in childhood.[17, 24] The exact CpGs from those studies were either not given or were not present on the 450 K Illumina array and could thus not be examined in our data. However, none of the CpGs in our dataset that annotated to these genes were associated with BMI in our analyses.[17, 24] Also, methylation at CpGs in hypoxia-inducible factor 3A (*HIF3A),* previously reported to be differentially methylated in relation to BMI in adults and children, did not show any association with BMI in childhood or adolescence in our data.[7, 20, 21, 23] This finding is in concordance with two recently published studies, both in approximately 1000 participants, which did not find an association between childhood BMI and methylation at *HIF3A*.[21, 22]

Strengths and limitations

This EWAS is much larger than the previous genome-wide studies of the association between DNA methylation and BMI in childhood and adolescence. Other strengths of this study are the extensive analyses from 2-18 years, both longitudinal and cross-sectional. We also used a harmonized analysis plan and robust methods in the PACE consortium. However, compared to studies in adults, the sample size of this meta-analysis is still modest. All participating studies used the Infinium Human Methylation 450K array, which covers only 1.7% of all CpG sites in the genome.[78] Thus, we cannot exclude that methylation at other, non-measured CpGs could be associated with childhood BMI. The 450K BeadChip has now been replaced by the EPIC BeadChip which includes > 850,000 CpG sites (Illumina, San Diego, California, USA).[78, 79] Some previous literature included one of the participating studies in this manuscript. We analyzed the associations between DNA methylation and BMI at different times in childhood and adolescence but did not study longitudinal changes in DNA methylation in the same individuals from early life until adulthood in relation to BMI. A recent study among 1,485 adults performed a cross-lagged analyses of DNA methylation and BMI, both measured at two time points.[26] These analyses showed significant unidirectional paths from BMI to DNA methylation, in line with other, cross-sectional adult studies.[5, 7] We used blood to measure DNA methylation patterns in relation to BMI, which may not be the most relevant tissue. As overweight and obesity are associated with an inflammatory phenotype in blood and

may affect the white blood cell composition, blood may be a relevant target tissue.[80] However, there are many potentially relevant target tissues related to BMI, including the brain, adipocytes, pancreas liver, and many others, and associations of DNA methylation with BMI may differ between these tissues. In large population-based studies, it is virtually impossible to collect samples from these tissues. A study among adults examined whether the associations of DNA methylation at a specific CpG in blood and adipose tissue in relation to BMI were comparable and showed similar findings between the tissues.[7] We adjusted our childhood and adolescent analyses for estimated cell type proportions using an adult reference dataset, which is likely not an optimal way to adjust for white blood cell proportions at these ages. However, to the best of our knowledge, no childhood- or adolescent-specific reference panels exist.[37, 40] Thus, we may have been unable to fully account for potential differences in biology of blood at the different ages, which may have had some influence on our results. Specific cord blood reference datasets only became available after completion of the cohort-specific analyses.[41, 81] However, we observed no substantial differences in results in two of our largest studies, Generation R (maximum N = 789) and ALSPAC (maximum N = 669), when comparing our main analyses using the adult reference with the same analyses using cell counts estimated with a cord blood-specific reference panel.[37, 41] Correlation coefficients of the effect estimates of the analyses using the adult and cord blood-specific reference panel across all 450k CpG sites were r = 0.98 and r = 0.89, respectively. Childhood BMI is influenced by genetic, prenatal and postnatal environmental factors. We adjusted for a large number of potential confounding factors. However, residual confounding due to other, non-measured factors, might still be present. Individual studies contributing to this meta-analysis performed their own preferred quality control and methylation normalization process. We have previously shown that this does not have a large effect on the associations of interest compared to the use of non-normalized methylation data.[82] Meta-analyzing results of 23 studies may introduce between-study heterogeneity. We ran multiple sensitivity analyses, which showed results that were comparable with the main findings. Based on I^2 values, most top CpGs did not show large between-study heterogeneity, although three FDR-significant findings did. These three CpG sites had I^2 values of 50.2, 52.7 and 61.8. Forest plots and leave-one-out plots did not show large heterogeneity or an extreme effect of one study (forest plots, **Supplementary Information, Figures S3H, I and K**). The current analyses cannot determine whether any of the associations are causal. Future research using methods such as Mendelian Randomization could shed further light on causality, already used by some studies in adults.[5, 9, 21, 83] Analyzing associations of BMI with DNA methylation assessed with the EPIC BeadChip could provide new insights, as it interrogates almost twice the number of CpG sites compared to the 450K BeadChip, and particularly focuses on CpG sites in potential regulatory regions.[78, 79] Also, bisulfite sequencing methods to measure DNA

methylation could provide more detailed information. In the current study, we analyzed differential methylation at single CpGs. Future studies could analyze regional patterns of differential methylation (differentially methylated regions, DMRs) and their associations with BMI to provide further biological insights. We studied BMI mostly in general population samples. If exposure to overweight already changes the DNA methylation profile in childhood or adolescence, it would be interesting to analyze the associations in a population with a more extreme phenotype of obesity in childhood or adolescence. To examine effects of potential interventions, studies of DNA methylation before and after weight loss in children or adolescents could be useful. In adults, weight loss has been shown to be associated with significantly different DNA methylation patterns.[84-86] Analyzing longitudinal trajectories of DNA methylation and BMI at various time-points in the same population from birth to adolescence would help to understand further the biological relevance of DNA methylation level changes and patterns of change.[26, 87]

CONCLUSIONS

In this large epigenome-wide association study meta-analysis among children and adolescents, we observed little evidence for associations between DNA methylation at individual CpGs and childhood or adolescent BMI. With advancing age across childhood and adolescence, we observed increasing effect estimates, increasing correlations between adult effect sizes and those in our analyses, and increasing enrichment of CpGs previously identified for their associations with adult adiposity. These findings may be compatible with the hypothesis that DNA methylation differences are mostly a consequence rather than a cause of obesity, but this remains to be confirmed.

Detailed acknowledgements and Supplementary Data can be found in the published article online: https://pubmed.ncbi.nlm.nih.gov/33239103/

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SUPPLEMENTARY INFORMATION

Figure S1A-D. QQ plots and genomic inflation factors (lambdas) of all four analyses: the associations of DNA methylation in cord blood with early childhood BMI (Fig. S1A) and late childhood BMI (Fig. S1B), of DNA methylation in whole blood in childhood with childhood BMI (Fig. S1C) and of DNA methylation in whole blood in adolescence with adolescent BMI (Fig. S1D).

Figure S2A-D. Volcano plots showing methylation levels in association with childhood or adolescent BMI of all four analyses: the associations of DNA methylation in cord blood with early childhood BMI (Fig. S2A) and late childhood BMI (Fig. S2B), of DNA methylation in whole blood in childhood with childhood BMI (Fig. S2C) and of DNA methylation in whole blood in adolescence with adolescent BMI (Fig. S2D).

Figure S3A-L. Forest plots for the genome-wide Bonferroni- and FDR- significantly associated CpGs in the analyses of cord blood DNA methylation and later childhood BMI (Fig. S3A), DNA methylation in whole blood in childhood and childhood BMI (Fig. S3B-K) and DNA methylation in whole blood in adolescence and adolescent BMI (Fig. S3L).

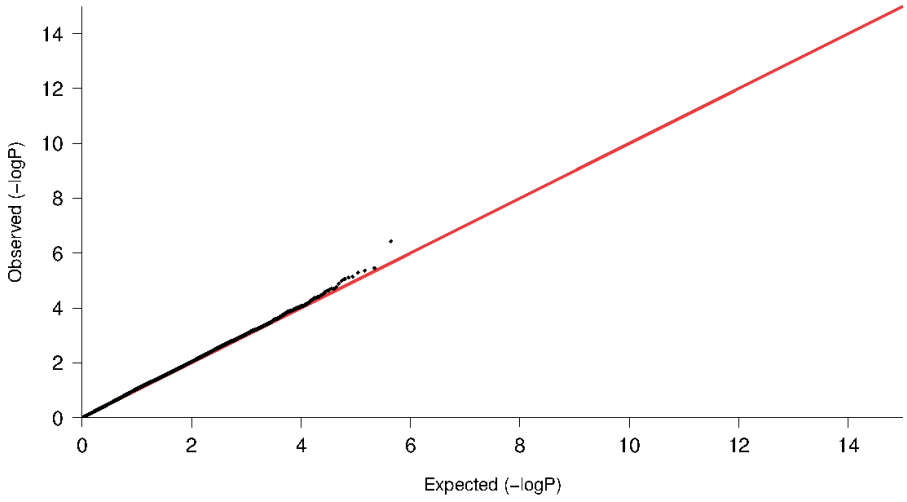
Figure S4A-L. Leave-one-out plots for the genome-wide Bonferroni- and FDR- significantly associated CpGs in the analyses of cord blood DNA methylation and later childhood BMI (Fig. S4A), DNA methylation in whole blood in childhood and childhood BMI (Fig. S4B-K) and DNA methylation in whole blood in adolescence and adolescent BMI (Fig. S4L), showing the results after omitting one study at a time.

Figure S5. Boxplots showing the distribution of effect sizes of the 187 CpGs significantly associated with adult BMI in a previous study for the four analyses for the four models, separately for CpGs with positive and negative effect estimates in the original analysis.[1]

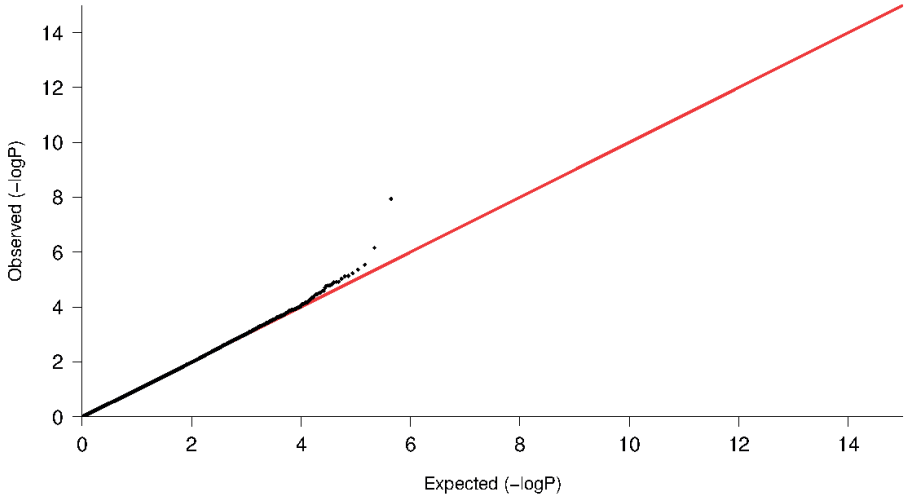
Figure S6A-D. Density plots performed within the Generation R Study for those FDR-significant CpGs that are flagged in the main meta-analyses tables as potential polymorphic sites.

Figure S1A-D. QQ plots and genomic inflation factors (lambdas) of all four analyses: the associations of DNA methylation in cord blood with early childhood BMI (Fig. S1A) and late childhood BMI (Fig. S1B), of DNA methylation in whole blood in childhood with childhood BMI (Fig. S1C) and of DNA methylation in whole blood in adolescence with adolescent BMI (Fig. 1D).

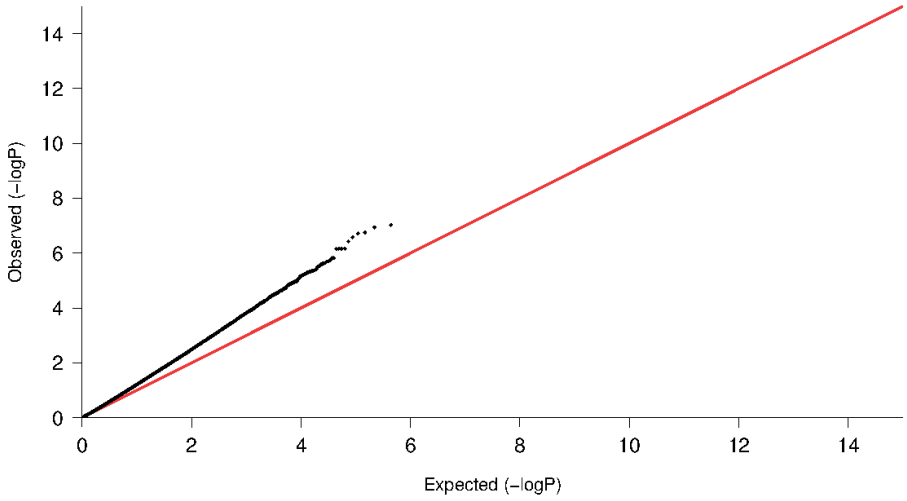
A



B



C


Observed (−logP)
Expected (−logP)
0
2
4
6
8
10
12
14

D

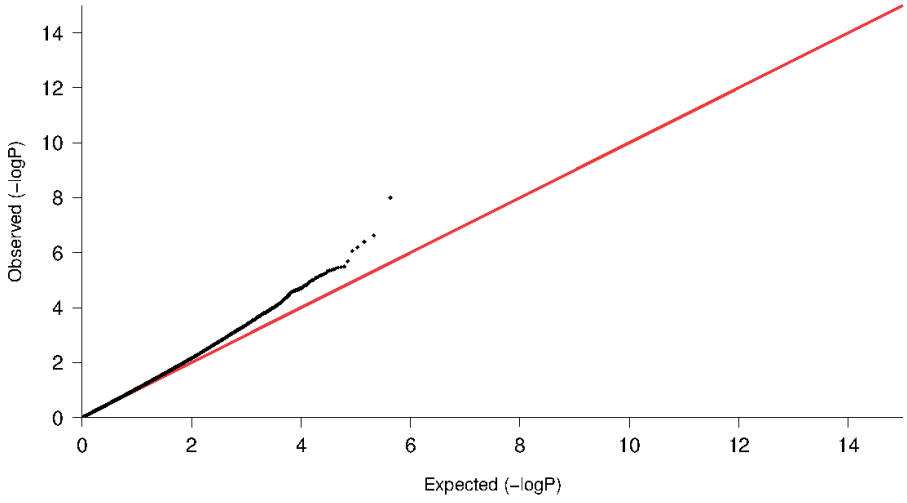
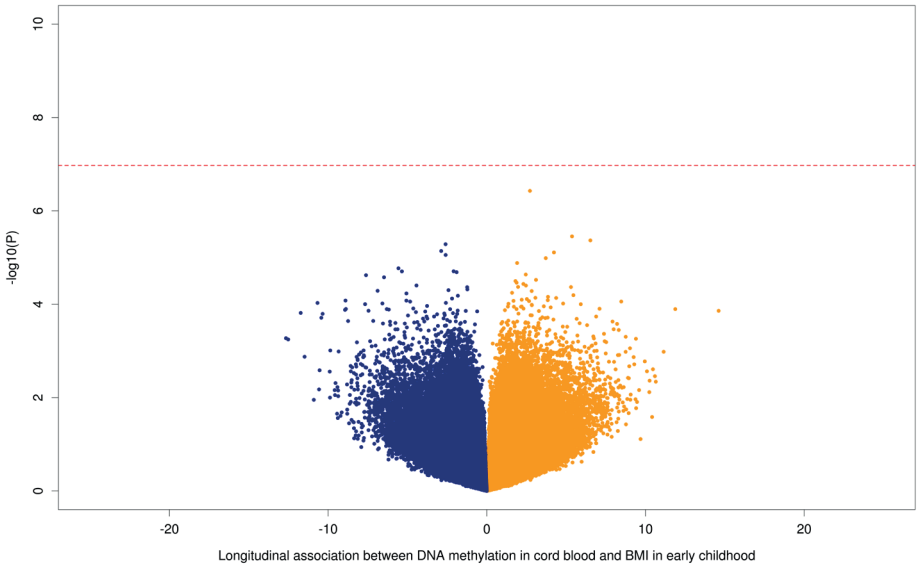

Observed (−logP)
Expected (−logP)
0
2
4
6
8
10
12
14

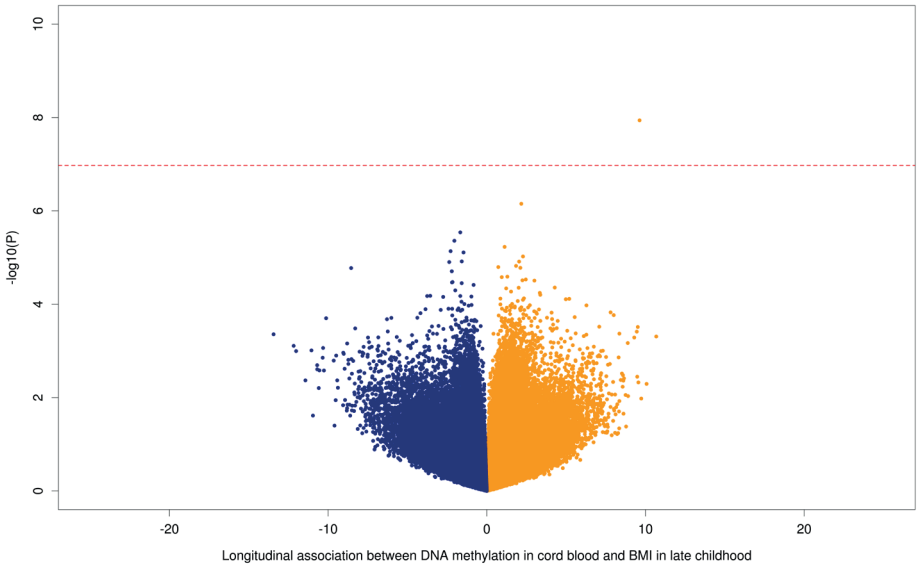
Figure S2A-D. Volcano plot for differential DNA methylation for all four analyses. The x-axes show the mean DNA methylation difference, while the y-axis shows the -log10 of the adjusted p-value for each CpG-site, representing the strength of the association. Above the dashed line indicates the CpGs that are statistically significant after Bonferroni correction ($p< 1.06x10^{-7}$).

A



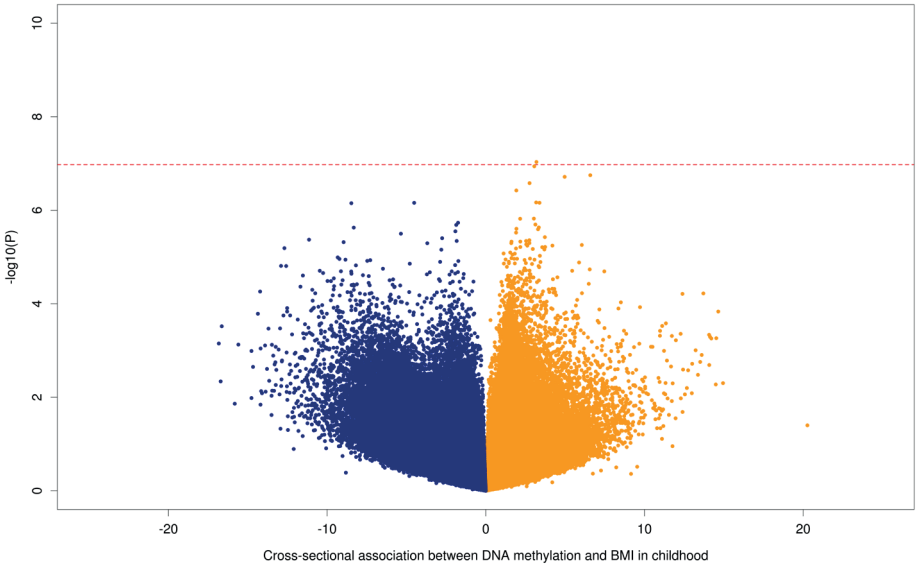
In analysis A, associations of DNA methylation in cordblood with early childhood BMI were positive for 222,659 CpGs (51.8%) and negative for 207,279 (48.2%).

B



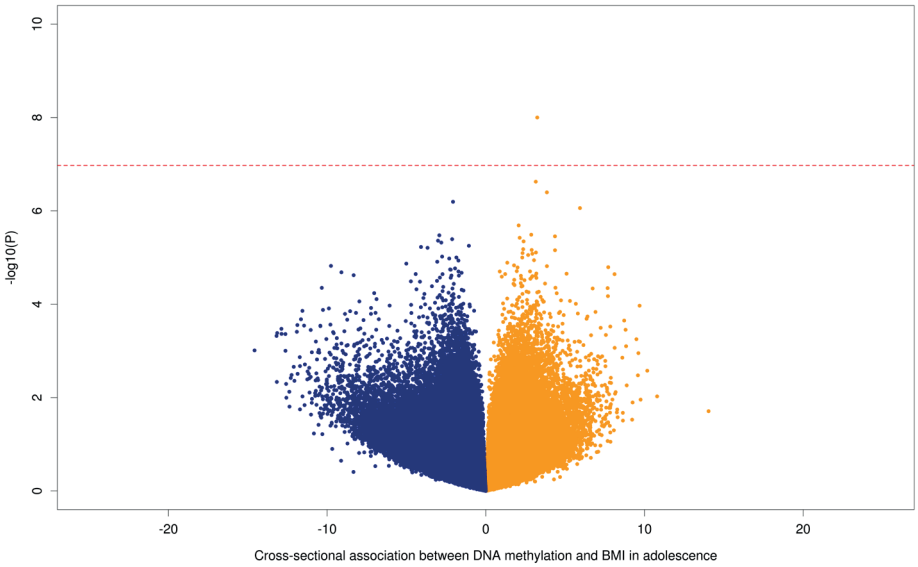
In analysis B, associations of DNA methylation in cordblood with late childhood BMI were positive for 201,602 CpGs (46.9%) and negative for 228,327 CpGs (53.1%).

C



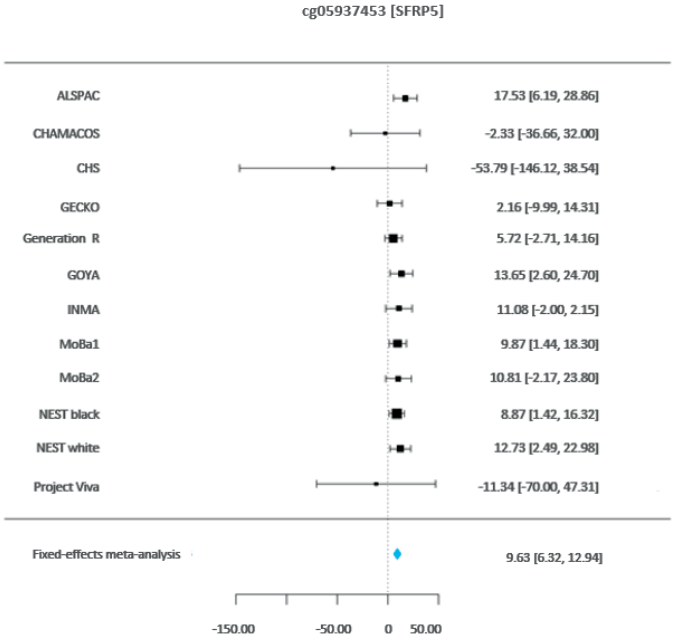
In analysis C, associations of DNA methylation in childhood with childhood BMI were positive for 217,326 CpGs (50.5%) and negative for 212,604 CpGs (49.4%).

D



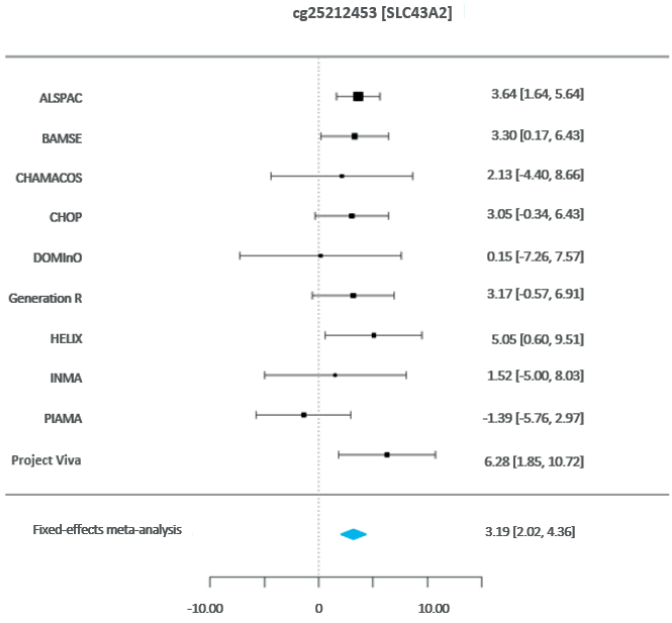
In analysis D, associations of DNA methylation in adolescence with adolescent BMI were positive for 18,716 CpGs (51.0%) and negative for 210,229 CpGs (49.0%).

Figure S3A. Forest plot for the genome-wide significantly associated CpG (cg05937453) in the analysis of cord blood DNA methylation and late childhood BMI.



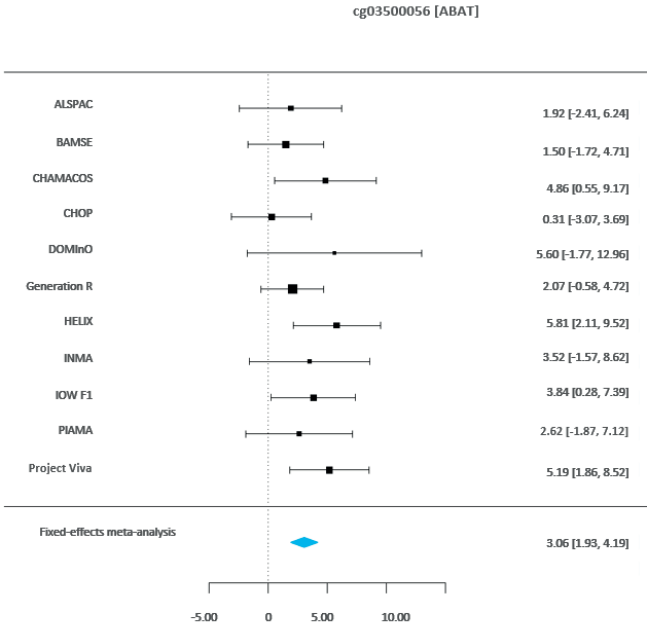
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3B. Forest plot for the genome-wide Bonferroni-significantly associated CpG (cg25212453) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



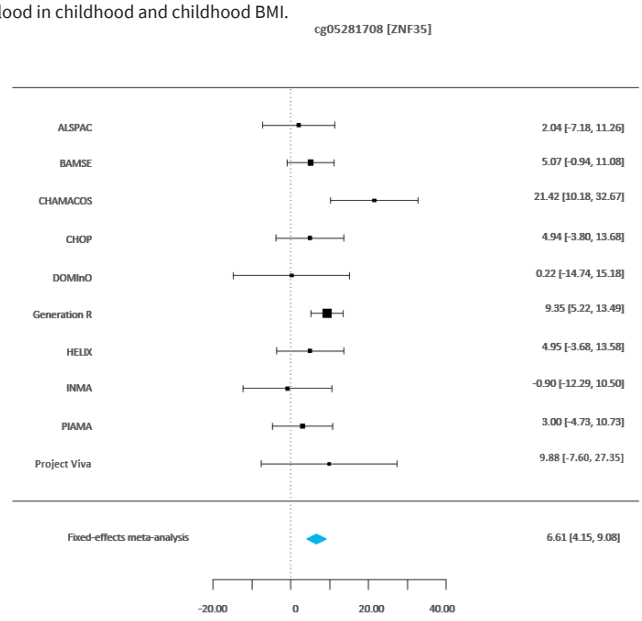
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3C. Forest plot for the genome-wide FDR-significantly associated CpG (cg03500056) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



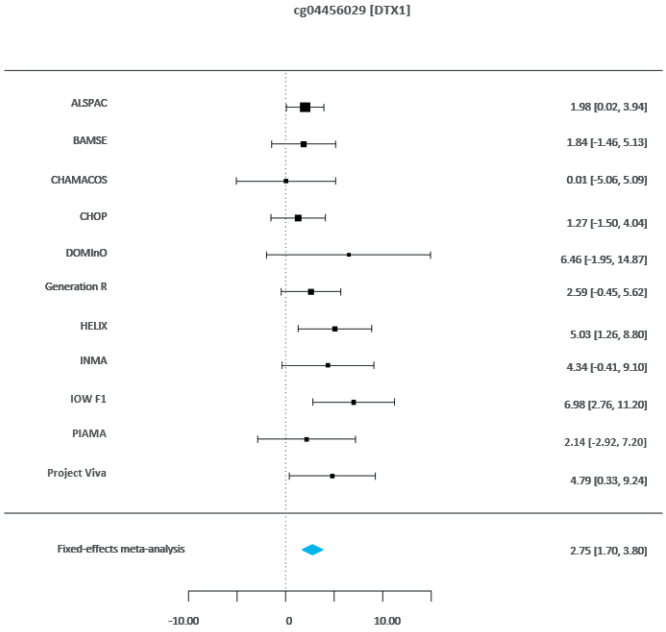
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3D. Forest plot for the genome-wide FDR-significantly associated CpG (cg05281708) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



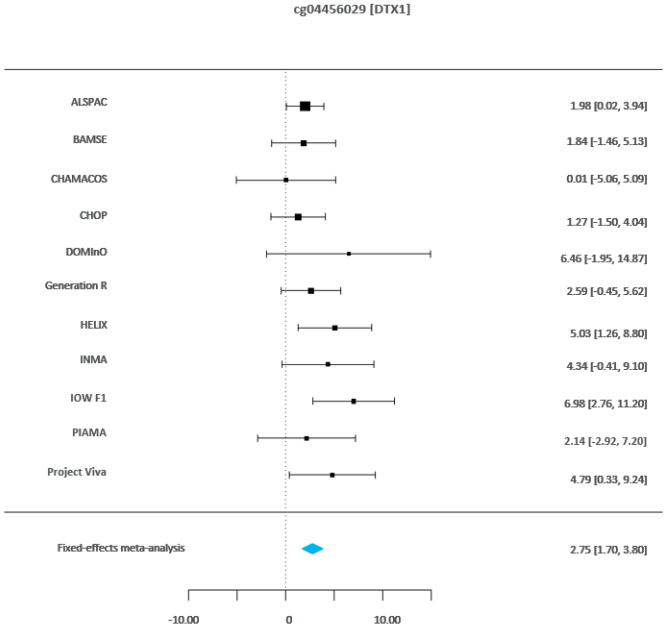
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3E. Forest plot for the genome-wide FDR-significantly associated CpG (cg15125798) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



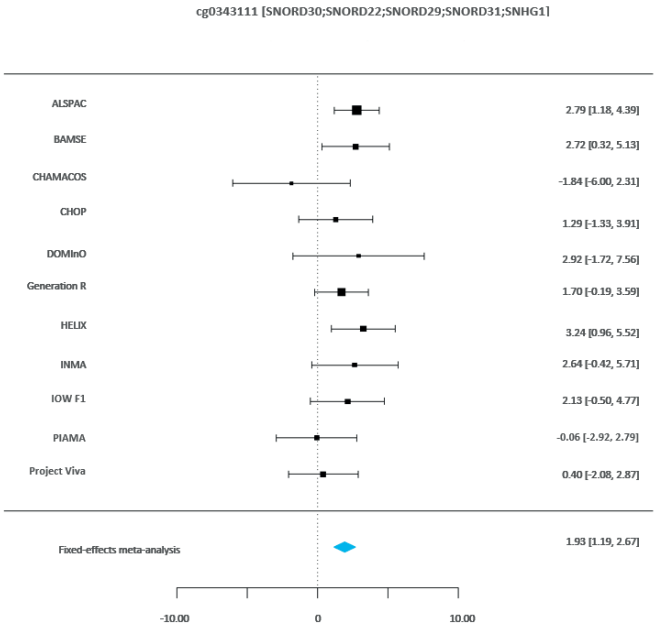
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3F. Forest plot for the genome-wide FDR-significantly associated CpG (cg04456029) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



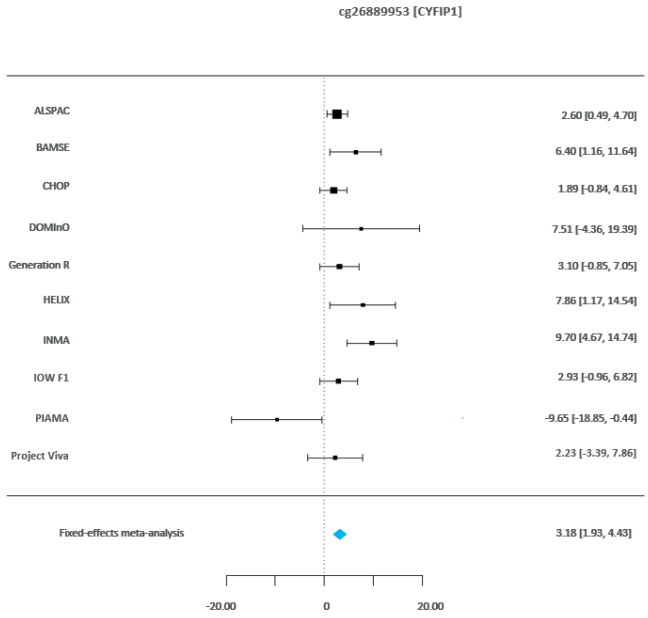
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3G. Forest plot for the genome-wide FDR-significantly associated CpG (cg0343111) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



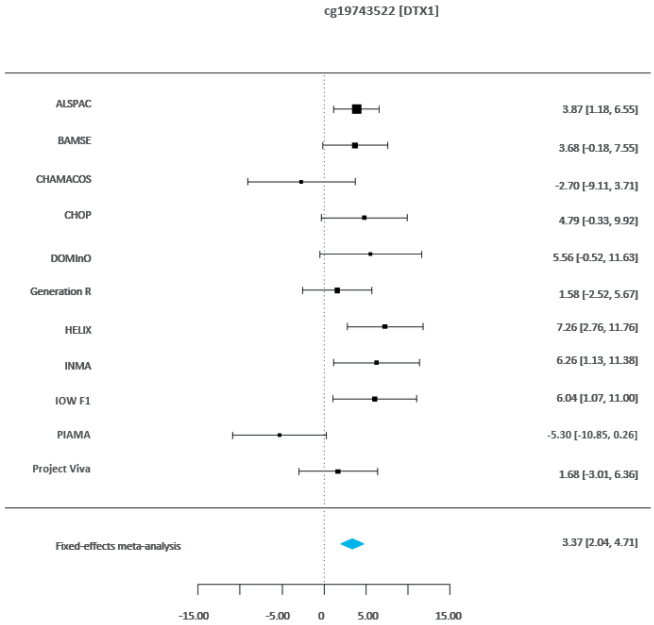
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3H. Forest plot for the genome-wide FDR-significantly associated CpG (cg26889953) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



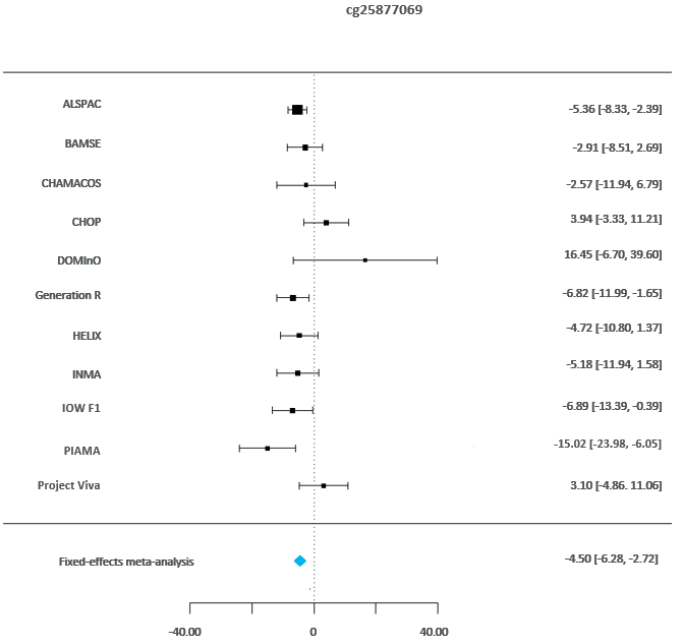
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3I. Forest plot for the genome-wide FDR-significantly associated CpG (cg19743522) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



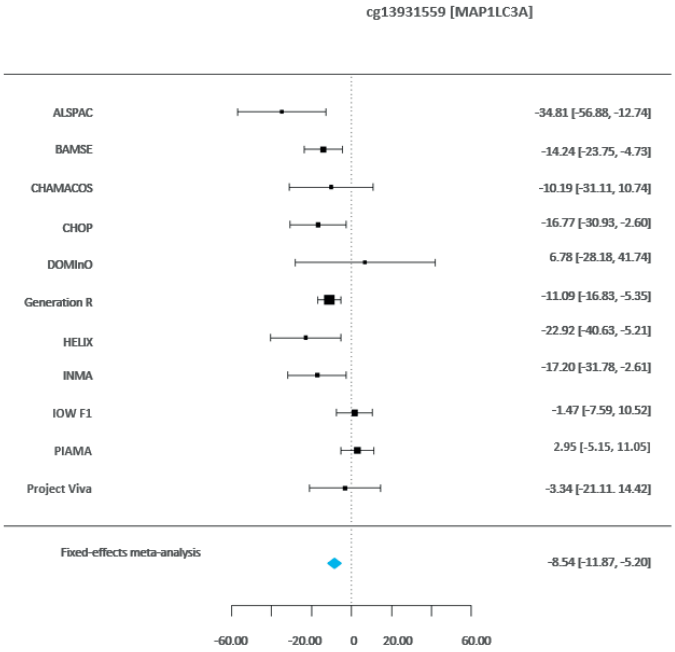
Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3J. Forest plot for the genome-wide FDR-significantly associated CpG (cg25877069) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



Change in childhood BMI-standard deviation score per 100% increase in childhood DNA methylation

Figure S3K. Forest plot for the genome-wide FDR-significantly associated CpG (cg13931559) in the analysis of DNA methylation in whole blood in childhood and childhood BMI.



Change in adolescent BMI-standard deviation score per 100% increase in adolescent DNA methylation

Figure S3L. Forest plot for the genome-wide significantly associated CpG (cg10040131) in the analysis of DNA methylation in whole blood in adolescence and adolescent BMI.

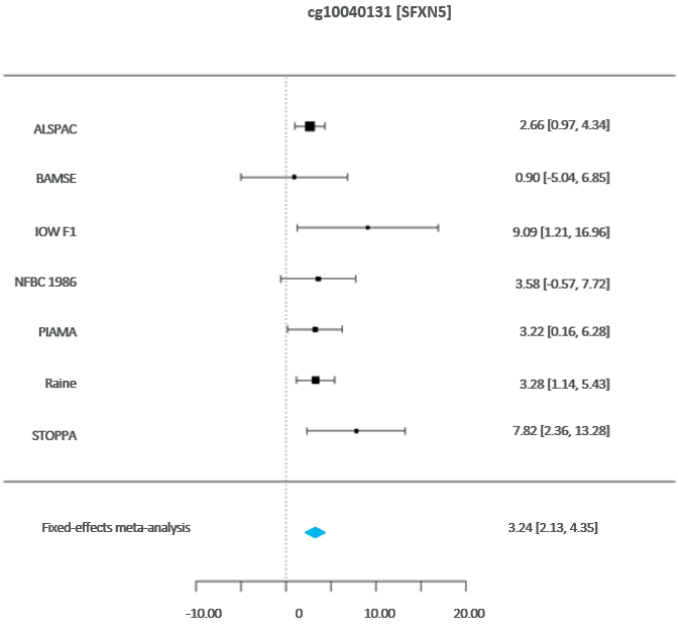


Figure S4A. Leave-one-out plot for the genome-wide Bonferroni-significantly associated cg05937453, showing the association of methylation levels in cord blood with late childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line represents the effect size of the full meta-analysis beta and the dotted red lines indicate the 20% range around that effect size.

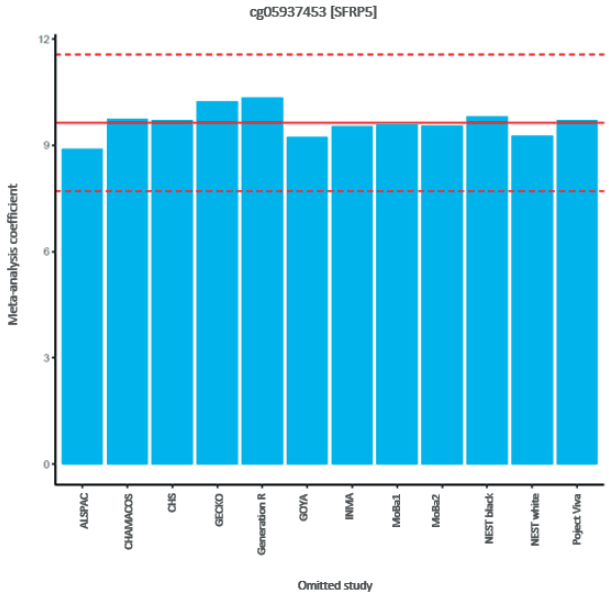


Figure S4B. Leave-one-out plot for the genome-wide Bonferroni-significantly associated cg25212453, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

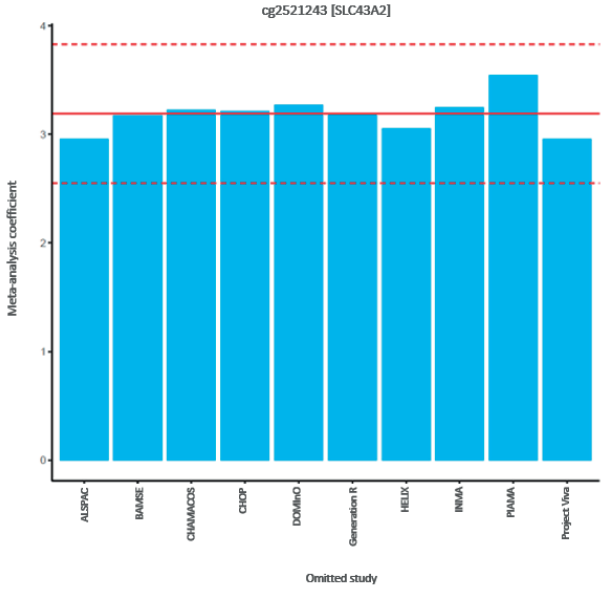


Figure S4C. Leave-one-out plot for the genome-wide FDR-significantly associated cg03500056, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

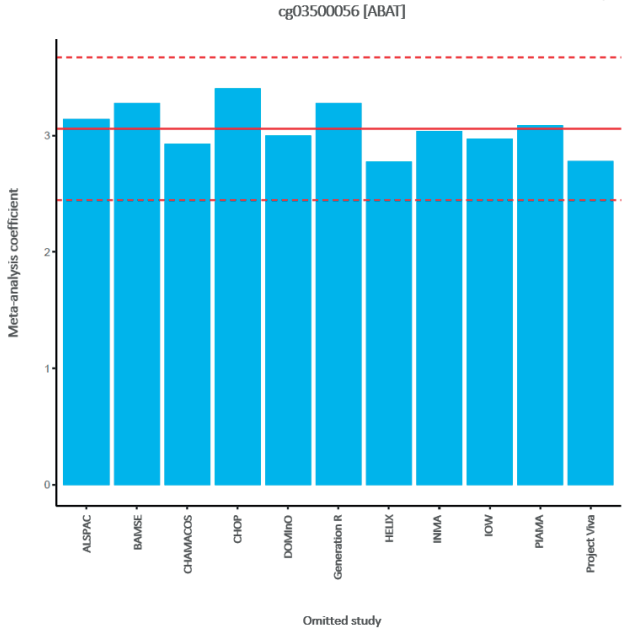


Figure S4D. Leave-one-out plot for the genome-wide FDR-significantly associated cg05281708, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

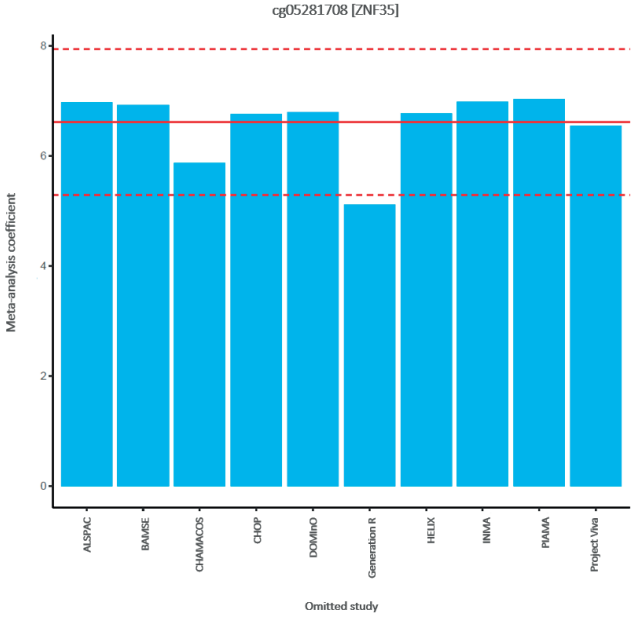


Figure S4E. Leave-one-out plot for the genome-wide FDR-significantly associated cg15125798, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

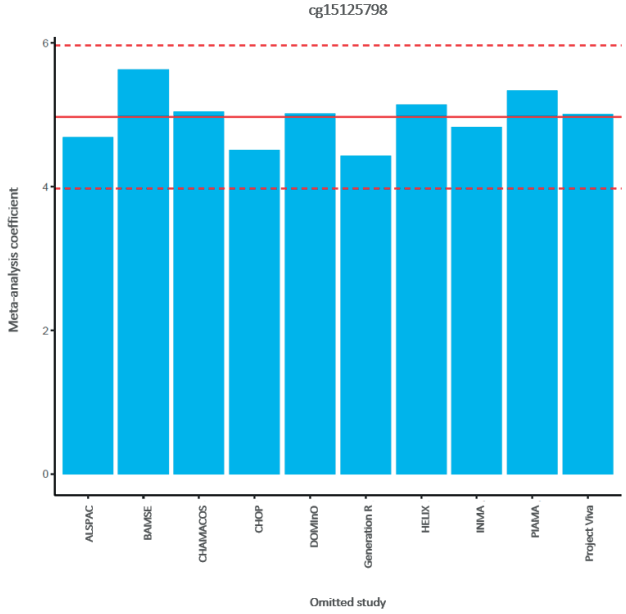


Figure S4F. Leave-one-out plot for the genome-wide FDR-significantly associated cg04456029, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

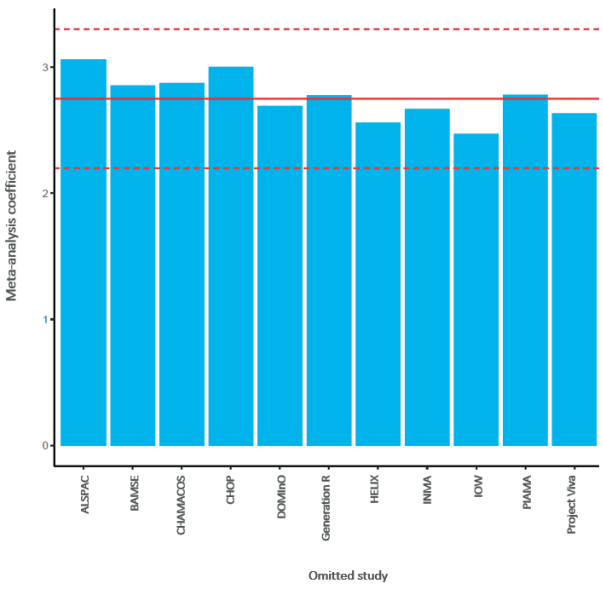


Figure S4G. Leave-one-out plot for the genome-wide FDR-significantly associated cg0343111, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

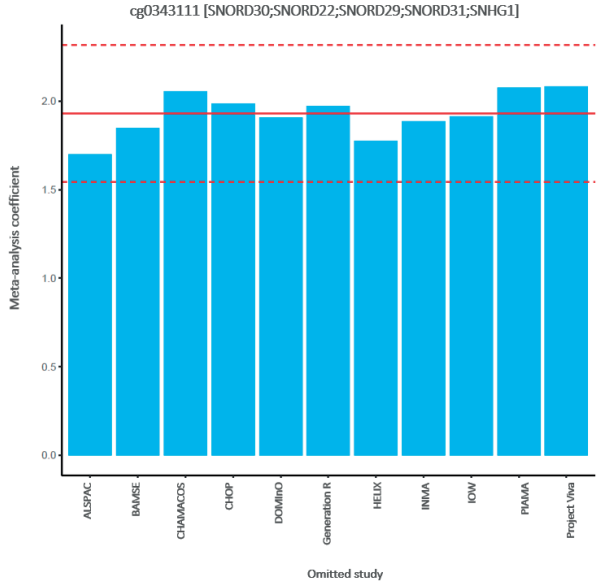


Figure S4H. Leave-one-out plot for the genome-wide FDR-significantly associated cg26889953, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

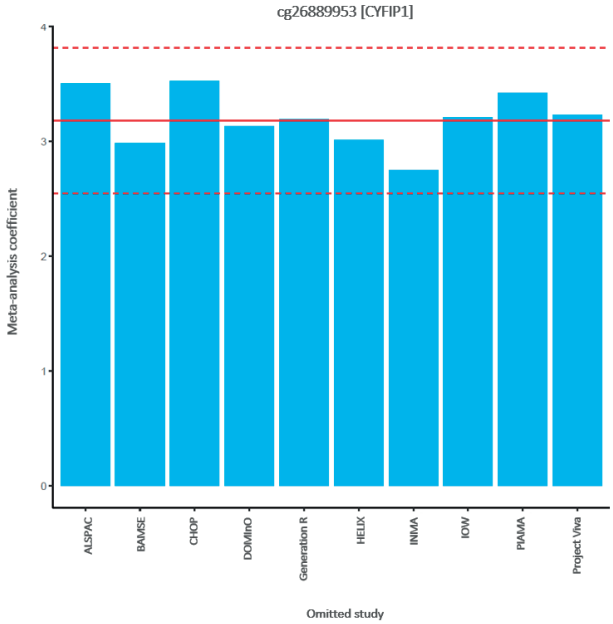


Figure S4I. Leave-one-out plot for the genome-wide FDR-significantly associated cg19743522, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

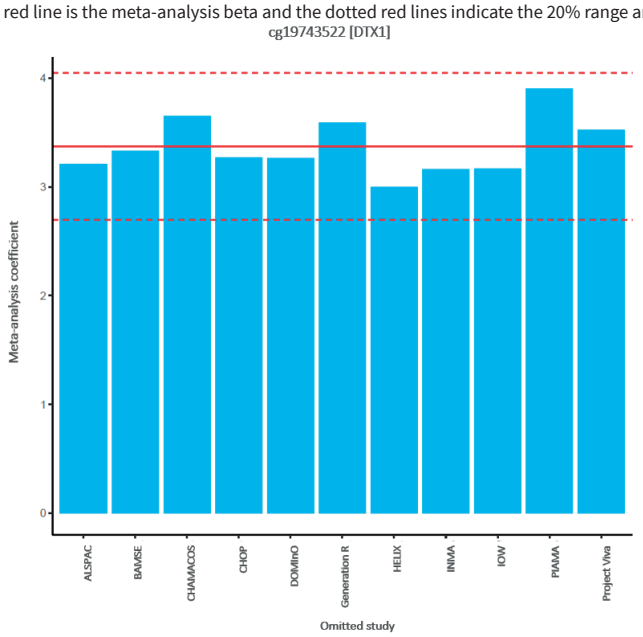


Figure S4J. Leave-one-out plot for the genome-wide FDR-significantly associated cg25877069, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

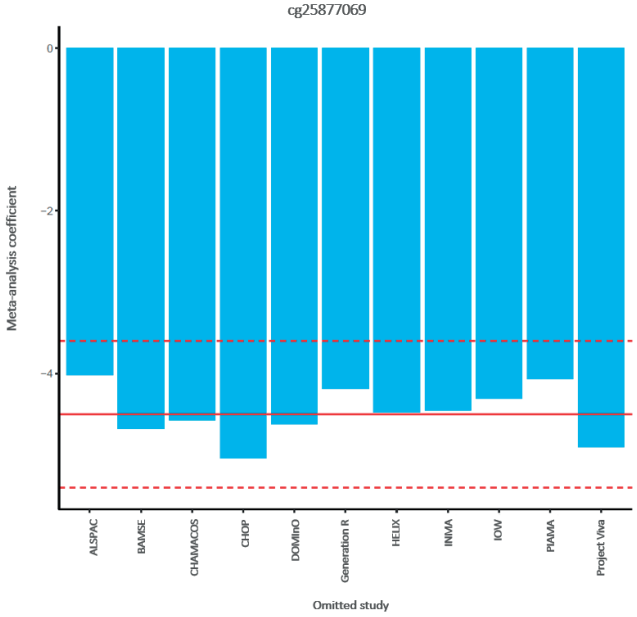


Figure S4K. Leave-one-out plot for the genome-wide FDR-significantly associated cg13931559, showing the association of methylation levels in whole blood in childhood with childhood BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

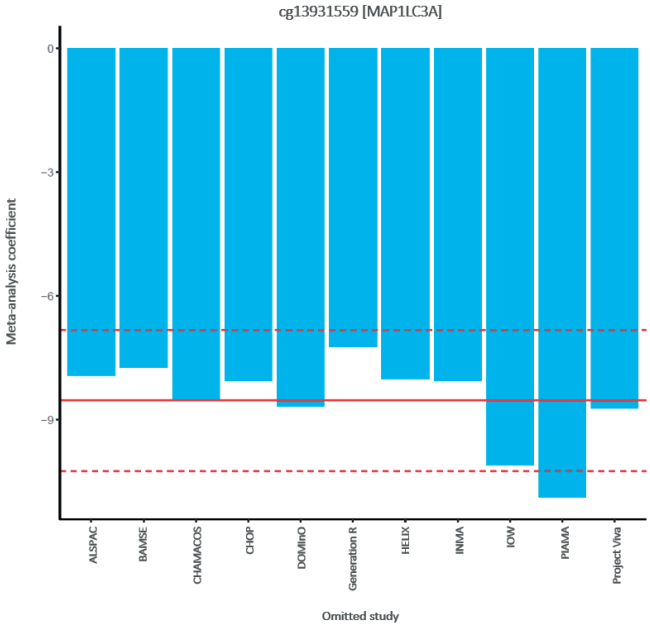


Figure S4L. Leave-one-out plot for the genome-wide Bonferroni-significantly associated cg10040131, showing the association of methylation levels in whole blood in adolescence with adolescent BMI, if the indicated study would be omitted from the meta-analysis. The red line is the meta-analysis beta and the dotted red lines indicate the 20% range around the beta.

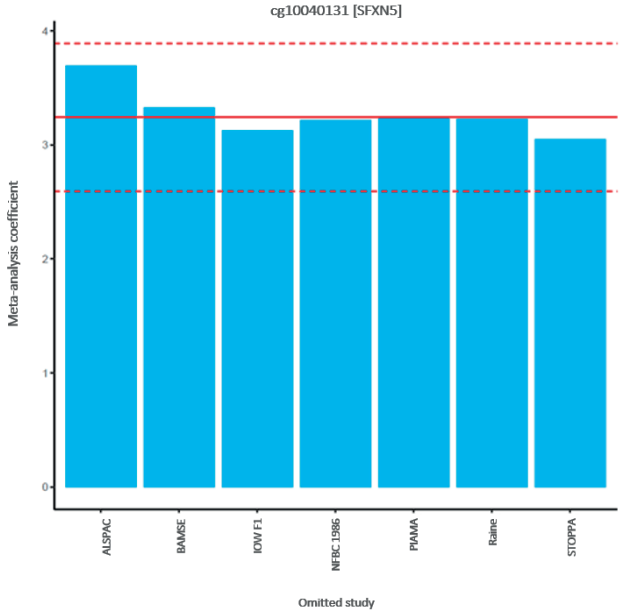


Figure S5. Boxplots showing the distribution of effect sizes of the 187 CpGs significantly associated with adult BMI in a previous study for the four analyses: the associations of DNA methylation in cord blood with early childhood BMI (Analysis A) and late childhood BMI (Analysis B), of DNA methylation in whole blood in childhood with childhood BMI (Analysis C) and of DNA methylation in whole blood in adolescence with adolescent BMI (Analysis D). Results are shown separately for CpGs with positive and negative effest estimates in the original analysis.[1]

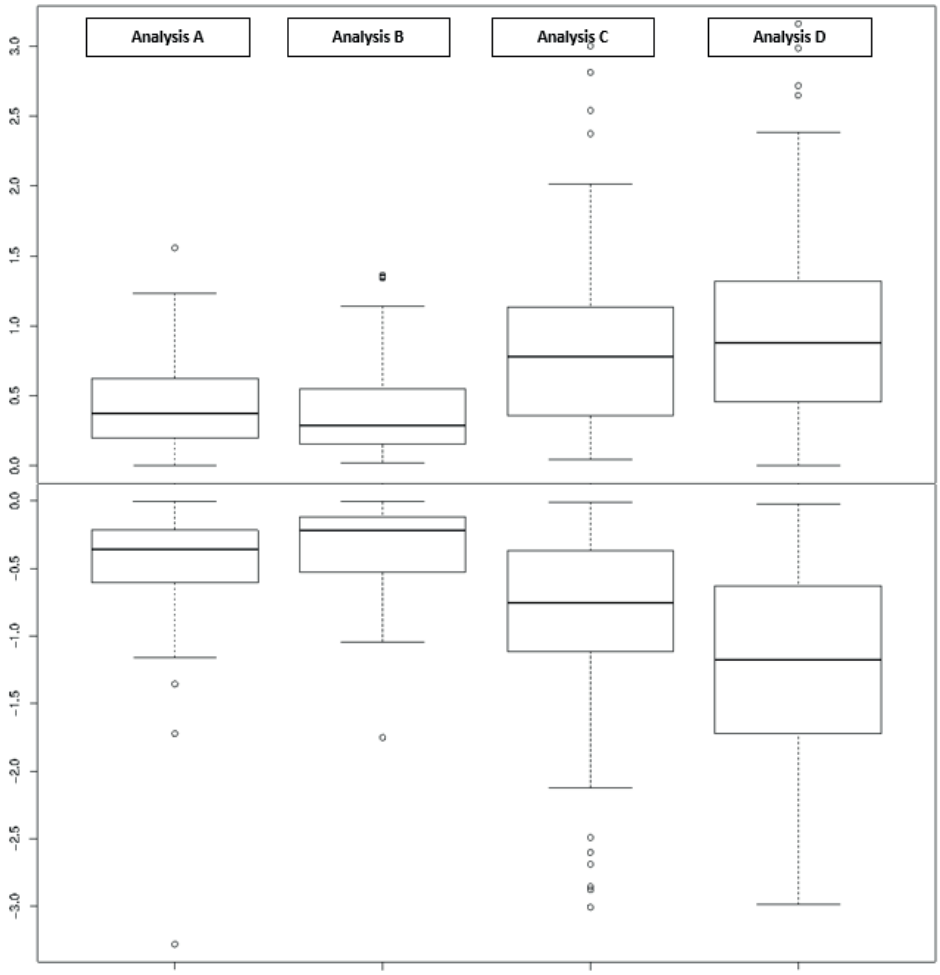


Figure S6A. Density plot performed within the Generation R Study for the potentially polymorphic probe, the genome-wide FDR_significantly associated cg03431111 in analysis C (cross-sectional analysis of DNA methylation and BMI in childhood), showing no indication of a non-unimodal distribution.

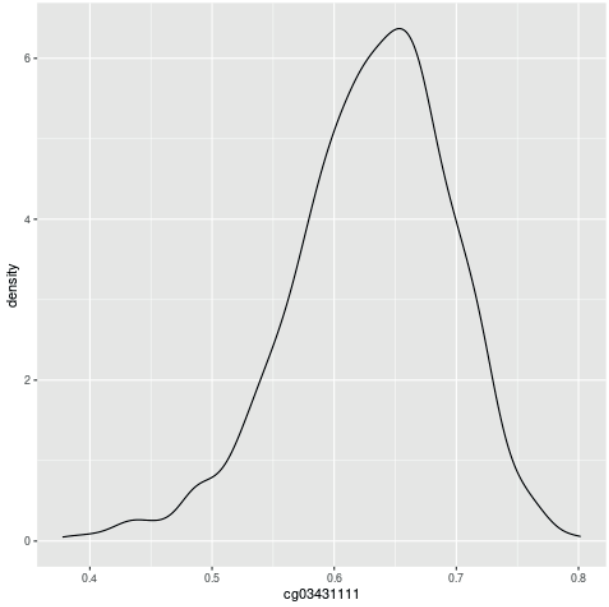


Figure S6B. Density plot performed within the Generation R Study for the potentially polymorphic probe, the genome-wide FDR_significantly associated cg04456029 in analysis C (cross-sectional analysis of DNA methylation and BMI in childhood), showing no indication of a non-unimodal distribution.

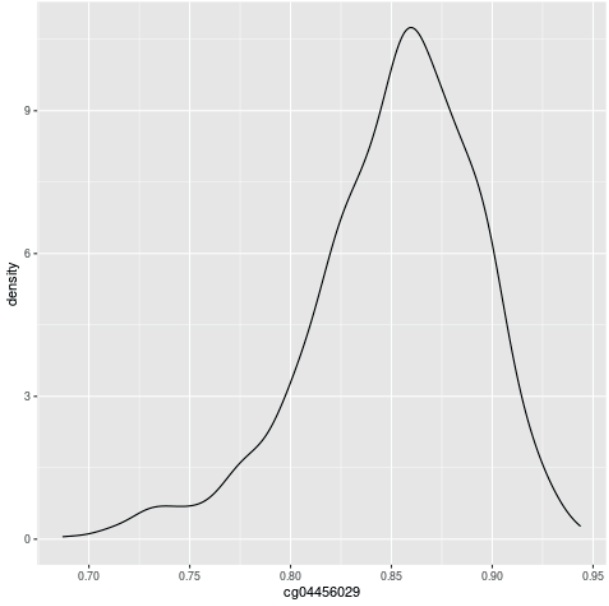


Figure S6C. Density plot performed within the Generation R Study for the potentially polymorphic probe, the genome-wide FDR_significantly associated cg05281708 in analysis C (cross-sectional analysis of DNA methylation and BMI in childhood), showing no indication of a non-unimodal distribution.

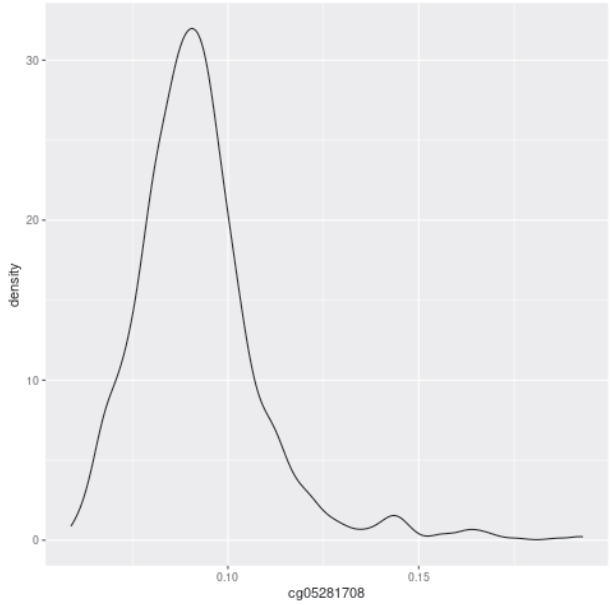
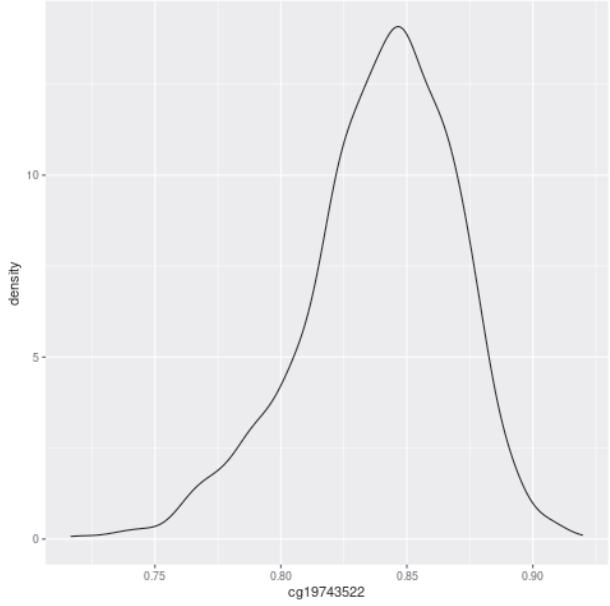


Figure S6D. Density plot performed within the Generation R Study for the potentially polymorphic probe, the genome-wide FDR_significantly associated cg19743522 in analysis C (cross-sectional analysis of DNA methylation and BMI in childhood), showing no indication of a non-unimodal distribution.



References

1. Wahl S, Drong A, Lehne B, Loh M, Scott WR, Kunze S, et al. Epigenome-wide association study of body mass index, and the adverse outcomes of adiposity. Nature. 2017;541(7635):81-6.

5

General Discussion

INTRODUCTION

Every individual will experience stress, both psychological and physical, to some extent in life. The adaptive response to stress is critical for the survival of the individual. Stressful situations trigger physical reactions such as increased blood pressure and heart rate.[1] These responses are very useful in the short term, but may have harmful effects in the long term. Chronic stress is associated with many adverse outcomes in adults such as adiposity, heart failure and metabolic syndrome.[2,3] Thus, long term exposure to elevated cortisol concentrations seems to have deleterious effect on the function of the cardiovascular and metabolic systems. Less is known about the effect of early-life stress on cardio-metabolic health in childhood. According to the 'Developmental Origins of Health and Disease' (DOHaD) hypothesis, adverse exposures in fetal life and infancy are suggested to have an important role in the development of diseases in later life.[4,5] Exposure to physical or psychological stress in early-life may permanently alter the activity of the hypothalamic-pituitary-adrenal (HPA) axis and induce changes in growth, organ structure, neurological- and endocrine function and metabolism.[6]

The main hypothesis for this thesis was that the associations of chronic stress with adverse cardio-metabolic outcomes originate in early life. The key objectives of this thesis were to assess the associations of maternal psychological distress in pregnancy and childhood hair cortisol concentrations with cardio-metabolic outcomes in childhood. DNA methylation may be a pathway underlying associations between early-life stress and outcomes in children. Therefore, we assessed the association of maternal haemoglobin concentrations in pregnancy with DNA methylation and of DNA methylation with adiposity in childhood and adolescence. The main results and limitations of the studies presented in this thesis have been discussed in the previous chapters. This chapter provides a general overview and interpretation of the main findings presented in this thesis, discusses general methodological issues and suggests directions for further research and potential implications for clinical practice.

INTERPRETATION OF MAIN FINDINGS

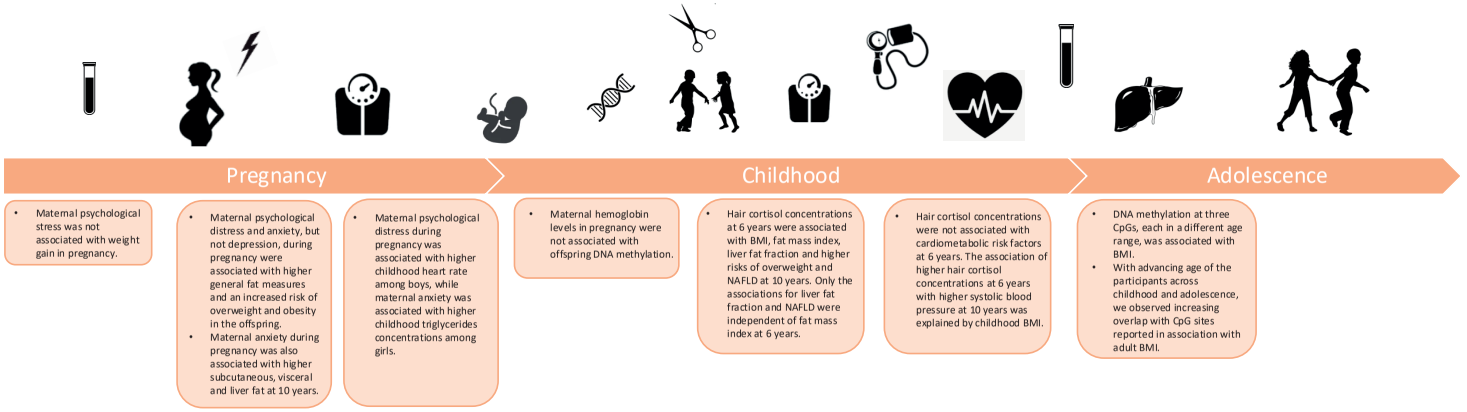
Figure 1 presents the most important findings presented in this thesis.

Maternal psychological distress during pregnancy

Background

Pregnancy is one of the most vulnerable periods in a woman's life as it brings along numerous physical, social and psychological changes. In previous literature, the preva-

Figure 1. Presentation of the most important findings presented in this thesis.



lence of experienced psychological stress, depressive or anxious feelings in pregnancy differs widely and ranges from 5-80%.[7-10] This wide range may be explained by differences in screening tools and populations. However, most studies report that 10-20% of pregnant women experience some kind of psychological distress.[7-9] In our population-based study, we observed that 7-10% of all pregnant women reported psychological distress, depressive or anxious feelings.[11-13] This is slightly lower than numbers in the literature, which might indicate a selection towards a healthier population.

Pregnancy is a critical period for psychological distress, for mother and offspring. Psychological distress is generally defined as general stress, depressive symptoms, anxiety or experiencing an adverse life event.[7, 14] Risk factors for psychological distress symptoms during pregnancy include history of (perinatal) depression as well as psychosocial stressors, such as unmarried status, poor social support, and lower socioeconomic status.[15] In a narrative review in **Chapter 2.1,** we provided an overview of the literature about the associations of maternal psychological distress on child health outcomes.[16] In this review, we reported that intra-uterine exposure to maternal stress is associated with multiple adverse fetal and child health outcomes. However, studies were not always of high quality and results were sometimes inconsistent (**Table 1**). Therefore, it is important to gain more knowledge on the associations of maternal psychological distress during pregnancy with child health outcomes.

Gestational weight gain

Previous literature suggests that psychological distress during pregnancy is associated with adverse maternal outcomes such as an increased risk of gestational hypertension and pre-eclampsia.[17] Whether stress during pregnancy is also associated with gestational weight gain is less clear, as two large systematic reviews reported contradictory findings.[18, 19] These reviews compiled studies with a modest sample size and different definitions of psychological distress, depression and anxiety. Studies among non-pregnant participants have shown that weight status and psychological wellbeing are related. However, this relationship is complex and most likely bidirectional.[20 21] In pregnancy, both inadequate and excessive weight gain are associated with adverse maternal and offspring outcomes such as increased risk of obstetric complications, impaired growth and preterm birth.[16, 22] Gestational weight gain is a potential modifiable factor and therefore it is important to obtain more knowledge about the associations between stress during pregnancy and gestational weight gain. However, in line with a systematic review among 35 studies, we found no clear association of maternal psychological distress, depression or anxiety with weight gain in pregnancy (**Chapter 2.2**).[11,19] Thus, based on the current study there is no evidence to support the inclusion of psychological stress reduction in prevention strategies to optimize gestational weight gain.

Table 1. Summary findings of discussed literature

	Maternal psychological distress*
Fetal outcomes	
Low birth weight	++
Small head circumference	+
Intrauterine growth retardation	+
Preterm birth	++
Child cardio-metabolic outcomes	
Adiposity	++
Fetal heart rate	+/-
High blood pressure	+/-
Adverse lipid profile	-
Increased inflammatory markers	+/-
Impaired insulin/growth homeostasis	+/-
Child respiratory and atopic outcomes	
Wheezing	++
Asthma	++
Lower lung function	+
Allergy	+/-
Eczema	+
Child neurodevelopmental outcomes	
Lower academic functioning	+/-
Delayed language development	+/-
Internalizing and externalizing problems	+

**defined as general stress, depression, anxiety or experiencing an adverse life event during pregnancy*
Quality of evidence:
++ Good evidence for an association based on consistent results from multiple studies and meta-analyses
+ Moderate evidence for an association based on multiple studies, but with some inconsistencies
+/- Insufficient evidence for an association based on only a few studies, or with substantial inconsistencies
- No or very few studies on the association

General and organ fat measures in childhood

We examined whether maternal psychological distress is associated with childhood general and organ fat measures (**Chapter 2.3**). Maternal stress is suggested to increase maternal cortisol concentrations, which may partly cross the placenta and lead to altered HPA axis activity in the fetus and thereby increased risk of obesity in the offspring.[23] We observed that maternal psychological distress and anxiety during pregnancy were associated with an increased risk of childhood obesity and higher child fat mass index. Maternal anxiety was additionally associated with higher childhood body mass index (BMI) and an increased risk of overweight at 10 years.[12] In a previous study in the same study population no association was observed between prenatal stress of the mother and offspring BMI in children aged 3 months to 3 years.[24] Thus, it seems that

the associations of maternal psychological distress during pregnancy with childhood adiposity measures become more apparent in later childhood. This finding is consistent with a study among over 65,000 mother- child pairs, which also found an association between maternal stress during pregnancy, defined by bereavement in the mother, and an increased risk of overweight in the offspring at 10 to 13 years of age, but not in children younger than 10 years.[25] Large cohort studies such as the Framingham Heart Study and the Jackson Heart Study have reported that excess visceral adiposity and ectopic fat deposition are related to various cardio-metabolic abnormalities, independently of total or subcutaneous fat.[26-29] Therefore, it is important to obtain more information on the association between maternal distress in pregnancy and organ fat in the offspring. However, to our knowledge no studies reported about this association yet. In our study, we observed that maternal anxiety, but not psychological distress and depression, was associated with higher subcutaneous fat index, visceral fat index, and liver fat fraction in 10-year-old children.[11] Even though the anxiety and depression scale of the BSI are strongly correlated and the scales, at least partly, measure similar concepts, results were non-significant for depression. From previous studies we know that anxiety and depression share a common biological and genetic background but are not identical emotional states.[30] Thus, maternal psychological distress and anxiety, but not depression, during pregnancy might be risk factors for developing excess general and organ fat measures in childhood. Future studies, ideally randomized controlled trials and Mendelian randomization studies, are needed to obtain further insight into the causality of the observed associations, and the underlying biological mechanisms. Mendelian randomization approaches use genetic variants, which are robustly associated with the exposure of interest and not affected by confounding, as an instrumental variable for a specific exposure, to examine whether an exposure is causally related to the outcome.[31] If proven causal, maternal psychological health in pregnancy could be a modifiable target in prevention strategies in order to improve child well-being.

Cardio-metabolic risk factors in childhood

In **Chapter 2.4**, we investigated whether maternal psychological distress during pregnancy is related to cardio-metabolic health in childhood, beyond the associations with fat measures described above. Exposure to increased maternal cortisol levels due to increased psychological distress may cause permanent metabolic abnormalities in the offspring, and a potential concomitant vulnerability to cardio-metabolic disease, also called 'metabolic programming'. [32] We identified associations of maternal psychological distress, depression and anxiety with offspring blood pressure, cholesterol, insulin, glucose and C-reactive protein concentrations, but these were largely explained by family based socio-demographic factors.[13] For the remaining associations, we did observe sex-specific differences. After adjustment for potential confounders, maternal

psychological distress, depression and anxiety during pregnancy were associated with higher childhood heart rate at 10 years in boys, but not in girls. Also, maternal anxiety, but not overall psychological distress and depression during pregnancy, was associated with higher triglycerides in girls but not in boys, after adjustment for confounders. These findings suggest that the effects of prenatal stress exposure on subsequent cardiometabolic outcomes differ by sex of the offspring. Several other studies, both in animals and humans, report sex-specific differences in response to prenatal stress.[33, 34] Placental adaptation to increased glucocorticoid levels due to maternal stress may be different in female and male fetuses.[35] Different mechanisms have been proposed such as female, but not male placenta, adjustment of their glucocorticoid metabolic activity in the presence of high maternal glucocorticoid concentrations.[33, 35, 36] An alternative explanation is the different placenta-regulated growth strategies of male and female foetuses: male foetuses are more vulnerable during disturbed pregnancies than female foetuses, due to their faster growth rate and diminished flexibility[34]. However, the precise mechanisms of sex-specific susceptibility to prenatal stress are not completely clarified. Further studies are needed to further explore and replicate the sex-specific associations.

Underlying mechanisms

The best described mechanism underlying the associations between maternal stress in pregnancy and childhood outcomes encompasses fetal exposure to increased cortisol levels due to altered activation of the maternal HPA axis in response to physiological and psychological distress.[37 38] The placenta is the barrier between mother and fetus and produces 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts active cortisol into its inactive form, cortisone.[39] However, several factors may challenge this mechanism such as decreased levels or variable efficiency of 11β-HSD2, which may be induced by prenatal stress itself.[37, 39] Therefore, not all active cortisol may be inactivated and some cortisol may cross the placenta and elevate fetal glucocorticoid levels.[40] Cortisol can perturb the development of the fetal HPA axis during vulnerable periods by resetting the set point of the HPA axis 's negative feedback mechanism, potentially resulting in permanent changes in the postnatal activity of the fetal HPA axis.[41] Exposure of the fetus to stress or high levels of glucocorticoids can also permanently affect glucocorticoid receptor expression. A reduction of glucocorticoid receptors would be expected to reduce glucocorticoid negative feedback and lead to an overactive HPA axis.[41] However, trans-placental transfer of maternal cortisol is not the only mechanism that may explain the effects of maternal stress on offspring health.[37] Another main pathway of the stress system is the activation of the sympathetic nervous system and inactivation of the parasympathetic nervous system in response to stress.[42] Within seconds after a stressor, catecholamines such as epinephrine and norepinephrine are produced, which increase heart rate and lead to higher glucose levels.[43] Catecholamines are also

linked to the HPA axis which responds within minutes to hours and supports the action of the catecholamines.[44] Thus, the autonomic nervous system and HPA axis are highly coordinated and physically interconnected.[45] Also, oxidative stress and altered maternal microbiota may play a role as mediators of maternal stress effects on fetal development and later life cardio-metabolic health.[37]

Main findings

- Maternal psychological distress, depression and anxiety were not associated with weight gain in pregnancy.
- Maternal psychological distress and anxiety, but not depression, during pregnancy were associated with higher general fat measures and an increased risk of overweight and obesity in the offspring. Maternal anxiety during pregnancy was also associated with higher subcutaneous, visceral and liver fat at 10 years.
- Maternal psychological distress was associated with higher childhood heart rate among boys, while maternal anxiety was associated with higher childhood triglycerides concentrations among girls.

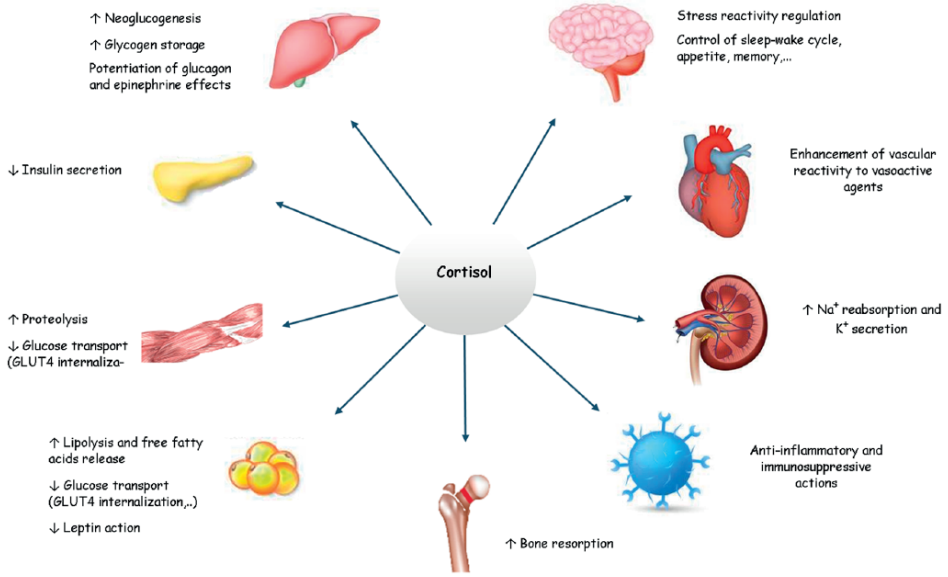
Hair cortisol concentrations in childhood

Background

Endogenous overproduction of cortisol, in patients with Cushing's disease, results in impaired glucose tolerance, dyslipidaemia, abdominal fat distribution and hypertension.[46-48] This led to the hypothesis that increased cortisol concentrations in childhood may also be associated with adiposity and an adverse cardio-metabolic risk profile in childhood. Many studies have examined these relationships with sometimes contradictory results.[49, 50 51] Most studies measured circulating cortisol in saliva, serum or urine. These measures are representative of acute or short-term (e.g. 24h) cortisol concentrations and are influenced by the circadian rhythm and acute stressors such as psychosocial threats, intense exercise and food intake.[52] In the past two decades, scalp hair analysis has been introduced as a marker for long-term cortisol concentrations. Because head hair grows at an average of 1 cm per month, assessment of cortisol and cortisone in hair can reflect on several weeks or months. Cortisol and its inactive form cortisone are both glucocorticoids. Cortisol is the end product of the HPA axis and is produced from cholesterol by the adrenal glands, in response to a (perceived) psychological or physiological stressor.[53] Increased serum cortisol concentrations in turn decrease the activity of the HPA axis, forming a negative feedback loop.[53, 54] Cortisol effects are mainly mediated through glucocorticoid receptors, which are expressed in almost all tissues. Some of the most

important effects of cortisol are increased gluconeogenesis, decreased insulin sensitivity, anti-inflammatory effect, increased lipids concentrations, and reduced immune-system activity (**Figure 2**).[55] Individuals with hypercortisolism have increased abdominal adipose tissue which adversely affects the cardio-metabolic risk profile in adults.[26, 28, 29]

Figure 2. Physiological effects of cortisol. Adapted from Oprea et al.[55]



General and organ fat measures in childhood

Previous studies in adults reported positive associations between hair cortisol concentrations and adiposity measures.[52, 56-58] A recent review of child studies reported inconsistent findings on the association of hair cortisol concentrations and BMI.[59] However, studies were of modest sample size, used a cross-sectional design and did not look into the association of cortisol with organ fat measures. In line with previous studies in adults, we observed in **Chapter 3.1** an association of higher hair cortisol concentrations at 6 years with higher BMI, fat mass index, liver fat fraction, and higher risks of overweight and non-alcohol fatty liver disease (NAFLD) at 10 years.[60] After adjustment for BMI or fat mass index at 6 years, only the associations for liver fat fraction and NAFLD remained, suggesting that these associations were not explained by the association between hair cortisol and adiposity measures already present at 6 years. In a previous study in the same population, cross-sectional associations of higher hair cortisol concentrations with higher BMI and fat mass index at 6 years were reported.[61] Opposite of expected, we observed no association of increased hair cortisol concentrations with visceral fat in children. Increased glucocorticoid levels are known to mobilize fat from the periphery

to the central region and cause increased visceral and abdominal fat in adults.[48, 62-64] Additionally, in adipose tissue in obese patients, an elevation of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), which converts inactive steroids into active glucocorticoids, is observed.[56, 65, 66] It may be that this association is not present yet in childhood or that there is not enough variation in visceral fat levels in childhood to be able to see an association.

Thus, there are associations of hair cortisol concentrations and adiposity measures at 6 years of age, which remain when the outcomes are measured at 10 years of age. Due to the observational design of our study we are not able to draw conclusions on the causality and directionality of the observed associations. Even, a bidirectional association, in which changes in the cortisol metabolism are consequences of metabolic changes accompanying obesity and liver fat, might be present.[67] These findings warrant further research since we know that body fat distribution tracks from childhood into adulthood and should be identified and treated in an early stage. Also, increased cortisol is associated with liver fat and NAFLD at 10 years, independent of fat mass index. If this association is causal, there might be a separate mechanism next to the pathway of cortisol with BMI and fat mass, from increased cortisol to increased liver fat. A previous study observed that cortisol concentrations are altered in adult patients with hepatic steatosis, obesity-associated liver disease.[68] Based on our study, we observed that these associations are already present in childhood. This finding may be of importance for the development of preventive strategies for liver diseases. Future research should explore the potential of reversed causation and examine underlying mechanisms of these associations.

Cardio-metabolic risk factors in childhood

Large studies in adults have found that stress-related disorders are robustly associated with cardio-metabolic outcomes such as hypertension, heart failure and metabolic syndrome.[1-3] Cardio-metabolic diseases are common health consequences of overweight/obesity and both track from childhood into adulthood.[69] Recent literature reported positive cross-sectional associations of hair cortisol concentrations and cardio-metabolic outcomes including hypertension, diabetes, metabolic syndrome, coronary heart disease and stroke among adults.[56, 70] Similar to the association between cortisol and obesity, the associations of cortisol with cardio-metabolic risk factors may also originate in early-life. In **Chapter 3.2**, we did not find evidence for cross-sectional or longitudinal association of hair cortisol concentrations with cardio-metabolic risk factors at 6 or 10 years.[71] These findings are in line with three studies in children, which did not find associations between hair cortisol concentrations and blood pressure, heart rate, lipids, C-reactive protein or glucose metabolism. Three cross-sectional studies reported a positive association of serum cortisol concentrations with systolic blood

pressure in children, independent of BMI, but not, or less clearly, with diastolic blood pressure. In our study, we did observe an association between increased hair cortisol concentrations and higher systolic blood pressure. However, the association attenuated into non-significance after adjustment for childhood BMI at 6 years. Based on our study, we cannot conclude whether BMI is a mediator or confounder in this association. The role of BMI warrants further research.

Underlying mechanisms

Based on our observational studies, we cannot establish causality of the observed associations. Previous studies suggested a bidirectional association between adiposity and cortisol. On the one hand, increased cortisol concentrations increase appetite with a preference for calorie-dense food, stimulate adipogenesis, induce insulin resistance and negatively affect brown adipose tissue, which all may lead to obesity.[72-75] On the other hand, adiposity may cause metabolic changes affecting the cortisol metabolism, such as the regulation of 11β-HSD1 expression.[67] Also, it was found that there is a significantly higher expression of 11β-HSD1 in adipose tissue of obese people compared to the adipose tissue in non-obese people, causing increased regeneration of cortisol from the inactive cortisone.[66] Moreover, experiencing weight stigma may be stressful. Psychological stress in its turn may negatively affect eating behavior leading to more weight gain, creating a "vicious cycle". [76] Lastly, it is likely that there are inter-individual differences in this complex relationship, potentially due to varying levels of glucocorticoid exposure or sensitivity.[44] This suggestion is supported by the fact that not all people with obesity have elevated hair cortisol levels; however the obese patients with high cortisol levels may be more prone to metabolic syndrome and cardiovascular disease.[74]

Main findings

- Hair cortisol concentrations at 6 years were associated with BMI, fat mass index, liver fat fraction and higher risks of overweight and NAFLD at 10 years. Only the associations for liver fat fraction and NAFLD were independent of fat mass index at 6 years.
- Hair cortisol concentrations were not associated with cardio-metabolic risk factors at 6 years. The association of higher hair cortisol concentrations at 6 years with higher systolic blood pressure at 10 years was explained by childhood BMI.

DNA methylation studies

Background

Epigenetics is of increasing interest as an underlying mechanism in the DOHaD hypothesis.[77] Environmental influences in early-life may induce epigenetic changes, of which DNA methylation is the best described mechanism, and thereby, in interplay with genetic factors, affect metabolism and chronic disease risk.[78] From animal studies we know that experimentally induced epigenetic changes produce lifelong physiological changes of relevance to human disease, such as metabolic alterations and exaggerated stress responses.[77, 78] In placenta tissue, inter-individual variation of the DNA methylation pattern is seen in the third trimester relative to first and second, supporting an accumulation of environmentally induced or stochastic changes.[79] Several prenatal exposures, such as maternal smoking, maternal pre-pregnancy BMI and maternal plasma folate levels during pregnancy, have been found to be associated with offspring DNA methylation.[80-82] Some of the identified cytosine-phosphate-guanine (CpG) sites were in or near genes with known roles in diseases associated with the exposures.[80] These findings suggest that epigenetic modifications induced by environmental factors in early-life may have consequences for health throughout the life course. Changes in DNA methylation patterns may underlie the associations of early-life exposures with adiposity and cardiometabolic outcomes in childhood.

Maternal haemoglobin levels in pregnancy and childhood DNA methylation

Altered maternal haemoglobin levels during pregnancy are associated with adverse perinatal outcomes such as preterm delivery and intrauterine growth restriction.[83] Fetal growth restriction is associated with an increased risk of obesity, higher blood pressure levels, and insulin resistance in childhood and adulthood.[84, 85] Changes in DNA methylation patterns may underlie the association of maternal haemoglobin concentrations in pregnancy and health outcomes in the offspring. To our knowledge, no studies explored associations of maternal haemoglobin levels in pregnancy with offspring DNA methylation. In a meta-analysis including ten studies presented in **Chapter 4.1**, we did not observe associations of maternal haemoglobin concentrations during pregnancy with differential DNA methylation in the offspring.[86] Despite the relatively large sample size and low between-study heterogeneity, the study has several limitations such as the relatively low number of individuals with haemoglobin concentrations outside the normal range. Since all studies were performed in high income countries, participants with abnormal haemoglobin concentrations were most likely treated. It remains unclear whether more extreme values of maternal haemoglobin concentrations may influence DNA methylation patterns of the offspring.

DNA methylation and body mass index from birth to adolescence

Most evidence regarding associations of DNA methylation with BMI stems from adult studies. The largest study identified cross-sectional associations between DNA methylation at 187 CpGs and BMI in over 10,000 adult participants.[87] DNA methylation may underlie the associations of early-life exposures and childhood adiposity.[87] Previous studies of BMI and DNA methylation among children were small and inconclusive. In meta-analyses of epigenome-wide association studies including up to 4133 children from 23 studies presented in **Chapter 4.2**, we found very little evidence for associations of DNA methylation with childhood and adolescent BMI.[88] DNA methylation at three CpGs only (cg05937453, cg25212453, and cg10040131), each in a different age range, was associated with BMI.[88] However, we did observe increasing enrichment and increasing point estimates of CpGs previously reported in relation to adult adiposity, with increasing age of participants in our study. [87] These findings are in line with the findings of several recent studies in adults, using methods such as Mendelian randomization, which posit that alterations in DNA methylation are predominantly the consequence of adiposity, rather than the cause. The mechanisms linking adiposity to its cardio-metabolic consequences are poorly understood. If differential DNA methylation is the result of exposure to higher BMI, it may be part of the pathway that links adiposity to cardio-metabolic diseases. Findings of our study support this hypothesis. Further studies in childhood and adolescence using methods such as Mendelian randomization, could provide more insight on causality and direction of effect.

Main findings

- Maternal haemoglobin levels in pregnancy were not associated with offspring DNA methylation.
- DNA methylation at three CpGs, each in a different age range, was associated with BMI. With advancing age of the participants across childhood and adolescence, we observed increasing overlap with CpG sites reported in association with adult BMI.

METHODOLOGICAL CONSIDERATIONS

The strengths and limitations for each study are described in the respective chapters of this thesis. In the following paragraphs, general methodological considerations regarding internal and external validity, causality, and DNA methylation studies are discussed. When designing or interpreting epidemiologic studies, there are two main concerns: the external and internal validity of the study. External validity refers to the generalizability

of the results and includes scientific and statistical generalisation.[89] Internal validity is considered a prerequisite for external validity and is determined by how well a study can rule out alternative explanations for its findings.[90] Most violations of internal validity can be classified into three general categories: selection bias, information bias and confounding.[90, 91] Bias and confounding can lead to inaccurate estimates of association, and over- or underestimation of results. These three concepts are discussed below in the context of the studies performed in this thesis.

Selection bias

Selection bias refers to systematic errors that result from procedures used to select subjects and from factors that influence study participation.[90] Selection bias is present if associations between exposure and outcome variables are different in subjects who participate in the study and subjects who were eligible for the study, but do not participate. As a consequence, the obtained results are not representative for the population intended to be analysed. Selection bias may occur due to selective non-response at baseline. In the Generation R study, 61% (N = 9,749) of all children eligible at birth, participated at baseline.[92] Most likely, this non-response was not at random. Accordingly, comparison between characteristics of Generation R study participants with population figures in Rotterdam showed that participants were more often from European ancestry, had a higher socio-economic status and fewer adverse birth outcomes such as low birth weight.[93] Selection bias may also arise from selective loss to follow-up, which is the major source of selection bias in cohort studies.[94] Loss to follow-up would lead to selection bias if associations between exposure and outcome variables are different between those included in the analyses and those loss to follow-up. Non-response and loss to follow-up may reduce statistical power, due to lower prevalence rates of exposures and outcomes. We performed non-response analyses in our studies to examine differences between participants and non-participants. Overall, non-participants were more often of non-European origin, more frequently lower educated and more often had unhealthy lifestyle habits, and their children were more often born with a lower birth weight. Both selective non-response at baseline and selective loss to follow-up may lead towards a more affluent and healthy population, which may have led to lower prevalence rates of maternal psychological distress, childhood obesity and cardio-metabolic risk factors in childhood, and subsequently reduced statistical power. Also, a healthier population may affect the generalizability of findings to less healthy populations. Previous analyses in cohort studies showed that the studied associations were not strongly influenced by selection bias, and therefore it seems unlikely that the results of this thesis are biased due to the selection procedures.[94-96] We applied multiple imputation, limiting the risk of selection bias due to missing values in covariates, which otherwise may require complete case analysis.[97, 98]

Information bias

Information bias refers to systematic errors due to misclassification of participant data, both for exposure and outcome measurements. Misclassification can be classified as differential or non-differential. Misclassification is differential (non-random) when the misclassification is different for those with and without the exposure or outcome of interest. This type of misclassification can lead to underestimation or overestimation of the effect estimates. Misclassification is non-differential (random) when it is unrelated to the occurrence or presence of the exposure or outcome of the study. This type of misclassification can lead to bias towards the null. For the studies in this thesis, information on exposures and outcomes was obtained prospectively by maternal questionnaires, physical examinations, blood and hair analyses, measurements of blood pressure and heart rate and imaging using dual-energy X-ray absorptiometry scan (DXA scan) and magnetic resonance imaging (MRI) scan. The risk of differential misclassification in the studies in this thesis is small because exposure data were collected longitudinally and before assessment of the outcome data, the data collectors were blinded to the exposure status when assessing the outcomes and both parents and data collectors were unaware of the specific research questions under study. However, non-differential misclassification may have occurred. In some of the studies included in this thesis, information on psychological wellbeing during pregnancy was self-reported. A previous study reported that women with certain psychiatric disorders are inclined to underestimate their own psychological problems, which may lead to an underestimation of the observed effects. [99] Yet, studies reported a high reliability and validity of the Brief Symptom Inventory (BSI), the questionnaire used to examine psychological distress at 20 weeks of gestation.[100-102] Also, information on weight before pregnancy and maximum weight in pregnancy was self-reported. Women tend to underestimate their pre-pregnancy weight and overestimate their maximum gestational weight gain, which may lead to an overestimation of total weight gain during pregnancy.[103, 104] However, the correlations between self-reported weight and measured weight were high ($r \geq 0.95$, $p < 0.001$) , which suggests that bias due to non-differential misclassification is unlikely. Moreover, pregnant women may have been more aware of their weight status because of their participation in the study, minimizing the error of self-reported weight. For the outcome measures in our child studies we relied on measurements of weight, height, blood pressure and heart rate, imaging of total body fat using a DXA scanner, imaging of visceral and organ fat using MRI scans. The use of thirty-minutes fasting blood samples may have resulted in non-differential misclassification. However, previous studies reported that insulin resistance and sensitivity in semi-fasted blood samples are moderately correlated with fasting values and that non-fasting lipids are associated with risk of cardiovascular disease. Thus, semi-fasted samples of cardio-metabolic markers may be used in large population-based studies where fasting samples are not available.[105-107] In

summary, information bias is unlikely to have influenced the findings of this thesis and, if present, may have led to underestimation of the observed associations.

Confounding

A confounding factor is a variable, which is associated with both exposure and outcome, and which explains all or part of the association.[90] If not taken into account, confounding may lead to either under- or overestimation of the effect. To take account for confounding, we adjusted all analyses for multiple potential confounders. We selected potential confounders based on previous studies, their associations with the exposures and outcomes, or a change in the effect estimate of more than 10%.[108] Variables were not considered confounders when affected by exposure or outcome, and particular they cannot be an intermediate step in the causal pathway between exposure and outcome.[90, 108] In some of the studies presented in this thesis, adjustment for potential confounders resulted in an attenuation of the effect, which suggests that observed associations were partly explained by these variables. Many of the potential confounders were derived from questionnaires, and therefore misclassification bias of these variables may have occurred, which may have affected the observed effects. Although information about many potential confounders was available in the studies performed, residual confounding by lifestyle or genetics may still be an issue, which may have led to an over- or underestimation of the effect estimates.

Causality

Due to the observational design of all studies reported in this thesis, we cannot draw conclusions on causality of the assessed associations. The Bradford Hill criteria may be used to determine the causality of observed associations.[109] These criteria include the strength, consistency, specificity, temporality, dose-response relationship, biological plausibility, coherence, experiment and analogy.[109] Most effect sizes (strength) observed in our studies were small which does not mean there is no causal effect, however the larger the effect sizes, the more likely they are causal. The main findings of this thesis were consistent with previous studies. Specificity of the association, and thereby the likelihood of causation, is increased when the association is limited to a specific population at a specific site and disease with no other likely explanation.[109] This is not applicable to the setting of our epidemiological studies. Part of our studies were longitudinal supporting the temporality between exposures and outcomes, which indicates that the effect has to occur after the cause. Although not explored in all studies presented in this thesis, we did observe a tendency for dose-response effects for maternal stress, depression and anxiety with childhood adiposity. The differences in childhood adiposity for mothers who reported severe compared to moderate stress, depression or anxiety were not statistically significant which may partly be explained by a low number of women re-

porting severe levels of stress, compromising the statistical power. Additionally, several potential biological mechanisms underlying the observed associations, with coherence in animal studies and for example Cushing's disease studies, have been described. As this thesis is based on observational research, the criterion of experiment is not addressed in this thesis. Studies that assessed analogue factors for the endogenous stress assessed in this thesis provide further evidence for the observed associations. For example, treatment with local and systemic corticosteroids, which could be considered analogues for increased endogenous cortisol, have been associated with a higher BMI.[110] Although the studies in this thesis were not designed to clarify the causality of the associations, our observational studies seem to provide some evidence for causal relationships based on the Bradford Hill criteria.

Methodological issues in epigenetic studies

Epigenome-wide association studies (EWAS) have the potential to examine associations of a large number of CpG-sites across the genome with exposures and outcomes.[111] However, some limitations need to be addressed. In the study in **Chapter 4.2** all participating studies used the Infinium Human Methylation 450K array and in the study in **Chapter 4.1** some studies used the 450K BeadChip and others used the EPIC BeadChip. The 450K BeadChip covers approximately 485.000 CpGs (1.6%) and the EPIC BeadChip covers around 850.000 (3.0%) of all CpGs located in the human genome.[112, 113] Thus, we cannot exclude that methylation at other, non-measured CpGs could be associated with maternal haemoglobin or childhood BMI. DNA methylation patterns are largely tissue-specific. We used cord blood and peripheral blood to measure DNA methylation in relation to maternal haemoglobin and childhood BMI, which may not be the most relevant tissue in the case of BMI. In large population-based studies, it is virtually impossible to collect samples from other tissues such as brain, adipocytes, pancreas and liver. However, a study comparing blood and adipose tissue in patients undergoing surgery, showed some cross-tissue concordance in DNA methylation patterns.[114] DNA-methylation differences between blood samples are strongly influenced by cellular heterogeneity. Therefore, we adjusted our childhood and adolescent analyses for estimated cell type proportions. In **Chapter 4.1** we used a cord blood-specific reference panel for the cord blood analyses, whereas in **Chapter 4.1** for the childhood and adolescent analyses and for all analyses in **Chapter 4.2**, we used an adult reference dataset because no other reference panels were available, which is likely not an optimal way to adjust for white blood cell proportions at these ages. [115-117] In both chapters most study participants were of European ancestry. This limits the generalizability to populations of other ancestries. Future studies of DNA methylation should include populations of different ethnic backgrounds. The technology to measure DNA methylation in the human genome relies on hybridization of genomic fragments to probes on the chip. [118] However, certain genomic factors may

compromise the ability to measure methylation using the array such as single nucleotide polymorphisms (SNPs) and small insertions and deletions (INDELs).[118] Therefore we decided to exclude probes, next to probes that were measured in only one study and that mapped to X and Y chromosomes, that co-hybridized to alternate sequences since we don't know to what extend these probes confound associations or lead to spurious results.[118, 119] Too stringent exclusion of probes may result in false-negative results, therefore we flagged the polymorphic probes. Further studies are needed to gain more insight in these probes and their potential effect on associations. Since DNA methylation is a dynamic process, influenced by genetic and environment factors, it may be a cause, consequence or a by-product of the phenotype under study. [120] Observational studies, especially those with a cross-sectional design (some analyses in **Chapter** 4), may introduce the possibility of reverse causation. To address causality recent studies applied causal inference methods, such as two-step Mendelian randomization, and suggested that alterations in DNA methylation are predominantly a consequence of adiposity, rather than a cause.[87, 121, 122] Future research using methods such as Mendelian randomization should focus on the causality of these associations already in childhood. Analysis of EWAS data involves testing all the CpG sites simultaneously, leading to a multiple testing problem. We accounted for multiple testing by using both the Bonferroni correction and the less stringent false discovery rate (FDR) threshold using the method by Benjamini and Hochberg. [123] The field of population epigenetics is still under development but has the potential to provide light on mechanisms underlying associations of early-life adverse exposures and later life health.

FUTURE RESEARCH

Assessment of exposures

In this thesis, information on maternal psychological distress during pregnancy was obtained by questionnaires. The BSI questionnaire was used to examine overall psychological distress, depression and anxiety symptoms at approximately 20 weeks of gestation.[100, 102] Studies reported good reliability and validity of the BSI in identifying women with symptoms of overall distress, depression and anxiety.[102, 124] Several studies investigated whether cortisol, measured in hair was correlated with self-reported stress measured with various tools and stress-linked mental health measures such as depression and anxiety.[125] Results of these studies are not consistent and warrant further research. Ideally, studies should look at both self-reported stress and cortisol measures to have objective and perceived measures of stress. Self-reporting of maternal stress only may result in underreporting of psychological symptoms and subsequent underestimation of observed effects. The BSI refers to the previous 7 days only and was measured

once in mid-pregnancy. Studies with repeated measurements of psychological distress are needed to provide more insight into the intensity and persistence of psychological symptoms during pregnancy. Most previous studies used other methods than the BSI to assess psychological distress, making it more difficult to compare results. Future studies should use harmonized methods to assess psychological distress in pregnancy to enable better comparison of results from different studies. Most early-life cohorts begin during pregnancy and do not have data on the preconception and early pregnancy periods, which are known to be important for offspring health and which are periods with an opportunity for intervention.[126] Preconception cohorts, like Generation R *Next* (www.generationr.nl/next), will provide important information on exposures in these very early life stages. In Generation R, measurements of hair cortisol concentrations are available in a large population-based child cohort. The availability of such measures in other, comparable cohorts is limited. However, we only have data on hair cortisol concentration at 6 years. To obtain more information on how cortisol concentrations develop over time, future studies should measure hair cortisol concentrations frequently from childhood into adulthood.

Assessment of outcomes

We used self-reported maternal pre-pregnancy weight and maximum weight in pregnancy to calculate total weight gain in pregnancy. Future studies with measured pre-pregnancy weight and maximum gestational weight are needed to reduce the risk of misclassification. Childhood outcomes studied in this thesis included BMI, blood pressure, lipid, CRP and insulin levels, and total, abdominal and organ fat, measured by MRI at 10 years. MRI is considered the gold standard for the measurement of intra-abdominal and organ fat deposition because of its accurate and reproducible technique. Since we know that organ fat outcomes measured by MRI are important cardio-metabolic risk factors and track from childhood into adulthood, it would be useful to have several MRI measurements from birth onwards to assess the association with cortisol levels throughout childhood. Future studies should consider to assess the associations between more refined cardiac measures obtained by ultrasound or MRI from early embryonic life onwards with hair cortisol concentrations, to gain more knowledge about the cardiovascular development in relation to glucocorticoids.

DNA methylation

The field of population epigenetics is still in its infancy and has challenges in terms of methodology and genetic, environmental and technical confounding. Cohort studies with measures of epigenome-wide DNA methylation, outcomes and confounding variables at multiple time points during childhood are scarce and needed. A longitudinal design may allow more detailed examination of direction of effect and causality. The

longitudinal design of both epigenetic studies in this thesis reduces the risk of reverse causation. However, we did not study the associations between trajectories of DNA methylation and BMI or maternal haemoglobin and DNA methylation in the same individuals from early life onwards. Ideally, future studies should use causal inference approaches, such as Mendelian randomization, to gain more insight into the causality of associations in childhood. This approach has already been used by some recent studies in adults.[87, 122] The analyses in the EWAS of childhood BMI were adjusted for cell type proportions using an adult reference dataset, which was the only available method at the time of the analyses.[116, 117] Since then, cord blood specific reference panels have become available, but we observed no substantial differences in results when comparing both datasets in two large cohorts.[115] As cell type composition varies with age, future studies should, if possible, use reference sets specific for the age of participants under study. The tissue-specificity of DNA methylation will remain a major challenge for future studies since many tissues that are potentially relevant for disease development are not accessible for population researchers. However, even if associations with DNA methylation in blood are not causal, identified CpGs may be useful as biomarkers of either exposure or outcome. Increased collaboration between population science and basic science may also contribute to better understanding of mechanisms. Lastly, in the emerging field of population epigenetics, the role of genetics warrants more research, as studies reported that a substantial proportion of the variation in DNA methylation between individuals is caused by genetic variants.

Causality

Despite the extensive adjustment for potential confounders in the studies performed in this thesis, the causality of the associations observed remains unclear due to the observational design. Randomized controlled trials (RCT) are the gold standard for studying causal relationships as randomization eliminates much of the bias inherent with observational study designs. However, randomized exposure to externally regulated stress in pregnancy or glucocorticoids in childhood without a medical reason, would be unethical. Instead, experimental interventions promoting psychological wellbeing during pregnancy and preventing increased cortisol levels in childhood, may provide more insight into the potential causal association of these exposures with childhood adiposity and cardio-metabolic health. An RCT in pregnant women showed that weekly exercise sessions reduced the level of prenatal depressive symptoms.[127] Also, studies on the effect of mindfulness-based interventions on reducing perceived stress, anxiety and depression show promising results.[128] Future studies should evaluate the effect of stress reduction in pregnancy on offspring health in larger groups of participants with long-term follow-up. Comparing the associations of maternal to those of paternal psychological distress in pregnancy with childhood obesity may further disentangle underlying

mechanisms. Stronger associations for maternal psychological distress suggest direct intrauterine mechanisms, whereas similar or stronger associations for paternal stress suggest a role for extra-uterine exposures such as shared genetic or lifestyle-related characteristics.[129] Most birth cohorts did not perform extensive measures in the (future) fathers. Ideally, measures in mothers and fathers would, if applicable, be equal in terms of methods, frequency and time of assessments in order to fully compare them. Another approach that may be used to control for environmental characteristics as well as maternal genotype that are shared among siblings, is the use of sibling comparison studies. However, this study design may introduce bias due to differences in major exposures of interest and other lifestyle-related characteristics between siblings.[130] Studies using Mendelian randomization in large sample sizes are needed in observational studies like the studies in this thesis, to shed further light on causality and avoid confounding.

CLINICAL IMPLICATIONS

Early-life stress may contribute to the burden of childhood obesity at a population level. Findings from this thesis suggest that maternal psychological distress during pregnancy and increased hair cortisol concentrations in childhood are associated with childhood general and organ fat measures. The research presented in this thesis is mainly based on observational data. As discussed previously, this limits the identification of causality and translation of the findings to clinical settings. The observed effect estimates were small to moderate, but are of interest from a developmental perspective since obesity risk factors tend to track from childhood into adulthood. Also, even subclinical differences in cardiovascular risk factors during childhood are related to the development of cardiovascular disease in later life. Thus, a healthy mental wellbeing in pregnancy and childhood may be important to reduce the burden of obesity throughout the life course. Despite the limitations our studies have some important clinical implications.

Maternal psychological distress during pregnancy is associated with several adverse outcomes in the offspring. We observed that maternal psychological distress and anxiety in pregnancy were associated with higher general fat measures and an increased risk of obesity, and that maternal anxiety during pregnancy was additionally associated with higher organ fat measures. If the observed associations are causal, although effect estimates were small, they imply that reducing the prevalence of psychological distress among pregnant women may lower the prevalence of adiposity among their offspring. Health care providers involved in obstetric care should be aware of the importance of healthy mental wellbeing during pregnancy. These findings support the recommendation of the American College of Obstetrics and Gynecology in 2006 to consider screening pregnant women for depression.[131] Our findings are in line with the Dutch guideline

for obstetricians and gynecologists for fetal growth restriction, in which psychological distress is mentioned as a risk factor for preterm birth with many other potential health consequences.[132] Also, if causal, these findings underline the importance of the first 1,000 days of life, from conception until one's second birthday, a critical window of opportunity when the foundations for optimal health and growth are established.[133] Early, ideally before conception, identification and guidance of women at risk for developing psychological distress symptoms may improve offspring health. Since preconception consultations are available but not standard in the Netherlands, health care providers should address the importance of mental wellbeing during pregnancy during regular appointments to increase awareness among future parents. Our findings also highlight the importance of a program such as "Kansrijke Start" which aims to advice and guide vulnerable women through their pregnancies.[134]

The sex-specific differences observed in the association between maternal psychological distress and both heart rate and triglycerides, support the hypothesis of sex-specific differences in response to prenatal stress and warrant further research. Health care professionals should be aware of these potential sex-specific associations.

Our findings on the association of hair cortisol concentrations and adiposity measures in childhood provide valuable information on the relation between cortisol and adverse body composition. Further studies are needed to draw conclusions on the causality and directionality of the observed associations. Health care providers including general physicians, family physicians, and pediatricians, but also teachers and parents should be aware of this relation and aim to prevent both adiposity and stress in children. Interventions may involve reducing stress in children but warrant further research first.

Conclusion

Findings from this thesis suggest that maternal psychological distress during pregnancy and increased hair cortisol concentrations in childhood are associated with childhood general and organ fat measures. Although the observed associations are relatively small and causality needs to be proven, our findings suggest that early-life stress may be important for the burden of childhood obesity at a population level.

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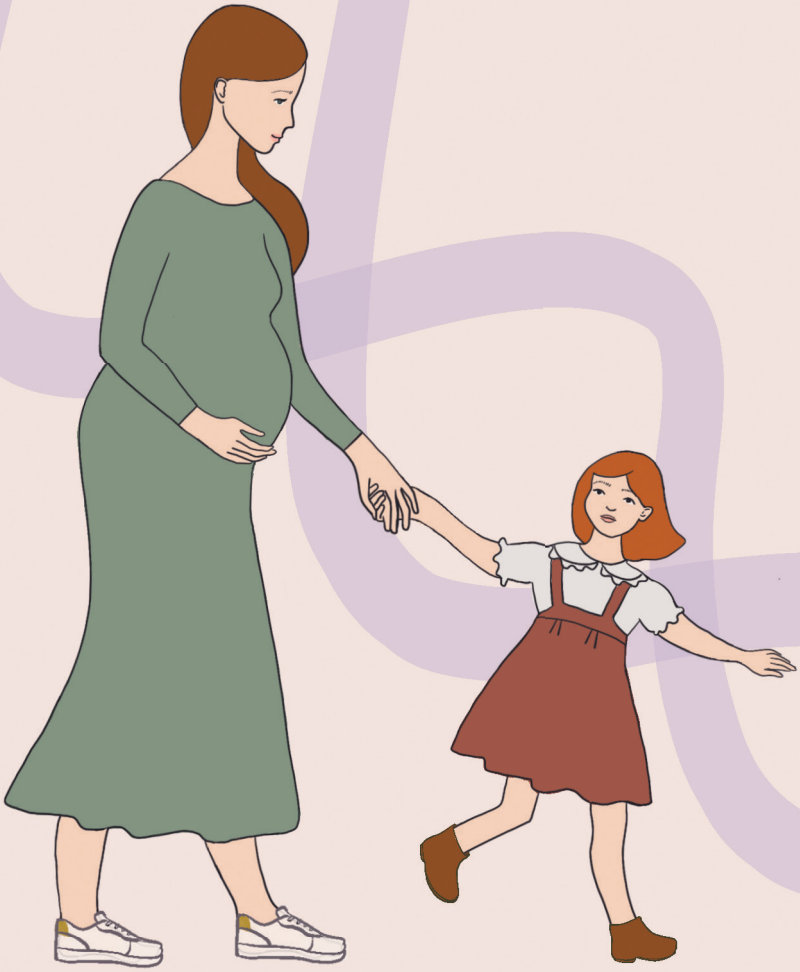

6

Summary
Samenvatting

SUMMARY

Chronic stress is associated with several cardio-metabolic outcomes in adults such as hypertension, heart failure and the metabolic syndrome. Long-term exposure to elevated concentrations of cortisol, also known as the most important stress hormone, seems to have a deleterious effect on the function of cardiovascular and metabolic systems. The main hypothesis for this thesis was that the associations of chronic stress with cardio-metabolic outcomes may originate in early life. Identifying adverse exposures in early life is important for the development of preventive strategies and interventions aiming to reduce the burden of disease in later life. During fetal life, the hypothalamic-pituitary-adrenal (HPA) axis, responsible for the regulation of cortisol, is under construction and therefore susceptible to prenatal programming influences. Exposure to physical or psychological stress in early-life may induce permanent changes in growth, organ structure and metabolism, and thereby have an important role in the development of diseases in later life.

The first objective of this thesis was to assess the associations of maternal psychological distress in pregnancy with body fat development and cardio-metabolic outcomes in childhood. The second objective was to assess the associations of childhood hair cortisol concentrations with body fat development and cardio-metabolic outcomes in childhood. As third objective, we assessed the associations of maternal haemoglobin concentrations and DNA methylation in the offspring. Lastly, as fourth objective, we aimed to identify whether DNA methylation in newborns, children and adolescents was associated with BMI in childhood and adolescence. The studies presented in this thesis used data from the Generation R Study, a population-based cohort study from fetal life onwards in Rotterdam, the Netherlands, and the Pregnancy And Childhood Epigenetics Consortium (PACE).

In **Chapter 1** we provide the background, hypothesis, aims and design for the studies presented in this thesis.

Chapter 2 describes studies on the associations of maternal psychological distress during pregnancy with maternal and child cardio-metabolic outcomes. In ***Chapter 2.1*** we summarized the available evidence on the associations of maternal psychological distress during pregnancy on child health outcomes. We reported that intra-uterine exposure to maternal stress is associated with multiple adverse fetal and child health outcomes. However, not all reviewed studies were of high quality and studies showed contradictory results. These findings warrant more studies on the associations of maternal psychological distress during pregnancy with child health outcomes. In ***Chapter 2.2*** we examined the associations of maternal psychological distress with weight gain in pregnancy. Overall, psychological distress, depression and anxiety were not associated with continuous measures of gestational weight. Only women with anxiety symptoms

had a lower risk of excessive weight gain. The findings of our study do not strongly support the hypothesis that psychological distress, depression and anxiety affect weight gain in pregnant women. In ***Chapter 2.3***, we evaluated the associations of maternal psychological distress during pregnancy with childhood general and organ fat measures. Children of mothers with psychological distress during pregnancy had a higher fat mass index and a higher risk of obesity. Maternal anxiety in pregnancy was associated with a higher BMI, fat mass index and higher risks of overweight and obesity in the offspring. Maternal anxiety in pregnancy was also associated with higher subcutaneous and visceral fat indices and liver fat fraction in childhood. We observed no associations for maternal depression during pregnancy. These findings suggest that lowering psychological distress and anxiety in pregnant women may help to prevent childhood adiposity. In ***Chapter 2.4,*** we studied the associations of maternal psychological distress during pregnancy and childhood cardio-metabolic risk factors. We did not observe an association in the whole group. However, psychological distress during pregnancy was associated with higher childhood heart rate in boys only, whereas anxiety during pregnancy was associated with higher childhood triglyceride concentrations among girls only. These findings suggest that effects of prenatal stress exposure on subsequent cardio-metabolic outcomes may differ by sex of the offspring.

Chapter 3 focuses on the associations of hair cortisol concentrations with cardio-metabolic outcomes in children. In ***Chapter 3.1*** we assessed the associations of hair cortisol concentrations in children with childhood general and organ fat measures. We observed that higher hair cortisol concentrations at 6 years of age were associated with higher BMI, fat mass index, liver fat fraction and higher risks of overweight and non-alcoholic fatty liver disease (NAFLD) at age 10 years. Only the associations for liver fraction and NAFLD were independent of fat mass index at 6 years. These results warrant future studies to assess the direction of effects. In ***Chapter 3.2*** we examined whether hair cortisol concentrations in children were associated with cardio-metabolic risk factors such as blood pressure, lipid levels, insulin and C-reactive protein (CRP) concentrations. Higher hair cortisol concentrations at age 6 years were associated with higher systolic blood pressure at age 10 years. However the association attenuated into non-significance after adjustment for childhood BMI at age 6 years. Higher hair cortisol concentrations at age 6 years were associated with an increase in total and LDL-cholesterol between 6 and 10 year of age, 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. These results suggest that higher cortisol in hair is not associated with cardio-metabolic risk factors in childhood. These associations may develop at later ages.

Chapter 4 describes studies on the potential epigenetic mechanisms linking adverse exposures in early life to later-life health. In ***Chapter 4.1*** we examined associations of maternal haemoglobin concentrations in pregnancy and offspring DNA methylation.

There was no statistical support for associations of maternal haemoglobin levels with offspring DNA methylation either at individual methylation sites or clustered in regions. These results suggest that maternal haemoglobin in pregnancy does not affect the DNA methylation of the offspring. However, maternal haemoglobin levels were within the normal range for most participants, whereas adverse outcomes often arise at the extremes. Studies including participants with more extreme values of haemoglobin, both low and high, are needed to provide more insight. Lastly, in ***Chapter 4.2*** we assessed whether DNA methylation in cord blood and whole blood in childhood and adolescence was associated with BMI in childhood and adolescence. DNA methylation at only three CpGs, each in a different age range, was associated with BMI. With advancing age of the participants across childhood and adolescence, we observed increasing overlap with altered DNA methylation loci reported in association with adult BMI. These results support, but do not prove, the hypothesis that DNA methylation differences are mostly a consequence rather than a cause of obesity.
Finally, in **Chapter 5**, a general discussion of all studies included in this thesis, suggestions for future research and potential implications for clinical practice are provided.

SAMENVATTING

Chronische stress is geassocieerd met nadelige cardio-metabole uitkomsten bij volwassenen, zoals hypertensie, hartfalen en het metabool syndroom. Langdurige blootstelling aan verhoogde concentraties cortisol, het belangrijkste stress hormoon, heeft schadelijke effecten op de functie van de cardiovasculaire en metabole systemen in het lichaam. In dit proefschrift onderzochten we de hypothese dat de associaties van chronische stress met cardio-metabole uitkomsten al vroeg in het leven ontstaan. Het identificeren van deze associaties zal bijdragen aan het begrip en de kennis over het ontstaan van cardio-metabole ziekten. Dit is belangrijk voor de ontwikkeling van preventiestrategieën en interventies gericht op het verminderen van het vóórkomen van ziekten op latere leeftijd. De hypothalamus-hypofyse-bijnier-as, verantwoordelijk voor onder andere de regulering van cortisol, wordt aangelegd tijdens het foetale leven en is in die periode vatbaar voor prenatale programmering. Blootstelling aan fysieke of psychologische stress in het vroege leven kan permanente veranderingen in groei, orgaan structuur en metabolisme veroorzaken en op die manier bijdragen aan het ontstaan van ziekten later in het leven.

Het eerste doel van dit proefschrift was het bestuderen van de associaties van maternale psychologische stress tijdens de zwangerschap met de ontwikkeling van lichaamsvet en cardio-metabole risicofactoren bij kinderen. Het tweede doel was het bestuderen van de associaties van cortisol concentraties in het haar van kinderen met de ontwikkeling van lichaamsvet en cardio-metabole risicofactoren bij kinderen zoals bloeddruk, lipiden en CRP. Het derde doel van dit proefschrift was te onderzoeken of maternale hemoglobine concentraties geassocieerd zijn met DNA methylatie in het kind op verschillende leeftijden. Tenslotte, als vierde doel van dit proefschrift, hebben we onderzocht of DNA methylatie in pasgeborenen, kinderen en adolescenten geassocieerd was met BMI in de kindertijd en in de adolescentie. Voor de studies, beschreven in dit proefschrift, is gebruik gemaakt van data van het Generation R onderzoek, een prospectieve cohort studie onder zwangere vrouwen en hun kinderen in Rotterdam, en het Pregnancy And Childhood Epigenetics Consortium (PACE).

Hoofdstuk 1 beschrijft de achtergrond, hypotheses en onderzoeksdoeleinden van de studies die beschreven worden in dit proefschrift.

Hoofdstuk 2 beschrijft onderzoeken naar de associaties van maternale psychologische stress tijdens de zwangerschap met cardio-metabole uitkomsten in moeders en kinderen. In ***Hoofdstuk 2.1*** hebben we de beschikbare studies over maternale psychologische stress tijdens de zwangerschap en de gezondheid van het kind samengevat. Het merendeel van de studies laat een associatie zien tussen maternale psychologische stress tijdens de zwangerschap en foetale, cardio-metabole, respiratoire en neurologische uitkomsten. Echter, de beschikbare studies waren niet altijd van hoge kwaliteit en bevin-

dingen waren niet altijd consistent tussen de studies. Deze resultaten vragen om meer gedegen onderzoek naar de gevolgen van psychologische stress bij zwangere vrouwen op de gezondheid van hun nageslacht. In ***Hoofdstuk 2.2*** hebben we de associaties bestudeerd van maternale psychologische stress en gewichtstoename tijdens de zwangerschap. Zowel psychologische stress, als depressie en angst waren niet geassocieerd met continue maten van gewichtstoename tijdens de zwangerschap. Alleen zwangere vrouwen, die symptomen van angst rapporteerden, hadden een lager risico op excessieve gewichtstoename. Deze bevindingen ondersteunen de hypothese dat psychologische stress, depressie en angst gewichtstoename in zwangere vrouwen beïnvloeden, niet. In ***Hoofdstuk 2.3*** laten we zien dat kinderen van moeders, die psychologische stress rapporteerden tijdens hun zwangerschap, een hogere totale lichaamsvetmassa en een hoger risico op obesitas hadden. Daarnaast beschrijven we dat angstklachten van moeder tijdens de zwangerschap geassocieerd waren met een hoger BMI, hogere totale lichaamsvetmassa en een hoger risico op overgewicht en obesitas bij hun kinderen. Angst in de zwangerschap was ook geassocieerd met een hogere subcutane en viscerale vetmassa en verhoogde hoeveelheid levervet bij het kind. We vonden geen associaties voor maternale depressie tijdens de zwangerschap. Deze bevindingen suggereren dat het verlagen van psychologische stress en angst bij zwangere vrouwen mogelijk bijdragen aan het voorkomen van adipositas bij kinderen. In **Hoofdstuk 2.4** hebben we de associaties bestudeerd van maternale psychologische stress tijdens de zwangerschap en cardio-metabole risicofactoren in de kindertijd. We vonden geen associaties in de hele onderzoeksgroep. Psychologische stress van de moeder tijdens de zwangerschap was wel geassocieerd met een hogere hartslag bij jongens, terwijl maternale angst in de zwangerschap geassocieerd was met hogere triglyceride concentraties bij meisjes. Dit geeft aan dat de effecten van blootstelling aan prenatale stress mogelijk verschillend zijn voor jongens en meisjes.

In **Hoofdstuk 3** beschrijven we de associaties van cortisol concentraties in het haar en cardio-metabole uitkomsten in de kindertijd. In **Hoofdstuk 3.1** hebben we laten zien dat verhoogde cortisol concentraties in het haar op 6-jarige leeftijd geassocieerd waren met een hoger BMI, totale lichaamsvetmassa, levervet fractie en een verhoogd risico op overgewicht en non-alcoholische leververvetting op 10-jarige leeftijd. Alleen de associaties van levervet fractie en non-alcoholische leververvetting waren onafhankelijk van de totale lichaamsvetmassa op 6-jarige leeftijd. Nieuwe studies zijn nodig om de causaliteit en richting van de geobserveerde associaties te onderzoeken. In **Hoofdstuk 3.2** hebben we onderzocht of verhoogde haar cortisol concentraties op 6-jarige leeftijd geassocieerd zijn met cardio-metabole risicofactoren zoals bloeddruk, lipiden en CRP. Verhoogde haarcortisol concentraties waren geassocieerd met een hogere systolische bloeddruk op 10-jarige leeftijd maar deze associaties waren niet meer significant na correctie voor BMI op 6-jarige leeftijd. Hogere haarcortisol concentraties op 6-jarige

leeftijd waren geassocieerd met een toename van zowel het totale cholesterol als het LDL-cholesterol tussen 6- en 10-jarige leeftijd maar niet met deze metingen zelf. Deze resultaten laten zien dat de associaties tussen verhoogde cortisol waarden en verscheidene cardio-metabole risicofactoren nog niet aanwezig zijn op de kinderleeftijd, maar zich mogelijk op latere leeftijd ontwikkelen.

In **Hoofdstuk 4** beschrijven we twee studies waarin epigenetische mechanismen als mogelijke link tussen blootstelling aan nadelige factoren in het vroege leven en gezondheid in het latere leven worden onderzocht. In **Hoofdstuk 4.1** hebben we associaties van hemoglobine concentraties bij de moeder gedurende de zwangerschap met DNA methylatie in navelstrengbloed en op verschillende momenten in de kindertijd onderzocht. Hemoglobine concentraties in de zwangerschap waren niet geassocieerd met DNA methylatie op individuele locaties en ook niet wanneer DNA methylatie sites werden geclusterd in regio's. Deze resultaten suggereren dat maternaal hemoglobine tijdens de zwangerschap DNA methylatie van het kind niet beïnvloedt. Echter, hemoglobine concentraties in deze studie waren grotendeels binnen de normaalwaarden, terwijl nadelige uitkomsten zich meestal voordoen bij meer extreme waarden. Studies waarbij naar meer extreme waarden van hemoglobine, wordt gekeken, zijn nodig om meer duidelijkheid te geven. Tenslotte hebben we in **Hoofdstuk 4.2** gekeken of DNA methylatie in navelstrengbloed en bloed op de kinderleeftijd en in de adolescentie geassocieerd is met BMI in de kindertijd en adolescentie. DNA methylatie op 3 verschillende locaties in het DNA, elk in een andere leeftijdscategorie, was geassocieerd met BMI. Met de toename van de leeftijd van de deelnemers in de studie van kindertijd naar adolescentie zagen we een toename in de overlap met locaties in het DNA waar methylatie geassocieerd is met BMI in volwassenen. Deze resultaten ondersteunen de hypothese dat DNA-methylatie patronen met name een gevolg zijn van een veranderd BMI en niet de oorzaak, maar bewijzen dit niet.

Tot slot wordt in **Hoofdstuk 5** een overzicht en interpretatie van de studies die beschreven worden in dit proefschrift gegeven. Tevens worden methodologische overwegingen, klinische implicaties en suggesties voor toekomstig onderzoek besproken.

7

Appendices

List of publications
Phd portfolio
About the author
Dankwoord

LIST OF PUBLICATIONS

Vehmeijer FOL, Moudrous W, el Addouli H, Verboon C, Dippel WJ. Cerebellar infarction requires quick diagnosis and immediate treatment with intravenous thrombolytics - a case report. *Erasmus Journal of Medicine* (Dutch). 2015;4(2):41-2.

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PHD PORTFOLIO

Name PhD candidate	Florianne Olga Lucia Vehmeijer
Erasmus MC Department	The Generation R Study Pediatrics, Erasmus MC, Rotterdam
Medical school	Erasmus University Medical Center, Rotterdam (2006-2013)
Research school	Netherlands Institute for Health Sciences (NIHES), Rotterdam (2015-2017)
PhD Period	December 2015 – March 2021
Promotor	Prof. dr. V.W.V. Jaddoe
Copromotors	Dr. J.F. Felix, Dr. S. Santos

	Year	Workload (ECTS)
Master of Science in Clinical Epidemiology, NIHES, Rotterdam	2015-2017	70,2
Common core		
Study Design		
Biostatistical Methods I: Basic Principles		
Biostatistical Methods II: Classical Regression Models		
English Language		
Development Research proposal		
Introduction to Medical Writing		
Research period PIN Health Sciences		
Required		
Clinical Epidemiology		
Methodologic Topics in Epidemiologic Research		
Principles of Research in Medicine and Epidemiology		
Methods of Public Health Research		
Clinical Trials		
Health Economics		
The Practice of Epidemiologic Analysis		
Fundamentals of Medicine Decision Making		
Elective courses		
Repeated Measurements in Clinical Studies		
Missing Values in Clinical Research		
Genomics in Molecular Medicine		
Advances in Genome-Wide Association Studies		
Planning and Evaluation of Screening		
Courses for the Quantitative Researcher		
Human Epigenomics		
Genome Wide Association Studies		

Courses		
Scientific Integrity for PhD students, Erasmus MC, Rotterdam	2016	0,3
The Course on R	2016	1,4

Seminars and workshops		
Research meetings Generation R Study	2016-2021	1,0
Maternal and Child Health meetings	2016-2021	4,0
Molecular Epidemiology meetings	2016-2021	4,0
DynaHealth Consortium meetings (Oulu, Rotterdam, York)	2016-2018	2,0
LifeCycle Consortium meeting (Barcelona)	2015	1,0
PACE consortium meetings (Rotterdam 2x)	2017, 2019)	1,0

Conferences and presentations		
The Power of Programming, Munich, Germany Poster presentation	2016	1,0
Developmental Origins of Health and Disease (DOHaD), Rotterdam Oral presentation	2017	1,0
Epigenomics of Common Diseases, Cambridge, UK Poster presentation	2017	1,0
3rd Paula Rantakallio Symposium on Birth Cohorts and Longitudinal Studies, Oulu, Finland Poster presentation	2016, 2018	2,0
DynaHealth, annual General Assembly meeting, York, UK Oral presentation	2018	1,0

Teaching activities – supervising students		
S. Balkaran, Master Medicine	2016	3,0
P. Parmar, Research visit	2017	1,0
C. da Silva, Research project	2017-2018	3,0

Other activities	
Co-writing HORIZON 2020 grant application	2016
Project team member at Medical Business Projects	2016
Organizational committee DOHaD World Congress 2017	2017
Project Management LifeCycle Consortium	2020

Peer review	
Peer review of abstracts for DOHaD World Congress 2017	2017
Clinical Epigenetics	2019-2020

Scholarship	
Vereniging Trustfonds Erasmus MC University Medical Center, Rotterdam	2017,2018

*1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours

ABOUT THE AUTHOR

Florianne Vehmeijer was born on September 29th, 1986 in Rotterdam, the Netherlands. She grew up as the eldest child with two brothers, Caspar and Quirijn. She graduated from the Rotterdam Montesorri Lyceum in 2004. Her journey continued abroad, studying Spanish in Spain and Ecuador for one year. Because she was not selected for medical school, she studied International Business Administration at the Rotterdam School of Management, Erasmus University Rotterdam for 2 years. Fortunately, in 2006 she was admitted to medical school at Erasmus University Rotterdam. She went to Nepal and Argentina for (research) internships. After obtaining her medical degree in 2013, she worked as a resident in pediatrics (ANIOS) for 1 year in the Sint Franciscus Gasthuis and for 1 year in the Neonatology Intensive Care Unit at the Erasmus Medical Center – Sophia Children's Hospital. December 2015 she started with her PhD project focused on early-life stress and childhood cardio-metabolic health under supervision of Prof.dr. Vincent Jaddoe and dr. Janine Felix and dr. Susana Santos at the department of Generation R at the Erasmus MC Rotterdam. During her project, she obtained her Master of Science degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences (NIHES) in 2017. In March 2021 she started as a GP in training.
Florianne lives in Rotterdam together with Yorick and their twin boys Krijn and Boele.

DANKWOORD

De totstandkoming van dit proefschrift zou nooit geslaagd zijn zonder de hulp van velen.

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Commissie

Aan de leden van de kleine commissie: Prof. dr. van Meurs, Prof. dr. Rings en Prof. dr. ir. Seiddel, heel hartelijk dank voor het lezen en beoordelen van dit proefschrift en voor jullie bereidheid om plaats te nemen in de kleine commissie. Aan de overige leden van de grote commissie: Prof. dr. Van Rossum, dr. Vrijkotte, Prof. dr. Bindels, hartelijk dank voor jullie tijd en bereidheid om met mij van gedachte te wisselen.

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LifeCycle colleagues

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Dear colleagues from abroad. One of the great things of being part of the Generation R Study Group is the opportunity to meet people from so many different backgrounds. I really enjoyed working with all of you.

Musici
Schrijven zonder muziek is als fietsen zonder stuur. Vivaldi, Bach, Martin Garrix, OneRepublic, Antoon en vele anderen, bedankt voor jullie composities. Over smaak valt niet te twisten.

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Siem, het grootste deel van onze promotietijd hebben wij samen op de 29e gezeten. Qua werk hadden we weinig met elkaar te maken, we spraken elkaar meer over andere dingen, jouw relaxte en eerlijke manier van zijn ben ik de afgelopen jaren erg gaan waarderen. Mooi om te zien dat je zo je draai hebt gevonden bij de KNO. Voor een koffie of glas wijn hoef ik nu maar één straat verder te zijn.
Hert (aka Laura), jij hebt dit promotietraject van dichtbij meegemaakt. En dat terwijl wij elkaar vroeger niet eens mochten. We hebben alle tennisbanen van Rotterdam denk ik

wel uitgeprobeerd inmiddels. Altijd “even” bijkletsen aan het net, superfanatiek als we zijn. Dankjewel voor je vriendschap en nuchtere kijk op het leven.

Broers
Lieve Cas en Qui. Ik mag mezelf gelukkig prijzen met jullie als broers. Vroeger was ik de oudere zus, inmiddels doet leeftijd er niet meer toe. Ik ben supertrots op de mannen die jullie geworden zijn. Cas, je hebt zoveel enthousiasme en drive om je werk en honderd andere dingen tot een succes te maken. Je verhalen zijn aanstekelijk. Qui, wij lijken veel op elkaar wat zorgt voor gedeelde interesses en goede gesprekken, jij snapt me vaak meteen. Ik vind het heel gezellig dat we dichtbij elkaar wonen en elkaar vaak zien. Als jullie ooit denken concurrentie te zijn met kaarten, let me know. Lieve Mies, ik heb er een hele gezellige schoonzus bij! En de tweeling een heel leuke tante. ☺

Mamma en pappa
Lieve mams en paps, ik kan niet in woorden uitdrukken hoeveel jullie voor mij betekenen, maar toch doe ik een poging. Onbezorgd opgroeien in een warm, gezellig gezin is een groot geluk. Jullie zijn er altijd voor ons. Nu jullie Omi en Opi van Boele en Krijn zijn, zien we elkaar nog vaker. Dank voor jullie onvoorwaardelijke steun, interesse, tijd en zorgen, altijd.

Yorick
Liefste Eggie, eeuwige fiancée. Van jou kwam ooit het idee om te promoveren, dat heb ik geweten. Het co-schap Interne Geneeskunde startte ik op 5-Noord, waar het allemaal begon.. Inmiddels hebben we zoveel samen meegemaakt, veel mooie maar ook erg verdrietige dingen. Ik heb veel bewondering voor hoe jij in het leven staat. Je bent mijn voorbeeld als het gaat om nuchterheid en relativering. Dankjewel dat je zo geduldig mijn eeuwige getwijfel hebt aangehoord. En dankjewel voor de afgelopen maanden. Nu de verbouwing bijna klaar is, dit boek eíííndelijk af is en ik de bordjes op de A16 niet meer hoef te tellen, is er echt weer meer tijd! Tijd voor een avontuur in het buitenland dit jaar, ik kan niet wachten!

Krijn en Boele
Allerliefste jongetjes, jullie zijn het mooiste wat mij en ons is overkomen. Sinds jullie er zijn, is het leven nog zoveel leuker (en vermoeiender) geworden. Ik vind het fantastisch jullie moeder te mogen zijn!

Be Curious

Charlie Mackesy

If I learned one lesson, count your blessings
Look to the rising sun and run, run, run