Prenatal Influences on Foetal Growth and Children's Cognition and Behaviour: Epidemiological and Epigenetic Studies

Nina H. van Mil

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PhD thesis, Erasmus University Rotterdam, The Netherlands

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Proefschrift

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Chapter 1

General Introduction

For many years mental illnesses were, according to professional and public opinion, a sign of problematic relationships between children and their parents. Parent coldness, obsessiveness, or poor parenting skills were considered a major determinant of disorders as autism, schizophrenia or ADHD. In contemporary medicine, the child-parent relationship is often considered of only modest importance compared to biological and genetic factors in posing children at risk for mental disorders. The biopsychosocial model, in 1977 theorized by psychiatrist George L. Engel (1), is although not undisputed nowadays the primary model in mainstream Western psychiatry. According to this model biological, psychological and social factors all play a significant role in health and disease. A complex interplay of environment and genetics accounting for a person's susceptibility is conceptualized to alter neurodevelopmental processes involved in mental disorders (2). Brain plasticity occurs throughout life but the perinatal period is recognized as critical. The development of the central nervous system begins with the formation of the neural tube in the first four weeks after conception. In this phase the neuro-ectodermal layer is programmed and developed into brain structures and its first functions. This process is followed by overlapping processes of neurogenesis, migration and cytodifferentiation. Most of the brain's billions of neurons are generated by the second trimester. Yet, myelinisation of the cerebral commissures and long tracts is not completed before late childhood and synaptic density in the cortex increases until early adult life (3).

Several teratogens, such as heavy metals and infectious disease agents like Rubella virus are known causes of congenital malformations (4, 5). The Dutch Hunger Winter study suggested foetal undernutrition as a risk factor for schizophrenia, antisocial personality disorder and neurological defects (6). However, it became increasingly recognized that even small variations in the prenatal environment, for example subtle variations in the exposure to maternal diet or smoking, play a critical role in shaping the developing brain and contributing to postnatal behaviour (7, 8).

Previous population-based research repeatedly related birth weight, as indicator of prenatal development, to neurodevelopmental outcomes (9, 10). The same prenatal influences that impair foetal growth could on the long term also contribute to disrupted neurodevelopment. However, prenatal risk factors and birth weight are not always part of the same pathway. Sometimes (partly) independent relations exist were only foetal growth or neuro-development is affected.

In the current thesis we aimed to explore prenatal influences as risk factors and predictors of child prenatal growth, cognition and behaviour at ages 4-6 years. We also focused on mechanisms underlying the association between foetal growth and neurodevelopment. Specifically, three aims were defined: 1) to assess the association between prenatal influences and foetal growth and children's cognition and behaviour; 2) to explore the association between alterations in newborn DNA methylation and foetal growth and child behaviour, and 3) to investigate relationships between foetal growth and children's cognition and behaviour.

SETTING

The studies in this thesis were embedded within the Generation R Study, a population-based birth cohort, in Rotterdam the Netherlands (11). The longitudinal design of the study, tracking of children from foetal life onwards, provides the unique opportunity to unravel the relationship between the early prenatal environment and later cognition and behaviour. Between 2001 and 2005 all pregnant women living in Rotterdam were invited to participate in the Generation R Study.

In total, 9,778 pregnant women participated in the study, including 8,879 women with enrollment in pregnancy and 899 at birth of the child. During the two postnatal phases of the study (0-4 and 6 years), information was obtained in 7,893 and 8,305 children, respectively.

OUTLINE

PART II focusses on associations between prenatal exposures and foetal growth. In **chapter 2** we describe the association between maternal hypothyroxinaemia during pregnancy and growth of the foetal and infant head. In **chapter 3** we relate maternal pregnancy plasma n-3 and n-6 fatty acid concentrations to foetal growth velocity and pregnancy outcomes. We present the association between the maternal mid-pregnancy TFA-C18:1 plasma concentration and birth weight and pregnancy outcomes in **chapter 4**.

In **PART III** two studies are described on prenatal exposures and the associations with children's cognition and behaviour. In **chapter 5** we relate maternal urinary iodine concentrations, as a proxy for iodine intake, to child executive functioning. In **chapter 6** we test possible explanations for the association between seasonality of birth and child IQ.

The studies presented in **PART IV** address the relationship between DNA methylation and child development. **Chapter 7** describes profiles of DNA methylation of *IGF2DMR* and H19, two genes implicated in early growth, and foetal growth. In **chapter 8** we explore the association of candidate gene DNA methylation profiles at birth and ADHD symptoms of the child at 6 years of age. In **chapter 9** we identify determinants of maternal one carbon metabolism in early pregnancy and associations with DNA methylation profiles of the newborn.

In **PART V** we focus on the relationship between foetal growth and children's cognition and behaviour. A study of the relationship between birth weight and attention problems at age 6 years is presented in **chapter 10**. In **chapter 11** we report associations between duration of pregnancy and birth weight and non-verbal IQ at age 6 years.

Chapter 12 covers the general discussion of the main findings, some methodological issues involved in the studies and inference of the results. Potential implications of our findings and recommendations for future research are provided.

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Part I

Maternal Conditions During Gestation and Foetal Growth

Chapter 2

Maternal Hypothyroxinemia During Pregnancy and Growth of the Foetal and Infant Head

ABSTRACT

Severe maternal thyroid dysfunction during pregnancy affects foetal brain growth and corticogenesis. This study focused on the effect of maternal hypothyroxinaemia during early pregnancy on growth of the foetal and infant head. In a population-based birth cohort, we assessed thyroid status in early pregnancy (median 13.4, 90% range 10.8-17.2), in 4,894 women, and measured the prenatal and postnatal head size of their children at 5 time points. Hypothyroxinaemia was defined as normal thyroid-stimulating hormone levels and free thyroxine-4 concentrations below the 10th percentile. Statistical analysis was performed using linear generalized estimating equation. Maternal hypothyroxinaemia was associated with larger foetal and infant head size (overall estimate ß = 1.38, 95% confidence interval 0.56; 2.19, p = 0.001). In conclusion, in the general population, even small variations in maternal thyroid function during pregnancy may affect the developing head of the young child.

INTRODUCTION

Thyroid hormones are important for different developing organ systems such as the kidney, lung, and skeleton. Furthermore, thyroid hormones play a crucial role, particularly in foetal brain maturation (1). As supported by experimental data in animals, a shortage of maternal thyroid hormones impacts the cytoarchitecture and radial distribution of specific neurons in the somotosensory cortex and hippocampus of the foetus (2, 3). Other studies have suggested a detrimental effect of maternal thyroid dysfunction in pregnancy on the cognitive and behavioural development of the child (4). Recently, we found that a low maternal free thyroxine-4 (FT4) concentration in early pregnancy predicts the cognitive delay in children (5).

It has been shown that children with congenital hypothyroidism have larger head circumferences (6-9). Newborns of hypothyroid mothers are reported to have a smaller head circumference and lower birth weight (10). Impaired prenatal brain development, as indexed by foetal head growth may underlie this association. The consequences of subtle nonclinical variations in the maternal thyroid status during pregnancy on foetal head and brain development, however, are not clear. Moreover, birth outcomes such as birth weight or head circumference at birth are only crude summary measures of intrauterine growth and cannot provide information of the growth across different time periods in pregnancy. Neonates can reach the same birth weight by different foetal growth trajectories (11). Studies that investigate the influence of maternal thyroid status on foetal head growth during pregnancy are scarce (12).

Therefore, in the current study, we investigated whether maternal thyroid status assessed in early pregnancy is related to prenatal and postnatal head growth. We also explored whether any effect on the growth trajectories of the foetal head mediates the association between maternal thyroid function and postnatal cognitive infant development.

MATERIALS AND METHODS

Design and Study Population

This study was embedded in the Generation R Study, an ongoing population-based birth cohort from foetal life onward. The Generation R Study, designed to identify early environmental and genetic determinants of growth, development and health, has been previously described in detail (13). Assessments were performed with ultrasonography, physical examinations, biological samples, and detailed questionnaires.

In mothers of 5831 foetuses, thyroid-stimulating hormone (TSH) and FT4 concentrations were measured during early pregnancy. Twin pregnancies (n = 124) were excluded since maternal thyroid function and growth potentials for individual foetuses in multiple pregnancies are different from singleton pregnancies. We excluded pregnancies of mothers receiving any thyroid-related medication including thyroxin (n = 39) leaving 5276 children.

Totally, 392 mothers participated with 2 or 3 siblings. Since the results did not differ after random exclusion of 1 or 2 of the siblings, they were included in the analyses. We excluded 256 mothers with thyroid problems other than hypothyroxinaemia. Of the remainder, 4638 mothers had at least 1 prenatal or postnatal measurement of head circumference and were included in one or more analyses.

The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants before participation.

Assessment of Maternal Thyroid Function and Urinary Iodine

In early pregnancy (median 13.4, 90% range 10.8-17.2), venous blood samples were drawn in plain tubes. Subsequently, serum was transported to the regional laboratory for storage at –80°C within 3 hours after sampling. Maternal TSH and FT4 from the stored samples were assayed in batches of 50 to 150 over a 6-month period using a chemoluminescence assay on the Vitros ECI Immunodiagnostic System (ORTHO Clinical Diagnostics, Rochester, New York). The interassay coefficients of variation (CVs) for TSH and FT4 were <4.1% and <5.4%, respectively, whereas the intraassay CVs were <1.2% and <2.7%, respectively. Thyroid peroxidase (TPO) antibodies are measured using ImmunoCAP 250-assays (Phadia AB, Uppsala, Sweden). Maternal TPO-antibodies was measured using the Phadia 250 immunoassay (Phadia AB) and regarded as positive when >60 IU/mL.

Reference ranges for maternal TSH (0.03-4.56 mIU/L), FT4 (10.36-22.16 pmol/L) levels were defined as the range between the 2.5th and 97.5th percentiles, after exclusion of women with known thyroid disease, thyroid-related medication usage, and twin pregnancies. Hypothyroxinaemia was defined as normal TSH levels and FT4 concentrations below the 10th percentile (11.82 pmol/L). This definition has been applied to the current sample before (5).

Maternal subclinical hypothyroidism (TSH >97.5% and FT4 >2.5%; n = 31), overt hypothyroidism (TSH >97.5% and FT4 <2.5%; n = 5), and hyperthyroidism (TSH <2.5% and FT4 >97.5%; n = 14) were not studied as determinant due to the small number of children at risk.

At the same time of blood sampling, maternal single voided urine samples were collected at random moments over the day. In a subsample of 1082 women, urinary iodine was measured through the ceri-arsenite reaction following destruction by means of ammoniumpersulfate. After brief centrifugation, sodium arsenite solution (0.1 mol/L in 1 mol/L of sulfuric acid) was added. Subsequently, ceriammonium sulfate was added and color was allowed to develop at 250°C during 60 minutes. Optical density was assessed at 405 nm. At a level of 1.7 μmol/L the within-assay CV was 5.1% and the between-assay CV was 14.3%.

To adjust for total urinary volume, we used the iodine to creatinine ratio.

Foetal Ultrasound Examinations and Postnatal Assessments

Sonographers carried out foetal ultrasound examinations at the visits to the research centers in early, mid, and late pregnancy. Most assessments (88%) were performed at the Generation R research center in Rotterdam. The remaining assessments were carried out in 1 of 5 collaborating hospitals. The foetal ultrasound examinations were used to establish gestational age and to assess the foetal growth characteristics. Crown-rump length was used for pregnancy dating until a gestational age of 12 weeks. The measurement of the biparietal diameter in the first trimester was used for pregnancy dating after 12 weeks.

The median (90% range) gestational age for the foetal ultrasound examinations in early, mid, and late pregnancy was 13.4 (10.8-17.2) weeks, 20.5 (19.1-22.6) weeks, and 30.3 (29.0-32.2) weeks, respectively. Postnatal measurement in early and mid-infancy was performed at 2.1 (1.0-3.8) and 6.3 (5.5-11.3) months of infant age, respectively.

To control for possible symmetric growth restriction, we corrected for abdominal circumference in additional analyses.

Measurements used for the present study, including head circumference and abdominal circumference, were performed using standardized techniques. The head circumference was measured at the level of the biparietal diameter and represents the outer perimeter of the foetal skull. Sonographers were blinded to the thyroid status of the pregnant women.

The intraobserver and interobserver reliability of foetal biometry measurements in early pregnancy within the Generation R Study is good (intraclass correlation coefficients for head circumference 0.995 [intraobserver] and 0.988 [interobserver] and for biparietal diameter 0.995 [intraobserver] and 0.994 [interobserver] with CVs between 1.8% and 3.8%) (14).

Postnatal measurements of child head circumference were collected at community health centers as part of routine health care using standardised procedures of health centers in the Netherlands (15).

Cognitive Assessment

Parent Report of Children Abilities (PARCA) was used as an indicator of cognitive development. The PARCA compromises 48 parent-reported items on the ability of their children to perform specific nonverbal cognitive tasks at age 30 months. A delay was defined as nonverbal cognitive scores below the 15th age- and gender-specific percentile. Previously, we showed a consistent association of maternal hypothyroxinaemia with delayed cognitive functioning (5).

Covariates

Information on maternal age, parity, national origin, education, prenatal smoking and alcohol use, folic acid use, and prenatal psychological problems was obtained by questionnaires during pregnancy. Educational level of the mother was assessed by the highest completed education and reclassified into 3 categories: primary school, secondary school, and higher education. National origin of the mother was based on the country of birth of the parents.

Maternal prenatal smoking and alcohol use were classified as "no use," "use until pregnancy was confirmed," and "continued use during pregnancy." Height and weight were measured without shoes and heavy clothing; body mass index (BMI) was calculated from height and weight (weight/height^2). At 20 weeks of pregnancy, we measured maternal prenatal psychological problems using the Brief Symptom Inventory (16). Child's gender, birth weight, Apgar scores 1 minute after birth, and the mode of delivery were derived from medical records completed by gynecologists and midwives. To define the gestational age at birth, we used the last menstrual period of the mother and the ultrasound examination at the first prenatal visit.

Statistical Analysis

Maternal thyroid status was a categorical determinant in our analyses. First, to examine the associations between maternal thyroid status during early pregnancy and foetal or infant head size at different ages, linear regression models (ANOVA) were used. To enable comparison, these analyses were restricted to children with all 3 prenatal ultrasounds and 2 postnatal measurements of mothers with hypothyroxinaemia of reference thyroid status (n = 2,621).

Linear generalized estimating equation (GEE) analyses (17) were used to study the longitudinal effects of maternal hypothyroxinaemia on foetal and child head size in all 4,638 children. With GEE analyses, repeatedly measured head size were analysed over time, taking correlations within the same participant into account. We calculated the overall effect of maternal hypothyroxinaemia on head size throughout the longitudinal period.

Additionally, to test a dose–response relation with head growth in the lower tail of the FT4 distribution, *P* for trends were calculated. Aimed on this, hypothyroxinaemia was divided in 2 categories: FT4 levels within the 5th to 10th percentile and FT4 <5th percentile.

All analyses were adjusted for maternal age, BMI, parity, education, national origin, smoking, alcohol use, prenatal psychological problems, child's gender, intrauterine position, and gestational age at ultrasound measurement. For postnatal analyses, age at birth and birth weight were also added as confounders to the model. The choice of potential confounders was determined a priori based on earlier literature (18, 19). Further covariates were included in the analyses if the effect estimates of maternal thyroid function changed (≥ 5%). Pregnancy complications and birth weight did not pass this threshold.

Furthermore, we explored whether the foetal head growth mediated any observed association between thyroid status and cognitive delay. Aimed on this, we first tested whether the thyroid status increased the risk of cognitive delay in the current sample of children. Next, we used linear regression models (ANOVA) to study if head circumference during pregnancy is related to nonverbal cognitive functioning. Finally, we tested possible mediation by studying the effect estimate change if head size is added to the model of thyroid status and cognitive delay.

RESULTS

Characteristics of pregnant women and their children categorized by thyroid status are presented in Table 1. Based on the criteria described above, 476 (9.7%) mothers had hypothyroxinaemia. Hypothyroxinaemic women are older, less educated, more often of non-Dutch national origin, and more often smoking in pregnancy. Urinary iodine concentration did not differ among hypothyroxinaemic and nonhypothyroxinaemic mothers. Children born to women with hypothyroxinaemia have higher birth weight.

Table 1: Study population by maternal thyroid status (total n = 4,638)

Thyroid status	**Reference** n = 4,162	**Hypothyroxinaemia** n = 476	**P-value**
Maternal characteristics			
Gestational age intake, wks median (90% range)	13.2 (10.1-17.5)	14.8 (10.9-17.8)	<0.001
Age, yrs. mean (SD)	29.7 (5.0)	30.2 (5.1)	0.09
Parity (nulliparous %)	58.4	51.0	<0.001
Body mass index, kg/m^2	24.3 (4.3)	26.1 (4.7)	<0.001
Educational level (%)			<0.001
Primary level	4.6	7.4	
Secondary level	41.4	48.8	
Higher education	53.9	43.8	
National origin (%)			<0.001
Dutch	55.0	42.5	
Other Western	12.2	12.1	
Non Western	32.8	45.4	
Smoking in pregnancy (%)			0.50
No	72.5	66.9	
Yes, stopped	8.6	9.8	
Yes, continued	18.9	23.3	
Iodine concentration, µg/mg creatinine median (90% range)	272 (112-619)	296 (105-731)	0.90
<10th percentile (%)	10.0	8.8	0.70
Child characteristics			
Gender (% boys)	51.4	50.4	0.67
Gestational age birth, wks. mean (sd)	39.9 (1.8)	39.8 (2.2)	0.66
Birth weight, g. mean (sd)	3411.4 (557.9)	3499.9 (562.6)	0.001

Maternal characteristics were assessed at intake. Child characteristics were assessed at birth. Values represent percentage (%) within column, mean (SD) or median (90% range). Independent students *T*-test, Mann-Whitney *U* and Chi Square tested for differences in baseline.

Table 2. Foetal and infant head size by maternal thyroid status during pregnancy

Foetal and infant head size in mm stratified by maternal thyroid status. n = 2,621			
Foetal head circumference	**early pregnancy** *median 13.4 weeks*	**mid pregnancy** *median 20.5 weeks*	**late pregnancy** *median 30.3 weeks*
reference, mm (95%CI)	89.6 (89.4; 89.7)	178.9 (178.7; 179.2)	285.3 (284.9;285.6)
hypothyroxinaemia, mm (95%CI)	90.3 (89.8; 90.8)	179.1 (178.4; 179.9)	286.1 (285.1;287.2)
p-value for adjusted difference	$p = 0.008$	$p = 0.60$	$p = 0.14$
Infant head circumference	**early infancy** *median 2.1 months*	**mid infancy** *median 6.3 months*	
reference, mm (95%CI)	391.9 (391.5; 392.4)	438.9 (438.4; 439.9)	
hypothyroxinaemia, mm (95%CI)	394.3 (393.0; 395.6)	440.9 (439.4; 442.3)	
p-value for adjusted difference	$p = 0.001$	$p = 0.01$	

Values are obtained from linear regression models. Adjusted for maternal age, BMI, height, educational level, national origin, parity, smoking, alcohol use and maternal psychopathology and foetal intra uterine position and gender. Prenatal analyses are adjusted for gestational age at assessment based on ultrasound; postnatal analyses are adjusted for infant age at assessment. Postnatal analyses are also adjusted for gestational age at birth.

Table 2 presents the association between hypothyroxinaemia and foetal head size by gestational age. In early pregnancy, head circumference of foetuses of hypothyroxinaemic mothers was 0.7 mm (95% confidence interval [CI] 0.2-1.2) larger as compared to the reference group (89.6 mm; 95%CI 89.4-89.7, p = 0.008). Later in pregnancy, there were no significant differences in foetal head circumference.

Postnatal children born to hypothyroxinaemic mothers had larger head circumference compared to mothers with reference thyroid status (2.4 mm; 95%CI 1.0-3.8, p = 0.001 in early infancy and 2.0 mm; 95%CI 0.4-3.5, p = 0.01 in mid-infancy).

The GEE analysis, which summarizes the effect over time, showed a significant increased head size over time in children born to hypothyroxinaemic mothers as compared to mothers with reference thyroid status (ß = 1.38, 95%CI 0.56-2.19, p = 0.001).

Figure 1 depicted the associations between hypothyroxinaemia and head size at individual time points and during follow-up. The figure suggests that the effect of hypothyroxinaemia on head size differs over time. Significant effects for individual time points in early pregnancy and infancy were observed, however, the differences between time points were not significant; indeed, the GEE model did not show any time differences (ß = –0.64, 95%CI –1.52-0.24, p = 0.16).

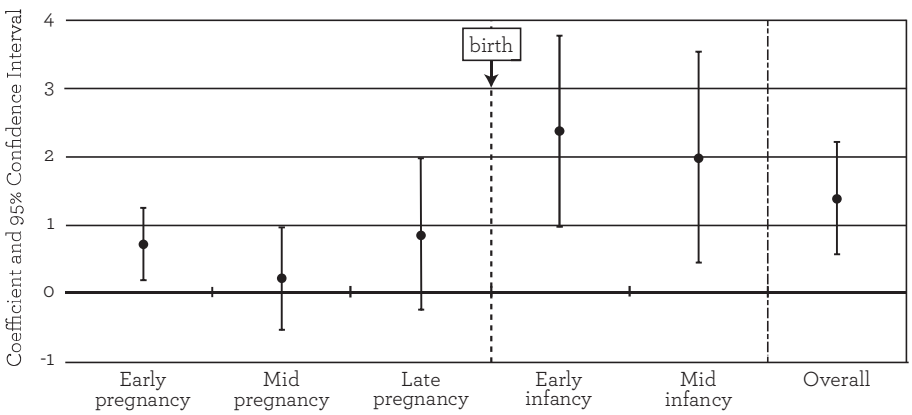

Figure 1. Head size hypothyroxinaemia compared to reference thyroid status.

Estimated estimates in head circumference in foetuses or infants of mothers with hypothyroxinaemia compared to reference group as obtained from ANOVA. GEE-analysis was used to calculate overall estimate. All models were adjusted for maternal age, BMI, height, educational level, national origin, parity, folic acid supplement use, smoking, alcohol use and maternal psychopathology and child's gender, intrauterine position and (gestational) age at assessment. Postnatal and longitudinal analyses were also adjusted for gestational age at birth.

To distinguish between decreased head growth and a more general symmetrical growth restriction including decreased head circumference, we additionally adjusted for abdominal circumference measured at the same gestational age. The effect of hypothyroxinaemia

on foetal head circumference remained present (difference in head circumference for hypothyroxinaemia early in pregnancy 0.7 mm, 95%CI 0.2-1.2, p = 0.01).

Next, we explored whether the effect of hypothyroxinaemia was dose responsive. There was a dose–response relation in the lower range of the FT4 distribution between the degree of hypothyroxinaemia and foetal head growth in early pregnancy (ß for linear contrast 0.64, 95%CI 0.14-1.13, p for trend = 0.01).

Finally, we tested whether head size early in pregnancy mediated the relation between maternal thyroid status and cognitive delay. In the current sample, hypothyroxinaemia was associated with an increased risk of cognitive delay (odds ratio [OR] = 1.63, 95%CI 1.06-2.52, p = 0.03). Head circumference measured during early pregnancy showed no association with cognitive delay (ß = –0.007, 95%CI –0.04-0.03, p = 0.99). This means that children with larger heads, early in pregnancy, did not have a higher risk of cognitive delay. The relation between thyroid status and cognitive delay can thus not be explained by early foetal head size. Using continuous measures of cognitive delay or curvilinear models of head size did not provide any evidence for mediation by foetal head size either (data not shown).

DISCUSSION

In our study, maternal hypothyroxinaemia in early pregnancy was associated with a larger size of the foetal and infant head. In early pregnancy, the effect was dose–response; severe hypothyroxinaemia was associated with more severe deviations in head growth than mild hypothyroxinaemia.

The growth of the foetal head did not primarily differ due to foetal dating differences in early pregnancy. In principle, the observed effects could reflect differences in foetal dating in early pregnancy. This seems unlikely since both sonographers and patients were blinded to thyroid status and hypothyroxinaemia is an asymptomatic condition. The observed growth difference showed a consistent dose–response pattern in early pregnancy and the difference in head size continued after birth. In our study, hypothyroxinaemia was not due to lower iodine concentrations suggesting that thyroid problems in this iodine sufficient area mostly have other origins. However, iodine excretion was measured only in a subsample.

Different explanations may help to understand the untoward effects of low FT4 levels in pregnancy on prenatal and postnatal head growth.

First, foetal growth may be impaired by low FT4 levels that affect placentation in early pregnancy. This is supported by the associations between maternal thyroid dysfunction and pregnancy complications related to malplacentation, including preeclampsia, intrauterine growth retardation, and still birth. It has been suggested that uteroplacental tissues are sensitive to minor perturbations in maternal thyroid function (20). A relation between placental insufficiency and a smaller head size in childhood with increasing divergence after puberty has been reported (21). On the other hand, our study also showed reduced growth of the foetal head circumference, even after controlling for the effects of low maternal FT4

levels on abdominal circumference. This indicates that thyroid hormones have a differential effect of on foetal head size.

A second explanation for the effect of the prenatal maternal hypothyroxinaemia is the direct effect of thyroid hormones on the developing brain. Such a mechanism has mainly been shown in animal studies. Ausó et al reported that FT4 deficits in rats alter normal migration of cortical neurons and the cytoarchitectonic organization of the neocortex and hippocampus in the offspring (2). If we infer the critical period from animal studies, the human foetal brain should be most vulnerable to maternal hypothyroxinaemia in late first trimester or midgestation. In that period, neurogenesis, myelination, and apoptosis take place that might explain the observed differences in size in early pregnancy (22, 23). Around birth synaptogenesis starts and the number of synapses growths exponential till the first year of life (24). Synaptogenesis starts to accelerate at the beginning of the third trimester of pregnancy and increases rapidly in the first 2 years of life. However, we argue as no statistically significant differences were observed between time points this interpretation is very speculative.

Apoptosis is important for normal brain development as 20% to 50% of neurons are known to undergo programmed cell death (25). In rats, neonatal administration of thyroxin produces smaller cerebellum with fewer folia (22, 23). Studies on the in vivo effects of thyroid hormone upon neurons of the cerebral cortex show that thyroid hormone produces an increase of proteasome–ubiquitin–protein conjugates and an increase in apoptosis (26, 27). This implies that a decrease in thyroxin need not lead to a decrease in brain tissue.

Third, as animal studies have shown, maternal thyroid hormones may affect foetal programming by epigenetic mechanisms in the structures and functions of organs. This is substantiated by studies showing acute changes in the maternal thyroid hormones that directly affect the expression of selected and stage-specific genes in the foetal brain (28, 29). A decrease in T4 resulted in less expression of neuroendocrine-specific protein. This protein inhibits axonal growth action of central myelin in growth cone collapse and neurite outgrowth (30) that probably results in increased brain mass. Again, this is in line with our observation that lower thyroid levels in early life are associated with a larger head circumference.

A fourth explanation for our observation might be that a lack of thyrotropin caused abnormal skull development, and abnormalities in thyrotropin metabolism are known to affect the skeletal development. However, the described delay in ossification and reduction of bone mineralization typically decrease rather than increase growth velocity (31). Furthermore, experimental work has shown that the growth of the vault of the skull depends on and is controlled by the growth of the brain (32).

As head circumference is known to correlate to brain volume, it is tempting to speculate that increased head growth is one of the mediators in the relationship between maternal thyroid status in pregnancy and psychological and behavioural child development (33). However, children with autism spectrum disorders also show increased growth in head circumference during postnatal life. While there are probably some differences in the

brain growth trajectories between these 2 conditions (ie, the exact onset of altered head growth in children with autism, (34) it is tempting to speculate that related neurobiological mechanisms, such as the postnatal acceleration of formation of cortical neurones, (35, 36) underlie both conditions.

Besides, data from the present cohort showed that maternal hypothyroxinaemia negatively affected behaviour and cognitive functioning in infants (5, 37). Inverted U-shaped associations between mid and late pregnancy head size and infant alertness have been reported previously indicating that both smaller and larger heads are negatively associated with this indicator of early neurobehavioural development (38). However, here we found no indication that a larger head in the first trimester is related to cognitive functioning. Possibly, structural changes not expressed by changes in head size may underlie this cognitive delay. Alternatively, a large head can reflect healthy and untoward effects, and this covers the negative impact of maternal thyroid status. Second, a catch-up and compensatory effect of brain development may make any early pregnancy effect not observable in toddlers anymore.

The strengths of this present study include our combination of ultrasound and postnatal measurements, which enabled us to determine the growth trajectories throughout gestation and infancy.

The large population-based prospective cohort enabled us to control for important confounding factors, including lifestyle factors, socioeconomic factors, and known determinants of foetal and infants' growth. Despite this, it cannot be ruled out that the associations are confounded by diet-related determinants or other unknown factors. Unfortunately, postnatal head circumference was not measured routinely at the Dutch child health care centers after the first year anymore. Longer follow-up studies are necessary to determine whether maternal hypothyroxinaemia affects child head growth later in life. Finally, since data were more complete in higher-educated mothers, we cannot rule out that selective nonresponse influenced our findings.

In conclusion, in the general population, even small variations in maternal thyroid function during pregnancy may affect the developing head of the young child.

Low maternal FT4 increases the head circumference in the first trimester of pregnancy and in early infancy. However, the effect of altered head growth on cognitive functioning is not yet clear. Further studies of the possible effect of screening and intervention based on maternal thyroid function are needed.

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PART II

Prenatal Influences on Children's Cognition and Behaviour

Chapter 5

Low Urinary Iodine Excretion During Early Pregnancy is Associated with Alterations in Executive Functioning in Children

ABSTRACT

The rare but deleterious effects of severe iodine deficiency during pregnancy on cognitive functioning of children are well known. Reports on possible associations between mild-to-moderate maternal iodine deficiency and child development, however, are scarce. In a population-based cohort we examined the association between maternal urinary iodine during early pregnancy and executive functioning in children at 4 years of age. In addition, we investigated the modification of this association by maternal diet and thyroid function. During pregnancy, we measured urinary iodine and thyroid hormone concentrations in 1156 women. In 692 of their children impairment of executive functioning was assessed by the Behaviour Rating Inventory of Executive Function. Five hundred mothers of Dutch national origin completed an FFQ. Analyses were performed by using regression models. The children of mothers with low urinary iodine showed higher scores on the problem scales of inhibition [ß = 0.05 (95%CI: 0.01, 0.10), p = 0.03] and working memory [ß = 0.07 (95%CI: 0.02, 0.12), p = 0.003]. Although maternal dietary intake and thyroid hormone concentration did not significantly modify these associations, the associations between urinary iodine and problems of inhibition were attenuated after adjustment for maternal psychological symptoms. In addition, the consumption of bread [ß = 0.61 (95%CI: 0.27, 0.95), p<0.001] and eggs [ß = 1.87 (95%CI: 0.13, 3.62), p = 0.04] was associated with higher urinary iodine. Thus, low maternal urinary iodine during pregnancy is associated with impaired executive functioning in children. Because these symptoms were subclinical and occurred at an early age, future studies are needed to show whether these children are more vulnerable to develop later clinical disorders.

INTRODUCTION

Iodine is required for the synthesis of thyroid hormones, which play an essential role in foetal and early postnatal growth and development of most organs, especially of the brain (1). This micronutrient is mainly obtained by the consumption of foods that contain natural or synthetic iodine. Because during pregnancy the production of thyroxin physiologically increases by 50%, this increased need is recommended to be compensated with a 50% increase in daily iodine requirement to prevent the onset of thyroid dysfunction (2).

Despite considerable progress over the past decades in developing countries, the prevalence of inadequate iodine intake is estimated at >20% in industrialized countries previously considered to be iodine sufficient (3, 4). Surveys indicate that especially girls and women of reproductive age may have deficient iodine consumption (5, 6). This also raises concern about a poor iodine intake during pregnancy in the United States and Europe for which changing dietary habits, especially low fish and milk consumption, are suggested to be responsible.

Severe iodine deficiency during pregnancy detrimentally affects maternal thyroid function and child neurobehavioural development (7). The severity of maternal iodine deficiency during pregnancy is related to the degree of impaired functioning in children (8). It is unknown, however, whether the increasing mild-to-moderate iodine deficiencies during pregnancy, especially in industrialized countries, detrimentally affects maternal thyroid function and neurodevelopment in offspring (9, 10).

Whereas cognition provides global insight of brain functioning, executive functioning represents different structures and functions of the brain involved in the cognitive regulation of behaviour (11). Executive function is defined as a group of processes, e.g., inhibition, working memory, and the ability to plan and organize, that are dependent on and influence more basic cognitive abilities, such as attention, language, and perception (12).

Iodine concentration in urine and excreted by the kidneys is a good marker of the dietary intake of iodine during the previous days. It is the measure of choice for assessment of iodine status because of its noninvasiveness (13). In epidemiologic studies urinary iodine concentrations of spot samples are used to define the iodine status in individuals and in populations (2).

Against this background the aims of our study were to examine in a population-based cohort with available assessments of maternal diet and urinary iodine in early pregnancy the associations between the following: 1) maternal diet and urinary iodine; 2) maternal urinary iodine and thyroid function; and 3) maternal urinary iodine, diet, and executive functioning in children at the age of 4 years.

PARTICIPANTS AND METHODS

Design and Study Population

This study was embedded in the Generation R Study, an ongoing population-based birth cohort from foetal life onward. Mothers with a delivery date from April 2002 until January 2006 were enrolled in the study. The Generation R Study was designed to identify early environmental and genetic determinants of growth, development, and health. The data obtained comprised detailed questionnaires, ultrasonography, and biological samples. The study has been previously described in detail (14).

The flow chart of the study population is presented in Figure 1. For this study we selected all mother-child pairs (n = 1,316) with available measurements of urinary iodine and thyroid hormone concentrations in early pregnancy. The sample for iodine measurements was selected semi-randomly from the total cohort with measurements of thyroid hormones (n = 5,831), with oversampling of women who had free thyroxin 4 (FT4) concentrations below the 10th percentile: Of the sample, 21.4% (n = 282) of the women had low FT4 concentrations, whereas 78.6% (n = 1,035) of the sample consisted of women with higher FT4 concentrations. To account for this oversampling, cases were weighted on the ratio of the population proportion on the sample proportion.

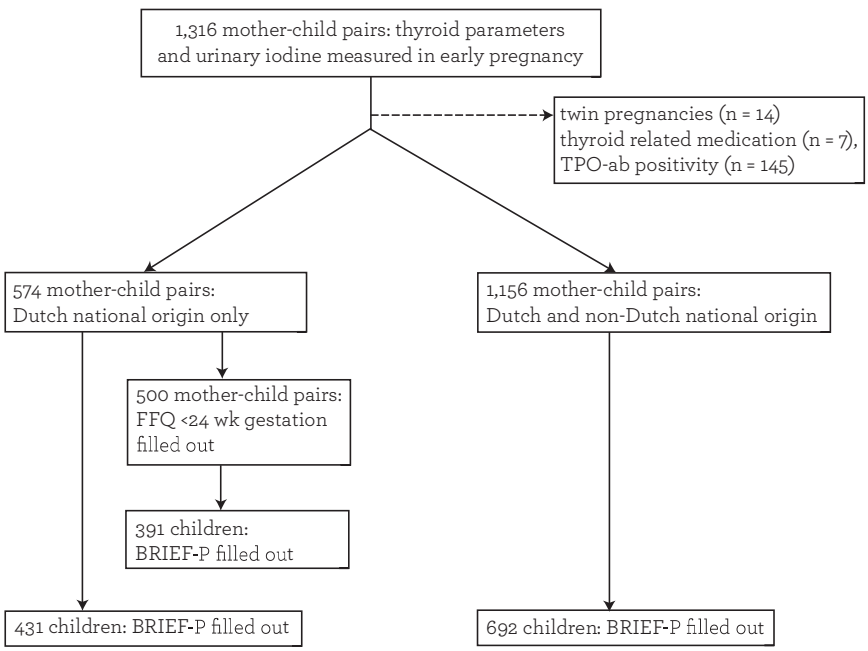

Figure 1

Selected characteristics of mothers and children by iodine intake. Thirty-seven mothers with ≥2 children participated. BRIEF-P, Behaviour Rating Inventory of Executive Function for Preschoolers; TPO-ab, thyroid peroxidase antibody.

No instance of fertility treatment was reported in this sample. Twin pregnancies (n = 14) were excluded because thyroid variables in multiple pregnancies are different from those in singleton pregnancies (15). In addition, we excluded mother-child pairs in which mothers received any thyroid-related medication including thyroxin (n = 7) or who were thyroid peroxidase antibody–positive (n = 145), which left 1156 mother-child pairs. A total of 574 mothers were of Dutch national origin; 500 of these completed an FFQ, 431 completed the Behaviour Rating Inventory of Executive Function for Preschoolers (BRIEF-P) for the child, and 391 completed both an FFQ and the BRIEF-P. Of women who were not of Dutch national origin (n = 583), 354 completed an FFQ, 261 completed the BRIEF-P, and 185 completed both questionnaires.

The study was approved by the Medical Ethics Committee of the Erasmus MC, University Medical Centre, Rotterdam, The Netherlands. Written informed consent was obtained from all individuals before participation.

Maternal Dietary Intake

In early pregnancy (median: 13.2 weeks; 95% range: 10.2-17.6) the nutritional intake of the previous 3 months was assessed by using a modified version of a validated semiquantitative FFQ (16). The FFQ consists of 293 food items and is structured according to meal patterns. Questions on the FFQ include consumption frequency, portion size, preparation method, and additions of the foods. Portion sizes were estimated by using household measures and photographs (17). To calculate average daily nutritional values, the Dutch food composition table 2006 was used (18).

The 293 food items were reduced to 19 food groups, according to the European Prospective Investigation into Cancer and Nutrition classification, on the basis of origin, culinary usage, and nutrient profiles (19).

To extract dietary patterns from food consumption data in the selected study population, we used principal components analysis (PCA) as previously described by Hu (20) and applied in a number of recent studies of dietary patterns and foetal and child development (21, 22). PCA was performed in the total Generation R cohort of women of Dutch national origin (n = 3,463). Because of the larger number of cases this gives a more accurate estimate.

Each woman was given a score for each of the factor or dietary patterns, calculated as the product of the food group value and its factor loadings summed across foods. For convenience we termed this score "adherence to dietary pattern." The 3 most prevalent dietary patterns were selected for further analysis.

Spearman correlation coefficients were used to correlate the dietary patterns after the PCA with the original food groups.

Maternal Urinary Iodine and Thyroid Function

At the same time of the assessment of nutritional intake, maternal single voided urine samples were collected at random times during the day. Urinary iodine was measured by the ceri-arsenite reaction after digestion by means of ammonium persulfate. After brief

centrifugation, sodium arsenite solution (0.1 mol/L in 1 mol/L of sulphuric acid) was added. Subsequently, ceri-ammonium sulfate was added, and color was allowed to develop at 250°C over 60 min. Optical density was assessed at 405 nm. At a concentration of 1.7 μmol/L iodine the within-assay CV was 5.1% and the between-assay CV was 14.3%.

To adjust for total urinary volume we used the iodine to creatinine ratio. Spot urine sampling is considered to be a reliable and practical laboratory technique available to quantify iodine excretion in individuals (23). Because >90% of iodine intake is excreted in the urine, urinary iodine excretion is considered the most appropriate indicator of iodine intake of the previous days as well as of iodine status (13). We defined low urinary iodine as an iodine:creatinine ratio below the 10th percentile of the study sample [0.04-0.12 mmol/mol creatinine (48.6-136.1 μg/g creatinine)].

To assess maternal thyroid function, at the same time of urine sampling venous blood samples were collected in plain tubes. Serum was transported to the regional laboratory for storage at -80°C within 3 h after sampling (24). Thyroid-stimulating hormone (TSH) and FT4 from the stored samples were assayed in batches of 50–150 over a 6-month period by using a chemiluminescence assay on the Vitros ECI Immunodiagnostic System (Ortho Clinical Diagnostics). The interassay CV for TSH and FT4 were <4.1 and <5.4%, respectively, and the intraassay CV for TSH and FT4 were <1.2 and <2.7%, respectively.

Thyroid peroxidase antibodies were measured by using ImmunoCAP 250 assays (Phadia AB) and regarded as positive when >0.06 IU/L.

Assessment of Executive Functioning

We measured impairment of executive functioning in children at 4 years of age by using the BRIEF-P (25). The BRIEF-P is a standardized rating scale developed to provide a window into behaviours associated with specific domains of executive functioning in children aged 2 to 5 years.

The BRIEF-P consists of a single rating form, completed by parents or other caregivers, with 63 items in 5 scales: inhibition (to stop own behaviour), shifting (to make a transition and change focus from one mindset to another), emotional control (to modulate emotional responses), working memory (to hold information in mind for the purpose of completing a task), and planning/organization (to manage current and future-oriented task demands within the situational context). The scales can be combined into the global executive composite. Raw scale scores are transformed to age- and gender-normed T-scores [50 ± 10 (mean ± SD)] to make scores comparable. Higher scores indicate more problems with executive functioning. In the present study, the parents were asked to rate how often a particular behaviour of the child was problematic in the preceding month.

Other researchers have shown the content validity of the BRIEF-P (26). The subscales of BRIEF-P show adequate to high test-retest reliability, indicating suitability for research purposes.

Covariates

Information was obtained by questionnaires during pregnancy on maternal age, national origin, education, parity, prenatal tobacco and alcohol use, and the use of folic acid supplements or (iodine- and non-iodine-containing) multivitamin supplements. The season of completing the FFQ was registered. National origin of the mother was based on the country of birth of her parents. The educational level of the mother was assessed by the highest completed grade and reclassified into 3 categories: primary school, secondary school, and higher education (e.g., higher vocational education or higher).

Maternal smoking and alcohol use were classified as "no use," "use until pregnancy was confirmed," and "continued use during pregnancy." Women were asked about the use of any multivitamin supplement or folic acid supplement during the past 6 months.

Height and weight were measured without shoes or heavy clothing; BMI was calculated from height and weight (kg/m^2). At 20 weeks pregnancy, we measured maternal psychological problems by using the Brief Symptom Inventory (27).

Child gender, birth weight, Apgar score 1 min after birth, and the mode of delivery were derived from the medical records completed by gynecologists and midwives. To define gestational age at birth, we used the last menstrual period of the mother and the ultrasound examination at the first prenatal visit. In case these methods disagreed, pregnancy was dated on the ultrasound data.

The following covariates were considered as potential confounders: maternal age, national origin, education, BMI, parity, prenatal psychological problem score, smoking, alcohol use, energy intake, use of any multivitamin, folic acid supplement use, season of completing FFQ, and child's gender, gestational age at birth, birth weight, Apgar score 1 min after birth, and the mode of delivery.

Statistical Analysis

Because the FFQ used is only validated in Dutch populations, all analyses with food intake were primarily restricted to mothers of Dutch national origin (n = 500).

First, associations between separate food groups as independent variables and urinary iodine as an outcome variable were analysed by using multivariable regression analyses. Second, Pearson's correlation coefficients between urinary iodine and maternal TSH and FT4 were calculated. Prior to analyses, TSH concentrations were transformed by the natural logarithm to achieve normal distribution. Third, associations between urinary iodine as a categorical determinant (below or above the 10^{th} percentile) and BRIEF-P problem scores were calculated with multivariate regression analyses. Because BRIEF-P scores were non-normally distributed, scores were transformed by the natural logarithm. Associations between urinary iodine and BRIEF-P problem scores were further explored by adding FT4 in the model. Maternal psychological problems were added separately in the model to assess the change in estimate due to psychological problems. In addition, we stratified the analysis on executive functioning for gender, and tested interactions between gender and low urinary iodine. For any observed association between urinary iodine and executive functioning the

model was further explored by adjustment for maternal intake of food groups. To reduce the number of comparisons, we tested only food groups that were associated with urinary iodine as mediators. To test whether the estimates were influenced by maternal national origin, all analyses were repeated among children of pooled Dutch and non-Dutch mothers (n = 692). Finally, to test whether results depended on the choice of the 10th percentile cutoff for urinary iodine, all analyses were repeated using a 5^{th}- and 15^{th}-percentile threshold.

All analyses were adjusted for gestational age at blood and urine sampling and estimated protein intake. A covariate was selected as a confounding variable if the effect estimates changed by ≥5% in the exploratory regression analyses. By using this criterion, maternal age, national origin, education, prenatal smoking, and child's birth weight and gestational age at blood sampling were included as confounders in the final multivariable analyses.

Differences between characteristics of mothers and children were tested by using Student's *t* test or Mann-Whitney *U* test for continuous variables and Pearson's chi-square test for categorical variables.

Missing data of covariables were completed by using the Markov chain Monte Carlo multiple imputation technique, creating 5 data sets. Subsequently, multivariable regression analyses were performed separately on each completed data set and thereafter combined to one pooled estimate (28). For all analyses, results including imputed missing data are presented. All analyses were performed by using SPSS software, version 17.0 (SPSS, Inc.).

RESULTS

Characteristics of mothers and children categorized by urinary iodine are presented in Table 1. In comparison to mothers with urinary iodine above the 10th percentile, mothers with low urinary iodine (mothers of Dutch national origin only, n = 56; all mothers, n = 117) were younger, had a higher BMI, and less often experienced an instrumental delivery. They presented more often with psychological symptoms and showed lower TSH concentrations.

Associations between the separate food groups and urinary iodine were analysed by using multivariable regression analyses. In mothers of Dutch national origin, cereal products [ß = 0.61 (95%CI: 0.27, 0.95), $p<0.001$] and eggs [ß = 1.87 (95%CI: 0.13, 3.62), $p = 0.04$] were significantly associated with higher urinary iodine (Supplemental Table 1).

Three factors were derived from the PCA as the most prominent dietary patterns used in the study group of women of Dutch national origin. The first factor was labeled the Mediterranean dietary pattern and explained 8.1% of the variance of dietary intake of the total study group. It comprised high intakes of vegetables, fruit, cereal products, vegetable oil, and fish and shellfish. The second factor, which explained 6.9% of the total variance, was labeled a traditionally Dutch dietary pattern because it was characterized by high intakes of potatoes, fresh and processed meat, and margarine and a low intake of fruit. The third pattern, a confectionary dietary pattern, explained 6.1% of the variance and was characterized by a high intake of cakes, sugar and confectionary, and tea (all $r\geq0.20$

and *p*-<0.05) (Supplemental Table 2). No significant association was established between adherence to the dietary patterns and urinary iodine (Supplemental Table 3).

Table 1. Selected characteristics for mothers and children by maternal urinary iodine excretion[1]

Total n = 1,156	**Low urinary iodine <10 percentile** (n = 117)	**Urinary iodine ≥10 percentile** (n = 1,039)	**P-value**
Mother			
Age, years	27.4 ± 5.4	30.2 ± 5.0	<0.001
Gestational age at enrolment, weeks	13.2 (9.2; 17.7)	13.2 (10.2; 17.6)	0.55
National origin, %			0.19
Dutch	48.2	50.7	
Western other	8.9	14.0	
Non western	42.9	35.3	
Parity, % primiparous	61.1	62.8	0.95
BMI, kg/m^2	25.4 ± 5.6	24.4 ± 4.3	0.05
Educational level, %			0.18
Primary school	31.3	23.9	
Secondary school	48.2	52.5	
High education	20.5	23.6	
Psychological symptoms	0.2 (0.0; 1.6)	0.2 (0.0; 1.3)	0.003
Smoking during pregnancy, %			0.30
Never	67.6	74.9	
Until pregnancy was confirmed	12.0	9.9	
Continued	20.4	15.2	
Multivitamin use, % yes	27.1	30.8	0.50
TSH, mU/L	1.3 ± 0.8	1.5 ± 1.0	0.001
FT4, pmol/L	15.0 ± 3.3	14.6 ± 3.4	0.14
Children			
Gender, % boys	47.0	50.8	0.33
Birth weight, kg	3.4 ± 0.5	3.5 ± 0.5	0.31
Gestational age, weeks	40.1 ± 1.6	40.1 ± 1.6	1.00
Apgar score 1 min after birth	8.5 ± 1.1	8.6 ± 1.1	0.44
Mode of delivery, %			0.04
Spontaneous vaginal	87.2	77.4	
Instrumental vaginal	9.2	14.9	
Caesarean section	3.7	7.7	

[1]Values are mean ± SD, median (95% range), or percentage.
BMI, Body Mass Index; FT4, free thyroxin 4; TSH, Thyroid stimulating hormone.

Urinary iodine showed no correlation with FT4 and a borderline correlation with TSH [Pearson's rank correlation coefficients: -0.04 (p = 0.17) and 0.06 (p = 0.05), respectively]. For children from mothers of Dutch national origin with the lowest decile of urinary iodine, the problem scores on inhibition [ß = 0.08 (95%CI: 0.02, 0.14), p = 0.008], working memory [ß = 0.07 (95%CI: 0.01, 0.12), p = 0.03], and global executive composite [ß = 0.06 (95%CI: 0.00, 0.12), p = 0.04] were significantly higher than those from mothers with urinary iodine at or above the 10th percentile p10 (Table 2). After adjustment for maternal psychological problems in pregnancy, associations between urinary iodine and problems of child executive functioning became smaller [inhibition ß = 0.06 (95%CI: 0.00, 0.12), p = 0.046; working memory ß = 0.05 (95%CI: -0.01, 0.11), p = 0.11; and global executive composite ß = 0.04 (95%CI: -0.02, 0.10), p = 0.19]. As expected, adjustment of the association between urinary iodine and executive functioning for maternal FT4 did not change the effect estimates (data not shown).

When analyses were stratified by gender, the association between urinary iodine on inhibition did not reach significance in these smaller subpopulations. The effect on working memory and global executive composite was, if anything, more prominent in girls [ß = 0.12 (95%CI: 0.05, 0.20), p = 0.002, and ß = 0.09 (95%CI: 0.009, 0.17), p = 0.03, respectively] and was not significant in boys. However, an interaction effect of gender was not found (data not shown).

Because of the association between urinary iodine and cereals, bread, and eggs, we tested whether maternal intake of these separate food groups modified the association between urinary iodine and executive functioning. The addition of these food groups did not significantly change the effect estimates (data not shown).

Finally, after pooling of mothers of Dutch and non-Dutch national origin (n = 692) we showed associations between urinary iodine and higher problem scores of inhibition [ß = 0.05 (95%CI: 0.005, 0.10), p = 0.03], working memory [ß = 0.07 (95%CI: 0.02, 0.12), p = 0.003], and global executive composite [ß = 0.05 (95%CI: 0.00, 0.10), p = 0.05] in children (Table 2). These results changed slightly after adjustment for maternal psychological symptoms [inhibition ß = 0.04 (95%CI: -0.004, 0.09), p = 0.07; working memory ß = 0.06 (95%CI: 0.01, 0.10), p = 0.02; and global executive composite ß = 0.03 (95%CI: -0.01, 0.08), p = 0.16].

All analyses were repeated using 5th and 15th percentile cutoffs instead of the 10th percentile cutoff as an indicator of low urinary iodine excretion. Results were essentially the same (data not shown).

Table 2. Associations between low maternal urinary iodine and children's score on BRIEF-P stratified by maternal national origin[1]

BRIEF problem scale	Dutch women (n = 431)				All women (n = 692)			
	Adjusted[2]		Additionally adjusted[2,3]		Adjusted[2,4]		Additionally adjusted[2,3,4]	
	ß (95%CI)	P-value	ß (95%CI)	P-value	ß (95%CI)	P-value[5]	ß (95%CI)	P-value
Inhibition	0.08 (0.02; 0.14)	0.008	0.06 (0.00; 0.01)	0.05	0.05 (0.01; 0.10)	0.03	0.04 (-0.00; 0.09)	0.07
Shifting	-0.01 (-0.06; 0.04)	0.76	-0.02 (-0.07; 0.03)	0.51	-0.01 (-0.05; 0.03)	0.64	-0.02 (-0.06; 0.02)	0.41
Emotional Control	0.02 (-0.05; 0.08)	0.59	0.00 (-0.07; 0.07)	0.99	0.01 (-0.04; 0.06)	0.63	0.00 (-0.05; 0.05)	0.94
Working Memory	0.07 (0.01; 0.12)	0.03	0.05 (-0.01; 0.11)	0.11	0.07 (0.03; 0.12)	0.003	0.06 (0.01; 0.10)	0.01
Planning/ Organization	0.05 (-0.01; 0.11)	0.11	0.03 (-0.03; 0.10)	0.28	0.03 (-0.02; 0.08)	0.19	0.02 (-0.03; 0.07)	0.43
Global Executive Composite	0.06 (0.00; 0.12)	0.04	0.04 (-0.02; 0.10)	0.19	0.05 (0.00; 0.10)	0.05	0.03 (-0.01; 0.08)	0.16

[1]Results from multivariable regression analyses. The five scales of executive function were analysed using log-transformed standardized scores (T-scores) to achieve normal distribution. [2]Adjusted for gestational age at blood and urine sampling, maternal age, education, BMI, and smoking, alcohol use, protein intake, and child's birth weight. [3]Additionally adjusted for maternal psychological symptoms. [4]Additionally adjusted for maternal national origin. BMI, Body Mass Index; BRIEF-P, The Behaviour Rating Inventory of Executive Function-Preschool Version.

DISCUSSION

This study showed that children of mothers with low urinary iodine, a marker of low iodine status, and independent of maternal thyroid concentrations in early pregnancy have higher scores of impaired executive functioning at 4 years of age. Although maternal urinary iodine was positively associated with maternal intake of specific food groups, these intakes could not explain the association between urinary iodine and impaired executive functioning in children.

Food groups for which intake was associated with higher urinary iodine in early pregnancy were cereals, bread, and eggs. In The Netherlands, consumption of bread, meat, vegetables, potatoes, and eggs is relatively high (19). Our results suggest therefore that in the Dutch population the major sources of iodine are bread and bread replacements, which are voluntarily fortified with iodized salt, and eggs. This is in line with other Western countries in which dairy products, bread, seafood, eggs, meat, and poultry are the main sources of iodine (10).

The issue of iodine deficiency during pregnancy is also related to the advisability of iodine supplementation of women as relates to the need for fortification of the food supply. Worldwide, the use of iodized salt is the most important method for preventing iodine deficiencies. Before 2008 the most important source of iodine in The Netherlands was bread, which provides 50% of average iodine intake (29). After 2008 the number of foods containing iodized salt was increased because of the decreasing consumption of bread, especially among teenagers and adolescents. At the same time, however, the iodine content in iodized salt was reduced to avoid overintake, the use of salt in processed foods was reduced to prevent hypertension, and food producers limited the use of iodized salt. This resulted in a 25% decrease in iodine intake as compared with before 2008 (30). Because our data sampling was performed between 2001 and 2006, iodine deficiency might currently be even more prevalent in this population.

In contrast to studies performed in other Western countries (31, 32), dairy foods were not associated with urinary iodine, which might be due to the Dutch legislation on the limitation of iodine in these foods. The content of iodine in milk, poultry, and meat depends on the iodine supplementation of animal foods. In addition, the use of iodophor disinfectants in milking equipment contributes to the iodine concentration of dairy products (33, 34). In The Netherlands only small regional differences in the iodine content of milk were observed that were explained by the type of soil (35). This might explain that, in a study in children aged 6-18 years no differences in urinary iodine were observed (36). Fish, fruit, and vegetables are other iodine-rich sources due to the iodine content of soil and fertilizers and irrigation practices (37). The intake of these foods, however, is low (38). Because urinary iodine reflects short-term iodine status, foods with a low frequency of intake are less reflected by urinary iodine. In addition, we did not establish effect modification by iodine-rich food groups of the association between maternal iodine status and executive functioning in children. This may also be explained by the low frequency of intake of iodine-rich foods.

The amount of variance (21.1%) explained by dietary patterns is suggested to be rather small, but the estimates are comparable with previous dietary studies in pregnant women (39, 40). Moreover, the explained variance of dietary patterns by definition is dependent on the number of included food groups for the factor analyses (20). We used 19 predefined food groups, which allowed more variance in the model, but at the same time reduced the explained variance of the identified dietary patterns.

Because iodine is released from the body through the urine, the measurement of the amounts of iodine in urine samples is a reliable method to determine iodine deficiency across a large population. The median urinary iodine concentration in our population was 203 µg/L (1.6 mmol/L), which meets the WHO recommendations for pregnant women of 150-249 µg/L (1.2-2.0 mmol/L) (41). However, our estimated range of 9.3-1743.5 µg/L (0.07-13.7 mmol/L) for iodine was very large, which supports its high variability (42).

In the same population-based cohort, we previously reported an association between mothers' hypothyroxinaemia in early pregnancy and cognitive delay in their children at age 3 years (43). In the current analysis, the association between low maternal urinary iodine and impairment of executive function in offspring could not be explained by derangements of the biomarkers of hypothyroxinaemia; FT4 and TSH were both low. There may be other explanations to understand this finding. The current analysis was performed in a study population of the same cohort who had a very low expected frequency of impaired in thyroid function, because women using thyroid medication were excluded for analysis. This implies that we examined associations in mothers with a relatively mild iodine deficiency, as one would expect in an iodine-sufficient area. Our findings are supported by others, which show no relationship between urinary iodine and TSH (44) and FT4 (45, 46). A shortage of maternal iodine intake may result in iodine deficiency in the mother and foetus, but both respond differently, with the mother preserving euthyroidism and the foetus becoming hypothyroid (8). This may explain why the foetus is more affected by iodine deficiency during pregnancy than the mother, resulting in impaired executive functioning of the child and normal maternal biomarkers of thyroid function.

In our study low maternal urinary iodine was associated with problems of inhibition, working memory, and global executive composite in children at 4 years of age. Impairments of executive functioning are consistently associated with attention-deficit hyperactivity disorder (ADHD) (45). Children with ADHD are rated higher than controls on all scales of executive functioning, with the largest effect sizes on inhibition and working memory (46). However, deficits in inhibition are not uniquely associated with ADHD but also with oppositional defiant disorder and conduct disorder (45). The children in our study population, however, are too young to be diagnosed with ADHD. Although hyperactive and impulsive symptoms typically are observed by the time the child is 4 years of age, they peak in severity at school age (47). Therefore, future follow-up of executive functioning in these children may show interesting associations.

A relationship between maternal iodine deficiency and poor mental and psychomotor development in the offspring has been described repeatedly (48). This association is suggested among others to be due to the induced derangement in maternal thyroid function. This is supported by the associations between maternal iodine deficiency, congenital hypothyroidism, and ADHD (49, 50). This is further substantiated by the reported higher incidence (70%) of ADHD in individuals with generalized resistance to thyroid hormones (51, 52). However, in these studies maternal thyroid dysfunction was not due to iodine deficiency, because they were conducted in iodine-sufficient populations (53). Because the full causal chain that links iodine and thyroid hormone to risk of developmental problems has not been established, the indirect evidence has to be considered carefully.

Part of the effect of low urinary iodine on executive functioning in our study was explained by maternal psychological symptoms. Maternal psychological distress during and after pregnancy is known to be a strong determinant of behavioural and cognitive functioning of the child (54). After adjustment for this important confounder only the association between urinary iodine and working memory remained. The correlation between diet and mental health is possibly bidirectional. Depression and stress may promote unhealthy dietary preferences (55), whereas an unhealthy diet, in turn, may affect the mental health of the mother (56).

Human studies showed that iodine supplementation trials in iodine-deficient areas were associated with more prominent cognitive improvement among girls (57, 58). Recently Murcia et al. (59) reported potentially deleterious effects of maternal iodine supplement use during pregnancy on psychomotor achievement, especially in girls. This is in line with our data showing a more prominent effect of low urinary iodine on executive functioning in girls as compared with boys. However, because no interaction effect was found, these findings should be interpreted with caution.

A strength of our study is that we examined the relationship between mild iodine deficiency during early pregnancy and executive functioning in children at 4 years of age, thereby including maternal nutrition and thyroid function as determinants of the same pathway. In addition, the large population-based prospective cohort enabled us to control for important confounding factors, including lifestyle factors, socioeconomic factors, and known determinants of foetal and infant development. However, this does not completely exclude residual confounding. Because data were more complete in more highly educated mothers, we cannot rule out that selective nonresponse influenced our findings.

The effect sizes in our study were rather small because executive functions were measured instead of clinical diagnosis of behavioural problems. Nevertheless, the continuous traits of executive functioning provide better statistical power because exposure and outcome are rare. More importantly, the BRIEF-P scale converges with a variety of clinical groups including traumatic brain injury, autism spectrum disorders (60), ADHD, and Tourette syndrome (61).

In conclusion, low maternal urinary iodine status during early pregnancy is associated with impairment of executive functioning in children at 4 years of age. This finding could not be explained by low nutritional iodine intake during pregnancy or maternal thyroid function and should be confirmed by others.

The observed impairments in executive function at an early age are considered to be subclinical symptoms. Only future studies may show whether these children have an increased vulnerability for developing clinical disorders later in life.

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Supplemental Table 1. Associations between the intake of food groups and urinary iodine in early pregnancy in mothers of Dutch national origin (n = 500).[1]

Food groups	ß (95%CI)	P-value[2]
Vegetables	0.02 (-0.28; 0.33)	0.89
Fruits	0.05 (-0.08; 0.17)	0.48
Potatoes	0.21 (-0.22; 0.64)	0.34
Legumes	2.08 (-0.46; 4.62)	0.11
Cereals, bread and other cereal products	0.61 (0.27; 0.95)	0.001
Cakes	-0.33 (-1.00; 0.33)	0.33
Sugar and confectionery	0.09 (-0.46; 0.64)	0.75
Vegetable oils	-2.31 (-6.02; 1.41)	0.22
Margarines	0.53 (-0.73; 1.79)	0.41
Butter	1.65 (-0.81; 4.11)	0.19
Milk	0.06 (-0.02; 0.14)	0.12
Dairy products	0.03 (-0.09; 0.15)	0.61
Fresh meat	-0.13 (-0.73; 0.47)	0.67
Processed meat	0.36 (-0.44; 1.15)	0.38
Eggs	1.87 (0.13; 3.62)	0.04
Fish and shellfish	-0.84 (-2.23; 0.55)	0.24
Sauces	0.01 (-0.82; 0.84)	0.99
Tea	0.02 (-0.03; 0.07)	0.36
Coffee	0.01 (-0.07; 0.09)	0.82
Soft drinks	-0.04 (-0.11; 0.04)	0.34
Fruit/vegetable juices	0.01 (-0.08; 0.1)	0.89
Alcoholic beverages	0.44 (-0.42; 1.3)	0.31
Soups and bouillon	-0.01 (-0.2; 0.17)	0.90
Miscellaneous	-0.04 (-0.87; 0.79)	0.92

[1]Results from multivariable regression analyses. [2]Adjusted for gestational age at urine sampling, maternal age, education, BMI, prenatal psychological problems and smoking and alcohol use. BMI; Body Mass Index.

Supplemental Table 2. Factor loadings of food groups in dietary patterns in mothers of Dutch national origin (n = 500).[1]

Food groups according EPIC	Mediterranean Pattern		Traditionally Dutch Pattern		Confectionary Pattern	
	r_s	P-value	r_s	P-value	r_s	P-value
Vegetables	0.65	<0.001	-0.10	0.02	0.01	0.77
Fruits	0.47	<0.001	-0.16	<0.001	0.34	<0.001
Potatoes	-0.15	<0.001	0.42	<0.001	-0.19	<0.001
Legumes	0.09	0.04	-0.21	<0.001	-0.08	0.06
Cereals, bread and other cereal products	0.23	<0.001	0.02	0.67	0.36	<0.001
Cakes	0.07	0.12	-0.08	0.08	0.72	<0.001
Sugar and confectionery	-0.18	<0.001	0.02	0.70	0.55	<0.001
Vegetable oils	0.56	<0.001	-0.01	0.77	-0.08	0.07
Margarines	-0.18	<0.001	0.24	<0.001	0.17	<0.001
Butter	0.12	0.01	-0.08	0.08	0.23	<0.001
Milk	-0.07	0.10	0.14	<0.001	-0.09	0.05
Dairy products	0.12	0.01	0.00	0.93	0.28	<0.001
Fresh meat	0.00	0.97	0.69	<0.001	-0.07	0.10
Processed meat	-0.21	<0.001	0.67	<0.001	0.10	0.02
Eggs	0.26	<0.001	-0.09	0.04	0.05	0.22
Fish and shellfish	0.61	<0.001	-0.16	<0.001	-0.01	0.86
Sauces	0.20	<0.001	0.15	<0.001	0.09	0.04
Tea	0.25	<0.001	-0.05	0.22	0.49	<0.001
Coffee	0.01	0.90	0.04	0.36	-0.02	0.59
Soft drinks	-0.03	0.51	0.19	<0.001	0.02	0.60
Fruit/vegetable juices	-0.19	<0.001	0.04	0.40	-0.05	0.24
Alcoholic beverages	0.11	0.01	-0.04	0.33	0.08	0.06
Soups and bouillon	0.10	0.02	0.10	0.03	-0.03	0.55
Miscellaneous	-0.05	0.23	-0.73	<0.001	-0.01	0.85

[1]PCA was used as an extraction method in which the Spearman's Rank correlation coefficients represent the relative contribution of that food group to the identified dietary pattern. EPIC, European Prospective Investigation into Cancer and Nutrition; PCA, Principle Component Analysis.

Supplemental Table 3. Associations between adherence to major dietary patterns, maternal thyroid hormones and urinary iodine in mothers of Dutch national origin (n = 500).[1]

Dietary pattern	Urinary iodine, mmol/mol creatinine		FT4, pmol/L		TSH, mU/L	
	ß (95%CI)	P-value	ß (95%CI)	P-value	ß (95%CI)	P-value
Mediterranean	-3.09 (-18.3; 12.2)	0.69	0.07 (-0.2; 0.3)	0.64	-0.02 (-0.1; 0.1)	0.73
Traditional Dutch	10.89 (-3.6; 25.3)	0.14	-0.26 (-0.5; 0.0)	0.06	0.08 (-0.0; 0.2)	0.12
Confectionary	5.6 (-6.9; 18.1)	0.38	-0.06 (-0.3; 0.2)	0.58	0.03 (-0.1; 0.1)	0.95

[1]Adjusted for gestational age at blood sampling, maternal age, education, BMI, psychological symptoms, smoking, alcohol use and protein intake. BMI, Body Mass Index; T4, free thyroxin 4; TSH, Thyroid stimulating hormone.

Part III

DNA Methylation and Foetal and Early Child Development

Chapter 7

DNA Methylation of IGF2DMR and H19 is associated with Foetal and Infant Growth: The Generation R Study

ABSTRACT

Changes in epigenetic programming of embryonic growth genes during pregnancy seem to affect foetal growth. Therefore, in a population-based prospective birth cohort in the Netherlands, we examined associations between foetal and infant growth and DNA methylation of *IGF2DMR*, *H19* and *MTHFR*. For this study, we selected 69 case children born small-for-gestational age (SGA, birth weight <-2 SDS) and 471 control children. Foetal growth was assessed with serial ultrasound measurements. Information on birth outcomes was retrieved from medical records. Infant weight was assessed at three and six months. Methylation was assessed in DNA extracted from umbilical cord white blood cells. Analyses were performed using linear mixed models with DNA methylation as dependent variable. The DNA methylation levels of *IGF2DMR* and *H19* in the control group were, median (90% range), 53.6% (44.5-61.6) and 30.0% (25.6-34.2) and in the SGA group 52.0% (43.9-60.9) and 30.5% (23.9-32.9), respectively. The MTHFR region was found to be hypomethylated with limited variability in the control and SGA group, 2.5% (1.4-4.0) and 2.4% (1.5-3.8), respectively. SGA was associated with lower *IGF2DMR* DNA methylation (ß = -1.07, 95%CI -1.93; -0.21, p = 0.015), but not with H19 methylation. A weight gain in the first three months after birth was associated with lower *IGF2DMR* DNA methylation (ß = -0.53, 95%CI -0.91; -0.16, p = 0.005). Genetic variants in the *IGF2/H19* locus were associated with IGF2DMR DNA methylation (p = <0.05), but not with H19 methylation. Furthermore, our results suggest a possibility of mediation of DNA methylation in the association between the genetic variants and SGA. To conclude, *IGF2DMR* and *H19* DNA methylation is associated with foetal and infant growth.

INTRODUCTION

One of the main causes of perinatal morbidity and mortality is being born small-for-gestational age (SGA) (1, 2). SGA can be the result of a poor prenatal environment, which could have been induced by an adaptive response during foetal life (3). These infants are particular at risk for early onset of non-communicable diseases when they also experience a period of catch up growth during the first months of life (4). One of the proposed underlying mechanisms are changes in epigenetic markings acquired and maintained during pregnancy, which are not directly related to the DNA sequence itself (5).

One of the best understood epigenetic mechanisms are modifications in DNA methylation which occur predominantly at cytosines of CpG dinucleotides (5). To establish and maintain DNA methylation patterns, methyl donors, such as folate and choline, acting as intermediates in the one-carbon pathway are required (6). Low intake of methyl donors and subtle genetic variations in methyltetrahydrofolate reductase (MTHFR) can derange the one carbon pathway resulting in a mild to moderate hyperhomocysteinemia. Associations have been reported between elevated maternal homocysteine levels during pregnancy and a lower birth weight and increased risk of SGA (7). Moreover, hyperhomocysteinemia also affects DNA methylation levels (8, 9).

Although DNA methylation patterns are believed to be relatively stable, it seems that exposures, particularly during the prenatal period, can permanently alter DNA methylation patterns in offspring (10). Especially genes that are expressed in a parent-of-origin-specific manner, known as imprinted genes, are of interest as they are essential during development and regulated through epigenetic mechanisms (11). *The IGF2/H19* imprinted region is one of the best studied loci. In humans, exposure to famine during the periconceptional period has been linked to altered DNA methylation patterns of the insulin-like growth factor 2 (*IGF2DMR*) in adulthood (12). Also, periconceptional folic acid supplement use is associated with increased methylation of the *IGF2DMR* in humans and *IGF2DMR* methylation was inversely associated with birth weight (13).

We hypothesized that changes in foetal growth and subsequent early infant growth are partly due to alterations in DNA methylation of genes implicated in foetal and infant growth. Therefore, we assessed DNA methylation in 2 imprinted genes (*IGF2* and *H19*) and 1 non imprinted folate gene (*MTHFR*). Our main focus was to investigate the association between SGA and DNA methylation. Next, we examined associations between foetal and infant growth and DNA methylation. In addition, we investigated whether genetic variations within the investigated genes are associated with DNA methylation. Furthermore, we explored the possible mediation of DNA methylation in the association between the genetic variants and SGA.

MATERIALS AND METHODS

Design and Study Population

We hypothesized that changes in foetal growth and subsequent early infant growth are partly due to alterations in DNA methylation of genes implicated in foetal and infant growth. Therefore, we assessed DNA methylation in 2 imprinted genes (*IGF2* and *H19*) and 1 non imprinted folate gene (*MTHFR*). Our main focus was to investigate the association between SGA and DNA methylation. Next, we examined associations between foetal and infant growth and DNA methylation. In addition, we investigated whether genetic variations within the investigated genes are associated with DNA methylation. Furthermore, we explored the possible mediation of DNA methylation in the association between the genetic variants and SGA.

The present study was carried out in a subset of the original Generation R cohort (n = 540). Analyses were restricted to Dutch singleton pregnancies (n = 4,829) and infants with available DNA extracted from umbilical cord white blood cells (n = 3,127). From this study group all infants born with a gestational-age and sex-adjusted birth weight below -2 standard deviation score (SDS) (n=69) were selected for analysis. The current study is part of a project, in which we investigate the hypothesis that both SGA and children with attention deficit hyperactivity disorder (ADHD) have a shared causality in DNA methylation of especially imprinted foetal growth genes. Therefore, 92 children were included with ADHD based on parent interview Diagnostic Inventory of Screening Children or Child Behaviour Checklist teacher report at age 6. Two of the infants with ADHD were also born with a gestational-age and sex-adjusted birth weight below -2 SDS. The remaining 381 control infants were randomly selected. Therefore, the control group consisted of 90 ADHD children and 381 infants who were randomly drawn.

Foetal and Infant Growth

Foetal ultrasound measurements were performed to assess gestational age and to estimate foetal growth (15). Crown-to-rump length was used for pregnancy dating until a gestational age of 12 weeks and 5 days (crown-to-rump length <65 mm), and biparietal diameter (BPD) for pregnancy dating thereafter (gestational age from 12 weeks and 5 days onwards, BPD >23 mm). Foetal growth characteristics included head circumference, BPD, abdominal circumference, and femur length and were measured trans-abdominal to the nearest millimeter using standardized ultrasound procedures in mid pregnancy (median 20.5 weeks of gestation, 90% range 19.1-22.4) and late pregnancy (median 30.4 weeks of gestation, 90% range 28.8-32.1). Estimated foetal weight (EFW) was calculated using the formula by Hadlock (16). A gestational age-adjusted SDS, based on reference growth curves from the entire study population, was constructed for EFW (15).

Information concerning date of birth, infant sex and birth weight was obtained from the medical records of community midwives and hospitals (14). Gestational age and sex-adjusted SDS were constructed for birth weight, according to the methodology of Niklasson et al. (17).

SGA was defined according to the International SGA Advisory Board Panel as a gestational age and sex-adjusted birth weight below -2 SDS (18). Born appropriate for gestational age (AGA) was defined as a gestational age and sex-adjusted birth weight ≥-2 SDS. Born large for gestational age (LGA) was defined as a gestational age and sex-adjusted birth weight ≥2 SDS. The growth rate of weight between mid-pregnancy and birth was calculated as (birth weight SDS – EFW SDS in mid pregnancy).

Trained staff in community health centers obtained information on infant weight during periodic visits scheduled at three (median 3.3 months, 90% range 3.0-3.9) and six months (median 6.2 months, 90% range 5.5-7.4) (14). For every visit, an age- and sex-adjusted SDS for weight was calculated with the use of Dutch reference curves (Growth Analyzer 3.0, Dutch Growth Research Foundation, Rotterdam, the Netherlands). Infant growth rates of weight gain in the first three and six months after birth were calculated as (weight at three or six months SDS – birth weight SDS).

Assessment of DNA methylation and Genotyping

Genomic DNA was isolated from umbilical cord white blood samples at birth as previously been described (19). Based on previous studies, three loci were selected for the assessment of DNA methylation (9, 20). DNA methylation of *IGF2DMR* and *H19* have been previously studied by other groups (12, 20). However, DNA methylation *MTHFR* has not been studied before. It was chosen as it is a key enzyme in the one carbon pathway. A CpG island outside the *MTHFR* gene was selected for analyses. Primers were designed using the online tool of MySequenom.com. Details of the measured amplicons can be found in Supplement table S1. Isolated genomic DNA (500 ng) was treated with sodium bisulphite for 16 hours using the EZ-96 DNA methylation kit (Shallow) (Zymo Research, Irvine, CA, USA), according to the manufactures' protocol. Samples were randomly distributed on six 96-well plates. The bisulphite treatment was followed by PCR amplification, fragmentation after reverse transcription and analysis on a mass spectrometer, according to the manufactures' protocol (Sequenom, Inc, San Diego, USA). This generated mass signal patterns that were translated into quantitative DNA methylation levels of different CpG sites of the selected genes by MassARRAY EpiTYPER Analyzer software (v1.0, built 1.0.6.88 Sequenom, Inc, San Diego, USA) (21, 22). Fragments containing one or more CpG sites were called CpG units. PCR and subsequent steps were done in triplicate.

Data quality control for methylation consisted of exclusion of CpG units with too low or too high mass or CpG units with overlapping or duplicate RNA fragments (e.g. silent signals) were excluded from further analysis. Furthermore, at least two out of three of the replicate measurements per CpG unit had to be successful, the standard deviation of the duplicates or triplicates had to be ≤0.10 and the success rate per CpG unit had to be ≥75%. Last, CpG units that contained a known SNPs with a frequency >5% were also excluded, as this could change the weight of the CpG unit and therefore interfere with the measurement. Details concerning the success rate of the amplicons can be found in Supplement table S2.

Based on literature (23), four SNPs in the *IGF2/H19* locus were identified that were associated with birth weight in Caucasian children. Subsequently, two genetic variants of the *MTHFR* gene which have been associated with global DNA methylation were identified (24). Genotypes were obtained using high-density SNP arrays (Illumina) and then imputed for ~2.4 million HapMap SNPs (Phase II, release 21/22). Frequency distribution conformed to the Hardy-Weinberg equilibrium. Details concerning the SNPs can be found in Supplemental table S3.

Covariates

From self-administered questionnaires, data was available on maternal age and maternal educational level, parity, smoking and folic acid supplement use before and during pregnancy. Maternal education level was assessed by the highest completed education and classified as 1) none/primary or 'low'; 2) secondary or 'medium'; 3) college/university or 'high'. Parity was classified as (1) nulliparous and (2) multiparous. Maternal smoking was assessed in each trimester. Women, who reported any or no smoking during pregnancy were respectively classified as 'smokers' and 'non-smokers'. Folic acid supplement use was categorized into 1) folic acid supplement use (pre- or postconceptional start); 2) no folic acid supplement use. At enrolment (median 13.5 weeks, 90% range 10.7; 21.6) maternal weight and height were measured to calculate body mass index (BMI, kg/m^2). From hospital medical records, the occurrence of hypertension and hypertension-related pregnancy disorders were obtained (25). Preeclampsia was defined according to the criteria described by the International Society for the Study of Hypertension in Pregnancy (ISSHP) (26).

Statistical Analysis

First, we tested for differences in maternal or infant characteristics between cases and controls using chi-square, Mann-Whitney *U* and *T*-tests. Second, linear mixed models were used to examine the associations between foetal or infant growth (independent variable) and DNA methylation (dependent variable). This model was chosen as it can account for correlation between CpG dinucleotides, incorporates relevant adjustments within the models and has the ability to accommodate missing data. The restricted maximum likelihood (REML) method was used for the model fitting. DNA methylation was treated as a continuous variable. To achieve normality, DNA methylation was square root transformed. Outliers per CpG (>3 SD) were excluded from further analysis. For all analyses, subject/person identifier was added as random effect and bisulphite batch and CpG site were added as fixed effects. In the crude analyses, the growth characteristic was entered as a fixed effect. Potential confounders were additionally entered to the model at the same time as fixed effects. In addition, the analyses were also repeated with exclusion of the ADHD and the LGA cases. Third, associations between genetic variants in the *IGF2/H19* locus and the *MTHFR* gene and DNA methylation levels (dependent variable) were investigated using a linear mixed model. The genotype was entered to the model continuously as a fixed effect. Subsequently, we explored the possibility of mediation of DNA methylation in the association between the genetic variant and SGA using logistic regression models.

Missing data of maternal educational level (1.3%), folic acid supplement use (18.5%) and smoking (6.3%) were completed using the Markov-Chain-Monte-Carlo multiple imputation technique (27). Ten imputed datasets were created. For all analyses, results including imputed missing data are presented. Multiple testing correction was performed according to the method developed by Bonferroni. The linear mixed model analyses were performed using the data measured in triplicates. All analyses were performed using the Statistical Package of Social Sciences version 20.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

The maternal and foetal characteristics are presented in Table 1. Mothers of children born SGA were lower educated, more often nulliparous, smoked more often during pregnancy, used less often a folic acid supplement during the periconceptional period and more frequently developed preeclampsia (all p = <0.05). The median (90% range) DNA methylation levels of *IGF2DMR*, *H19* and *MTHFR* were 53.2% (44.3-61.3), 30.1% (25.5-34.2) and 2.5% (1.4-4.0) respectively. The median (90% range) DNA methylation levels of *IGF2DMR*, *H19* and *MTHFR* in the AGA group were 53.6 (44.5-61.6), 30.0 (25.6-34.2) and 2.5 (1.4-4.0), respectively. The median (90% range) DNA methylation levels of *IGF2DMR*, *H19* and *MTHFR* in the SGA group were 52.0 (43.9-60.9), 30.5 (23.9-32.9) and 2.4 (1.5-3.8), respectively. As the *MTHFR* region was found to be hypomethylated with limited variability, no further analyses were conducted within this region.

Table 2 shows the associations between the occurrence of SGA (categorical) and DNA methylation levels of *IGF2DMR* and *H19*. SGA was associated with lower *IGF2DMR* DNA methylation (ß = -1.07, 95%CI -1.93; -0.21, p = 0.015). Expressed relative to the standard deviation, this difference corresponds with a standardized effect size in DNA methylation of -0.13 SDS units. No associations were observed between SGA and *H19* methylation. After multiple testing adjustment (three independent loci), the association of SGA with *IGF2DMR* methylation remained significant.

Table 3 shows the associations between foetal and infant growth and DNA methylation. As the SGA cases were oversampled in the data, the analyses were performed in the total study population, but were also stratified for SGA or AGA. In the total study population, lower *IGF2DMR* DNA methylation was associated with an increase in weight in the first three months after birth (ß = -0.46, 95%CI -0.86; -0.07, p = 0.022), corresponding to a standardized difference in DNA methylation of -0.06 SDS. In the total study population, no association was found with H19 methylation. After the analyses were stratified for SGA and AGA, higher *H19* methylation was associated with an increase in weight in the first three months after birth (ß = 0.35, 95%CI 0.03; 0.68, p = 0.034) in children born AGA, which corresponds with a standardized difference in DNA methylation of -0.05 SDS.

Table 1. Maternal and infant characteristics.

	All infants	**Controls**	**SGA**	**P-value**[1]
	n = 540	n = 471	n = 69	
Maternal characteristics				
Age at intake, years*	30.3 (5.1)	30.3 (5.1)	30.0 (5.4)	NS
Body mass index at intake (kg/m^2)	23.3 (19.3-32.4)	23.3 (19.4-32.4)	22.8 (18.9-30.9)	NS
Education (%)				0.003
Primary education	4.4	3.8	8.7	
Secondary education	45.9	45.9	46.4	
Higher education	48.3	49.3	42.0	
Missing	1.3	1.1	2.9	
Parity (%)				<0.001
0	66.9	64.1	85.5	
≥1	33.1	35.9	14.5	
Smoking status during pregnancy (%)				0.003
Yes	23.1	21.9	31.9	
Until pregnancy recognition	8.7	8.5	10.1	
No	61.9	63.3	52.2	
Missing	6.3	6.4	5.8	
Folic acid supplement use during pregnancy (%)				<0.001
Start preconception	43.5	45.2	31.9	
Start postconception	25.9	24.8	33.3	
No	12.0	11.5	15.9	
Missing	18.5	18.5	18.8	
Preeclampsia (%)	2.0	1.3	7.2	*<0.001*
Infant characteristics				
Gender (% boys)	57.8	57.3	60.9	NS
Estimated foetal weight, grams mid-pregnancy†	358.2 (257.7-537.4)	359.5 (364.3-544.7)	352.5 (238.1-502.4)	<0.001
Estimated foetal weight, grams late-pregnancy†	1538.2 (1199.1-2032.6)	1589.4 (1251.5-2038.2)	1362.9 (1118.6-1564.3)	<0.001
Gestational age at birth, weeks	40.3 (37.6-42.1)	40.3 (37.4-42.1)	40.3 (37.7-42.1)	NS
Birth weight, grams†	3418 (2491-4300)	3510 (2735-4325)	2625 (1930-2830)	<0.001
Weight, grams, 3 months	6215 (4990-7541)	6260 (5120-7613)	5690 (4823-6545)	<0.001
Weight, grams, 6 months	7690 (6320-9255)	7820 (6450-9330)	7085 (5890-9250)	<0.001
IGF2DMR methylation (%)	53.2 (44.3-61.3)	53.6 (44.5-61.6)	52.0 (43.9-60.9)	0.003
H19 methylation (%)	30.1 (25.5-34.2)	30.0 (25.6-34.2)	30.5 (23.9-32.9)	NS

Values are presented as *mean (standard deviation) and †median (90% range). [1]Student's *T*-test, Mann Whitney *U* and chi-square tests are used to test differences between the control group and the SGA group.

Table 2. Association between SGA and DNA methylation.

	***IGF2DMR* methylation** (n = 499)			***H19* methylation** (n = 510)		
	ß[1]	95%CI	**P-value**	ß[1]	95%CI	**P-value**
MODEL 1: adjusted for correlations between CpG sites, bisulphite batch, gestational age						
Small-for-gestational age (<-2 SDS)	**-1.26**	**-2.10; -0.42**	**0.003**	-0.15	-0.78; 0.48	0.635
MODEL 2: model 1 + maternal age, maternal educational level, parity and foetal gender						
Small-for-gestational age (<-2 SDS)	**-1.22**	**-2.07; -0.37**	**0.005**	-0.25	-0.90; 0.40	0.443
MODEL 3: model 2 + maternal BMI, folic acid supplement use, smoking and the occurrence of preeclampsia						
Small-for-gestational age (<-2 SDS)	**-1.07**	**-1.93; -0.21**	**0.015**	-0.27	-0.94; 0.39	0.422

Results from linear mixed model analyses with small-for-gestational age as independent variable and DNA methylation as dependent variable. [1]Analyses were performed with square-root transformed methylation data and values are presented as regression coefficients (95% confidence interval).

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Table 4 shows the associations between genetic variants in the *IGF2/H19* locus and DNA methylation. Genetic variants in the IGF2/H19 locus were associated with both higher (rs3741205, rs2251375) and lower (rs2067051) DNA methylation of *IGF2DMR* (rs3741205, C-allele: ß = 1.20, 95% 0.71; 1.69, $p = 2.0 \times 10^{-6}$, rs2067051, C-allele: ß = -0.44, 95%CI -0.86; -0.02, $p = 0.041$ and rs2251375, A-allele: ß = 0.48, 95%CI 0.04; 0.93, $p = 0.033$), which corresponds to a standardized difference in DNA methylation of 0.15 SDS (rs3741205), 0.06 SDS (rs2067051) and 0.06 SDS (rs2251375). The genetic variants was not associated with H19 methylation. The IGF2 SNP (rs3741205) showed a significant association with birth weight (beta 127 grams, 95%CI 40; 215, $p = 0.004$), but the H19 SNPs did not (rs2067051: beta -35 grams, 95%CI -109; 39, $p = 0.349$; rs2251375: beta 18 grams, 95%CI -60; 97, $p- = 0.643$; rs4929984: beta -21 grams, 95%CI -96; 53, $p = 0.572$). Next, we explored the possibility of mediation of DNA methylation in the association between the genetic variant rs3741205 and SGA, which is depicted in Table 5. The genetic variant rs3741205 was chosen, because of its strong association with IGF2DMR DNA methylation. The risk allele of the genetic variant rs3741205 was significantly associated with an increased risk of SGA (adjusted odds ratio (aOR) 1.41, 95%CI 1.24; 1.61, $p = 1.9 \times 10^{-7}$). Afterwards, the mean DNA methylation of IGF2DMR was added to the model and the effect was attenuated (aOR 1.26, 95%CI 1.10; 1.44, $p = 0.001$).

Table 3. Associations between foetal and infant growth parameters and DNA methylation.

	Crude[1]	P-value	Adjusted[2]	P-value	Crude[1]	P-value	Adjusted[2]	P-value
All infants	***IGF2DMR* methylation** (n = 499)				***H19* methylation** (n = 510)			
Birth weight SDS	0.15 (-0.08; 0.38)	0.206	0.05 (-0.21; 0.31)	0.704	-0.01 (-0.19; 0.16)	0.888	0.06 (-0.15; 0.26)	0.597
Δ weight 2nd trimester – birth	-0.12 (-0.60; 0.37)	0.642	-0.13 (-0.64; 0.38)	0.606	-0.27 (-0.61; 0.07)	0.116	-0.19 (-0.54; 0.16)	0.283
Δ weight birth-3 months	**-0.53 (-0.91; -0.16)**	**0.005**	**-0.46 (-0.86; -0.07)**	**0.022**	0.23 (-0.04; 0.50)	0.099	0.22 (-0.08; 0.52)	0.153
Δ weight birth-6 months	-0.23 (-0.53; 0.08)	0.142	-0.16 (-0.50; 0.18)	0.367	0.16 (-0.06; 0.38)	0.146	0.11 (-0.15; 0.37)	0.407
SGA infants	***IGF2DMR* methylation** (n = 65)				***H19* methylation** (n = 65)			
Birth weight SDS	1.55 (-0.31; 3.41)	0.103	1.18 (-0.83; 3.18)	0.251	-0.03 (-1.39; 1.33)	0.965	-0.08 (-1.80; 1.63)	0.924
Δ weight 2nd trimester – birth	0.89 (-1.03; 2.80)	0.363	-0.02 (-3.68; 3.64)	0.992	**1.71 (0.55; 2.87)**	**0.004**	-1.09 (-9.41; 7.22)	0.790
Δ weight birth-3 months	-1.26 (-2.92; 0.40)	0.136	-1.65 (-3.76; 0.47)	0.127	0.04 (-1.23; 1.31)	0.950	0.18 (-2.13; 2.48)	0.884
Δ weight birth-6 months	-0.18 (-1.12; 0.76)	0.704	-0.07 (-1.21; 1.07)	0.905	0.55 (-0.17; 1.27)	0.137	0.72 (-0.44; 1.88)	0.222
AGA infants	***IGF2DMR* methylation** (n = 434)				***H19* methylation** (n = 445)			
Birth weight SDS	-0.16 (-0.46; 0.15)	0.310	-0.27 (-0.60; 0.06)	0.111	-0.07 (-0.30; 0.16)	0.533	-0.00 (-0.27; 0.26)	0.982
Δ weight 2nd trimester – birth	-0.22 (-0.83; 0.39)	0.475	-0.25 (-0.88; 0.37)	0.428	**-0.51 (-0.94; -0.08)**	**0.020**	-0.39 (-0.81; 0.04)	0.076
Δ weight birth-3 months	-0.31 (-0.73; 0.11)	0.147	-0.26 (-0.69; 0.18)	0.255	**0.32 (0.01; 0.64)**	**0.042**	**0.35 (0.03; 0.68)**	**0.034**
Δ weight birth-6 months	0.03 (-0.34; 0.40)	0.868	0.05 (-0.36; 0.46)	0.826	0.20 (-0.07; 0.46)	0.143	0.13 (-0.18; 0.43)	0.414

Results from linear mixed model analyses with DNA methylation as dependent variable and the foetal growth parameters as independent variables. Results are presented for the whole study population and stratified for SGA/AGA. Analyses were performed with square-root transformed methylation data and values are presented as regression coefficients (95% confidence interval). [1]Crude values are adjusted for the correlations between CpG sites, bisulphite batch, and gestational age. [2]Adjusted values were additionally adjusted for maternal characteristics (age, educational level, parity, BMI, folic acid supplement use, smoking and the occurrence of preeclampsia) and foetal gender.

Last, the analyses were repeated with the exclusion of the ADHD cases and subsequently with the exclusion of LGA cases. The results of the ADHD cases are depicted in Supplement Table S4. After exclusion of the ADHD cases, the previous found associations remained and the effect estimates did not change substantially. The results of the LGA cases can be found in Supplement Table S5. After exclusion of the LGA cases, the previous observed associations remained and the effect estimates did not change substantially.

Table 4. Associations between SNPs in the *IGF2/H19* locus and DNA methylation.

		***IGF2DMR* methylation** (n = 499)			***H19* methylation** (n = 510)		
Genetic variant	**Gene**	ß[1]	95%CI	**P-value**	ß[1]	95%CI	**P-value**
rs3741205	*IGF2*	**1.20**	**0,71; 1.69**	**2.0 E-6**	-0.06	-0.42; 0.30	0.742
rs2067051	*H19*	**-0.44**	**-0.86; -0.02**	**0.041**	0.28	-0.02; 0.59	0.066
rs2251375	*H19*	**0.48**	**0.04; 0.93**	**0.033**	0.06	-0.26; 0.38	0.717
rs4929984	*H19*	-0.33	-0.75; 0.09	0.123	0.27	-0.04; 0.57	0.084
rs1801131	*MTHFR*	-0.03	-0.45; 0.40	0.898	-0.07	-0.38; 0.24	0.654
rs1801133	*MTHFR*	0.07	-0.35; 0.49	0.752	-0.05	-0.35; 0.25	0.750

Result from linear mixed model analyses with DNA methylation as dependent variable and the genetic variant as independent variable. [1]Analyses were performed with square-root transformed methylation data and adjusted for CpG, bisulphite plate, gestational age at birth and gender.

Table 5. Mediating effects of DNA methylation.

	Small for gestational age		
	aOR	95% CI	**P-value**
Model 1: adjusted for gender			
rs3741205	1.41	1.24; 1.61	**1.9×10^{-7}**
Model 2: model 1 + mean DNA methylation of *IGF2DMR*			
rs3741205	1.26	1.10; 1.44	**0.001**

Result from logistic regression analyses with small-for-gestational age as dependent variable and the genetic variant as independent variable. aOR: adjusted odds ratio, 95 CI and their corresponding P-value represent the effect of the minor allele (C-allele) on the risk of having a small-for-gestational age infant.

DISCUSSION

In 540 children, derived from a population-based birth cohort, we examined whether DNA methylation levels of the *IGF2DMR*, *H19* and *MTHFR* gene in cord blood were associated with foetal and infant growth.

The *IGF2* and *H19* loci have been studied extensively in the past and are strong candidate genes for influencing birth weight (23). In our study, lower DNA methylation of *IGF2DMR*

was observed in children born SGA. There have been conflicting results regarding the association between *IGF2/H19* DNA methylation and foetal and infant growth (28-31). A recent study by St-Pierre et al. (31) studied *IGF2/H19* DNA methylation at both the maternal and the foetal side of the placenta in relation to foetal birth anthropometrics. In this study, higher *IGF2DMR* DNA methylation was associated with higher birth weight. Moreover, they showed that alterations in *IGF2/H19* DNA methylation are likely to be functional, because of the established positive association with circulating *IGF2* concentrations in cord blood. In addition, Displas et al. reported loss of imprinting of *IGF2/H19* in placentas of children with intrauterine growth restriction (30). In contrast to our findings, Tobi et al. has found no association between *IGF2DMR* methylation and SGA (difference SGA and AGA -0.2%) (29), which might be explained by their study population of preterm born children (<32 weeks) and the use of a different definition for SGA (<-1 SDS) than ours (<-2SDS). In contrast to previous studies, we included postnatal growth parameters in addition to birth outcomes and found an association between an increase in weight as marker of postnatal growth in the first three months after birth and *IGF2DMR* methylation. As it has been shown that children born AGA and SGA have different postnatal growth patterns, we have shown the postnatal analyses separately. Postnatal growth acceleration has been previously identified as an important risk factor for the development of diseases in later life (32), which could partly be explained by epigenetic reprogramming. In our study, the SGA group is nearly 7 fold smaller than the AGA group. Therefore, a power problem seems a likely explanation for not finding this association in the SGA group. It would be interesting to address this question in future studies.

IGF2 methylation levels determined in umbilical cord white blood cells were positively associated with birth weight (31,33). Therefore, the association of SGA lower DNA methylation levels of *IGF2DMR* seems to be in contrast to the concept that increased methylation is associated with transcriptional silencing of the associated gene (5), but is in line with the increase in weight in the first three months after birth and lower *IGF2DMR* methylation levels. Adverse exposures have been previously linked to a decrease in methylation whereas advantageous exposures have been associated with increased methylation (12, 13). Therefore, DNA methylation can be regarded as a memory of previous exposures and alterations could have consequences in subsequent growth and development. St-Pierre et al. (31) showed that 31% of the variance of birth weight is explained by the *IGF2/H19* epigenotype and a genetic variant (rs2107425), which is in linkage disequilibrium (LD) with one of the SNPs that we have investigated, namely rs2251375 (R-squared 1.000).

IGF2 and *H19* are both imprinted genes and loss of imprinting of the *IGF2/H19* locus has been observed in the Beckwith-Wiedemann syndrome, a congenital overgrowth disorder (34). In this study, a significant positive association was observed between the polymorphism rs3741205 and *IGF2DMR* methylation. This genetic variant is a defining SNP of a CAGA haplotype in the *IGF2* DMR0 region which has been previously described in patients with a sporadic form of Beckwith-Wiedemann syndrome (35) and the presence of the C-allele has also been positively associated with birth weight (23). Recently, lower *IGF2*

DMR methylation has been associated with the minor allele of the *IGF2* SNP rs2239681, which is in LD with one of the SNPs that we have investigated, namely rs3741205 (R-squared 0.912) (20). In addition, our results suggest the possibility of mediation of DNA methylation in the association between the genetic variants and SGA. These observations could provide evidence for the complex interplay between the genome, epigenome and environmental factors in growth disorders.

In this study, we found the *MTHFR* region in both groups to be hypomethylated with limited variability. Therefore, no analyses were performed within this region. With the current methods for DNA methylation measurement, we do not recommend for others to investigate this region z.

Methodological Considerations

Some strengths and limitations of this study have to be addressed. This study was embedded in a large cohort from whom a selection of Dutch children was studied. As the SGA cases were oversampled in the data, the analyses were performed in all infants and SGA and AGA. The study population also consisted of 92 children with ADHD and 11 children born LGA, which could potentially influence our results. Therefore, all analyses were repeated without the ADHD and LGA children. As this did not change our results substantially, these children were not excluded.

This study showed modest changes in DNA methylation, which remained after multiple testing correction. Our findings are in line with the influences of periconceptional folic acid supplement use and adverse intrauterine exposures, such as the Dutch famine during 1944-45, showing modest epigenetic changes in early and adult life (12, 13). A recent study by Talens et al. (36) has demonstrated that epigenetic changes accumulate over time, both at imprinted (including *IGF2DMR*) and non-imprinted loci. Unfortunately, we were not able to assess whether the DNA methylation variations also result in changes in expression and long-term functional effects. In addition, the issue remains whether the measured DNA methylation differences reflect true methylation changes of the candidate genes of interest. The aim of this study was to estimate differences in the quantitative DNA methylation at selected individual CpG sites of candidate loci. This seems particularly relevant for the H19 locus, as this is measured around 30%, whereas 50% could be expected. Therefore, it would be valuable to replicate our findings and to validate the chosen methodology. However, others have investigated this region and found comparable levels (12, 20, 31). Therefore, we believe that our absolute DNA methylation levels are reliable, but most importantly the estimated differences seem to be valid.

DNA methylation was measured in umbilical cord white blood cells and not in other tissues. Therefore, it could be argued that DNA methylation patterns differ in various cell populations (37, 38). There is a possibility that the differences in DNA methylation could be attributed to the cellular heterogeneity in leukocytes. Unfortunately, no cell count was available for our study population. Therefore, we cannot assess to what extent this has influenced our results. It would be interesting to address this question in future studies.

DNA methylation patterns of IGF2DMR have been compared in blood and in buccal cells and showed a reasonable correlation (Spearman ρ~0.5) (37). It has also been reported that IGF2DMR methylation in blood may be informative as it marked the methylation patterns in colon tissue (39). However, DNA methylation can still differ between tissues. Therefore, it is important to establish in future studies correlations between DNA methylation in peripheral tissues, such as blood, and tissues that are directly involved in the disease. It would be informative to address this question with DNA methylation measurement at a genome-wide level.

CONCLUSIONS

Our analyses suggest that foetal and infant growth are associated with DNA methylation of *IGF2DMR* and *H19*. The observations in this paper could offer support for a potential functional link between DNA methylation in cord blood in the investigated genes and birth outcomes. The understanding how epigenetic control depends on early exposure may shed light on the link between foetal development and health over the lifespan and ultimately suggest new ways to prevent human disease.

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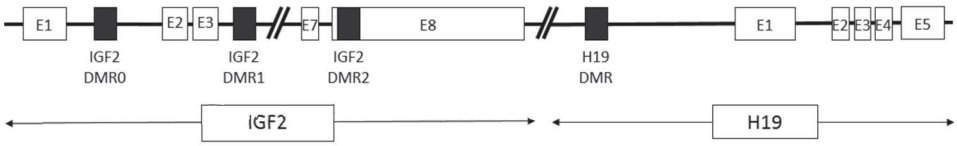
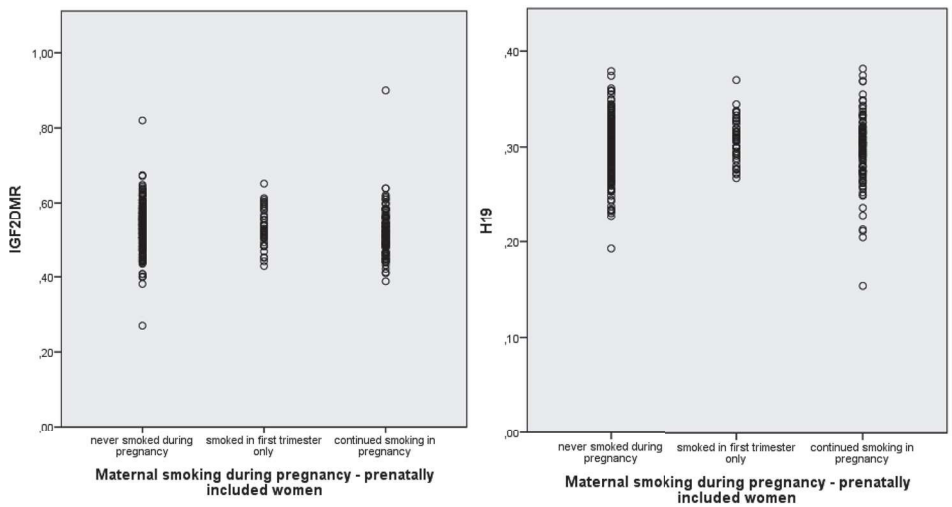


Supplemental Figure 1



Supplemental Figure 2

Supplemental Table 1. Parental tobacco smoking habits and DNA methylation, with exclusion of ADHD cases.

	IGF2DMR methylation			H19 methylation		
	ß[1]	95%CI	**P-value**	ß[1]	95%CI	**P-value**
MODEL1: adjusted for correlations between CpG sites, bisulphite batch, gestational age at birth						
Maternal tobacco smoking						
No (n = 277)		reference			reference	0.265
First trimester only (n = 37)	-0.82	-1.94;0.31	0.154	0.48	-0.37;1.33	0.265
Continued smoking, all (n = 104)	**-1.13**	**-1.87;-0.39**	0.154	0.48	-0.99;0.13	0.135
<5 cigarettes per day (n = 34)	**-1.25**	**-2.43;-0.08**	**0.037**	**-0.92**	**-1.80;-0.04**	**0.041**
≥5 cigarettes per day (n = 51)	-0.93	-1.94;0.07	0.068	-0.18	-0.91;0.56	0.637
P for trend		**0.021**			0.300	
Paternal tobacco smoking						
No (n = 176)		reference			reference	
Yes (n = 100)	-0.19	-0.98;0.60	0.639	0.17	-0.39;0.73	0.550
<5 cigarettes per day (n = 48)	-0.26	-1.30;0.78	0.621	0.16	-0.58;0.89	0.679
≥5 cigarettes per day (n = 51)	-0.05	-1.06;0.96	0.922	0.16	-0.56;0.89	0.658
P for trend		0.819			0.603	
No (n = 277)		reference			reference	
First trimester only (n = 37)	-0.89	-2.04; 0.25	0.127	0.40	-0.46; 1.25	0.366
Continued smoking, all (n=104)	**-1.18**	**-1.98; -0.37**	**0.004**	**-0.62**	**-1.24; -0.00**	**0.049**
<5 cigarettes per day (n = 34)	**-1.30**	**-2.50; -0.10**	**0.033**	**-0.93**	**-1.82; -0.03**	**0.043**
≥5 cigarettes per day (n = 51)	-0.81	-1.88; 0.26	0.136	-0.32	-1.11; 0.46	0.420
MODEL 2: model 1 + maternal characteristics (age, educational level, parity, BMI, periconceptional folic acid supplement use) and fetal gender						
Maternal tobacco smoking						
P for trend		**0.044**			0.184	
Paternal tobacco smoking		reference			reference	
No (n = 176)	-0.19	-1.01; 0.63			-0.51; 0.62	0.860
Yes (n = 100)	-0.23	-1.29; 0.83	0.646	0.05	-0.59; 0.86	0.712
<5 cigarettes per day (n = 48)	-0.07	-1.14; 1.00	0.670	0.14	-0.82; 0.67	0.849
≥5 cigarettes per day (n = 51)	-0.07	0.803	0.895	-0.07	0.958	0.849
P for trend		0.803			0.958	

Results from linear mixed model analyses with maternal or paternal tobacco smoking as independent variable and DNA methylation as dependent variable. [1]Analyses were performed with square-root transformed methylation data and values are presented as regression coefficients (95% confidence interval). Analyses on paternal smoking were restricted to non-smoking mothers.

Supplemental Table 1. Details of measured amplicons and PCR primers.

Gene	Genomic location[1]	Number of CpG units assessed	Primer sequence[2]	Source
Insulin-like growth factor 2 (IGF2)	Chr 11: 2169458-2169796	3 CpG units (4 CpG sites)	F: TGGATAGGAGATTGA-GGAGAAA R: AAACCCCAACAAAAA-CCACT	Heijmans, 2007
H19	Chr 11: 2019371-2019784	10 CpG units (13 CpG sites)	F: GGGTTTGGGAGAGTTT-GTGAGGT R: ATACCTACTACTCCCTA-CCTACCAAC	Heijmans, 2007

[1]Genome built: GRch 37.67

[2]Forward and reverse primer that will amplify the bisulphite converted genomic DNA. According to the MassARRAY EpiTYPER technology, taqs were added to the 5'end of the primers. Forward primer: 10mer spacer tag is added at the 5' primer end with the following sequence: 5'- AGGAAGAGAG + primer. Reverse primer: T7 promoter is added to the 5' primer end with the following sequence: 5'- CAGTAATACGACTCACTATAGGGAGAAGGCT + primer

Supplemental Table 3. Details quality control

Locus/unit	Reason for exclusion	Success rate (if included)
IGF2DMR		
IGF2_01	Excluded due to rs3741208	
IGF2_02	Excluded due to rs3741209	
IGF2_03		91.8%
IGF2_04		94.1%
IGF2_05	Excluded due to rs4930041	
IGF2_06.07		94.9%
IGF2_08	Excluded due to silent signal	
H19		
H19_01	Excluded due to duplicate H19_16 and silent signals	
H19_02		95.6%
H19_03-05	Excluded due to rs117916983, overlap H19_11 and silent signals	
H19_06	Excluded due to silent signals	
H19_07	Excluded due to silent signal	
H19_08	Excluded due to silent signal	
H19_09.10		95.0%
H19_11	Excluded due to overlap H19_03-05 and silent signals	
H19_12		95.4%
H19_13		95.1%
H19_14.15		96.7%
H19_16	Excluded due to duplicate H19_1 and silent signals	
H19_17		95.3%
H19_18.19		95.6%
H19_20		95.6%
H19_21	Excluded due to low mass and silent signal	
H19_22	Excluded due to silent signal	
H19_23	Excluded due to low mass	
H19_24	Excluded duet o>25% missing	
H19_25		94.7%

Chapter 8

DNA Methylation Profiles at Birth and Child ADHD Symptoms

ABSTRACT

Attention deficit/hyperactivity disorder (ADHD) is a common and highly heritable psychiatric disorder. In addition, early life environmental factors contribute to the occurrence of ADHD. Recently, DNA methylation has emerged as a mechanism potentially mediating genetic and environmental effects.

Here, we investigated whether newborn DNA methylation patterns of selected candidate genes involved in psychiatric disorders or foetal growth are associated with ADHD symptoms in childhood. Participants were 426 children from a large population based cohort of Dutch national origin. Behavioural data were obtained at age 6 years with the Child Behaviour Checklist. For the current study, 11 regions at 7 different genes were selected. DNA methylation levels of cord blood DNA were measured for the 11 regions combined and for each region separately. We examined the association between DNA methylation levels at different regions and ADHD symptoms with linear mixed models.

DNA methylation levels were negatively associated with ADHD symptom score in the overall analysis of all 11 regions. This association was largely explained by associations of DRD4 and 5-HTT regions. Other candidate genes showed no association between DNA methylation levels and ADHD symptom score. Associations between DNA methylation levels and ADHD symptom score were attenuated by co-occurring Oppositional defiant disorder and total symptoms.

Lower DNA methylation levels of the 7 genes assessed at birth, were associated with more ADHD symptoms of the child at 6 years of age. Further studies are needed to confirm our results and to investigate the possible underlying mechanism.

INTRODUCTION

Attention deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterised by delayed brain maturation (1). However, the exact underlying mechanism of ADHD is poorly understood. Twin studies have provided evidence that ADHD has a genetic basis and heritability is estimated to be around 0.76, ranging from around 60 to over 0.95 (2). Despite the high heritability, few definite risk genes have been identified (3). The numerous candidate gene studies and several genome wide association scans have largely been without consistent findings (4, 5). The complex phenotype, the very polygenic genotype or gene-environment interactions may explain this inconsistency between behavioural and molecular genetic studies (6).

Recent studies have demonstrated a possible role of DNA methylation in schizophrenia, depression and suicidal behaviour (7-9). Epigenetics may represent a mechanism explaining the occurrence of disease, in particular as a postulated mechanism linking environmental risk factors to biological mechanisms (10). Early onset, heritable neurodevelopmental disorders are candidates for epigenetic research as neurons are generated early during development. Epidemiological studies have associated prenatal exposure to adverse environmental factors like smoking (11), toxins (12) or maternal stress (13) with the risk of ADHD. These observed environmental risk factors exert an effect mainly during the prenatal or perinatal period, suggesting that timing of exposure is important for the susceptibility of the developing brain. Low birth weight, preceded by intrauterine growth restriction, can be seen as an indicator of a poor prenatal environment. Low birth weight also elevates the risk of adverse behavioural outcomes and is a known risk factor for ADHD (14).

Against this background, we investigated whether methylation patterns of neuronal genes observable at birth are associated with ADHD symptoms at age 6 years. As ADHD is observed more often in children born small-for-gestational age, we examined whether prenatal factors influence the occurrence of ADHD by alterations in DNA methylation.

DNA methylation patterns were assessed in cord blood to examine the association with ADHD prospectively. Although confounding cannot be excluded this design reduces the chance of reverse causation as it dissects whether differences in DNA methylation are a cause or a consequence of the disorder. However, unmeasured prenatal confounding factors cannot be ruled out.

Because of inaccessibility of brain tissue in living subjects, in the current study we examined DNA derived from leukocytes. It is hypothesized that environmental factors impacting the epigenetic marks in the foetal brain also induce peripheral epigenetic alterations (15). Based on the literature we selected a few genes, of which variations are assumed to affect nervous system development and metabolism (glucocorticoid receptor (NR3C1), methylenetetrahydrofolate-reductase (MTHFR)), presynaptic genes (Dopamine Receptor D4 (DRD4) and serotonin transporter protein (5-HTT)) or foetal growth (insulin-like growth factor 2 (IGF2DMR), H19, potassium channel protein (KCNQ1OT1)).

MATERIALS AND METHODS

Design and Study Population

This study was embedded in the Generation R Study, an ongoing population-based birth cohort from foetal life onwards. The Generation R Study, designed to identify early environmental and genetic determinants of growth, development and health, has been previously described in detail (16). Assessments with physical examinations, biological samples and detailed questionnaires were performed.

Participants comprised a subset (n = 540) of the original Generation R cohort. To reduce the effect of population stratification, the current study was restricted to children of Dutch national origin, which was based on the country of birth of the parents and grandparents. Of the selected children, sufficient quantity and quality of DNA derived from cord blood was available. 448 children were randomly drawn from the cohort. The sampling strategy was to use stratification with oversampling of children diagnosed with ADHD (n = 92). This improved power of the analyses and achieve a more normal distribution of ADHD symptoms, which are highly right skewed in the general population as most individuals have relatively few ADHD symptoms and only a minority have high ADHD symptoms (Supplementary Figures 1 and 2).

In addition, 100 children with the smallest birth weight for gestational age (<-1.75 SD) were selected to investigate the role of prenatal factors in the aetiology of ADHD. However, we also ran the analyses without this sample. In 426 of all selected children, parents provided data on ADHD symptoms by filling out the Child Behaviour Checklist.

The study has been approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam.

Measurements of DNA methylation and Genotyping

It is widely recognized that presynaptic genes are the most important components in the aetiology of ADHD (17). Based on literature, genes were selected on the basis of their potential involvement in neurotransmitter systems and neurodevelopment (Supplemental Table 1). The number of DNA methylation regions (n = 12) assessed was limited by the total amount of DNA (500 ng) used. Hence, we chose to determine the CpG islands of the genes, and selected the middle region or outer regions per island or the region previously investigated by other groups: Two regions of the 5-HTT gene were selected from a publication by Philibert where the relationship with depression and alcohol dependency has been investigated (18).

Previously, Wong et al. described epigenetic differences in twins in the region of the DRD4 gene and one of the regions in the 5-HTT gene (19). We additionally selected regions of the IGF2DMR and H19 gene that are implicated in foetal growth. Talens et al. reported about the variation and stability of DNA methylation of the IGF2DMR and H19 region (15). Epigenetic regulation of the NR3C1 has been associated with childhood abuse (20). Primers

of the other regions at the KCNQ1OT1, MTHFR and NR3C1 genes were designed using the online tool of EpiDesigner (epidesigner.com).

For convenience, we described the three regions at 5HTT and NR3C1 genes as region A, B or C (Supplemental Table 1). The assessment of the selected region near the gene CCNL1/LEKR1, implicated in foetal growth, did not succeed due to technical issues.

Isolated genomic DNA (500 ng) from cord blood samples was treated with sodium bisulphite for 16 hours using the EZ-96 DNA methylation kit (Shallow) (Zymo Research, Irvine, CA, USA). This was followed by PCR amplification, fragmentation after reverse transcription and analysis on a mass spectrometer (Sequenom, Inc, San Diego, USA). This generated mass spectra that were translated into quantitative DNA methylation levels of different CpG sites by MassARRAY EpiTYPER Analyzer software (v1.0, build 1.0.6.88 Sequenom, Inc, San Diego, USA). Samples were randomly divided over bisulphite conversion and PCR amplification batches. For each individual, the assays were amplified from the same bisulphite-treated DNA. All methylation measurements were done in triplicate from the same bisulphite-treated DNA.

Genotyping was performed for DRD4-48 base pair variable number tandem repeat (DRD4-48 bp VNTR) and a 44 bp insertion/deletion segment of the serotonin transporter gene 5-HTT (5-HTTLPR) as described previously (21). Frequency distributions conformed to the Hardy-Weinberg equilibrium.

Assessment of Behavioural Problems

We measured behavioural problems of the children at six years of age by using the Child Behaviour Checklist (CBCL/1,5-5). The CBCL is a parent report questionnaire that contains 99 problem items rated on a 3-point scale: 0 (not true), 1 (somewhat or sometimes true) and 2 (very true or often true). The 6-item DSM-oriented scale for ADHD was selected for our analyses since its items were chosen to closely map onto DSM-IV criteria for ADHD. The items included "Can't sit still, restless, or hyperactive," "Can't concentrate, can't pay attention for long" and "Quickly shifts from one activity to another". By summing scores of these selected items, a DSM-Oriented Scale of Attention-Deficit/Hyperactivity can be computed that has been used for continuous analyses. Higher scores represent higher severity. Good reliability and validity have been reported for the CBCL (22). The CBCL in general have shown to be a robust predictor of clinically-diagnosed ADHD (23).

For sample selection and dichotomous analyses the Diagnostic Interview Schedule for Children-Parent version (DISC-P) was used. The DISC-P is a structured interview with the parents, assessed during home visits, that define diagnoses according to the criteria specified by the Diagnostic and Statistical Manual of Mental Disorders (4th ed. DSM-IV, APA 1994). The reliability and validity of the DISC-P have been reported to be acceptable (24).

The DISC was only assessed in a selected risk and control group of the Generation R study group. For this reason, data from DISC was available in 116 children (mainly cases) of the current study population. To maximize the power for analyses we used CBCL scores as they were present in 426 children. Median CBCL ADHD symptom score in children that

fulfilled criteria of ADHD according to DISC-P was 8.0 (95%CI 7.2; 8.4), and 2.61 (95%CI 2.5; 3.0) in children that did not fulfill these criteria (Supplemental Figure 3).

Covariates

Possible determinants of DNA methylation and ADHD were derived from the literature (25-28). Information on maternal age, education, parity, prenatal smoking, alcohol use, folic acid supplement use and child national origin was obtained by questionnaires during pregnancy. Educational level of the mother was assessed by the highest completed education and reclassified into three categories: primary school, secondary school and higher education. Maternal prenatal smoking and alcohol use were classified as 'no use', 'use until pregnancy was confirmed' and 'continued use during pregnancy'. Height and weight were measured without shoes and heavy clothing; body mass index was calculated from weight and height (kg/m^2). At 20 weeks pregnancy, we measured maternal psychological problems using the Brief Symptom Inventory (29). Child gender, birth weight, Apgar score one minute after birth, and the mode of delivery were derived from medical records completed by gynecologists and midwives. To define gestational age at birth, we used foetal biometry measured at the first prenatal visit. In addition of selection of children of Dutch national origin, child genetic ancestry was determined by principle component analyses of genome wide association data, as described previously (30).

Statistical Analysis

First, we explored whether individual genetic variations may underlie variation in DNA methylation levels. The 5-HTT and DRD4 gene are characterized by a variable nucleotide repeat that have been linked to ADHD in childhood (17, 31). We examined the association between genetic variants DRD4-48 bp VNTR and 5-HTTLPR and DNA methylation levels in the measured regions in the DRD4 and 5-HTT gene. In the current population, we tested whether these variations are associated with ADHD symptoms at age 6 years using linear regression models.

Primarily, we investigated the association between DNA methylation and ADHD symptom score. To further explore our results we repeated the analyses using a dichotomous outcome defined by a cut-off of 2 on the ADHD symptom scale of the CBCL. We also examined the association between DNA methylation levels and ADHD diagnosis based on DISC-P interview.

In children with ADHD the risk for comorbid psychiatric disorders is high. We tested specificity for the association between DNA methylation on ADHD symptoms by adding symptom scores of the other CBCL syndrome scales and total symptom scores to the model. Additionally, to test whether results depended on the sampling strategy with an oversampling of children born small-for-gestational age, all analyses were repeated excluding this sample.

DNA methylation levels were treated as continuous variables. To achieve normal distribution, variables of DNA methylation levels were transformed by the square-root. For

the analyses, the triplicate measurements and DNA methylation levels of the separate CpG units within a region were treated as clustered variables and not as a mean.

For an initial overall analysis of methylation and ADHD symptom score, DNA methylation levels of all CpG units in the 11 regions together were combined in one analysis. We present the complete data from all regions selected and chose a hierarchical approach to reduce the risk of type I error rather than to report selectively. Next to specific effects of DNA methylation, general effects on the level of DNA methylation have been described. Besides, the specific regions are correlated; a combined analysis increases the power and helps detect small effects of DNA methylation. Consequently, we have to be very careful to interpret specific effects on DNA methylation as the focus was on the overall effect.

As most CpG units within one region are correlated (except for 5HTT region A, MTHFR and NR3C1, all CpGs within regions assessed show correlations varying from 0.23-0.90 (p<0.001)), the associations with DNA methylation level were calculated using Linear Mixed Models. Mixed models have the advantage to allow correlated random effects in individuals. Another advantage of this model is the ability to accommodate missing data points. Furthermore, using mixed models enables adjustment for relevant covariates on the raw data in the same model (32).

We also stratified the analysis on DNA methylation levels of DRD4 and 5-HTT regions by gender, and tested interactions between gender and ADHD symptoms.

To understand the effect of environmental factors on DNA methylation two models have been used. First, we only adjusted for bisulphite and PCR batch, child gender, genetic ancestry (principal components), gestational age at birth and age at assessment of CBCL. In the fully adjusted model, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use, child birth weight and Apgar score were added. This approach allowed us to evaluate the effect of exposures during pregnancy as possible explanatory factors behind any observational effect of methylation on ADHD symptoms (Supplemental Figure 4). Analyses of the association of DNA methylation of any 5HTT or DRD4 regions with ADHD outcomes were adjusted for the respective 5HTT or DRD4 genotypes.

All analyses were performed using SPSS software, version 20.0 (IBM-SPSS, Chicago, IL, USA).

RESULTS

Characteristics of the children and their mothers in the study population are presented in Table 1. The children were born after on average 40.2 (SD = 1.5) weeks of pregnancy. Mean birth weight was 3430 (SD = 580) grams. Mothers had a mean age of 30.1 (SD 4.7) years, 31.1% of the mothers was higher educated and 30.7% smoked during pregnancy.

Table 2 showed the association between adjacent DRD4-48 bp VNTR and 5-HTTLPR and DNA methylation levels. Children with one or two copies of the 7-repeat at the DRD4-48 bp

VNTR have significantly lower methylation levels than children without a 7-repeat. Likewise, having a short allele of 5-HTTLPR was also associated with lower DNA methylation levels of the measured 5-HTT regions. In the current population the presence of a short allele of 5-HTTLPR was associated with an increase of ADHD symptoms (per copy of short allele: ß = -0.07 95%CI -0.09; -0.05, p<0.001). DRD4-48 bp VNTR showed no association with ADHD symptom score (per copy of 7-repeat: ß = -0.01 95%CI -0.01; -0.04, p = 0.30).

Table 1. Maternal and child characteristics in the study population n = 426.

Child characteristics	
Gender, % boys	59.6
Birth order, % first born	68.9
Birth weight, g	3430 (580)
Gestational age, wk	40.2 (1.5)
Apgar score 1 min after birth	8.6 (1.3)
Mode of delivery, %	
Spontaneous vaginal	73.0
Instrumental vaginal	19.6
Caesarean section	7.4
Maternal characteristics	
Age, years	30.1 (4.7)
Body Mass Index, kg/m^2	24.3 (4.0)
Educational level. %	
Primary	13.5
Secondary	55.3
High	31.1
Psychological symptoms, score, median (95% range)	0.15 (0.00-1.08)
Smoking during pregnancy, %	
Never	69.3
Until pregnancy was confirmed	8.8
Continued	21.9

Values represent means (SD) unless otherwise indicated.

Next, we examined our primary hypothesis of an association between DNA methylation and ADHD symptom score measured at age 6 years (Table 3a). The table presents the models fully adjusted for bisulphite and PCR batch, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use, child gender, genetic ancestry (principle components), birth weight, Apgar score, gestational age at birth and age at assessment of CBCL. We found a negative relation between mean DNA methylation in the overall analysis of all regions measured. Children with higher methylation levels had less ADHD symptoms. This effect did not change meaningfully after

correction for DRD4-48 bp VNTR and 5-HTTLPR indicating an independent effect of DNA methylation level ADHD symptom score (ß = -0.13, 95%CI -0.22; -0.04, *p* = 0.01).

Table 2. Associations between haploid genotype in candidate genes and DNA methylation.

	Crude analyses		**Adjusted analyses**	
	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
DRD4, number of 7-repeats (n = 500)	**DRD4 region, % methylation**			
0 (n = 326)	*reference*		*reference*	
1 (n = 158)	-3.40 (-4.20; -2.60)	**<0.001**	-3.40 (-4.20; -2.60)	**<0.001**
2 (n = 16)	-7.10 (-9.20; -4.90)	**<0.001**	-6.70 (-8.80; -4.60)	**<0.001**
5-HTT, alleles-type (n = 512)	**combined 5-HTT regions, % methylation**			
LL (n = 171)	*reference*		*reference*	
SL (n = 239)	-0.23 (-0.32; 0.08)	0.25	-0.11 (-0.32; 0.09)	0.27
SS (n = 102)	-0.33 (-0.58; -0.08)	**0.01**	-0.33 (-0.58; -0.07)	**0.01**

ß, 95% confidence intervals and P-values. Analyses are adjusted for bisulphite and PCR batch, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use and child gender, genetic ancestry (principle components) , birth weight, Apgar score, gestational age at birth and age at assessment of CBCL.

In order to identify specific regions that contribute to the association between DNA methylation and ADHD symptoms in the overall analyses we subsequently explored specific regions. DNA methylation of specific regions that were negatively associated with ADHD symptom score were 5-HTT region B and DRD4 region. This direction is in line with the association between the ADHD risk variants and lower DNA methylation levels. Associations with KCNQ1OT1 (ß = -0.34 95%CI -0.63; -0.05, *p* = 0.02) were strongly attenuated and became non-significant after adjustment for maternal age, parity and educational level (Table 3a). To better understand the association between DNA methylation and ADHD symptoms, we repeated the analyses using a dichotomous variable of ADHD symptom score with a cut-off at 2 of the CBCL. The effects were consistent and remained significant after adjustment for potential confounders (Table 3b).

We additionally tested whether any interaction between child 5HTTLPR genotype and DNA methylation predicted ADHD symptoms. There was no interaction effect between the 5HTTLPR variation and the respective DNA methylation levels (Methylation 5HTT regions x 5HTTLPR: ß = -8.87 95%CI -21.97; 4.64, *p* = 0.20) nor between the DRD4 VNTR 7 repeat and the respective DNA methylation levels on the level of ADHD problems (Methylation DRD4 region x DRD4 VNTR 7 repeat: ß = -2.03 95%CI -5.20; 1.14, *p* = 0.21).

Table 3a. DNA methylation and ADHD symptom score.

n = 426	Overall methylation							
ADHD, symptom score (sqrt transformed)	All genes jointly analysed, methylation %							
	ß (95%CI)	**P-value**						
	-0.12 (-0.21; -0.02)	**0.02**						
	Neurotransmitter systems							
ADHD, symptom score (sqrt transformed)	DRD4, methylation %		5-HTT region A, methylation %		5-HTT region B, methylation %		5-HTT region C, methylation %	
	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
	-0.52 (-1.00; -0.05)	**0.03**	-0.00 (-0.15; 0.15)	1.00	-0.22 (-0.38; -0.06)	**0.006**	-0.09 (-0.26; 0.08)	0.30
	Metabolism							
ADHD, symptom score (sqrt transformed)	NR3C1 region A, methylation %		NR3C1 region B, methylation %		NR3C1 region C, methylation %		MTHFR5, methylation %	
	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
	-0.03 (-0.13; 0.06)	0.45	-0.08 (-0.20; 0.40)	0.18	0.03 (-0.08; 0.14)	0.56	0.05 (-0.04; 0.13)	0.28
	Foetal growth							
ADHD, symptom score (sqrt transformed)	H19, methylation %		IGF2DMR, methylation %		KCNQ1OT1, methylation %			
	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**		
	-0.07 (-0.33; 0.20)	0.62	0.05 (-0.30; 0.41)	0.77	-0.29 (-0.61; 0.02)	0.07		

The beta represent the change in methylation % per squareroot (sqrt) transformed ADHD symptom score as analysed with linear mixed models.
Analyses are adjusted for bisulphite and PCR batch, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use and child gender, genetic ancestry (principle components) , birth weight, Apgar score, gestational age at birth and age at assessment of CBCL. Analyses of DRD4 and 5-HTT regions are also adjusted for variable number tandem repeats.

Table 3b. DNA methylation and ADHD dichotomous symptom score.

n = 426	Overall methylation							
ADHD, symptom score (>2) dichotomous	All genes jointly analysed, methylation %							
	ß (95%CI)	**P-value**						
	-0.25 (-0.43; -0.06)	**0.009**						
	Neurotransmitter systems							
ADHD, symptom score (>2) dichotomous	DRD4, methylation %		5-HTT region A, methylation %		5-HTT region B, methylation %		5-HTT region C, methylation %	
	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
	-1.04 (-2.03; -0.48)	**0.04**	-0.07 (-0.37; 0.22)	0.63	-0.35 (-0.66; -0.03)	**0.03**	-0.07 (-0.40; 0.26)	0.68
	Metabolism							
ADHD, symptom score (>2) dichotomous	NR3C1 region A, methylation %		NR3C1 region B, methylation %		NR3C1 region C, methylation %		MTHFR5, methylation %	
	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
	-0.11 (-0.29; 0.07)	0.22	-0.10 (-0.34; 0.15)	0.44	0.03 (-0.19; 0.25)	0.78	0.07 (-0.10; 0.24)	0.44
	Foetal growth							
ADHD, symptom score (>2) dichotomous	H19, methylation %		IGF2DMR, methylation %		KCNQ1OT1, methylation %			
	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**		
	-0.20 (-0.73; 0.33)	0.46	0.15 (-0.55; 0.85)	0.68	-0.50 (-1.11; 0.12)	0.11		

The beta represent the change in methylation % per squareroot (sqrt) transformed ADHD symptom score as analysed with linear mixed models.
Analyses are adjusted for bisulphite and PCR batch, maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use and child gender, genetic ancestry (principle components) , birth weight, Apgar score, gestational age at birth and age at assessment of CBCL. Analyses of DRD4 and 5-HTT regions are also adjusted for variable number tandem repeats.

To test whether the results depend on the comorbid psychiatric symptoms, symptom scores of other CBCL syndrome scales and total symptoms were added to the model. Associations between DNA methylation levels and ADHD symptom score were attenuated by ODD and total symptoms (overall regions: ß = -0.10 95%CI -0.22; 0.03, p = 0.12 and ß = -0.08 95%CI -0.21; 0.06, p = 0.28 respectively). In contrast, the association between child DNA methylation level and ADHD symptoms could not be explained by internalizing, affective disorder, anxiety or PDD problem scale (results not shown).

Analyses of the associations between mean DNA methylation in cord blood and child ADHD diagnosis based on DISC-P showed no associations (overall analyses ß = 0.03 95%CI -0.17; 0.24, p = 0.77, other results not shown).

Next, we examined whether alterations in DNA methylation associated with ADHD symptoms, were influenced by prenatal environmental factors. To this aim, we also studied the associations without adjustment for indicators of maternal risks during pregnancy, including maternal educational level, age, parity, body mass index, psychological problems, smoking, folic acid supplement use, child birth weight and Apgar score. Results without adjustment for indicators of maternal exposure were very similar, none of these covariates change the effect estimates substantially. This indicates that adverse prenatal environmental factors do not explain the association between child DNA methylation and ADHD symptoms. The only covariates related to both DNA methylation and ADHD symptoms were genetic ancestry (principle components) and gender.

When analyses were stratified by gender, the association between DNA methylation of all regions measured and ADHD symptom score was not meaningful different. There was no interaction effect of gender (P-value for interaction 0.56).

When analyses were repeated with exclusion of children born small-for-gestational age, the associations between DNA methylation level and ADHD symptoms were very similar, however, some associations were not significant as the group size was smaller (Supplemental Table 2).

DISCUSSION

In this study we investigated the association of DNA methylation levels of neuronal and non-neuronal candidate genes with ADHD symptoms in childhood.

In an overall analysis across 11 regions, lower DNA methylation levels were associated with higher ADHD symptom scores. The results were independent of two selected DNA variants. However, we cannot rule out that other genetic variations in the vicinity of this region could directly influence DNA methylation measurements. The effect was largely explained by associations with DNA methylation levels in DRD4 and 5-HTT regions. However, this result should be interpreted with caution since our study had insufficient power to stringently correct for multiple testing in individual regions analyses.

DNA methylation was measured in cord blood collected directly after birth before any postnatal environmental influences exerted an effect. This created a unique opportunity to observe DNA methylation at baseline and investigate associations with ADHD in a prospective manner. However, since DNA methylation may be influenced by a variety of postnatal factors such as social environment, environmental toxins and drugs, this does not rule out environmental effects (33-36).

Several explanations may help to clarify the associations between lower levels of DNA methylation and more ADHD symptoms.

First of all, this association could be influenced by other confounding factors that might be in the causal pathway of environmental factors influencing the epigenome. We corrected our analysis for numerous potential confounding factors, however, there is little information from literature on what variables to control for.

Second, genetic factors could underlie the association between DNA methylation and ADHD symptoms. We corrected for the child genetic variables in the selected regions. In this study we reported lower DNA methylation levels in subjects with the risk allele of the DRD4-48 bp VNTR or the 5-HTTLPR polymorphism. The association between low DNA methylation levels of DRD4 and 5-HTT regions was largely independent of the genetic variants DRD4-48 bp VNTR or 5-HTTLPR. In previous studies, the presence of the DRD4-48 bp VNTR was shown to affect mRNA expression *in vitro* (37). However, we did not take into account other genetic variations than those two sites assessed, although several other polymorphisms exist in these regions.

Proper DNA methylation plays a critical role in embryonic development and cell differentiation. Methylation also plays a mediating role in gene expression (38). Although there is no simple relationship between DNA methylation and gene expression it is assumed that DNA methylation is associated with loss of gene expression (39). In reports of patients with psychiatric disorders, e.g. bipolar disorder, schizophrenia, anorexia nervosa and DNA methylation, patients were more likely to have hypomethylated (neuronal) candidate genes (40, 41). This is in line with our study, where we report a negative association between DNA methylation levels and ADHD symptoms.

The selection of our candidate genes was based on previous psychiatric epigenetic and candidate gene studies. It is postulated that ADHD may be caused by an imbalance of neurotransmitters (42). Research has focused in particular on the dopaminergic system, since effective medication was reported to block the reuptake of dopamine by the dopamine transporter molecule (43). Early reports of animal models of ADHD resembling the human condition demonstrated increased DRD4 expression (44, 45).

A study by Wang et al. suggests that peripheral DNA methylation of the serotonin transporter may be a marker of central serotonin transporter function (46). However, a meta-analyses of Gizer et al. indicated a modest but significant association between ADHD and the 5HTTLPR "long allele" (17).

In mice, prenatal protein restriction leading to intrauterine growth retardation, significantly increased expressed dopamine related genes (47). However, in the current study

the association between DNA methylation and ADHD symptoms was independent of birth weight. Possibly, although we corrected for many environmental factors, prenatal influences not reflected by changes in birth weight may underlie these behavioural symptoms.

In this study we did not observe any association between ADHD-associated prenatal exposures and child DNA methylation at birth. The observed effects are rather small, this makes it difficult to reveal a mediation by DNA methylation on the risk of ADHD symptoms. On the other hand, since no previous human study has succeed to link disease-associated exposures via DNA methylation to the occurrence of disease, it might be the case that both environmental exposure and DNA methylation have largely independent effects.

The association between DNA methylation patterns and ADHD diagnose based on DISC and CBCL ADHD symptom score show different results. An important explanation might be that using CBCL symptoms increases power as a symptom count was available in more children in the analyses and analysed continuously.

Second, there could be discrepancies in item specificity between the DISC and the CBCL instrument. Although the clinical utility of categorically defined ADHD is well established, there is also strong evidence that the liability to develop ADHD problems is continuous. Clustering of subjects in terms of subtypes, as DISC does, neglects variation in severity as children with moderately elevated symptom scores on several subtypes won't meet DSM-IV criteria for ADHD while children with elevated symptom scores on one subtype will (23).

As ADHD is known to have high psychiatric comorbidity, alterations in DNA methylation might be the result of other disorders. Alterations in DNA methylation are reported to play a role in the aetiology of bipolar disorder, autism and obsessive compulsive disorder, among others (48-50). In the current study, sensitivity analyses showed that ODD and total symptom scores attenuated the association between DNA methylation levels and ADHD symptom score. This suggests that the observed associations may not be specific for ADHD. Most likely, this reflects our choice of candidate gene sites for the methylation analyses. All psychiatric genes have been implicated in several developmental disorders, and candidate genes specifically related to ADHD have not been identified.

At the same time, the association between lower DNA methylation and more ADHD symptoms was independent of internalizing symptoms. This finding is in line with the observations from Kendler et collegues that internalizing and externalizing disorders originate partly from different genetic mechanisms (51, 52), whereas symptoms of ADHD and aggression are not only frequently co-morbid reflecting a more substantially shared genetic vulnerability (53). However, we have to be careful to translate findings in genetic studies to epigenetics and can only speculate about the parallels between underlying genetic and epigenetic structures in psychiatric disorders.

Epigenetics provide a possible mechanism for gene-environment interactions that have been observed in ADHD (54). Several models postulating a complex interplay between the genome, epigenome and environmental factors have been proposed. Some specific tandem repeats are associated with alterations in epigenetic patterns (55). Other possibilities are an increased susceptibility by genetic variations for environmental factors to alter DNA

methylation or, vice versa, an increased susceptibility for gene-environment interactions in the presence of altered DNA methylation. In the current study, we describe that DRD4-48 bp VNTR and 5-HTTLPR were associated with lower DNA methylation levels of the adjacent regions. The alterations in DNA methylation could be the result of interaction effects instead of direct genetic effects. However, testing interaction effects was not the core aim of the current study as we lacked hypotheses and, besides, sufficient power.

Several limitations of our study should be mentioned. First, in the current study attention problems were primarily assessed by means of a structured questionnaire and attention problems were considered as a continuous trait instead of a dichotomy. This has been shown to adequately represent attention problems on the population level and provides more power in the analyses. Further, the CBCL-ADHD symptom scale converges with the results of clinical interviews covering the DSMIV-criteria (56).

Second, our focus on selected regions in the genome made it unable to extrapolate our findings to other genomic regions. We performed an overall analysis of all regions assessed under the assumption that the variation of methylation has a uniform direction of effect. Separate analyses of the specific regions showed certain heterogeneity of effects across the different genomic regions, however, the direction of methylation effects at birth on ADHD symptoms was consistent.

Third, the DNA in the current study was isolated from leukocytes and not from brain tissue. The question rises how alterations in DNA methylation profiles in cord blood reflect DNA methylation profiles in other tissue of the child. This is a major problem in human studies. One post-mortem study analysed an average of approximately 1500 regions, from 12 different tissues from different brain parts and other organs obtained from adult individuals found that only 34 CpGs were differently methylated among neural and non-neuronal tissues (57). Another study analyzing DNA methylation variation across brain and blood found that, although between-tissue variation exceed between-individual variation, inter-individual variation was reflected across brain and blood. This implies that peripheral tissues may be of use for epidemiological studies (58). Talens et al. reported for four of the eight investigated regions strong correlations between DNA methylation profiles in blood and buccal cells, tissues from different germ layers (15). However, no studies have compared foetal brain and peripheral tissues.

Finally, our study relied on genomic DNA extracted from whole blood. Epigenetic differences across samples could be derived from different cellular leukocyte populations, although cellular heterogeneity is considered to have limited impact (15).

To conclude, consistent with the epigenetic hypothesis of ADHD, a number of regions were found in which lower methylation levels measured at birth were associated with more ADHD symptoms in childhood. However, we cannot distinguish whether genetic, non-genetic intragenerational transmission, or unknown environmental factors underlie this association. Moreover, the observed association with ADHD symptoms was partly explained by co-occurring ODD and total symptoms. Further studies are needed to confirm our results and to investigate the possible underlying mechanism.

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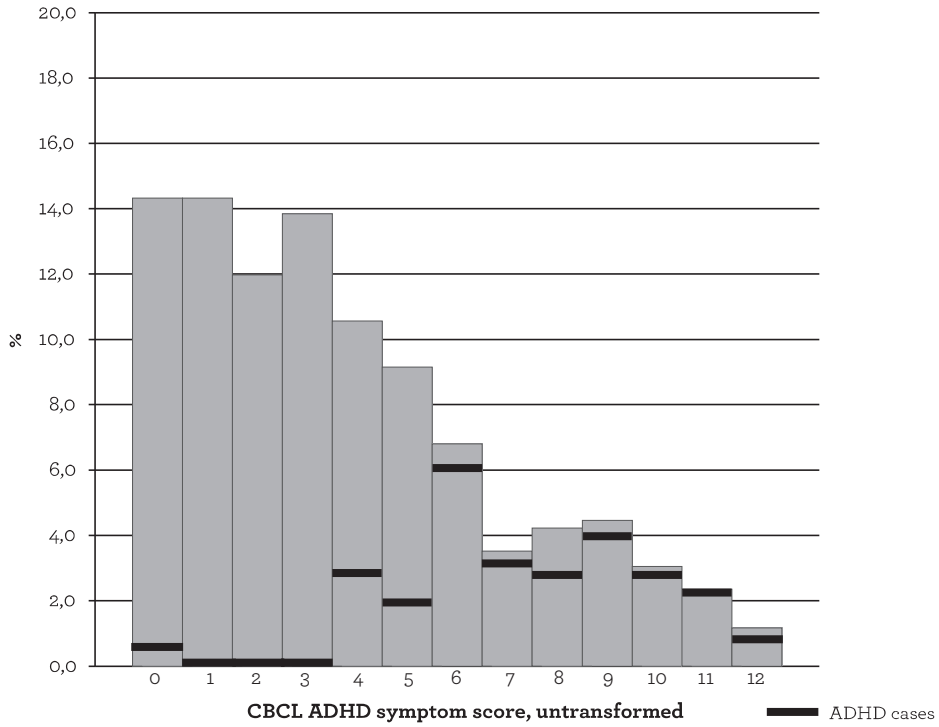
Supplemental Table 1. Summary of primer sequences for PCR amplification*.

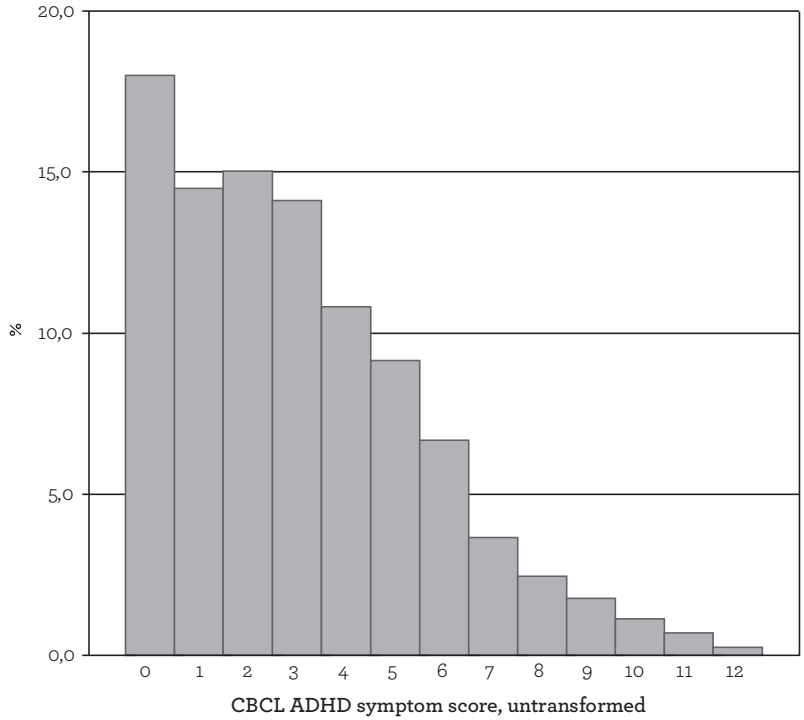
Gene	Candidate function	Genomic Region	Number of CpG units assessed	Primer sequence** (5' to 3')	Source	Mean % methylation (SD)
Dopamine D4 Receptor (DRD4)	Neurotransmitter system	chr11:636508-636904	11 CpG units (19 CpG sites)	F:GGGATTTTTTGTTTAGGGTTAGAGG R:CACCCTAATCCACCTAATATCTAACA	Wong, 2010 (19)	46.6 (5.2)
Serotonin Transporter (5-HTT) region A	Neurotransmitter system	chr17:28562435-28562812	6 CpG units (13 CpG sites)	F:GGTTAGTTTTAGTTTTGGTTTTTGTT R:CAAAAATTCTTCAAAAACTCTTTAAC	Wong, 2010 (19)	14.6 (1.3)
Serotonin Transporter (5-HTT) region B	Neurotransmitter system	chr17: 28562751-28563084	12 CpG units (22 CpG sites)	F:GTTTTTATATGGTTTGATTTTTAGATAG R:CCTACTCCTTTATACAACCTCCCCC	Philibert, 2008(18)	22.8 (1.8)
Serotonin Transporter (5-HTT) region C	Neurotransmitter system	chr17: 28562358-28562783	13 CpG units (25 CpG sites)	F:GTTATTTAGAGATTAGATTATGTGAGGGT R:CCTACAACAATAAACAAAAAAACCCC	Philibert, 2008(18)	18.8 (1.8)
Potassium channel protein (KCNQ1OT1)	Development growth and metabolism	chr11: 2721823-2722182	4 CpG units (6 CpG sites)	F:GTTAGGGAAGTTTTAGGGTGTGAAT R:TTCTAAAACCCCCACTACTATACCT	Designed in EpiDesigner	62.4 (3.0)
Insulin-like growth factor 2 (IGF2)	Growth and insulin signaling	chr11: 2169458-2169796	3 CpG units (4 CpG sites)	F:TGGATAGGAGATTGAGGAGAAA R:AAACCCCAACAAAAACCACT	Talens, 2010(15)	72.8 (3.8)
H19	Growth	chr11: 2019371-2019784	10 CpG units (13 CpG sites)	F:GGGTTTGGGAGAGTTTGTGAGGT R:ATACCTACTACTCCCTACCTACCAAC	Talens, 2010(15)	56.1 (3.0)
Methylenetetrahydrofolate reductase (MTHFR)	Homocystein metabolism	chr1: 11866184-11866627	12 CpG units (14 CpG sites)	F:GTTTGTAGTTATTTTTGGTTTTAGTTTT R:TAACCTAAATTCTCCCTCAAATTCC	Designed in EpiDesigner	5.0 (0.8)
Glucocorticoid receptor (NR3C1) region A	Development, metabolism and immune response	chr5: 142783585-142783906	10 CpG units (19 CpG sites)	F:TTTTTGAAGTTTTTTTAGAGGG R:AATTTCTCCAATTTCTTTTCTC	McGowan (20)	13.9 (1.6)
Glucocorticoid receptor (NR3C1) region B	Development, metabolism and immune response	chr5: 142782046 142782472	11 CpG units (22 CpG sites)	F:AAAGGGGTTATTTAGAAATTTTAGG R:AAAATCCTAACCTCTTTTCTCCCC	Designed in EpiDesigner	11.2 (0.9)
Glucocorticoid receptor (NR3C1) region C	Development, metabolism and immune response	chr5:142784559 142784950	10 CpG units (20 CpG sites)	F:TTTTTAGTTTAAGGGGAAGGG AAT R: CTTCAAAATATCAAAACAAAAAAAACC	Designed in EpiDesigner	13.0 (1.2)

** UCSC February 2009 GRCh37 Genomic Assembly*

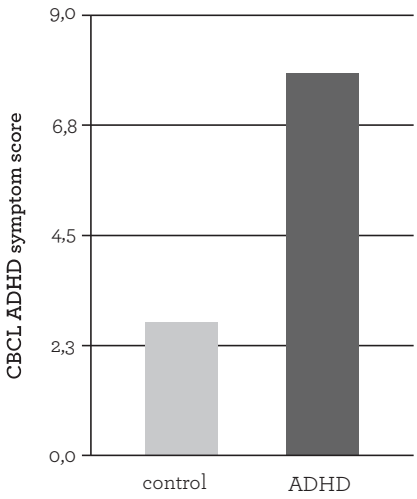
**Primers were delivered with standard Sequenom MassCleave tags added to the 5'end of the primers (10-mer at forward primer (F): aggaagagag, T7 at reverse primer (R) :cagtaatacgactcactatagggagaaggct).



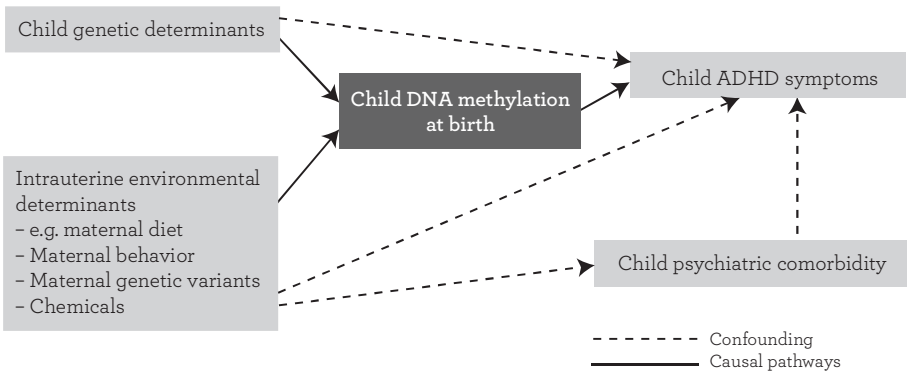
Supplemental Figure 1. Distribution of CBCL ADHD symptom score at age 6 years in the current study population n = 426.



Supplemental Figure 2. Distribution of CBCL ADHD symptom score at age 6 years in the total Generation R cohort n = 6,231.



Supplemental Figure 3. Mean CBCL ADHD symptom score at age 6 years in children with ADHD diagnose based on DISC and randomly selected controls n = 426.



Supplementary Figure 4. Proposed relationships between prenatal environmental influences, DNA methylation and ADHD symptoms.

Chapter 9

Determinants of Maternal One Carbon Metabolism in Early Pregnancy and the Epigenome of the Newborn: The Generation R Study

ABSTRACT

Maternal one-carbon (1-C) metabolism provides methylgroups for foetal development and programming by DNA methylation as one of the underlying epigenetic mechanisms. We aimed to investigate maternal 1-C biomarkers, folic acid supplement use and *MTHFR* C677T genotype as determinants of 1-C metabolism in early pregnancy in association with newborn DNA methylation levels of foetal growth and neurodevelopment candidate genes.

Participants were 463 mother-child pairs of Dutch national origin from a large population-based birth cohort in Rotterdam, The Netherlands. In early pregnancy (median 13.0 weeks, 90% range 10.4-17.1), we assessed the maternal folate and homocysteine blood concentrations, folic acid supplement use, and the *MTHFR* C677T genotype in mothers and newborns. In newborns, DNA methylation was measured in umbilical cord blood white blood cells at 11 regions of the 7 genes *NR3C1, DRD4, 5-HTT, IGF2DMR, H19, KCNQ1OT1* and *MTHFR*. The associations between the 1-C determinants and DNA methylation were examined using Linear Mixed Models.

An association was observed between maternal folate deficiency and lower newborn DNA methylation, which attenuated after adjustment for potential confounders. The maternal *MTHFR* TT genotype was significantly associated with lower DNA methylation. However, maternal homocysteine and folate concentrations, folic acid supplement use and the MTHFR genotype in the newborn were not associated with newborn DNA methylation. The maternal *MTHFR* C677T genotype, as determinant of folate status and 1-C metabolism, is associated with variations in the epigenome of a selection of genes in newborns. Research on the implications of these variations in methylation on gene expression and health is recommended.

INTRODUCTION

Epigenetics has gained interest as it is proposed to be an interface between the dynamic environment and the genome. DNA methylation, one of the best known epigenetic mechanisms, is essential for embryogenesis, foetal programming and development (1, 2).

One-carbon (1-C) metabolism plays a crucial role in DNA methylation as it determines the flux of methylgroups towards synthesis or methylation of DNA. Folate and choline are the main sources of methyl groups in 1-C metabolism that enable the conversion of homocysteine into methionine, which in turn leads to the production of *S*-adenosylmethionine (SAM) as principal methyldonor for DNA methylation (3). The 1-C metabolism is influenced by common polymorphisms, in particular the methylene tetrahydrofolate-reductase (*MTHFR) C677T polymorphism,* which interacts with the folate status and DNA methylation, in particular in situations of folate deficiency (4). In addition to genetic variants, dietary folate intake, folic acid supplement use, lifestyle and medication are known to influence the folate status (5). Our group previously reported higher levels of *IGF2* DMR methylation in white blood cell DNA from very young children after periconceptional exposure to maternal folic acid supplement use (6). This is supported by human and animal studies also emphasizing the importance of the 1-C pathway in foetal programming (5, 7).

A low maternal folate status during pregnancy has been associated with an increased risk of pregnancy complications, intrauterine growth restriction and developmental disorders including learning disabilities and emotional problems (8-11). In addition, polymorphisms in the *MTHFR* gene are suggested to contribute to the risk of developing psychiatric disorders, including depression, schizophrenia and bipolar disorder (12).

It can be concluded that alterations in the availability of folate during pregnancy may lead to variations in DNA methylation patterns in the offspring, which sometimes also change gene expression and outcomes of disease (13-15). Against this background, we investigated determinants of maternal 1-C metabolism in early pregnancy, i.e., folate and homocysteine concentrations, polymorphisms of the folate metabolizing gene *MTHFR* C677T (rs1801133), and folic acid supplement use in association with the newborn epigenome.

We related these factors to the overall DNA methylation status of 7 genes that were selected based on literature. Variations in these genes are assumed to influence neuronal development (glucocorticoid receptor (*NR3C1*), presynaptic genes (Dopamine Receptor-D4 (*DRD4*) and serotonin transporter protein (*5-HTT*)) or foetal growth (insulin-like growth factor 2 (*IGF2DMR*), H19, potassium channel protein (*KCNQ1OT1*)) and 1-C metabolism, i.e. *MTHFR*.

MATERIALS AND METHODS

Design and Study Population

This study was embedded in the Generation R Study, an ongoing population-based birth cohort from fetal life onwards in Rotterdam, The Netherlands. The Generation R Study, designed to identify early environmental and genetic determinants of growth, development and health, has been previously described in detail (16).

The study population comprised a subset (n = 540) of mother-child pairs of the original Generation R cohort. To reduce the effect of population stratification, we first restricted the study population to newborns of Dutch national origin based on the country of birth of the parents and grandparents. Next, we selected newborns with a sufficient quantity and quality of DNA derived from umbilical cord blood white blood cells. Finally, we oversampled newborns diagnosed with ADHD (n = 92) in childhood and those born small for gestational age (SGA, n = 100), because of previous study questions (17, 18), and 348 newborns randomly selected from the original cohort. We ran the analyses with and without the oversampled ADHD and SGA newborns.

The current study assessed multiple determinants of the 1-C metabolism (i.e. maternal and cord blood folate and homocysteine concentrations, maternal folic acid supplement use and maternal and newborn MTHFR C677T genotype) in relation to the outcome. To avoid the loss of power and bias due to complete case selection we performed the analyses in samples of varying size: of the 540 mother-child pairs 413 (76%) had data on maternal early pregnancy (median 13.0 weeks of gestation, 90% range 10.4-17.1) plasma folate and homocysteine concentrations. Plasma folate and homocysteine concentrations of the newborn were measured in 420 (77%) mother child-pairs.

Information on maternal folic acid supplement use was present in 440 (81%) of the 540 mother-child pairs. Maternal MTHFR C677T genotype was available in 463 (86%) mother-child pairs and information about the newborn MTHFR C677T genotype in 473 mother-child pairs (88%). The study has been approved by the Medical Ethics Committee of the Erasmus MC, University Medical Centre, Rotterdam.

Assessment of Biomarkers of 1-C Metabolism

In early pregnancy (median gestational age 13.0 weeks, 90% range 10.4-17.1) venous blood samples were drawn in plain tubes for the determination of plasma folate and homocysteine. Samples were transported to the regional laboratory for storage at -80°C within 3 hours after sampling. Ethylenediaminetetraacetic acid plasma samples were picked and analyzed using an immunoelectrochemoluminescence assay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands). The between-run coefficients of variation for plasma folate was 8.9% at 5.6 nmol/L, 2.5% at 16.6 nmol/L and 1.5% at 33.6 nmol/L, with an analytical range of 1.8-45.3 nmol/L. The same coefficients of variation for plasma homocysteine were 3.1% at 7.2 μmol/L, 3.1% at 12.9 μmol/L and 2.1% at 26.1 μmol/L, with an analytic range of 1-50 μmol/L (10).

Maternal Folic Acid Supplement Use

In early pregnancy, at the same moment of blood sampling, self-administered questionnaires were filled in by the mothers to obtain information on the use and initiation of folic acid and multivitamin supplements. In the Netherlands, a daily supplements of 0.4-0.5 mg folic acid is advised to the general population of women planning pregnancy up to 10 weeks of gestation (19). Mothers were categorised according to the moment of initiation of periconception folic acid supplement use as follows: 1) preconception initiation, 2) initiation after conception but before ten weeks of gestation or 3) no use. This categorisation has been applied to previous publications from the same study cohort (20).

MTHFR Genotyping

To assess the genotypes, DNA was extracted from white blood cells obtained from mothers in early pregnancy and newborns at birth. Genotyping of the *MTHFR* C677T polymorphism (rs1801133) performed by using TaqMan allelic discrimination assay (Applied Biosystems) and Abgene QPCR ROX mix (Abgene). The genotyping reaction was amplified by using the GeneAmp PCR system 9700 (95°C for 15 min), then 40 cycles of 94°C (15 s) and 60°C (1 min). The fluorescence was detected on the 7900HT Fast Real-Time PCR System (Applied Biosystems), and individual genotypes were determined by using SDS software (version 2.3; Applied Biosystems). Frequency distributions agreed with the Hardy-Weinberg equilibrium (Supplementary Table 2).

Measurements of DNA methylation

Based on literature, we selected genes implicated in fetal growth and neuronal development *5-HTT, DRD4, IGF2DMR, H19, KCNQ1OT1, NR3C1, CCNL1/LEKR1* and 1-C metabolism, i.e., *MTHFR* (Supplementary Table 1). Hence, we chose to investigate the CpG islands of the genes. We selected the middle region or outer regions per CpG island or the region previously investigated by other groups.

Two regions of the *5-HTT* gene were selected based on publication by Philibert where the relationship with depression and alcohol dependency has been investigated (21).

Previously, Wong et al. (22) described epigenetic differences in twins in the region of the *DRD4* gene and one of the regions in the *5-HTT* gene. Epigenetic regulation of the *NR3C1* gene has been associated with childhood abuse (23). We selected the same regions of the *IGF2 DMR* and *H19* gene as Talens et al, that are implicated in fetal growth (24). Primers of the other regions in the *KCNQ1OT1, MTHFR* and *NR3C1* genes were designed using the online tool of EpiDesigner (www.epidesigner.com).

Isolated genomic DNA (500 ng) from cord blood samples was treated with sodium bisulphite for 16 hours using the EZ-96 DNA methylation kit (Shallow) (Zymo Research, Irvine, CA, USA). This was followed by PCR amplification, fragmentation after reverse transcription and analysis on a mass spectrometer (Sequenom, Inc, San Diego, USA). This generated mass spectra that were translated into quantitative DNA methylation levels

of different CpG sites by MassARRAY EpiTYPER Analyzer software (v1.0, build 1.0.6.88 Sequenom, Inc, San Diego, USA).

Samples were randomly divided over bisulphite conversion batches. For each individual, the assays were amplified from the same bisulphite-treated DNA. All methylation measurements were performed in triplicate from the same bisulphite-treated DNA. Fragments containing one or more CpG sites were called CpG units. The following procedure has been followed for the quality control of the methylation data: 1) exclusion of CpG units with too low or too high mass or CpG units with overlapping or duplicate RNA fragments (e.g. silent signals), 2) for inclusion of CpG units at least two out of three replicate measurements per CpG unit had to be successful, 3) for inclusion of CpG units the standard deviation of the triplicates (or duplicates) had to be ≤0.10 and the success rate per CpG unit had to be ≥75% and 4) exclusion of CpG units with interference of single nucleotide polymorphisms (CEU) SNPs with a frequency >5% as this could interfere with the measurement by a change of weight of the CpG unit. Because the assessment of the selected region near the gene *CCNL1/LEKR1* was unsuccessful due to measurement errors, this data were not analysed any further.

Covariates

The following possible confounders were based on expertise and literature review (6, 25, 26).

Information on maternal education, prenatal smoking and alcohol consumption was obtained by questionnaires during early pregnancy. Educational level of the mother was assessed by the highest completed education and reclassified into three categories: primary school only, secondary school and higher education. Maternal prenatal was classified as 'non-smoking', 'smoking until pregnancy was confirmed' and 'continued smoking during pregnancy'. Newborn gender, birth weight and gestational age at birth derived from medical records completed by midwives and gynecologists. Birth weight was expressed as gestational age and gender adjusted standard deviation scores (27).

Statistical Analysis

First, we examined the association between a derangement in maternal 1-C metabolism, reflected by a low plasma folate or high homocysteine, and newborn DNA methylation of all genes combined. Therefore, we tested for a threshold effect of folate deficiency defined as a plasma concentration of less than 7 nmol/L (percentile 6.4) (28). In a similar way, we tested for associations between maternal plasma homocysteine concentration of >11 μmol/L (percentile 95.2) and child DNA methylation levels (29).

Next, we used plasma folate and homocysteine as a continuous variable in the equation.

To understand the effect of potential confounders on DNA methylation we used a crude and an adjusted model with gestational age at the moment of blood sampling, maternal smoking and gestational age at birth as covariates. Covariates were selected and included in the models if they changed the effect estimates of the main association (maternal folate concentration and newborn DNA methylation level) >10% (30). Maternal alcohol

consumption was not included in models because this did not materially change effect estimates.

In addition, we tested for an association between folic acid supplement use and newborn DNA methylation, with mothers who initiated the use of folic acid supplements preconceptionally as the reference category.

Finally, we used the maternal *MTHFR* rs1801133 genotype as proxy for base line folate status to study the association between folate status and child DNA methylation. Based on studies investigating folate and homocysteine concentrations in individuals heterozygous and homozygous for the 677T allele (31, 32), we used a recessive model using both the CC and CT genotype as reference with the TT alleles as risk genotype. To check whether the genetic effects were specific for the mother, we additionally tested for an association between child *MTHFR* genotype and DNA methylation.

As single nucleotide polymorphisms (SNPs) have previously been found to be correlated with local DNA methylation patterns, mostly within a distance of 149 kb of CpG dinucleotides (33, 34), we also tested the associations after excluding the *MTHFR* region in the analysis. Additionally, to test whether results were dependent on the sampling strategy with an oversampling of newborns with childhood ADHD or those born SGA, all analyses were repeated excluding these samples.

For an initial overall analysis of methylation and ADHD symptom score, DNA methylation levels of all CpG units in the 11 regions together were combined in one analysis. We present the complete data from all regions selected and chose a hierarchical approach to reduce the risk of type I error rather than to report selectively. Next to specific effects of DNA methylation, general effects on the level of DNA methylation have been described. Besides, the specific regions are correlated; a combined analysis increases the power and helps detect small effects of DNA methylation. Consequently, we have to be very careful to interpret specific effects on DNA methylation as the focus was on the overall effect.

DNA methylation levels were treated as continuous variables. To approximate normal distribution, variables of DNA methylation levels were transformed by the square-root. In the analyses, the triplicate measurements and DNA methylation levels of the separate CpG sites within a region were treated as clustered variables in a restructured data file and not as a mean.

As CpG sites within one region might be correlated, the associations with DNA methylation level were calculated using Linear Mixed Models. Mixed models have the advantage to allow correlated random effects in individuals. Another advantage of this model is the ability to accommodate missing data points. Furthermore, using mixed models enables adjustment for relevant covariates on the raw data in the same model (35).

To further illustrate the effect of maternal MTHFR C677T genotype we tested the association between the different maternal alleles and newborn cordblood concentrations of folate and homocysteine using ANOVA. In addition we tested the association between maternal periconceptional folic acid supplement use and maternal early pregnancy plasma folate and homocysteine concentrations.

Finally, to test whether our sampling strategy influenced our results we ran the analyses with and without the oversampled ADHD and SGA newborns. All analyses were performed using SPSS software, version 20.0 (IBM-SPSS, Chicago, IL, USA).

RESULTS

Characteristics of the newborns and their mothers in the study population are presented in Table 1. Mothers had a mean age of 30.1 (SD 4.8) years, 29.5% was higher educated and 32.9% smoked during pregnancy. Children were born after a mean of 40.1 (SD 1.5) weeks of gestation, 71.8% was born after spontaneous vaginal delivery and the mean birth weight was 3,404 (SD 582) grams.

The associations between maternal folate and homocysteine concentrations in early pregnancy and overall DNA methylation levels in the 11 regions of 7 fetal growth and neuronal developmental genes in the newborn are shown in respectively tables 2a and 2b. The continuous analysis of the maternal biomarkers showed no significant associations with the DNA methylation levels in newborns. However, early pregnancy exposure to folate deficiency, resulting in a deranged 1-C metabolism, revealed an overall lower DNA methylation level (ß = -0.39, 95%CI -0.74; -0.03, p = 0.03) that was attenuated after adjustment for potential confounders. The homocysteine concentration was not associated with newborn DNA methylation levels in the overall analyses of all regions measured.

Newborns of mothers who initiated folic acid supplement use in the first 10 weeks of gestation or did not use folic acid supplements did not show different DNA methylation levels compared to newborns of mothers who initiated folic acid supplement use preconceptionally, the reference category (Table 3).

As presented in Table 4, the maternal *MTHFR* risk genotype (TT) predicted a lower overall DNA methylation level in the newborn (ß = -0.31, 95%CI -0.59; -0.02, p = 0.03). As SNPs can influence DNA methylation in the adjacent region, we checked whether this association was dependent on methylation levels in the measured *MTHFR* region. The association between the maternal *MTHFR* C677T genotype and DNA methylation level in the newborn was also observed after excluding the *MTHFR* region from the analyses (ß = -0.37, 95%CI -0.68; -0.04, p = 0.02). There was no significant association between the newborn *MTHFR* C677T genotype and overall DNA methylation.

We were not able to investigate whether the effect of the folate concentration was modified by the maternal *MTHFR* C677T genotype due to the small number of newborns (n = 4) exposed to folate deficiency in mothers carrying the *MTHFR* TT genotype.

Maternal MTHFR C677T genotype was not associated with alterations in newborn cordbood folate or homocysteine levels (Supplemental Table 3). Plasma folate concentrations measured in early pregnancy were highest in women starting folic acid supplement use preconceptionally (mean 21.92 nmol/L, 95%CI 20.76; 23.01) compared to women starting folic acid supplement use in the first 10 weeks of gestation (18.33 nmol/L, 95%CI 16.81; 19.86,

p = <0.001) or women not using folic acid supplements (mean 8.59 nmol/L, 95%CI 6.24; 10.93, *p*<0.001). Early pregnancy homocysteine concentrations were lower in women using folic acid supplements preconceptionally (mean 7.11 umol/L, 95%CI 6.80; 7.43) as compared to women starting folic acid supplements in the first 10 weeks of gestation (mean 7.46 umol/L, 95%CI 7.05; 7.88, *p* = 0.19 or women not using folic acid supplements (mean 9.37 umol/L, 90% range 8.87; 10.02, *p*<0.001) (Supplemental Table 4).

When analyses were repeated with exclusion of newborns SGA or with ADHD, the results did not change materially (results not shown).

Table 1. Characteristics of the mothers and their newborns n = 420.

Newborn	
Gender, % boys	60.2
Birth weight, grams	3,404 (582)
Mother	
Age, years	30.1 (4.8)
Parity, % nulliparous	66.3
Gestational age at study entry weeks, median (90% range)	13.0 (10.4-17.1)
Gestational age at delivery, weeks	40.1 (1.5)
Body Mass Index, kg/m^2	24.1 (4.0)
Educational level, %	
Primary	19.8
Secondary	50.7
High	29.5
Smoking during pregnancy, %	
Never	67.1
Until pregnancy confirmation	9.8
Continued	23.1
Periconceptional folic acid supplement use, %	
Start preconceptionally	55.9
Start within first 10 weeks of gestation	31.4
No use	12.7
Plasma folate concentrations in early pregnancy, nmol/L	19.1 (9.0)
Plasma homocysteine concentrations in early pregnancy, µmol/L, median (90% range)	7.0 (4.9-10.9)
Mode of delivery, %	
Spontaneous vaginal	71.8
Instrumental vaginal	21.7
Caesarean section	6.5

Values represent means (SD) unless otherwise indicated.

Table 2a. Associations between maternal early pregnancy plasma folate and newborn DNA methylation levels n = 426.

Overall methylation						
	All genes assessed, ethylation			All genes assessed, methylation		
Unadjusted	ß (95%CI)	**P-value**	**Adjusted**	ß (95%CI)	**P-value**	
Continuous, µmol/L	-3.98 (-13.75; 5.80)	0.42	**Continuous,** µmol/L	-9.08 (-20.0; 1.85)	0.10	
Deficient, <7 nmol/L (n = 27)	-0.39 (-0.74; -0.03)	**0.03**	Deficient, <7 nmol/L (n = 27)	-0.01 (-0.40; 0.38)	0.95	
Neurotransmitter systems						
	DRD4, methylation		5-HTT_a, methylation		5-HTT_b, methylation		5-HTT_c, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Continuous, µmol/L	-8.81 (-60.27; 42.67)	0.74	7.56 (-6.87; 21.99)	0.30	-6.66 (-23.08; 9.76)	0.43	-13.04 (-30.00; 3.90)	0.13
Deficient, <7 nmol/L (n = 27)	-1.71 (-3.57; 0.15)	0.07	-0.11 (-0.64; 0.41)	0.67	-0.23 (-0.85; 0.38)	0.46	0.03 (-0.05; 0.10)	0.59
Adjusted								
Continuous, µmol/L	-51.59 (-105.37; 2.20)	0.06	7.99 (-7.76; 23.75)	0.32	-14.73 (-31.37; 2.42)	0.09	-17.82 (-35.85; 0.20)	0.05
Deficient, <7 nmol/L (n = 27)	-0.07 (-1.99; 1.86)	0.94	-0.17 (-0.73; 0.39)	0.54	0.12 (-0.51; 0.74)	0.72	-0.05 (-0.68; 0.59)	0.89
Metabolism								
	NR3C1_a, methylation		NR3C1_b, methylation		NR3C1_c, methylation		MTHFR5, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Continuous, µmol/L	9-1.72; 16.37)	0.11	4.74 (-6.84; 16.32)	0.42	2.73 (-7.95; 13.41)	0.62	-1.35 (-9.84; 7.14)	0.76
Deficient, <7 nmol/L (n = 27)	-0.51 (-0.84; -0.19)	**0.002**	-0.36 (-0.77; 0.06)	0.09	-0.05 (-0.43; 0.34)	0.81	0.02 (-0.29; 0.32)	0.91
Adjusted	7.33							
Continuous, µmol/L	8.69 (-1.22; 18.60)	0.09	3.53 (-9.10; 16.16)	0.58	3.69 (-7.95; 15.34)	0.53	-4.07 (-13.23; 5.09)	0.38
Deficient, <7 nmol/L (n = 27)	-0.60 (-0.94; -0.25)	**0.001**	-0.21 (-0.65; 0.24)	0.37	-0.03 (-0.44; 0.38)	0.89	0.09 (-0.24; 0.42)	0.59

Table 2a. *(Continued)*

	Foetal growth					
	H19, methylation		IGF2DMR, methylation		KCNQ1OT1, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Continuous, µmol/L	-18.32 (-44.38; 7.74)	0.17	7.72 (-28.15; 43.59)	0.67	-23.74 (-53.56; 6.07)	0.12
Deficient, <7 nmol/L (n = 27)	-23.08 (-1.18; 7.17)	0.63	-1.08 (-2.39; 0.24)	0.11	-0.24 (-1.33; 0.85)	0.66
Adjusted						
Continuous,µmol/L	15.70 (-44.25; 12.84)	0.28	3.32 (-35.32; 41.95)	0.87	-21.90 (-54.45; 10.65)	0.19
Deficient, <7 nmol/L (n = 27)	-0.27 (-1.29; 7.47)	0.60	-0.74 (-2.13; 0.65)	0.30	-0.41 (-1.58; 0.75)	0.48

The beta represent the change in methylation % per 1 µmol/L increase of folate concentration (continuous) or the change in methylation % in folate deficiency (<7 µmol/L) compared to adequate concentrations ≥ 7 µmol/L as analysed with linear mixed models. Values represent Beta, 95% confidence intervals and P-values. Adjusted analyses are adjusted for gestational age at the moment of blood sampling, maternal educational level, smoking, child gender, birth weight and gestational age at birth

Table 2b. Associations between maternal early pregnancy plasma homocysteine and newborn DNA methylation levels n = 426.

				Overall methylation				
	All genes assessed, methylation				All genes assessed, methylation			
Unadjusted	ß (95%CI)	**P-value**		**Adjusted**	ß (95%CI)	**P-value**		
Continuous, µmol/L	-0.01 (-0.05; 0.02)	0.48		Continuous, µmol/L	0.00 (-0.03; 0.04)	0.87		
Elevated homocysteine, >11 µmol/L (n = 30)	-0.05 (-0.39; 0.29)	0.76		Elevated homocysteine, >11 µmol/L (n = 30)	0.06 (-0.27; 0.39)	0.73		
				Neurotransmitter systems				
	DRD4, methylation		5-HTT_a, methylation		5-HTT_b, methylation		5-HTT_c, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Continuous, µmol/L	-0.06 (-0.26; 0.14)	0.53	0.03 (-0.03; 0.09)	0.29	0.00 (-0.06; 0.07)	0.89	-0.05 (-0.11; 0.02)	0.16
Elevated homocysteine, >11 µmol/L (n = 30)	-0.45 (-2.22; 1.33)	0.62	-0.03 (-0.52; 0.47)	0.91	-0.10 (-0.67; 0.47)	0.73	-0.37 (-0.96; 0.23)	0.23
Adjusted								
Continuous, µmol/L	0.03 (-0.17; 0.23)	0.74	0.02 (-0.04; 0.08)	0.46	0.03 (-0.04; 0.09)	0.40	-0.04 (-0.11; 0.03)	0.24
Elevated homocysteine, >11 µmol/L (n =30)	0.26 (-1.51; 2.03)	0.77	-0.04 (-0.55; 0.47)	0.87	0.09 (-0.47; 0.64)	0.76	-0.28 (-0.88; 0.31)	0.35

Table 2b. *(Continued)*

	Metabolism							
	NR3C1_a, methylation		NR3C1_b, methylation		NR3C1_c, methylation		MTHFR5, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Continuous, µmol/L	-0.04 (-0.08; -0.01)	**0.02**	-0.04 (-0.08; 0.01)	0.11	-0.02 (-0.06; 0.02)	0.40	-0.03 (-0.07; 0.00)	**0.04**
Elevated homocysteine, >11 µmol/L (n = 30)	-0.27 (-0.58; 0.04)	0.09	-0.38 (-0.78; 0.02)	0.06	-0.29 (-0.66; 0.09)	0.13	-0.16 (-0.46; 0.13)	0.27
Adjusted								
Continuous, µmol/L	-0.04 (-0.08; -0.04)	**0.03**	-0.03 (-0.08; 0.02)	0.20	-0.02 (-0.06; 0.03)	0.45	-0.03 (-0.07; 0.00)	0.07
Elevated homocysteine, >11 µmol/L (n = 30)	-0.26 (-0.58; 0.06)	0.11	-0.39 (-0.80; 0.02)	0.07	-0.33 (-0.71; 0.06)	0.10	-0.11 (-0.41; 0.19)	0.48

	Metabolism					
	H19, methylation		IGF2DMR, methylation		KCNQ1OT1, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Continuous, µmol/L	0.09 (-0.01; 0.19)	0.07	-0.06 (-0.21; 0.08)	0.41	0.06 (-0.06; 0.18)	0.33
Elevated homocysteine, >11 µmol/L (n = 30)	1.00 (0.10; 1.90)	**0.03**	-0.29 (-1.55; 0.96)	0.65	0.10 (-0.95; 1.15)	0.85
Adjusted						
Continuous, µmol/L	0.09 (-0.01; 0.20)	0.09	-0.08 (-0.23; 0.07)	0.30	0.05 (-0.07; 0.18)	0.40
Elevated homocysteine, >11 µmol/L (n = 30)	0.92 (-0.01; 1.86)	**0.05**	-0.32 (-1.60; 0.96)	0.62	0.05 (-1.03; 1.14)	0.92

The beta represent the change in methylation % per 1 µmol/L increase of homocysteine concentration (continuous) or the change in methylation % in elevated homocysteine concentration (>11 µmol/L) compared to adequate concentrations ≤ 11 µmol/L as analysed with linear mixed models. Values represent Beta, 95% confidence intervals and P-values. Adjusted analyses are adjusted for gestational age at the moment of blood sampling, maternal educational level, smoking, child gender, birth weight and gestational age at birth.

Table 3. Associations between maternal early periconceptional folic acid supplement use and newborn DNA methylation levels n = 440.

Overall methylation						
	All genes assessed, methylation			All genes assessed, methylation		
Unadjusted	ß (95%CI)	**P-value**	**Adjusted**	ß (95%CI)	**P-value**	
Start preconceptionally	*reference*		Start preconceptionally	*reference*		
Start within first 10 weeks of gestation	0.08 (-0.11; 0.27)	0.42	Start within first 10 weeks of gestation	0.07 (-0.16; 0.31)	0.56	
No use	-0.14 (-0.39; 0.11)	0.27	No use	0.20 (-0.15; 0.54)	0.26	
Neurotransmitter systems						
	DRD4, methylation		5-HTT_a, methylation		5-HTT_b, methylation		5-HTT_c, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Start preconceptionally	*reference*		*reference*		*reference*		*reference*	
Start within first 10 weeks of gestation	0.35 (-0.64; 1.35)	0.49	-0.14 (-0.20; 0.37)	0.55	0.10 (-0.22; 0.42)	0.54	0.07 (-0.27; 0.40)	0.69
No use	-5.56 (-1.87; 0.74)	0.40	-0.14 (-0.51; 0.23)	0.46	0.28 (-0.16; 0.71)	0.21	0.15 (-0.29; 0.59)	0.51
Adjusted								
Start preconceptionally	*reference*		*reference*		*reference*		*reference*	
Start within first 10 weeks of gestation	0.94 (-0.06; 1.94)	0.07	0.08 (-0.21; 0.38)	0.57	0.16 (-0.16; 0.48)	0.32	0.09 (-0.24; 0.43)	0.58
No use	0.67 (-0.72; 2.06)	0.34	-0.20 (-0.61; 0.21)	0.33	0.47 (0.02; 0.92)	**0.04**	0.17 (-0.30; 0.64)	0.48

Table 3. *(Continued)*

	Metabolism							
	NR3C1_a, methylation		NR3C1_b, methylation		NR3C1_c, methylation		MTHFR5, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Start preconceptionally	*reference*		*reference*		*reference*		*reference*	
Start within first 10 weeks of gestation	0.05 (-0.23; 0.12)	0.56	0.20 (-0.04; 0.43)	0.11	0.08 (-0.13; 0.29)	0.46	-0.01 (-0.18; 0.16)	0.91
No use	-0.16 (-0.39; 0.07)	0.17	-0.07 (-0.37; 0.24)	0.67	-0.10 (-0.38; 0.18)	0.47	0.13 (-0.09; 0.35)	0.25
Adjusted								
Start preconceptionally	*reference*		*reference*		*reference*		*reference*	
Start within first 10 weeks of gestation	0.06 (-0.24; 0.12)	0.52	0.18 (-0.06; 0.43)	0.15	0.09 (-0.13; 0.31)	0.43	0.02 (-0.16; 0.19)	0.87
No use	-0.29 (-0.54; -0.03)	**0.03**	0.01 (-0.33; 0.35)	0.96	-0.11 (-0.42; 0.20)	0.48	0.13 (-0.11; 0.38)	0.29

	Foetal growth					
	H19, methylation		IGF2DMR, methylation		KCNQ1OT1, methylation	
Unadjusted	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Start preconceptionally	*reference*		*reference*		*reference*	
Start within first 10 weeks of gestation	0.42 (-0.09; 0.93)	0.10	-0.03 (-0.73; 0.67)	0.94	0.48 (-11.48; 1.08)	0.11
No use	0.29 (-0.37; 0.95)	0.39	-0.87 (-1.79; 0.04)	0.06	0.12 (-0.67; 0.91)	0.76
Adjusted						
Start preconceptionally	*reference*		*reference*		*reference*	
Start within first 10 weeks of gestation	0.44 (-0.09; 0.97)	0.10	0.23 (-0.49; 0.94)	0.53	0.56 (-0.06; 1.19)	0.08
No use	0.41 (-0.32; 1.14)	0.27	-0.41 (-1.40; 0.59)	0.42	0.03 (-0.84; 0.90)	0.95

The beta represent the change in methylation % per 1 µmol/L increase of folate concentration (continuous) or the change in methylation % in folate deficiency compared to adequate folate concentrations (≥ 7 µmol/L) as analysed with linear mixed models. Values represent Beta, 95% confidence intervals and P-values. Adjusted analyses are adjusted for gestational age at the moment of blood sampling, maternal educational level, smoking, child gender, birth weight and gestational age at birth

Table 4. Associations between the *MTHFR* C677T (rs1801133) genotype in mothers and newborns and newborn DNA methylation, mothers n = 463, newborns n = 523.

			Overall methylation			
	All genes assessed, methylation			All genes assessed, methylation		
Maternal genotype	ß (95%CI)	**P-value**		**Child genotype**	ß (95%CI)	**P-value**
CC (n = 210)/CT (n = 203)	*reference*			CC (n = 210)/CT	*reference*	
TT (n = 50)	-0.31 (-0.59; -0.02)	**0.03**		(n = 203) TT (n = 50)	0.01 (-0.20; 0.35)	0.57

	DRD4, methylation		5-HTT_a, methylation		5-HTT_b, methylation		5-HTT_c, methylation	
Maternal genotype	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
CC (n = 210)/CT (n = 203)	*reference*		*reference*		*reference*		*reference*	
TT (n=50)	-1.56 (-3.00; -0.08)	**0.04**	-0.22 (-0.65; 0.21)	0.32	0.22 (-0.21; 0.66)	0.31	0.01 (-0.48; 0.50)	0.97
Child genotype								
CC (n = 210)/CT (n = 203)	*reference*		*reference*		*reference*		*reference*	
TT (n = 50)	-0.03 (-1.43; 1.37)	0.97	0.03 (-0.44; 0.38)	0.89	0.10 (0.37; 0.57)	0.67	-0.15 (0.63; 0.33)	0.54
			Metabolism					
	NR3C1_a, methylation		NR3C1_b, methylation		NR3C1_c, methylation		MTHFR5, methylation	
Maternal genotype	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
CC (n = 210)/CT (n = 203)	*reference*		*reference*		*reference*		*reference*	
TT (n = 50)	0.10 (-0.16; 0.38)	0.45	0.08 (-0.28; 0.44)	0.67	-0.16 (-0.48; 0.16)	0.34	0.12 (-0.15; 0.38)	0.38
Child genotype								
CC (n = 210)/CT (n = 203)	*reference*		*reference*		*reference*		*reference*	
TT (n = 50)	0.25 (-0.02; 0.51)	0.07	-0.01 (-0.36; 0.34)	0.97	0.06 (-0.24; 0.37)	0.69	0.15 (-0.45; 0.75)	0.62

Table 4. *(Continued)*

	Foetal growth					
	H19, methylation		IGF2DMR, methylation		KCNQ1OT1, methylation	
Maternal genotype	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
CC (n = 210)/CT (n = 203)	*reference*		*reference*		*reference*	
TT (n = 50)	-0.01 (-0.80; 0.78)	0.98	-0.55 (-1.62; 0.52)	0.31	-0.89 (-1.80; 0.02)	0.05
Child genotype						
CC (n = 210)/CT (n = 203)	*reference*		*reference*		*reference*	
TT (n = 50)	-0.47 (-1.20; 0.26)	0.21	0.50(-0.53; 1.52)	0.34	0.04 (-0.81; 0.89)	0.92

The beta represent the change in methylation % in individuals with the *MTHFR677TT* genotype compared to *MTHFR677CT* or CC genotype as analysed with linear mixed models. Values represent Beta, 95% confidence intervals and P-values. Analyses are adjusted for newborn birth weight.

DISCUSSION

Main Findings

There is a substantial interest in the impact of derangements in periconception maternal 1-C metabolism on newborn DNA methylation profiles with implications for birth outcome and health (5). In this study we observed a significant association between the maternal *MTHFR* 677TT genotype and newborn overall DNA methylation levels of fetal growth and neuronal developmental genes. The association between maternal folate deficiency measured in early pregnancy and newborn DNA methylation was attenuated after correction for maternal educational level, smoking, child gender, birth weight and gestational age at birth.

Strengths and Limitations

Some strengths and limitations have to be addressed. This study was embedded in a large cohort from which a selection of Dutch newborns was studied. Detailed information on maternal folate and homocysteine concentrations, folic acid supplement use and *MTHFR* C677T genotype in mothers and newborns was prospectively collected. Furthermore, to correct for possible confounding factors, including information on maternal educational level, smoking and child birth weight have been carefully documented.

A first limitation is the unsolved question how alterations in DNA methylation profiles in umbilical cord blood white blood cells reflect DNA methylation profiles in other tissues. This is a problem in most human studies. Gosh et al. (36) analyzed DNA methylation variation across brain and blood and found that, although between-tissue variation exceed between-individual variation, inter-individual variation was reflected across brain and blood. This implies that peripheral tissues may be of use for epidemiological studies. Talens et al. (24) reported for four of eight investigated regions, including *IGF2 DMR* and *H19* used in the current study, strong correlations between DNA methylation profiles in blood and buccal cells, tissues from different germ layers. Recently, we described tissue specificity of *IGF2 DMR* and *H19* gene regions among four umbilical cord blood tissues and concluded that the mononuclear cell fraction of umbilical cord blood is a rather homogeneous cell population for methylation studies (37). However, no studies have compared yet the agreement between umbilical cord blood white blood cells and peripheral tissues.

Second, folate and homocysteine concentrations were determined in blood samples obtained in early pregnancy at the same time when information on maternal folic acid supplement use was collected. A limitation of the study is the absence of information on the duration of maternal folic acid use and repeated measurements of these concentrations during gestation. Although it is not known how DNA methylation of the fetus and placenta develop throughout pregnancy the epigenetic system is thought to be primarily sensitive to environmental factors during the period of developmental plasticity (38).

Third, in the current study we describe several associations between determinants of maternal 1-C metabolism and alterations in newborn DNA methylation profiles. However, it is difficult to describe the potential influence of this effects on health outcomes. It is difficult

to translate our findings on newborn DNA methylation to health outcomes as literature describing the effects of alterations in DNA methylation is limited. Unfortunately, in the current study we were unable to relate our findings to gene expression data. Therefore, it would be valuable to replicate our findings and to validate *DNA methylation* markers.

Fourth, for an initial overall analysis of determinants of the maternal 1C metabolism and newborn DNA methylation, methylation levels of all CpG units in the 11 regions together were combined in one analysis. We present the complete data from all regions selected and chose a hierarchical approach to reduce the risk of type I error rather than to report selectively. Next to specific effects of DNA methylation, general effects on the level of DNA methylation have been described. Besides, the specific regions are correlated; a combined analysis increases the power and helps detect small effects of DNA methylation. Consequently, we have to be very careful to interpret specific effects on DNA methylation as the focus was on the overall effect.

Finally, our study relied on genomic DNA extracted from white blood cells, which consists of many distinct populations in varying proportions. Therefore, cell heterogeneity may act as a potential confounder when investigating DNA methylation differences, if cell distribution itself differs by one of investigated determinants of the 1-C metabolism. Glossop et al demonstrated that DNA methylation varied dramatically between matched-pairs T- and B-lymphocytes in a population of healthy volunteers (39). On the other hand, cellular heterogeneity is considered to have limited impact when the outcome and the genes are not involved in inflammation (24). Reinius et al. (40) evaluated genome wide DNA methylation of approximately 450 000 CpG sites in in different peripheral blood mononuclear cells of six healthy adult male donors. In selected genes implicated in immune-related disorders, CpG methylation differed between mononuclear cells and granulocytes and B-cells showing the most distinct profile. Nevertheless, in future studies it could be of added value when performing DNA methylation analyses using whole blood, to adjust the DNA methylation levels for differential cell counts or preferably to evaluate cell specific patterns for the locus of interest.

Interpretation

The association between maternal folate deficiency and lower DNA methylation in newborns attenuated after adjustment for maternal educational level, child gender, birth weight and gestational age at birth. It seems likely that these determinants influence the newborn epigenome also via the maternal 1-C metabolism (5). This is supported by the observed gene-specific effects of gestational age on newborn umbilical cord white blood cell DNA and the reported relationship between maternal smoking and lower methylation levels in the child (41).

Our data support the hypothesis that environmental factors influence the epigenome, which can alter the programming of gene expression and thus potentially contributes to health and disease in later life (42). However, causality is difficult to establish in observational studies and therefore confounding or presence of an epiphenomenon cannot be excluded.

Information on the factors which might influence the fidelity of DNA methylation profiles is limited. The association between maternal folate status and DNA methylation in newborns could be subject to confounding. This is illustrated by the attenuation of the association after adjustment for confounders. On the other hand this is also a strength of our study, because in most studies the control for socio-economic factors and maternal smoking is lacking (43).

Folate is an important provider of methyl groups to 1-C metabolism and hence a major determinant of SAM for the establishment of the human epigenome. Human studies of DNA methylation and folate concentration vary widely in study design, timing and level of exposure, tissue tested, and assays. Our data are supported by the accumulating evidence on the association between a low folate status and global DNA hypomethylation (3). Studies of the Dutch hunger winter showed alterations in offspring DNA methylation patterns 60 years after intrauterine exposure to famine, which might be an indicator of a poor folate status (44, 45). In addition, intervention studies in postmenopausal women have shown that moderate folate deficiency reduces genome-wide DNA methylation (46, 47).

Of interest is the finding that both folate deficiency and higher homocysteine concentrations were associated with a decreased newborn DNA methylation level of one of the glucocorticoid receptor gene loci. This locus covers the noncoding exon 1F promoter that contains the transcription factor NGFI-A binding site and is specific to the hippocampus (48). It is suggested that glucocorticoid receptor expression can be fine-tuned in response to the early life environment. Previous studies investigating DNA methylation in peripheral blood have shown increased levels of NR3C1 methylation in response to neonatal influences (49-51).

The results of studies addressing folic acid supplement use are sometimes contradicting. We showed that periconception maternal folic acid use significantly increased *IGF2 DMR* methylation in the very young child (6). In mice, low folate supply in utero resulted in gene and locus specific epigenetic effects in the offspring gut. Lower overall methylation was only in the *Slc39a4* CGI1 locus and not in *Esr1*, *Igf2* DMR1 or *Slc39a4* CGI2 loci (52). Haggarty et al. however suggested that preconception folic acid supplement use has no effect on human child methylation (53). Here, we did not observe any association between maternal folic acid supplement use and DNA methylation of the combined regions of 7 genes in the newborn.

An approach that uses genetic variants as surrogate for measuring exposure has the advantage to provide a relatively unbiased assessment whether modifiable risk factors are causally related to the outcome. Low folate status increases the homocysteine concentration in particular in individuals with the *MTHFR* 677TT genotype (54). Consistent with the epigenetic hypothesis, we observed an association between the maternal *MTHFR* 677TT genotype and lower DNA methylation level in newborns. This may suggest that the maternal genotype is more reliable to reflect the basic folate status than a single measurement of the plasma folate concentration reflecting the folate status of the previous 1-3 days. Unfortunately, we were unable to test interactions or mediation effects due to the small number of mothers and newborns with carrying the *MTHFR* 677CT/TT genotype and folate

deficiency. In the current study we observed that maternal MTHFR 677TT genotype is related to newborn DNA methylation, but newborn homozygosity for the C677T mutation was not significantly associated with newborn DNA methylation. From studies on associations between folate and neural tube defects we know that the odds ratio was almost 1.5 times higher if not only the mother, but also the fetus carried the TT genotype (55) suggesting that the fetal genotype has an additional effect on the maternal-fetal 1-C metabolism. Other studies have previously reported the fetal ability to concentrate vitamins. Active placental transport resulted in higher levels of folate and vitamin B12 in the fetus than in the mother (56). Probably, because of this mechanism the fetus is able to maintain a sufficient folate status regardless of its genotype. Furthermore, the maternal 1-C metabolism is essential for DNA methylation reprogramming during the early embryonic period (3). Probably, this process of demethylation and remethylation takes place shortly after fertilization, a long time before the fetal genotype could exert its effect.

In the current study we did not observe an association between maternal folic acid supplement use and DNA methylation in the newborn. Possibly the timing of the start of folic acid supplement use could have influenced our findings. DNA methylation reprogramming in the early embryo could benefit most from the use of supplements if they are taken before conception (5). Another possibility is that the effects of folic acid supplement use also depends on the MTHFR genotype. Shelnutt et al. (57) performed a trial in 41 young women with either the MTHFR 677CC or TT genotype. A 7-week folate depletion diet led to lower global DNA methylation in women with either genotype. However, the following 7-week folate repletion diet only led to an increase in global DNA methylation in women with the mutant TT genotype. It is possible that natural folate as part of food exerts different effects on biological processes than synthetic folic acid. It is not elucidated whether the increased uptake of folic acid rather than 5-methyltetrahydrofolate in the 1-C pathway at a critical time can impact on DNA methylation (58).

Experimental evidence is suggesting that epigenetic marks serve as a memory of exposure in early life and can increase the vulnerability to develop disease in later life. In this study, changes in DNA methylation level in response to the maternal the *MTHFR* genotype, although statistically significant, were relatively small. The magnitude of differences in DNA methylation is difficult to interpret and most importantly the thresholds of methylation to influence the expression of the gene are largely unknown. The majority of studies of others only examined a limited number of genetic regions and show contradicting results (3). Results from the current study reflect pooled data from DNA methylation in different genomic regions. So far the biological basis and implications of these altered profiles remains unknown. Fetal growth and neuronal development are characterized by widespread cell division. Inadequate folate status led to several types of birth defects, including certain heart defect, limb malformations and in particular neural tube defects. Pangilinan et al. (59) reported common genetic variants in 82 candidate genes selected from 1C-pathway, including MTHFR, as risk factors for neural tube defects. Other variants are involved in for example choline metabolism, reabsorption of vitamin B12 and mitochondrial folate

transport. To date, evidence from literature suggested that low folate status is associated with decreases in global DNA methylation, which has been associated with adverse health outcomes, in particular cancer (3). However, as yet it is unknown how specific genomic regions respond to lower or higher folate intakes.

CONCLUSION

This study suggests that the maternal MTHFR C677T genotype is a determinant of the DNA methylation status associated with some genes of the epigenome of the newborn. Confounding factors including maternal socio-demographic factors and pregnancy characteristics however, attenuated the association between maternal folate deficiency and lower DNA methylation in newborns. Therefore, further research to confirm our results and to investigate the influence of the expression of these genes and the implications for health is recommended.

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Supplemental Table 1. Summary of primer sequences for PCR amplification*.

Gene	Candidate function	Genomic Region	Number of CpG units assessed	Primer sequemce** (5' to 3')	Source	Median % methylation (90% range)
Dopamine D4 Receptor (DRD4)	Neurotransmitter system	chr11:636508-636904	11 CpG units (19 CpG sites)	F:GGGATTTTTTGTTTAGGGTTAGAGG R:CACCCTAATCCACCTAATATCTAACA	Wong, 2010 (20)	47.8 (37.4-55.1)
Serotonin Transporter (5-HTT) region A	Neurotransmitter system	chr17:28562435-28562812	6 CpG units (13 CpG sites)	F:GGTTAGTTTTAGTTTTGGTTTTTGTT R:CAAAAATTCTTCAAAAACTCTTTAAC	Wong, 2010 (20)	2.7 (2.0-3.8)
Serotonin Transporter (5-HTT) region B	Neurotransmitter system	chr17: 28562751-28563084	12 CpG units (22 CpG sites)	F:GTTTTTATATGGTTTGATTTTTAGATAG R:CCTACTCCTTTATACAACCTCCCCC	Philibert, 2008(19)	7.1 (5.2-9.1)
Serotonin Transporter (5-HTT) region C	Neurotransmitter system	chr17: 28562358-28562783	13 CpG units (25 CpG sites)	F:GTTATTTAGAGATTAGATTATGTGAGGGT R:CCTACAACAATAAACAAAAAAACCCC	Philibert, 2008(19)	5.6 (4.5-7.3)
Potassium channel protein (KCNQ1OT1)	Development growth and metabolism	chr11: 2721823-2722182	4 CpG units (6 CpG sites)	F:GTTAGGGAAGTTTAGGGTGTGAAT R:TTCTAAAACCCCCACTACTATACCT	Designed in EpiDesigner	39.0 (29.5-48.8)
Insulin-like growth factor 2 (IGF2)	Growth and insulin signaling	chr11: 2169458-2169796	3 CpG units (4 CpG sites)	F:TGGATAGGAGATTGAGGAGAAA R:AAACCCCAACAAAAACCACT	Talens, 2010(22)	54.0 (42.7-62.7)
H19	Growth	chr11: 2019371-2019784	10 CpG units (13 CpG sites)	F:GGGTTTGGGAGAGTTTGTGAGGT R:ATACCTACTACTCCCTACCTACCAAC	Talens, 2010(22)	31.7 (-24.2-39.2)
Methylenetetrahydro-folate reductase (MTHFR)	Homocystein metabolism	chr1: 11866184-11866627	12 CpG units (14 CpG sites)	F:GTTTGTAGTTATTTTTGGTTTTAGTTTT R:TAACCTAAATTCTCCCTCAAATTCC	Designed in EpiDesigner	0.9 (0.4-1.5)
Glucocorticoid receptor (NR3C1) region A	Development, metabolism and immune response	chr5: 142783585-142783906	10 CpG units (19 CpG sites)	F:TTTTTGAAGTTTTTTTAGAGGG R:AATTTCTCCAATTTCTTTTCTC	McGowan (21)	2.2 (1.4-3.1)
Glucocorticoid receptor (NR3C1) region B	Development, metabolism and immune response	chr5: 142782046 142782472	11 CpG units (22 CpG sites)	F:AAAGGGGTTATTTAGAAATTTTAGG R:AAAATCCTAACCTCTTTTCTCCCC	Designed in EpiDesigner	2.6 (1.8-3.6)
Glucocorticoid receptor (NR3C1) region C	Development, metabolism and immune response	chr5:142784559 142784950	10 CpG units (20 CpG sites)	F:TTTTTAGTTTAAGGGGAAGGG AAT R: CTTCAAAATATCAAAACAAAAAAAACC	Designed in EpiDesigner	3.1 (1.8-6.1)

* *UCSC February 2009 GRCh37 Genomic Assembly*

**Primers were delivered with standard Sequenom MassCleave tags added to the 5'end of the primers (10-mer at forward primer (F): aggaagagag, T7 at reverse primer (R) :cagtaatacgactcactatagggagaaggct).

Supplemental Table 2. Distribution of C677T polymorphism of the MTHFR gene in mothers and their newborns.

Maternal genotype (n = 463)	
Normal (C/C)	210 (45.4%)
Hyterozygote (C/T)	203 (43.8%)
Mutant (T/T) Hardy Weinberg Equilibrium	50 (10.8% 0.93
Newborn genotype (n = 523)	
Normal (C/C)	255 (48.8%)
Hyterozygote (C/T)	209 (40.0%)
Mutant (T/T) Hardy Weinberg Equilibrium	59 (11.3%) 0.11

Part IV

Foetal Growth and Children's Cognition and Behaviour

Chapter 10

Low and High Birth Weight and the Risk of Child ADHD Symptoms

ABSTRACT

Objective: To study the prospective association between birth weight and Attention Problems and to explore the role of maternal body mass index (BMI) in this association.

Study design: In 6,015 children of a population-based cohort (Rotterdam, the Netherlands, 2001-2005), information on birth weight was collected and gestational age-adjusted standard deviation (SD)-scores were calculated. At age 6 years, parents assessed Attention Problems with the Child Behaviour Checklist. We used linear regression to study the association of birth weight with Attention Problem score and examined the modification of this association by maternal early pregnancy BMI.

Results: The observed association between birth weight and Attention Problem score was curvilinear (adjusted ß per birth weight SD-score$^{2:}$ 0.02, 95%CI 0.00; 0.03, p = 0.008); the turning point equals 3.6 kg at term. In analyses of the extreme tails of the birth weight distribution, the associations with Attention Problem score disappeared after adjustment for socio-economic confounders. Maternal early pregnancy BMI moderated the association of child birth weight with Attention Problem score (*P-interaction* = 0.007, with curvilinear term in model).

Conclusions: Higher birth weight was related to less Attention Problems but from a birth weight of about 3.6 kg or more, a higher birth weight did not reduce the risk of Attention Problems any further. However, in children of obese mothers (BMI >30kg/m^2), high birth weight may increase the risk of Attention Problems.

INTRODUCTION

For decades the high risk of children born small for gestational age to develop mental health problems has intrigued scientists. Low birth weight has been linked to depression, anxiety (1), and schizophrenia (2, 3), and in particular to childhood attention-deficit/hyperactivity disorder (ADHD) (4). Children with low birth weight are at a greater risk of symptoms of inattention and to a lesser extend at risk of hyperactivity/impulsivity (5, 6).

A full understanding of the association between birth weight and ADHD symptoms is hampered by several limitations in the literature. First, with a few exceptions, previous studies have focused on the lower end of the birth weight distribution. Children with a very low birth weight (<1.5 kg) or moderate low birth weight (<2.5 kg) were repeatedly reported to have an increased risk of ADHD symptoms *(4)*. Some studies suggested nonlinear associations between birth weight and cognitive and behavioural functioning (7, 8), but a relation between birth weight within the entire range and ADHD symptoms is not well established. Studies that modeled birth weight as a continuous exposure include investigations of Kelly et al., Linnet et al., Hultman et al. and Schlotz et al. (9-12). Whereas these studies observed an inverse relationship between birth weight and risk of ADHD symptoms or ADHD diagnosis, Lahti et al. (13) did not observe an association.

Second, since these studies were conducted, the population distribution of birth weight has changed. In the last decades, a further rise in median birth weight was observed (14). It has been suggested that the increase in birth weight is explained by, among other factors, higher maternal body mass index (BMI) and altered smoking patterns (15). However, studies have not investigated the association between child birth weight across continuum and ADHD symptoms in children born in the last two decades.

Against this background, we postulated that because of time trends in mother's weight the relationship between child birth weight and Attention Problems may have changed. In a large population-based cohort, we addressed the following aims: First, we investigated the linear association between birth weight and Attention Problem score at age 6 years and also determined if the association between birth weight and Attention Problem score at age 6 years is curvilinear. In addition to this aim we studied the associations between low and high birth weight with Attention Problem score. We also examined the role of maternal early pregnancy BMI in these associations. We tested whether maternal early pregnancy BMI precedes high child birth weight and accounts for its association with ADHD using a mediation analyses. In addition, we tested whether the association between child birth weight and Attention Problems is moderated by maternal early pregnancy BMI by using an interaction model, i.e. we examined whether the association between birth weight and Attention Problems depended on maternal BMI. We hypothesized that low and very high birth weight confer a higher risk of Attention Problems than average birth weight. No hypothesis for the effect of maternal early pregnancy BMI on this association was formulated.

METHODS

Design and Study Population

This analysis was embedded in the Generation R Study, an ongoing population-based birth cohort from foetal life onwards (16). All pregnant women were enrolled between 2001 and 2005 in Rotterdam, the Netherlands. Assessments during pregnancy and childhood comprised physical examinations, ultrasonography, biological sampling, and parental questionnaires. The study was approved by the Medical Ethical Committee of the Erasmus Medical Center in Rotterdam. Written consent was obtained from all participating women.

In total, 8,301 mother-child pairs participated in the postnatal phase of the Generation R study. As depicted in the flow chart of the study population (Supplemental Figure 1; online only) of 8,009 mothers who gave birth to singleton live-born children we had information on child weight and gestational age at birth available. Twin pregnancies (n = 200) were excluded since growth potentials for individual foetuses in multiple pregnancies are not comparable to singleton pregnancies. Parents of 6,015 children (75%) provided behavioural data of the child at age 6 years by completing Child Behaviour Checklist (CBCL/1.5-5). In 5,448 mother-child pairs, both information on birth weight, maternal early pregnancy BMI as well as Attention Problem score was available.

Assessment of Birth Weight and Gestational Age

To estimate gestational age, crown-rump length (until a gestational age of 12 weeks and 5 days), or biparietal diameter (from 12 weeks and 5 days onwards), measured by foetal ultrasound examination, as previously described (17), were used. Inter- and intra-observer intra-class correlation coefficients were all >0.98 (17). Information on birth weight of the child was obtained from community midwifery and hospital registries. Birth weight was established directly postpartum and expressed in kilograms (kg).

To disentangle the effects of birth weight from gestational age we express birth weight in units adjusted for gestational age and gender, i.e. birth weight standard deviation (SD)-score. The birth weight SD-scores were constructed based on distributions in the Generation R cohort (18).

Assessment of Attention Problem score

We measured Attention Problem score of the child at 6 (range 4.9-8.0) years of age by using the Attention Problems subscale of the CBCL/1,5-5. The CBCL is a parent report questionnaire that contains 99 problem items rated on a 3-point scale: 0 (not true), 1 (somewhat or sometimes true) and 2 (very true or often true). By summing the raw scores, seven syndrome scales, including the continuous Attention Problems scale, consisting of 5 items, can be computed (Cronbach's alpha 0.70). Higher scores represent higher severity of problems. For the CBCL good reliability and validity have been reported (19).

Maternal Anthropometrics

In early pregnancy (median gestational age 14.4 weeks, interquartile range 12.5-17.8), maternal height and weight were measured without shoes and heavy clothing. BMI (kg/m^2) was calculated using weight (kg) and height (cm) in 5,448 women. Throughout the manuscript we refer to this variable as 'early pregnancy BMI'. The correlation between early pregnancy BMI and prepregnancy BMI (n = 4,619) obtained by questionnaire in early pregnancy was very good (Pearson correlation 0.95 (p<0.001)).

Covariates

Possible confounders of the association between birth weight and Attention Problem score were derived from the literature (20, 21). Child gender, Apgar score, mode of delivery, presence of gestational diabetes, and pre-eclampsia were derived from medical records completed by midwives and gynecologists. At enrollment (median gestational age 14.7 weeks (SD 3.6) we obtained information on maternal age, national origin, educational level, parity, prenatal smoking, alcohol use, and folic acid supplementation by questionnaire. National origin of the mother was based on the country of birth of the parents. Educational level of the pregnant woman was assessed by the highest completed education and categorized as primary school only, secondary school, or higher education. Maternal prenatal smoking and alcohol use were classified as 'no use', 'use until pregnancy was confirmed' and 'continued use during pregnancy'. Folic acid supplementation was classified as 'no use,' 'use started during the first 10 weeks of pregnancy' or 'use started preconceptional'. At 20 weeks pregnancy, we measured maternal psychological symptoms using the Brief Symptom Inventory (22). In this study, the total sum scale of maternal psychological symptoms was tested as a confounder, as maternal psychopathology may affect both foetal growth and may independently be related to child behavioural problems. Moreover, as this study is based on parent report information on Attention Problems, maternal psychological symptoms may influence the report. All analyses were also adjusted for age at Attention Problem assessment.

Statistical Analysis

Attention Problems were studied as a continuous outcome using linear regression models. To approximate a normal distribution, the CBCL attention problem-scale is square root transformed. In the first step of our analyses we studied whether birth weight of the child was linearly related to Attention Problem score at age 6 years in our population. Second, we explored a curvilinear association with Attention Problem score by adding a squared term of birth weight to the model. Third, we investigated low birth weight (as defined by <10th and <20th percentile SD-score) and high birth weight (as defined by >90th and >80th percentile SD-score) in relation to Attention Problems. We report the results of the 10% and 20% extremes on both ends of the birth weight distribution to test whether results depended on any choice of cut-off .We defined low and high birth weight based on population specific percentiles rather than using pre-defined cut-off points derived from other settings with different birth weight distributions.

Next, we examined whether the association between maternal early pregnancy BMI and child Attention Problem score was mediated by child birth weight. Towards this aim, we tested whether maternal early pregnancy BMI was associated with child birth weight as previously reported among 6,959 mothers and their children, in the current study population. Subsequently, we studied the association between maternal early pregnancy BMI and Attention Problem score.

The Preacher and Hayes' Bootstrapping Procedure (23) was followed to formally assess whether an indirect or mediation effect of child birth weight was present. This procedure involves taking 5000 random samples from the obtained data, sampling with replacement, and calculating the indirect effect for each sample by multiplying the coefficient for the a-path (determinant to potential mediator association) by the coefficient of the b-path (potential mediator to outcome association). Confidence intervals were obtained using Preacher and Hayes' SPSS macro. In the current analysis, child Attention Problem score was entered as the dependent variable, maternal early pregnancy BMI was entered as the independent variable, and child birth weight (SD-score[2]) as the mediator. Emerging perspectives pose that assessing mediation does not require the presence of a direct or total association between determinant and outcome (24).

Finally, we tested whether maternal early pregnancy BMI moderated the association between child birth weight and Attention Problem score by adding an interaction term of birth weight and maternal early pregnancy BMI (both linear terms) to the model. We followed the approach by Ganzach (25) and kept the linear and curvilinear term of birth weight in the model. This approach ensures that the model correctly indicates whether an interactive relationship is present and describes the correctly modeled relation between the independent variables (e.g. offsetting or synergistic).

Children born to mothers with diabetes have an elevated risk for adverse development, owing to hyperglycemia and other associated intrauterine factors (26). Moreover, high maternal weight is associated with a substantially higher risk of gestational diabetes (27). Therefore we further explored the role of maternal gestational diabetes on child Attention Problem score.

In all these analyses, we carefully evaluated socio-economic factors and pregnancy characteristics. Model 1 is adjusted for child gender and age at Attention Problem assessment. In model 2 we adjust for child gender, age at assessment of Attention Problems and Apgar score one minute after birth, mode of delivery, maternal age, educational level, parity, psychological symptoms, smoking, alcohol use, and folic acid supplementation, gestational diabetes, and pre-eclampsia. Model 3 is comparable to model 2 but additionally controls for child birth weight (SD-score). Model 4 is comparable to model 2 but does not include gestational diabetes as an additional covariate. As the CBCL Attention Problem scale remained skewed after transformation we also present the results of analyses of the association between birth weight and Attention Problems using a dichotomous outcome. We chose a median split to minimize the loss of statistical power in this dichotomous analysis.

Analyses were performed using SPSS software, version 21.0 (IBM-SPSS, Chicago, IL, USA).

RESULTS

Characteristics of the children and their mothers in the study are presented in Table 1. The children were on average born after 39.9 (SD 1.7) weeks of pregnancy. Median birth weight was 3.5 (interquartile range 3.1-3.8) kg.

First we investigated the linear association between birth weight and Attention Problem score at age 6 years. As shown in Table 2 the association between birth weight studied continuously and Attention Problem score showed a negative relationship if only adjusted for child age and gender (unadjusted ß per birth weight SD-score -0.05, 95%CI -0.07; -0.03, p<0.001). This association was substantially attenuated after adjustment for possible confounders.

Table 1. Characteristics of mothers and their children (n = 6,015).

Child characteristics	
Sex, % boys	50.3
Birth weight, kg, median (interquartile range)	3.4 (0.6) 3.5 (3.1-3.8)
Gestational age at birth, weeks	39.9 (1.7)
Apgar score 1 minute after birth	8.6 (1.2)
Maternal and pregnancy characteristics	
Age, years	31.0 (4.9)
National origin, %	
Dutch	60.3
Western other	12.2
Non Western	27.5
Body mass index, kg/m^2	24.6 (4.3)
Smoking during pregnancy, %	
Never	76.1
Until pregnancy was known	8.7
Continued	15.2
Educational level, %	
Primary	18.8
Secondary	52.7
Higher	26.9
Nulliparous, %	57.3
Mode of delivery, %	
Spontaneous vaginal	72.1
Instrumental vaginal	15.0
Caesarean section	12.9
Gestational diabetes, %	1.1

Values represent means standard deviation (SD) unless otherwise indicated.

Next, we explored the possibility of a curvilinear association between birth weight and Attention Problem score. We found a curvilinear association of birth weight with Attention Problem score (ß per birth weight SD-score^2 0.02, 95%CI0.01; 0.03, p = 0.002). That did not materially change after adjustment for potential confounders (ß per birth weight SD-score^2 0.02, 95%CI 0.00; 0.03, p = 0.008) (Table 2; Figure 2). The turning point of this curvilinear association was calculated at a SD-score birth weight of 0.3 (calculated as 0.01 / (0.02*2)); this equals a birth weight of 3.6 kg at 40 weeks of gestation. These analyses were adjusted for child gender, age at assessment of Attention Problems and Apgar score one minute after birth, mode of delivery, maternal age, educational level, parity, psychological symptoms, smoking, alcohol use, and folic acid supplementation, gestational diabetes, and pre-eclampsia.

Low birth weight (SD-score <10th percentile; <20th percentile) was associated with higher Attention Problem score in child age and gender adjustment models (model 1, see Table 2), but was considerably attenuated in the fully adjusted model (model 2). Further, the association between high birth weight (SD-score >80th and 90th percentile) and the level of Attention Problem score was tested. We found that high birth weight children (SD-score >90th percentile) had no increased risk of Attention Problems and the association between birth weight above the 80th percentile disappeared after adjustment for confounders in model 2 (Table 2). Confounders with a relatively large effect on the association between birth weight and Attention Problem score were maternal educational level, maternal psychological symptoms, parity, maternal early pregnancy BMI, and child gender (Table 3).

Table 2. Associations between birth weight and ADHD-symptoms at age 6 years (n = 6,015).

	Attention Problems, Model 1: age and gender adjusted[a]		**Attention Problems, Model 2: fully adjusted analyses[a,b]**	
Birth weight, SD-score	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Continuous	-0.05 (-0.07; -0.03)	**<0.001**	-0.01 (-0.03; 0.01)	0.37
Curvilinear:				
Continuous term	-0.05 (-0.07; -0.03)	**<0.001**	-0.01 (-0.03; 0.01)	0.40
Squared term	0.02 (0.01; 0.03)	**0.002**	0.02 (0.00; 0.03)	**0.008**
Dichotomous: low birth weight				
<10th percentile	0.13 (0.06; 0.20)	**<0.001**	0.04 (-0.03; 0.11)	0.26
<20th percentile	0.11 (0.06; 0.16)	**<0.001**	0.04 (-0.01; 0.10)	0.11
Dichotomous: high birth weight				
>90th percentile	-0.02 (-0.09; 0.05)	0.62	0.05 (-0.02; 0.12)	0.17
>80th percentile	-0.08 (-0.13; -0.03)	**0.002**	-0.01 (-0.07; 0.04)	0.61

CBCL attention problems-scale is square-root transformed to approximate normal distribution.

[a]Analyses were adjusted for child age at ADHD assessment and gender (Model 1).

[b]Analyses were adjusted for Apgar score one minute after birth, mode of delivery, maternal age, national origin, educational level, parity, body mass index, psychological symptoms, smoking, alcohol use, folic acid supplementation use, gestational diabetes, and pre-eclampsia (Model 2).

We tested whether child birth weight mediated the association between maternal early pregnancy BMI and child Attention Problem score. As reported previously in the current cohort (28), higher maternal early pregnancy BMI predicted higher child birth weight in the current sample (n = 5,448) (adjusted ß per 10 kg/m^2 0.42, 95%CI 0.35; 0.48, p<0.001) and was positively related to the squared term of child birth weight (ß per 10 kg/m^2 0.18, 95%CI 0.07; 0.28, p = 0.001). The association between maternal early pregnancy BMI and Attention Problem score of the child at age 6 years attenuated after adjustment for confounders (early pregnancy BMI: model 1 adjusted for child gender and age at ADHD assessment ß per 10 kg/m^2 0.09, 95%CI 0.04; 0.14, p = 0.001, model 3 fully adjusted ß per 10 kg/m^2 0.04, 95%CI -0.01; 0.10, p = 0.12). Results from the mediation analyses are presented in a graphical representation (Figure 3). Results indicated a mediation effect of child birth weight (SD-score^2) for Attention Problem score (ß = 0.003, 95%CI 0.001; 0.010).

Moreover, maternal early pregnancy BMI and child birth weight interacted (P-interaction of maternal early pregnancy BMI and child birth weight = 0.007, model 3 adjusted with linear and quadratic terms of birth weight in the model) (Table 4). To illustrate this interaction of continuous variables, we categorized maternal early pregnancy BMI in three groups: normal weight (BMI 18.5-24.9 kg/m^2; n = 3,354); overweight women (BMI 25-29.9 kg/m^2; n = 1,415) and obese women (BMI ≥30 kg/m^2; n = 577). Underweight women (BMI <18.5 kg/m^2) were not depicted in the figure due to the small number of women at risk (n = 102). Figure 4 shows the association between child birth weight and Attention Problem score in strata of maternal early pregnancy BMI. In particular when we restricted the analyses to obese women, we found a strong association between high birth weight and higher Attention Problem score (model 2 adjusted ß per birth weight SD-score 0.07, 95%CI 0.00; 0.14, p = 0.04 and ß per birth weight SD-score2 0.01, -0.03; 0.05, p = 0.64 (results not shown in table)).

We did not observe an association between maternal gestational diabetes and Attention Problem score (model 4 adjusted ß = 0.09, 95%CI -0.10; 0.29, p = 0.36) in the total study population. We performed an additional sensitivity analyses post hoc restricting the sample to those born with a birth weight in the range in which we observed a positive association between birth weight and Attention Problems. Thus we restricted analyses to children born with a birth weight SD-score >0.03 (the turning point of the curvilinear association between child birth weight and Attention Problem score). In these children the presence of gestational diabetes predicted more child Attention Problem score at age 6 years (model 4 adjusted ß = 0.50, 95%CI 0.32; 0.68, p<0.001). However, results from this post hoc analyses should be interpreted with caution.

To test the stability of the association between birth weight and Attention Problems, we repeated the analyses with a dichotomous categorization of Attention Problems using a median split. The effects were consistent with the analyses using a continuous variable of Attention Problems (Table 5).

Table 3. Contribution of selected covariates on the association between child birth weight and Attention Problems at age 6 years (n = 6,015).

	Child Attention Problems at age 6 years	
Determinant	**Effect estimate (ß) of child birth weight, unadjusted**	**Adjusted R^2**
Child birth weight, continuous	-0.050	0.004
Covariates **Maternal characteristics**	**Effect estimate (ß) of child birth weight after covariate inclusion**	**Adjusted R^2**
Educational level Low Mid High	-0.037	0.032
Psychological symptoms, score	-0.034	0.042
National origin Dutch Surinamese Turkish Moroccan Cape Verdean Dutch Antilles Other Western Other non-Western	-0.027	0.046
Body mass index, kg/m^2	-0.030	0.046
Parity Primiparous Multiparous	-0.014	0.056
Smoking during pregnancy Never Until pregnancy was known Continued	-0.010	0.060
Alcohol use Never Until pregnancy was known Continued	-0.007	0.062
Folic acid supplement use Started preconceptional Started postconceptional No use	-0.008	0.061
Gestational diabetes Gestational diabetes No gestational diabetes	-0.008	0.061
Pre-eclampsia Pre-eclampsia No pre-eclampsia	-0.007	0.061

Table 3. *(Continued)*

Covariates **Maternal characteristics**	**Effect estimate (ß) of child birth weight after covariate inclusion**	**Adjusted R^2**
Mode of delivery Spontaneous vaginal Instrumental vaginal Caesarean section	-0.008	0.063
Child characteristics		
Gender	-0.008	0.085
Apgar score	-0.008	0.085
Age at assessment	-0.008	0.086

Covariates were introduced stepwise in the order given here

Table 4. Interaction analyses of maternal BMI and child birth weight and Attention Problems at age 6 years (n = 5,448).

	Attention Problems, Model 1: gestational age, child age and gender adjusted[a]		**Attention Problems, Model 2: fully adjusted analyses[a,b]**	
	ß (95%CI)	**P-value**	ß (95%CI)	**P-value**
Variables in the interaction-model				
Maternal BMI, kg/m^2	0.01 (0.01; 0.02)	**<0.001**	0.00 (-0.00; 0.01)	0.26
Birth weight, SD-score, continuous	-0.25 (-0.37; -0.13)	**<0.001**	-0.16 (-0.27; -0.04)	**0.007**
Birth weight, SD-score, squared	0.01 (0.00; 0.03)	0.06	0.01 (0.00; 0.03)	**0.03**
Interaction: Maternal BMI, kg/m^2 x birth weight, SD-score, continuous	0.01 (0.00; 0.01)	**0.001**	0.01 (0.00; 0.01)	**0.008**

CBCL Attention Problem-scale is square-root transformed to approximate normal distribution. Estimates of effect size were obtained from a multiple regression model with a interaction term of maternal body mass index (BMI) during pregnancy and child birth weight. This model included linear and quadratic terms of birth weight.

[a]Analyses were adjusted for gestational age at measurement of BMI, child age at Attention Problem assessment and gender (Model 1).

[b]Analyses were adjusted for Apgar score one minute after birth, mode of delivery, maternal age, national origin, educational level, parity, BMI, psychological symptoms, smoking, alcohol use, folic acid supplementation use, gestational diabetes, gestational hypertension and pre-eclampsia (Model 2).

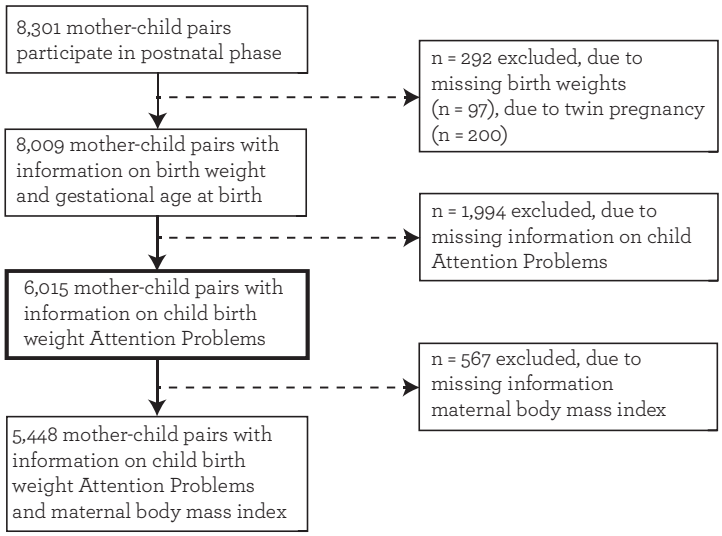

Figure 1. Flowchart of the study population.

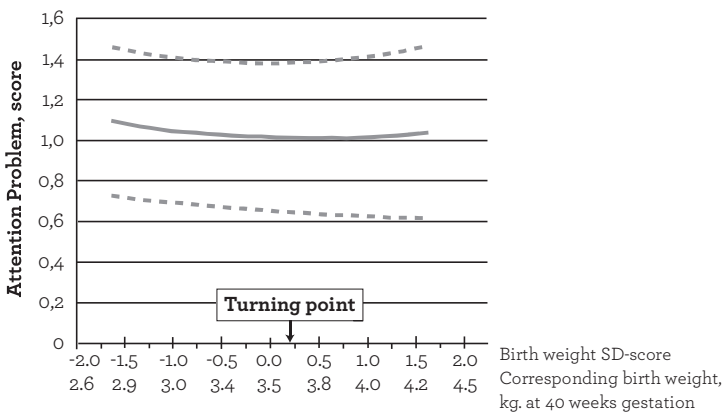

Figure 2. The association between child birth weight and Attention Problems at age 6 years (n = 6,015).

Estimated effects with 95% confidence intervals of gestational age-adjusted birth weight standard deviation (SD) score (90 percent range) on Attention Problems (score, square-root transformed). Estimates of effect size were obtained from a multiple regression model, adjusted for Apgar score one minute after birth, mode of delivery, maternal age, national origin, educational level, parity, body mass index, psychological symptoms, smoking, alcohol use, folic acid supplementation, gestational diabetes, and pre-eclampsia.

A birth weight SD-score of 0 at 40 weeks of gestational age equals a birth weight of 3.5 kg. The turning point of the curvilinear association between birth weight for gestational age and Attention Problems was at a birth weight SD-score of 0.3 (comparable with a birth weight of 3.6 kg at 40 weeks of gestation).

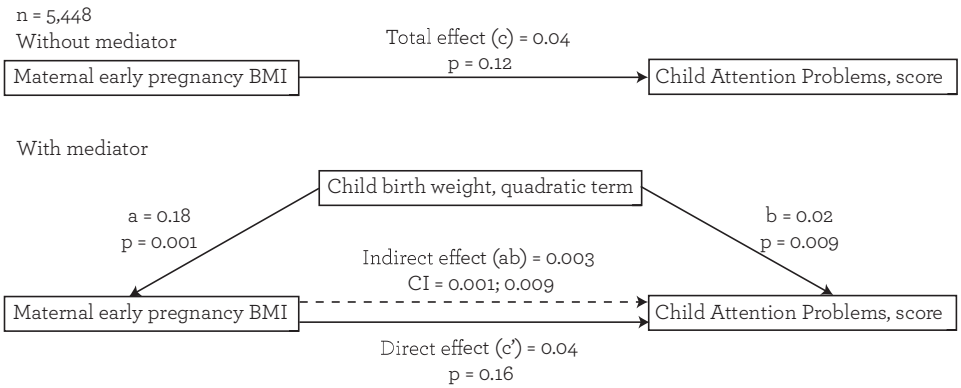

Figure 3. Model of maternal early pregnancy BMI as a predictor of child Attention Problems, mediated by child birth weight (SD-score[2]).

Results are obtained from Preacher and Hayes' Bootstrapping Procedure. Values represent unstandardized beta's, P-values. The confidence interval (CI) for the indirect effect is a bootstrapped CI based on 5000 samples.
Analyses are adjusted for child gender, age at assessment of Attention Problems, Apgar score one minute after birth and child birth weight (SD-score), mode of delivery, maternal age, educational level, parity, psychological symptoms, smoking, alcohol use, and folic acid supplementation, gestational diabetes, and pre-eclampsia.

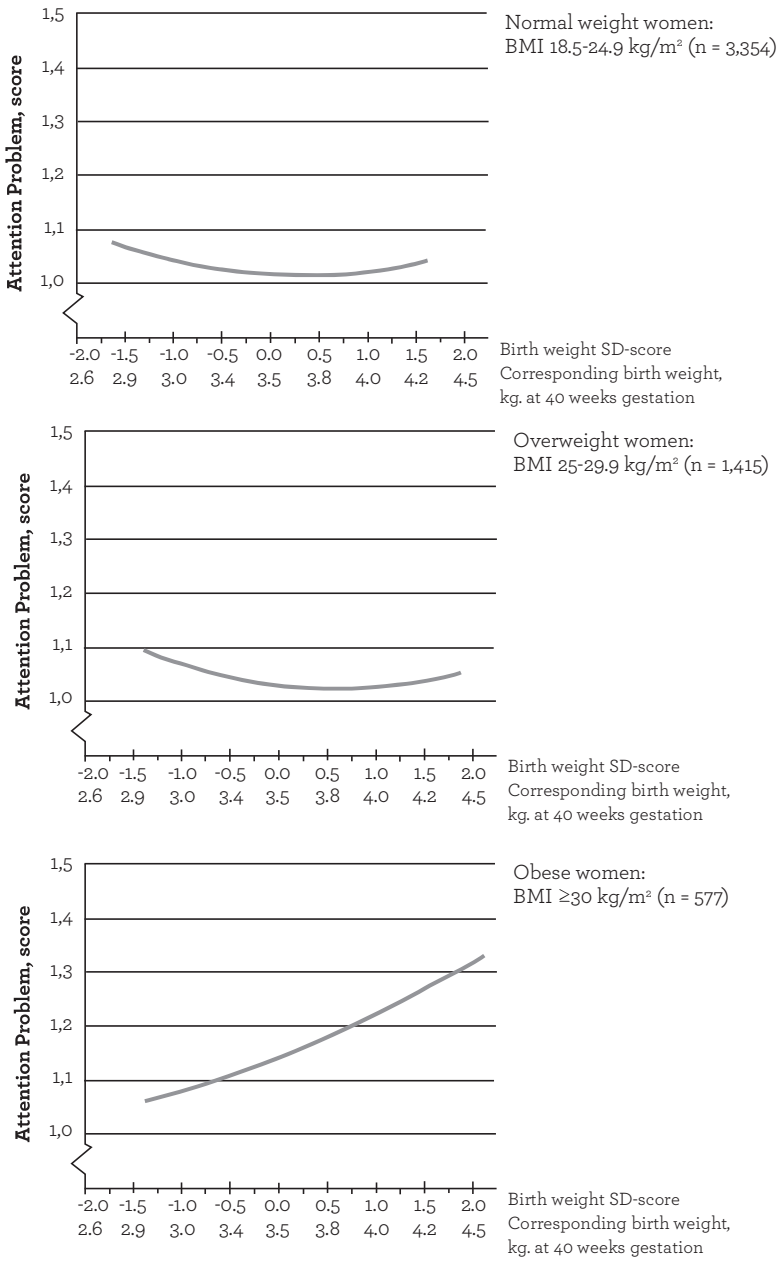

Figure 4. The association between child birth weight and Attention Problems at age 6 years stratified by maternal body mass index during pregnancy (n = 5,346), Rotterdam, the Netherlands, 2001-2005.

Estimated effects of gestational age adjusted birth weight standard deviation (SD) score (stratum specific 90 percent range) on Attention Problems (score, square-root transformed) in three strata of maternal early pregnancy body mass index (BMI). Estimates of effect size were obtained from a multiple regression model, adjusted for Apgar score one minute after birth, mode of delivery, maternal age, national origin, educational level, parity, early pregnancy BMI, psychological symptoms, smoking, alcohol use, folic acid supplementation, gestational diabetes, and pre-eclampsia. A birth weight SD-score of 0 at 40 weeks of gestational age equals a birth weight of 3.5 kg.

DISCUSSION

In this large, prospective, population-based cohort we observed a reverse J-shaped association of birth weight with Attention Problems. Higher birth weight was related to less Attention Problems but from a birth weight of about 3.6 kg or more, a higher birth weight did not reduce the risk of Attention Problems any further. In the model adjusted for the quadratic term of birth weight, maternal early pregnancy BMI moderated the effect of birth weight on Attention Problems: in children of obese mothers (BMI >30 kg/m^2), a high birth weight increased the risk of Attention Problems. Yet, if modeled dichotomously, low birth weight was not associated with Attention Problem score after adjusting for confounders such as maternal age, educational level, early pregnancy, psychological symptoms, and smoking during pregnancy.

We are not aware of any previous study showing that higher risks of Attention Problems can be found on both ends of the birth weight distribution. This curvilinear association may reflect that Generation R is a relatively young cohort; all children were born in the 21st century when the Western countries have experienced the obesity epidemic and lifestyle changes related to higher birth weights. Although birth cohorts traditionally have tested mostly linear associations between birth weight and health outcomes, nonlinear associations between birth weight and various other neurodevelopmental outcomes have been reported. Analyses after 4,503 singletons born between 1976 and 1990 with cerebral palsy revealed a reversed J-shaped rate variation (29). Gunnell et al. (30) reported in a 1973-1980 cohort of 246,655 male conscripts a reverse J-shaped association between gestation-adjusted birth weight and schizophrenia. Leonard and colleagues described that both children with lower and very high birth weight have an increased risk of intellectual disability (31). More recently, in the ALSPAC Cohort (born between 1991 and 1992) Wiles and coworkers (8) reported some evidence for nonlinear associations of birth weight and infant prosocial behaviour (inverted J-shape) and emotional problems (J-shape). In the current cohort our group previously reported an inverted J-shape association of measures of foetal size in both mid- and late pregnancy and infant alertness at three months of age (32).

The mechanism underlying the association between child birth weight and ADHD symptoms is not well understood. A few studies have attempted to investigate the causality of the effect of low birth weight on ADHD symptoms. Recently, a study in twins discordant for birth weight suggested a causal effect for birth weight on Attention Problems. The authors hypothesized that deficient nourishment *in utero* leads to impaired neurodevelopment and affects foetal brain development, which is reflected in ADHD symptoms (33). Some studies reported that the disturbance of the foetal maturation by intrauterine growth restriction leads to cerebral immaturity at birth and increased risk for cerebral palsy that represents a risk for neurodevelopment of low birth weight children (34, 35).

Most likely, different processes underlie the association of birth weight and ADHD symptoms in low birth weight and high birth weight children. A heterogeneity of pathogenesis underlying an association between birth weight and a disease has been

suggested among for type 2 diabetes. Namely, those born with low birth weight were more likely to be insulin resistant, whereas those born with a high birth weight were more likely to have metabolic syndrome (36). Another explanation that has increasingly been proposed is that the effect of low birth weight on later health risks might rather result from rapid early postnatal weight gain (37, 38).

The association between birth weight and Attention Problems is complex and likely to be subject to confounding. Previous studies controlled for several confounders such as socio-economic factors and maternal psychological symptoms but the control for pregnancy characteristics was typically less complete (39). Here, we also tried to account for intrauterine exposures by adjusting for maternal smoking, psychological symptoms, folic acid supplementation and pregnancy complications. Moreover, the use of a gestational age-adjusted birth weight variable prevents that the observed associations are driven by duration of gestation.

We found that maternal early pregnancy BMI moderated the association between child birth weight and Attention Problem score. Children born large for gestational age were at higher risk of Attention Problems if born to obese mothers. In general, a high birth weight reflects foetal and maternal health. Social deprivation, maternal disease and unhealthy life styles are all correlated negatively with birth weight (40). However, this might be different if a high birth weight results from high maternal weight. Maternal overnutrition and obesity may lead to metabolic alterations including elevated leptin and estrogen levels and insulin resistance. Insulin and insulin-like growth factors are known to affect neuronal differentiation, and survival, as well as neurite formation (41). In addition, studies have demonstrated that obesity in pregnancy is associated with a wide spectrum of peripartum complications including prolonged labor, increased caesarean section rates, and asphyxia of the child (42). However, we controlled for mode of delivery, gestational diabetes, pre-eclampsia, and child Apgar score to reduce the effects of confounding peripartum factors associated with maternal early pregnancy BMI.

A previous report of Rodriguez et al., investigating the association with maternal prepregnancy adiposity and child ADHD symptoms in three large Scandinavian pregnancy cohorts (43), described independent relationships with high maternal weight and child birth weight. Functional changes in the foetal brain of children exposed to excessive maternal weight, not related to high birth weight, may underlie these behavioural problems. Yet, this remains speculative, and no studies have described a very plausible pathway linking maternal obesity exposure and child health. Hence, the observed association may simply be the result of residual confounding since being overweight is known to correlate strongly with unfavorable socio-economic and behavioural characteristics like impulsivity (44, 45).

The strengths of the present study include the ability to investigate the relationship between birth weight and Attention Problems in a large, population-based sample of children with a birth weight distribution typically seen in the general population. Prospective measures of exposure and outcome and data on several important confounding factors are other strengths. Despite this, we cannot rule out that the observed association is the result of

residual confounding from socio-demographic and lifestyle related determinants. A second possible limitation of our study might be that child Attention Problems were assessed using the CBCL. Although behaviour problem scales do not provide a clinical diagnosis, continuous traits have been shown to represent adequately behavioural problems on the population level and provide better statistical power. Some studies have shown evidence that the CBCL-attention problem scale predicts ADHD well (46, 47), but is no measure of clinical ADHD symptoms. More importantly, many other studies have reported associations between reduced birth weight and increased Attention Problems instead of clinical diagnoses of ADHD. Our approach is thus in line with previous studies. Finally, since data were more complete in higher-educated mothers, we cannot rule out that selective non-response bias influenced our findings.

In conclusion, our results suggest that birth weight and Attention Problems are curvilinear related. Higher birth weight was related to less Attention Problems but from a birth weight of about 3.6 kg or more, a higher birth weight did not reduce the risk of Attention Problems any further. However, in children of obese mothers, a high birth weight may increase the risk of Attention Problems. Future research might focus not only on biological mechanisms and their effect of brain development in low birth weight children, but also high birth weight children.

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Chapter 11

How Important are Prenatal Influences for Neuro-development? The Association of Gestational Duration and Birth Weight with Child IQ: The Generation R study

ABSTRACT

Background: Several psychiatric disorders are considered to be neurodevelopmental in origin. Population-based research provides us with a unique opportunity to explore how prenatal factors influence neurodevelopment.

Aim: To analyse whether non-verbal child IQ is linked to gestational duration and birth weight and to find out whether these birth factors can be seen as to reflect the intra-uterine environment.

Method: Participants were 5,893 (73.6%) children enrolled in the Generation R Study. The gestational duration was determined on the basis of foetal ultrasound scans. Birth weight was measured and transformed into standard deviation scores corrected for gestational duration and gender. The non-verbal IQ was measured when the child reached the age of six.

Results: Even during in the a-term period, gestational duration showed a linear association with non-verbal IQ, whereas the association between birth weight and non-verbal IQ was found to be curvilinear.

Conclusion: Independently and showing different patterns, both gestational duration and birth weight were found to have a predictive value for childhood non-verbal iq. This finding illustrates the various mechanisms that could lead to neurodevelopmental disorders.

INTRODUCTION

Several psychiatric disorders are considered to be neurodevelopmental in origin (1). These disorders are characterized by onset at early age. Imaging, electrophysiological and neuropathological research after these disorders revealed anatomical and functional alterations in the brain. The rapid transformation of zygote into term neonate in just 270 days makes the prenatal phase a critical period, in particular for the complex central nervous system. Already during the 1970's, exposure to extreme factors during embryonic and foetal development, such as famine, iodine deficiency or viral infections (2) was linked to the occurrence of psychopathology. More recent, interest emerged for the hypothesis that also exposure to multiple, subtle variations in the prenatal environment could lead to psychiatric problems.

Several population based studies have been started that follow the development of children already from pregnancy onwards. One of these studies is the Generation R Study, a multi-ethnic cohort that, between 2002-2006, recruited 9.778 pregnant women in Rotterdam, The Netherlands. In this study, three foetal ultrasound examinations were performed and blood and urine of the mother was collected. Information about the pregnancy was derived from obstetric and medical records and information on numerous items was obtained via parental questionnaires (3). Much of the research in the Generation R Study was devoted to risk factors for a low birth weight, preterm birth or prenatal risk factors for neurodevelopmental disorders and in particular also on mechanisms underlying the association between foetal growth and neurodevelopment. A determinant – such as alcohol abuses during pregnancy – that affects foetal growth could also contribute to a disturbed neurodevelopment. Somethings, these are (partly) independent relationships, or only birth weight or neurodevelopment is influenced.

One of the studies embedded in the Generation R Study showed that both the lack of folic acid supplement use, as well as maternal folate deficiency, is associated with more child behavioural problems (3). We also found indications that maternal fatty acids are related to emotional and behavioural problems (4). In the Generation R study we also explored the effects of maternal overweight during pregnancy on child development. Given that the effect of paternal overweight was comparable to the maternal effect, we concluded that overweight of the mother did not have an independent intrauterine effect on neurodevelopment (3). The use of tobacco and cannabis of the mother during pregnancy was related to a decreased head growth of the foetus. Different from prenatal exposure to cannabis, smoking did not show a univocal intrauterine effect on the behavioural development of girls (3).

Within the Generation R Study, there was relatively much attention for the effects of thyroid metabolism on neurodevelopment. Thyroid hormones, transferred from mother to the embryo and foetus, are crucial for brain development as the foetal thyroid production does not start until the 10th week of pregnancy. We described a consistent association between maternal hypothyroxinaemia (decreased free T4 levels) and impaired cognitive development of children. Maternal hypothyroxinaemia was also related to a larger head

circumference during pregnancy and infancy. Severe maternal hypothyroxinaemia, in addition, was predictive of child autistic symptoms (3).
The use of selective serotonin inhibitors (SSRI's) during pregnancy is in general considered as safe. SSRI's could, however, cross the placenta where they could disturb the foetal serotonin balance. Mothers in the Generation R Study using SSRI's had less severe depressive symptoms, but the foetal head growth was further decreased as compared to women with depressive complaints that did not use SSRI's (3).

The ultrasound measurements and relatively precise determination of the duration of pregnancy within the Generation R Study gives us the possibility to distinguish between he effects of foetal growth and duration of pregnancy. Previously, we described an increased risk of behavioural problems in post term born children, born after 42 weeks of pregnancy (5). Recently, our research group described a curvilinear relationship between birth weight and attention problems. Higher birth weight was related to less attention problems but from a birth weight of about 3.6 kg or more, a higher birth weight did not reduce the risk of attention problems any further (3).

In the current study, we investigate the relationship between duration of pregnancy and birth weight with non-verbal intelligence quotient (IQ) at age 6 years. We hypothesized that these pregnancy outcomes were both, independently of each other, associated with non-verbal IQ.

METHODS

Children were 5,893 (73.6%) children born from singleton pregnancies participating in the Generation R Study. To define gestational age, ultrasounds were used. Gestational-duration- and gender adjusted birth weight standard deviation scores (SDS) were constructed.

During a laboratory visit, we assessed non-verbal intelligence of the child at age 6 years using two subtests of the Snijders-Oomen non-verbal intelligence (SON-R 2.5-7) test (3).

Statistical analyses were performed using linear regression models, adjusted for maternal age, national origin, education, parity, body mass index (BMI), smoking, alcohol use, folic acid supplement use, psychological symptoms, IQ, mode of delivery and child gender and Apgar score.

To gain insight in the specificity of the effects of the birth outcomes on cognition, we also explored the relationship between duration of pregnancy and birth weight with anxiety symptoms as reported by parents with the *Child Behaviour Checklist*, a behavioural questionnaire.

RESULTS

Children were born after on average 39.8 weeks of pregnancy (SD 1.8). 331 (5.6%) children were born preterm -before 37 weeks of pregnancy. Mean birth weight was 3.426 kilograms.

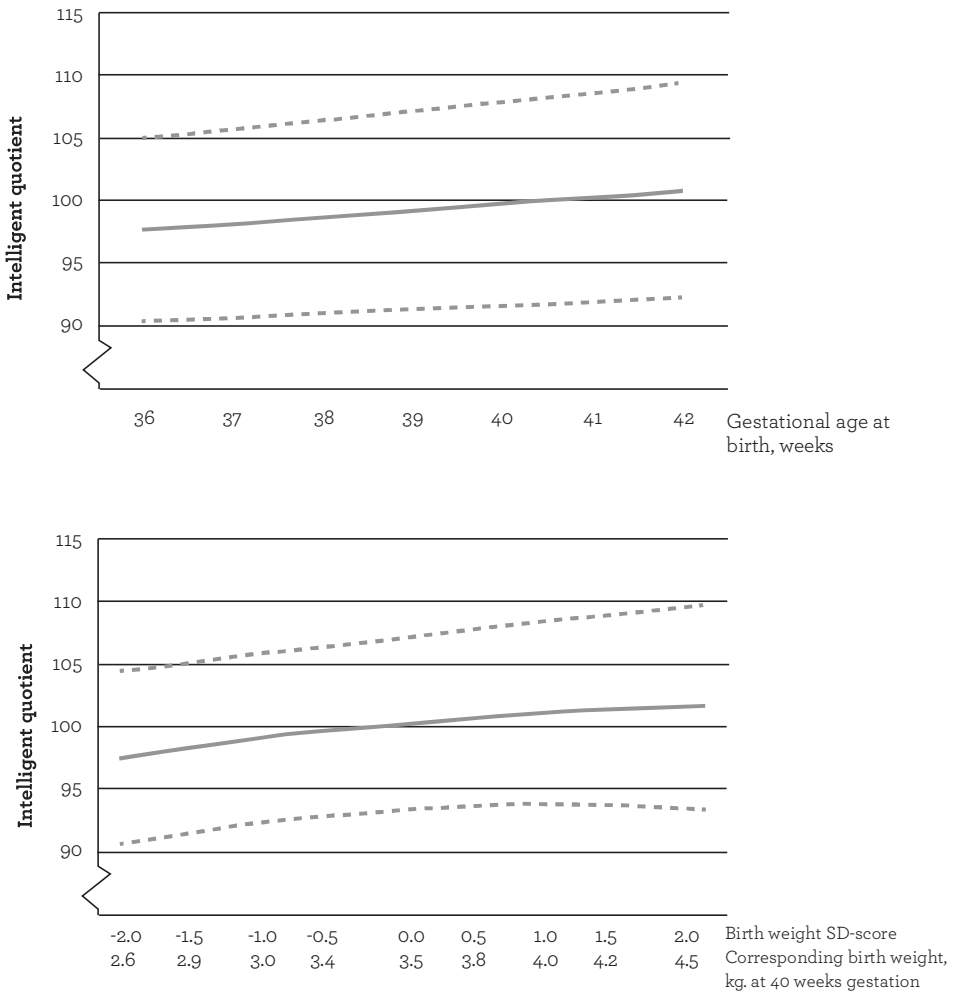
A longer gestational duration was associated with higher non-verbal IQ at age 6 years (ß per week of pregnancy duration 0.50. 95%CI 0.29; 0.70. *p* <0.001) (Figure) (Table). This association was also observed in children born at full term, after 39 weeks of pregnancy (adjusted ß per week gestational duration 0.48, 95%CI 0.02; 0.94, *p* = 0.04). Prematurity was associated with a lower nonverbal IQ (ß = -1.56, 95%CI -3,10; -0.01, *p* = 0.049). We did not observe any evidence for a curvilinear relationship between gestational duration and IQ.

Second, we investigated the association between birth weight and non verbal IQ. Being born small for gestational age (SD score <10th percentile = 2.9 kg was associated with lower IQ (adjusted ß = -2.94, 95%CI -4.19; -1.70, *p* <0.001). We observed a curvilinear relationship between birth weight and non verbal IQ (ß per birth weight SD-score[2] 1.11, 95%CI 0.73; 1.49, *p* <0.001). This curve showed a parabola with a ceiling, what indicates that above a birth weight above 3.5 kg a higher birth weight does not have an additional positive effect on non verbal IQ. There was no association between duration of pregnancy of birth weight and anxiety symptoms (results not shown).

Table. Associations between duration of pregnancy and birth weight with non verbal IQ at age 6 years, n = 5,893.

	IQ Gecorrigeerd[1] adjusted	
	ß (95%CI)	**P-value**
Duration of pregnancy, weeks		
Dichotomous: pre-term, <37 weeks duration of pregnancy	-1.56 (-3.10; -0.01)	**0.049**
Dichotomous: post-term, >42 weeks duration of pregnancy	1.26 (-0.43; 2.95)	0.14
Continuous:	0.52 (0.31; 0.72)	**<0.001**
Curvilinear:		
Continous term	3.45 (0.04; 6.86)	**0.047**
Quadratic term	-0.04 (-0.08; 0.01)	0.09
Birth weight, SD-score		
Dichotomomous: Low birth weight, <10^{e} percentile	-2.94 (-4.19; -1.70)	**<0.001**
Dichotomomous: High birth weight, >90^{e} percentile	1.68 (0.47; 2.88)	**0.006**
Continous	1.14 (0.76; 1.52)	**<0.001**
Curvilinear:		
Continuous term	1.11 (0.73; 1.49)	**<0.001**
Quadratic term	-0.26 (-0.48; -0.04)	**0.02**

[1]Analyses are adjusted for birth weight SD-scores (if duration of pregnancy is the determinant), gender, age at IQ assessment, Apgar-score, mode of develivery, maternal age, national origin, educational elvel, IQ, parity, BMI, length, psychological symptoms, smoking, alcohol use and folic acid supplement use.



Figures. Associations between duration of pregnancy and birth weight with non verbal IQ at age 6 years.

Estimated effects with confidence intervals of gender-and-gestational-duration adjusted birth weight SD-scores (95% range) on intelligence quotient at age 6 years. Estimates of effect size were obtained from a multiple regression model adjusted for child age at assessment of intelligence quotient, Apgar score one minute after birth, mode of delivery, maternal age, national origin, educational level, intelligence quotient, parity, body mass index, height, psychological symptoms, smoking, alcohol use and folic acid supplement use.
A birth weight SDS of 0 at 40 weeks of gestational duration is comparable to a birth weight of 3.5 kg.

DISCUSSION

A longer duration of pregnancy showed a positive relationship with non verbal IQ at age 6 years, even within the term period. These results suggest that a risk for lower cognitive functioning is not merely confined to preterm births, but that the effects of gestational duration appear similar across the whole range of gestational duration.

The positive relationship between birth weight and non verbal IQ, on the contrary, is strongest in children born with the lowest birth weights; a higher birth weight within the normal distribution hardly as an effect.

The returning question is how birth weight and gestational duration influence IQ. Women who give birth to preterm or small children are more likely to have less optimal socio-environmental factors and provide a less cognitively stimulation environment for the child. Here, we could control for numerous extraneous factors that have been implicated as risks for pregnancy and cognitive functioning of the child. And, as child cognitive development is strongly determined by maternal cognitive ability, we corrected for maternal IQ. Nevertheless, confounding is certainly a possible explanation for the current findings.

Birth before term is considered to interrupt brain maturation. At 34 gestational weeks, the overall brain weight is 65% of term weight (6). Another explanation could be that detrimental health outcomes are explained by the underlying causes of earlier birth that might themselves lead to suboptimal brain development. In the study of Bailit and colleagues (7) children born after a spontaneous onset of labor had a higher risk of ventilator use, sepsis and neonatal intensive care admissions compared to children born after induction of labor.

In the current study, we also describe a curvilinear relationship between birth weight and non verbal IQ. It is often suggested that exposure to a suboptimal intrauterine environment, leading to restricted growth, may lead to impaired brain development, which can have implications for the child in later life. Other researchers argue that birth weight is not on the causal pathway from the intrauterine environment and health outcomes. Alternative hypotheses, like the possibility that genes associated with metabolism and early growth also impact later neurodevelopment (8).

Duration of pregnancy seems, as compared to birth weight, a better predictor of neuro-developmental outcomes associated with neuro-maturation.

In the current study we did not observe an association between duration of pregnancy or birth weight and anxiety symptoms. Although anxiety can be considered a cognitive-affective disorder, here, different from cognition, the environment and more specific, in particular subcortical brain structures play an important role (9). This suggest that anxiety disorders are no neuro-developmental disorders.

From a clinical point of view, the effect sizes of birth weight found in our study are small. However, because of the large number of potentially affected infants, at population level a small derangement in neurodevelopmental outcome could result in physical, emotional and economic burden to individuals and society in general.

Based on previous population based research, like the Generation R Study, it ca be concluded that common prenatal factors, including overweight and no use of folic acid supplements, have a modest effect on child neuro development. Many of the observed associations could be explained by parental epiphenomena like educational level, family income and psychopathology.

With several specific prenatal factors like maternal SSRI use and thyroid malfunction we observed relative strong associations with behavioural problems. Here, also variations between the normal range had meaningful effects on neurodevelopment.

The understanding of etiology and pathogenesis of neurodevelopmental disorders is one of the major challenges within psychiatry. The interest in epigenetics and biomics among psychiatric researchers showed how much we depend on technological development to explore possible underlying mechanisms.

The current study contributes to unravel the relationship between the intra-uterine environment and cognitive development. We conclude that duration of pregnancy and birth weight, were independently and in a different way predictive of non verbal IQ at age 6 years. The effects were not limited to subgroups but applied to the total range of these birth outcomes. Underlying mechanisms in the association between pregnancy outcomes and cognitive development are not sufficiently known and require additional research.

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Part V

Chapter 12

General Discussion

This thesis brings together several studies conducted in a population-based study that explore prenatal influences on foetal growth and children's cognition and behaviour. The prenatal phase is a critical period as rapid cell division and tissue differentiation follow each other iteratively. Brain development requires an extraordinarily complex set of neurodevelopmental events including early synapses and neural circuits formation. This makes the foetus, and, in particular, its complex central nervous system, extremely sensitive to environmental exposures.

The vulnerability of the developing brain, however, not only depends on the teratogenic potential of the exposure, but in particular to the period of exposure. If exposure occurs during before or after organogenesis, adverse effects do not coincident with the ontogeny of developmental processes. If exposure occurs during organogenesis, it could potentially interfere with the cascade of developmental processes. Subtle alterations of the prenatal environment could have meaningful consequences for postnatal development.

In the current thesis, we focused on mechanisms underlying the association between foetal growth and children's cognition and behaviour. Prenatal influences that impair foetal growth could, on the long term, also contribute to disrupted neurodevelopment. As the human prenatal environment represent exposure to multiple non-specific prenatal influences difficult and often not feasible to simulate in experimental settings, birth weight, as the endpoint of foetal growth, is often used as a suitable biological marker to assess prenatal brain development. In large large-scale population-based studies, subtle variations in birth weight can be related to later-maturing differences on a cognitive or behavioural level.

It is not clear how the sub-optimal prenatal environment regulating foetal growth, affects neurodevelopment. Most likely, variations in embryonic and foetal neurogenesis are a consequence of epigenetic alterations such as DNA methylation. These epigenetic mechanisms could represent an interplay between environmental stressors and long-lasting molecular, cellular and complex behavioural phenotypes acquired during periods of developmental plasticity.

In this chapter, the main findings of this research are summarized and methodological aspects are discussed. Finally, I will discuss the potential implications of our findings and address considerations for future studies.

MAIN FINDINGS

Foetal Growth as Potential Intermediate between Prenatal Influences and Children's Cognition and Behaviour

The prenatal environment has been conceptualized as the "nexus of nutritional, metabolic, endocrinological, infectious, genetic epigenetic and socio-behavioural inputs" (1). These all come together in a dynamic interplay of the mother, the foetal environment and the sensitivity and plasticity of the foetus. Because growth of the foetus is suggested to be calibrated to the availability of maternal resources (2), birth weight is commonly used as an indicator of the conditions experienced prenatally. Reduced foetal growth is indicative of impaired organogenesis, and more specifically brain development, and could thus shape the health of the child and eventually the adult. Therefore, foetal growth could be seen as potential intermediate between prenatal influences and child outcomes cognition and behaviour. However, prenatal factors could also affect foetal growth without consequences for child neurodevelopment or vice versa.

The studies presented in this thesis suggest that prenatal exposure to maternal thyroid hormones and fatty acids, are related to foetal growth (**PART II**). Foetal growth, in turn, was associated with subsequent child behavioural problems and cognitive functioning (**PART V**).

Previous studies exploring the association between birth weight and mental health outcomes primarily focused on the consequences of low birth weight. In the last decades, however, a rise in median birth weight was observed (3-5). An increasing proportion of children is born large for gestational age (LGA) (6). This group of LGA children is growing rapidly in some countries. Higher birth weights, i.e., babies weighing more than 4.5 kg, account for less than 1% of total live births in some countries, e.g. France and Portugal, for 2.3% in the Netherlands, and for more than 4% in Finland, Denmark and Sweden (7). The increase in birth weight is be explained by, several factors such as increased maternal age (8), less women smoking (9), and higher maternal body mass index (BMI) (10). The latter may give rise to a public health concern as the prevalence of overweight in adult females increased markedly (11).

The observed relationship between birth weight and Attention Problems or IQ at age 6 years in the current thesis showed a (reverted) J-shaped pattern (**chapter 10 and 11**). The negative health effects are strongest in those born with the lowest birth weights but operate across the range of birth weights. In general a higher birthweight is considered an indication of a more favourable prenatal environment. However, this may be different for the extremes of the right tail of the birth weight distribution. A number of studies have reported a J-shaped or U-shaped relationship between birth weight and health outcomes what could illustrate less optimal development of infants born to diabetic or glucose intolerant mothers.

In **chapter 10**, we describe an interaction effect of birth weight with maternal BMI on child Attention Problems. In children of obese mothers (BMI >30 kg/m^2), high birth weight may increase the risk of Attention Problems. This finding add to the evidence of the negative

health effects of maternal obesity in pregnancy. Obesity of the mother promotes neural changes, that in turn, promotes the risk of obesity. If this is the case, an alarming situation of a vicious cycle is conceivable and obesity rates would rise in succeeding generations (12).

Although birth weight may be in a pathway from the exposure of interest to the neurodevelopmental outcome, several studies suggested that the association between birth weight and health outcomes may not be causal. In a series of papers (13-17), Allen Wilcox argues that reduced birth weights can be a sensitive marker of foetal growth restriction but not necessarily an indicator of poor health.

A remarkable characteristic of birth weight is the consistency of the shape of its relationship with various health, including mental health outcomes. The relation with cerebral palsy, intellectual disability, schizophrenia, and as presented in this thesis Attention Problems and IQ, follows the same (reverse) J-shaped pattern with a near-Gaussian distribution of birth weight (18). One of the striking phenomena Wilcox brings up is the existence of the low birth weight paradox; an apparently paradoxical observation relating to the lower infant mortality rate in low birth weight children born to smoking mothers if compared to mortality of low birth weight children of non-smokers. This phenomenon also applies to other populations at risk for adverse birth outcomes such as twins, high-altitude residents, and ethnic minorities in the United States. An adverse environment tend to change the level but not the shape of the curve. Specific causes of low birth weight are generally more harmful than being small at birth because of being part of a high-risk population identified with very global risk indicators.

Basso and colleagues simulated this association and suggest that the observed pattern of birth weight with mortality can be produced by a rare condition or by a cluster of conditions (43). Suggested candidate conditions that have both profound effects on birth weight and mortality were malformations, infections, or chromosomal disorders and genetic variations. Suggested candidate genes often put forward are those involved in the growth hormone and insulin-like growth factor receptor signaling pathway, because of its involvement in both early growth, cognitive functioning and psychiatric problems (19, 20). Many other candidate genes are currently being revealed by genome-wide association studies (GWAS), and the upcoming field of epigenome-wide association studies (EWAS).

Given the strong predictive value of birth weight for neurodevelopment it is an important factor in psychiatric epidemiological research, regardless the causality of such an association. Further research could be aimed to further map the genetic and environmental influences on early development and to distinguish when birth weight it is prognostic for impaired development (21).

The effects of common maternal prenatal exposures on children's problem behaviour and cognition are not clear. In **PART III** we examined the effects of prenatal influences of children's cognition and behaviour. Maternal low urinary iodine showed a modest association with children's impaired executive functioning. The children of mothers with low urinary iodine showed higher scores on the problem scales of inhibition and working memory. Maternal dietary intake and thyroid hormone concentration did not significantly modify

these associations. The associations between urinary iodine and problems of inhibition were attenuated after adjustment for maternal psychological symptoms (**chapter 5**).

Other prenatal exposures had effects best explained by confounding. In the current thesis this is illustrated by a study after season of birth and children's IQ (**chapter 6**). A birth day in spring has repeatedly been found to be a risk indicator for adverse neurodevelopmental outcomes. Mostly it is speculated that seasonally patterned biological exposures, including nutrition and exposure to influenza, during pregnancy explain this variation. Our results suggest that socio-demographic factors related to planning of pregnancy, and not biological confounders, are of particular importance to explain the association between season of birth and child development in the general population. Birth weight and gestational duration were of modest importance in this association as compared to maternal IQ and socio-demographic factors.

What underlays the association between a sub-optimal prenatal environment regulating foetal growth and adverse neurodevelopment is not clear. Epigenetic regulation is considered a major contributor of foetal growth and children's neurodevelopment representing a biological mechanism explaining the complex interaction between genetic factors and environmental influences. In the current thesis we focused on the concept that variations in DNA methylation may start as early as in utero following early prenatal exposures and could impact on children's neurodevelopment (**PART IV**). In newborns, DNA methylation was measured in umbilical cord blood white blood cells at 11 regions of the seven genes: *NR3C1, DRD4, 5-HTT, IGF2DMR, H19, KCNQ1OT1,* and *MTHFR*.

In **chapter 9**, we identified determinants of the maternal one carbon metabolism in early pregnancy and variations of newborn DNA methylation at these loci. An association was observed between maternal folate deficiency and lower newborn DNA methylation, which disappeared after adjustment for socio-economic confounders. Maternal homocysteine and folic acid supplement use were not associated with newborn DNA methylation. The maternal *MTHFR TT* genotype, associated with lower folate levels, was related to lower DNA methylation.

A low birth weight was associated with lower *IGF2DMR* DNA methylation, but not with *H19* methylation (**chapter 7**). Genetic variants in the *IGF2/H19* locus were associated with *IGF2DMR* DNA methylation. Our observations suggest a possibility of mediation by DNA methylation underlying the association between the genetic variants in the *IGF2DMR* gene and a low birth weight.

Lower DNA methylation levels were associated with more ADHD symptoms (**chapter 8**). This association was largely explained by associations of *DRD4* and *5-HTT* regions. Other candidate genes showed no association between DNA methylation levels and ADHD symptom score. Associations between DNA methylation levels and ADHD symptom score were attenuated by co-occurring Oppositional Defiant Disorder and total symptoms.

Our data support the hypothesis that prenatal environmental exposures influence the epigenome, which can alter the programming of gene expression and thus potentially contributes to health and disease in later life (22). However, as in other observational studies causality of observed associations cannot be ascertained. Our findings illustrate

the importance of careful adjustment for potential confounders. In previous epigenetic studies, control for socio-economic factors is typically less complete. In addition, our findings touch on the complex interplay between genetic variations and DNA methylation. Several common SNPs is in the loci assessed showed associations with variations in DNA methylation. However, detailed information on the factors which might influence the fidelity of DNA methylation profiles is lacking.

Thyroid Hormones

As the foetal thyroid gland does not reach maturity until the second trimester of pregnancy, the development of the foetal brain is sensitive to the maternal thyroid status. The detrimental effects of low maternal thyroid hormones levels on foetal brain development have been recognized for long.

Results from studies presented in the current thesis, as well as previous work from our study group, indicated that even subtle deviations in maternal thyroid function could have important implications for child neurodevelopment. We described altered foetal and infant head growth in children born to mothers with hypothyroxinaemia (**chapter 2**). This altered head size was not related to cognitive delays as previously reported in children from these hypothyroxinaemic mothers in the current cohort. In **chapter 5** we described the association of low maternal urinary iodine and child problems of executive functioning. This association was independent of maternal thyroid functioning. Because the full causal chain that links iodine and the different parameters of thyroid functioning to risk of developmental problems has not been established, the indirect evidence has to be considered carefully.

Although treatment of overt hypo- and hyperthyroidism during pregnancy is universally accepted, opinion differs for the subclinical forms. A large debate is going on regarding the definition of a normal thyroid function in pregnancy and selection of a population that would benefit from thyroid treatment. Observational studies, like ours, cannot demonstrate causality of the association between hypothyroxinaemia and neurodevelopment. Randomized controlled trials, with child neurodevelopmental functioning as main outcome, could clarify on the efficacy of screening and subsequently treatment for thyroid dysfunction in the general pregnant population. In 2012, Lazarus and colleagues published the results of the only randomized controlled trial that has been performed yet. Women with either a reduced thyroid function subclinical or overt hypothyroidism received 150 μg of levothyroxine per day initiated at up to 20 weeks' gestation. This treatment has no beneficial effects on global cognitive performance, as measured by an IQ test, at age 3 years. Although this lack of a significant effect of levothyroxine therapy may suggests that the observational studies reporting detrimental effects of subclinical thyroid dysfunction on offspring cognitive function were subject to confounding or some degree of selective reporting, several other explanations could be considered. The inclusion of pregnant women with milder hypothyroidism, the timing of initiation of levothyroxine administration, the type of assessment of cognitive functioning, and the length of the follow-up period to detect are methodological concerns (23).

Currently, we are awaiting the results of a second trial that administered levothyroxine to subclinical hypothyroid or hypothyroxinaemic women before 20 weeks of pregnancy (ClinicalTrials.gov number, NCT00388297). Here, the primary outcome will be intellectual function of children at 5 years of age, as measured by the Wechsler Preschool and Primary Scale of Intelligence. In addition, potential effects on the occurrence of attention deficits at 4 years of age and behavioural problems and 3 and 5 years of age will be taken into account. This, and other trials are needed to determine whether antenatal thyroid screening and treatment is justified in order to improve children's cognitive functioning or prevent neurodevelopmental alterations.

METHODOLOGICAL CONSIDERATIONS

Relationships between Interaction Terms and Non-linear Terms in Regression Models

In **chapter 10** we tested whether maternal early pregnancy BMI moderated the association between child birth weight and Attention Problem score by adding an interaction term of birth weight and maternal early pregnancy BMI (both linear terms) to the model. We followed the approach by Ganzach (24) and kept the linear and curvilinear term of birth weight in the model. In this seminal paper, Ganzach demonstrated the importance of including curvilinear terms when interactions are estimated. Here, he argued that curvilinear terms can partially account for the variance of an interaction term when the predictors are correlated. If the appropriate product terms are omitted, spurious effects could occur as interaction between two variables could be a proxy for a curvilinear effect of either variable alone (type I error). Another possible erogenous consequence of not including curvilinear terms in the model could be the inversion of the true nature of the relationship. The effects of the terms could either have minimizing or magnifying effects on each other what can result in the indication of a convex relationship when the true relationship is concave, or the other way around. In addition, not including the appropriate terms in the analysis may lead to a type II error; the failure to detect a true curvilinear relationship when one exists. However, others, including Aiken and West (25) and Shepperd (26) do not agree with Ganzachs recommendation. They argue that the use of a curvilinear term may lower the power for significant interactions. The curvilinear terms should be only included in the model unless it is theoretical justified. Against this background, inclusion of curvilinear terms should be carefully considered when testing interaction effects.

I suggest the following: If interaction effects remain present with introduction of a curvilinear term, this increases the likelihood of a true interaction effect. However, in case results change with introduction of a curvilinear term, I would suggest a more conservative approach is recommended when interpreting results. The presence of a interaction effect should be disregarded if such an effect is only observed without the curvilinear term included in the model.

DNA methylation Candidate Studies: What are we looking at?

In genetic research, the modest explanation of disease by genetic variation in the population, the marginal increase of biological relevant knowledge and the many spurious associations tempered high hopes. After the hype of candidate gen and gene-environment-interaction, the quick success of GWAS studies for many physiological traits and somatic disorders, a new postgenomic era has begun. More than a decade ago, in his keynote paper (27), Michael Meaney argued for epigenetic studies: "There are no genetic factors that can be studied independently of the environment, and there are no environmental factors that function independently of the genome. Phenotype emerges only from the interaction of gene and environment.."

The abilities of molecular epigenetics created high expectations to fill in some of the gaps in scientific knowledge, in particular 'the missing causality'. Epigenetic variations are, unlike genetic sequences, sensitive to and modifiable by environmental conditions, and could contribute to the phenotypic variation between individuals (28).

Given the complexity of epigenetic mechanisms, very little is known about the candidate loci in epigenetic studies. The differences in DNA methylation found in the epigenetic studies described in this thesis are modest, as one could expect if extrapolating previous findings. Although findings can vary between loci and populations, previously, differences in DNA methylation of about 20% have been related to a 2-fold change in gene expression (29). As observed in animal studies, alterations in gene expression in larger loci could lead to grave disorders or even lethality (30). However, what the functional relevance is of alterations in DNA methylation as described in our study for phenotypic outcomes has yet to be shown.

High expectations may lead to over-optimistic interpretation of results. The current evidence is still rather thin to form a solid basis for use in clinical studies. With measurement techniques often not validated and absence of a 'golden standard' the detection and validation of subtle alterations in DNA methylation leaves epigenetic research a conspicuous sense of uncertainty.

Currently, basic scientists are improving the understanding of molecular mechanisms and assessment of epigenetic processes. Major goals are the development of reliable measurement techniques with cell type specific analyses of the epitype within a complex tissue. Parallel, translational and clinical scientist are transmitting basic biology into clinical applications. The lesson learned from the early adapter genetic studies is that a rigorous, hypothesis free-approach in large international consortium-based settings is required. Replication of results, and limiting the number of outcomes and statistical models should be strongly considered for robust findings.

The epigenetic studies in the current thesis concern candidate-gene DNA methylation studies. By the time these studies were performed, although technical feasible, sufficiently powered studies of genome wide profiling of methylation were not in reach. A neuronal network approach, with genes that are known to interact functionally (network), e.g. nervous system development or neural facilitation, was chosen. This hypothesis-driven approach

could elaborate on existing knowledge. Ultimately, our results would be replicated in other cohorts or, if results could not be replicated, at best this contributes to the awareness of the methodological concerns. Nevertheless, if methodological criteria are met, the study of epigenetics in neurodevelopmental disorders holds a great promise for the future to reveal new insights into pathogenesis not identified by other research fields.

Assessment of Cognitive Abilities

It is generally agreed that IQ is related to executive functioning, but the nature of this relationship is not clear (31). Boring (1923) (32) defined intelligence as "what is measured by intelligence tests." Although this is debatable, the use of tests is to assess IQ is widespread. Traditionally IQ tests measured crystallized intelligence, which is the recall of knowledge and experience and the ability to use skills. More modern intelligence tests aim to capture fluid intelligence; ones capacity to think logically and solve novel problems, less dependent of acquired knowledge. Executive function comprises several domains including amongst others working memory, selective attention, and alertness that tap one's ability to pursuit and achieve a goal (33). In chapter 11 of the current thesis, a form of fluid intelligence was measured using the Snijders-Oomen non-verbal intelligence test (SON-R) in the children at age 6 years. Executive functioning was measured using the BRIEF-P in the current thesis (**chapter 5**).

There is no consensus on whether researchers should take executive function into account by statistical adjusting when assessing determinants of intelligence or whether they should take intelligence into account when studying executive functioning. A nice metaphor to distinguish between the two neuropsychological constructs IQ and executive functioning is to "Think of IQ as the engine in a car and Executive Function skills as the oil, fuel, belts and hoses that make it run effectively. A perfectly restored 1969 Pontiac GTO with a 330 horsepower engine has plenty of potential to cruise down the highway on a sunny Saturday, but see how far you get with faulty spark plug wires" (34). However, one could argue whether this metaphor can be justified from a scientific point of view.

Currently, the opinion of these two constructs being independent seems to predominate. Executive functioning predicts academic performance at early school age through university better than does IQ (35). In addition, as executive functions are impaired in many mental health disorders including ADHD, OCD and conduct disorder (36-38) they confer probably more than ones academic abilities. Indeed, in unaffected subjects, traditional executive tests scores correlate poorly with IQ scores (39-41). Others suggested that specific sub forms of executive functioning, as working memory capacity, are differentially related to intelligence (27). Inhibiting or shifting were not strongly related to IQ (31). Although this evidence creates a diffuse view, the relationship between these two constructs may be even more complex as studies of IQ and executive functioning in children with ADHD show that correlations differ by IQ (42). In patients with higher IQ, prefrontal component may be less impaired as both individuals with ADHD and controls scored better at executive function measures. In high IQ adults with ADHD, similar impairments of executive function are

observed as in patients on the wider spectrum of IQ scores (43). Remarkably, IQ scores accounted for a greater proportion of variance in executive functioning measures than the ADHD diagnosis.

To further complicate matters, there is also a developmental component in the relationship between these constructs. Research clearly shows that executive functions improve with child development as a functioning of age (44, 45) observed a strong correlation between IQ in children at ages 5 and 12, but a surprisingly weak correlation between executive functioning at age 5 and IQ performance at age 12.

In this research field full of uncertainties, more understanding of the association between executive functioning and intelligence is provided by neuroscientists studying brain laesion patients. While the prefrontal cortex plays a central role in executive control and intelligence, it was frequently observed that frontal laesion patients show a clear reduction in IQ (46). This was considered to suggested that executive functions are not related to intelligence (46). Alternatively, these findings may indicate that knowledge is preserved or that IQ tests are not sensitive to frontal laesions (47, 48). Neuroimaging suggested a strong neuroanatomic link between intelligence and executive functioning (49). Several structural features of grey matter in the frontal lobes showed considerable overlap across intelligence and cognitive factors.

In summary, the studies of either executive functioning or IQ, typically do not adjust for either of the other of these constructs. It is possible that introduction of these variables to the model lead to over-adjustment. Poor executive functioning could result in lower IQ scores by impairing of children's learning abilities. However, if executive functioning and IQ are determined independent of each other, then adjustment of executive functioning is theoretically justified. A careful interpretation is only possible if results of models with and without adjustment are presented.

CLINICAL IMPLICATIONS

The studies bundled in the current thesis highlight the importance of the prenatal environmental for optimal child neurodevelopment. Although the results presented in this thesis alone do not provide evidence strong enough to justify strong statements for clinical practice they do provide suggestions for both primary and secondary prevention strategies.

As the brain most prominently develops during pregnancy, preconception care could have a positive impact on child neurodevelopment. The provision of biomedical, behavioural and social health interventions to women and couples before conception aimed at improving their health status, and reducing behaviours and individual and environmental factors that contribute to poor maternal and child health outcomes. Previous research indicated that women that wish to become pregnant are more accessible for advice to improve lifestyle behaviours in favour of a healthy pregnancy (50).

Screening for intrauterine growth restriction is part of routine prenatal care. However, this approach is mainly focussed on detection of extreme deviance of foetal growth. There is less awareness of more subtle deviations of foetal growth and the possible implications of having a birth weight in the high extremes of the birth weight distribution as compared to have a birth weight in the lower tail of the birth weight distribution.

Several studies aimed at early intervention in low birth weight premature children to improve academic abilities. The study group of McCormick and colleagues (51) observed that preschool educational programs was related to better cognitive and behavioural development at age 18 years in moderate low birth weight children (2-2.5 kg). In very low birth weight children (<2.0 kg), no beneficial effects of the intervention program was observed. Probably, different pathogenic mechanisms underlay the foetal growth restriction in very low birth children as in moderate low birth weight children that cannot be compensated postnatally. In a recent study, Litt and colleagues (52) described that early gains in IQ from infant interventions did not protect children against the risk of special service use at school. This study furthermore indicated that only a limited proportion of low birth weight children with learning disabilities received special education. Given the vulnerability of preterm low birth weight children for academic and behavioural problems, these birth outcomes could represent an tool for screening for early intervention.

FUTURE RESEARCH

As the foetus is exposed to non-specific prenatal influences, that are difficult to simulate in laboratory conditions, large population-based studies, like the Generation R Study, remain of importance to explore the etiology of neurodevelopmental disorders. Upcoming research fields as epigenetics, biomics and neuro-imaging largely rely on epidemiological designs and large population-based studies. By embedding technological advantages new insights on pathological pathways could be produced.

One of the key challenges in epidemiology is causal inference. Not seldom, several risk factors co-occur in the same, most disadvantaged individuals. Influences considered environmental, could be actually mediated by the shared genetic predisposition of individuals and their parents. Some of the bias could be reduced by longitudinal designs. A time lag between exposure and outcome will help to reduce the likelihood of reverse causality. Currently, attempts are made to start preconceptional cohorts that par excellence could give insight on identification of critical periods for specific prenatal exposures. A longer follow-up of children will reveal which effects of the prenatal environment are transient and which will result in long lasting problems. Studying children longitudinally may also allow for the discovery of protective factors in neurodevelopment that compensate for prenatal adversities.

Most of the current research on prenatal exposures and child development focusses on maternal factors. (Neuro)developmental problems, however, can also result from

paternal exposures (53). Next to the genetic contribution, occupational exposures and adverse environmental and lifestyle factors may put the developing child at risk. Most of the contribution of the father will be indirect, by influencing the maternal environment. While it is already difficult to link the direct, maternal environment to child adversity, it is even harder to investigate possible paternal mechanisms. The inclusion of fathers in future studies will help to continue to disentangle genetic and pregnancy influences.

Ultimately, observational studies provide a first move to perform randomized controlled trials to establish causality. Currently, trials on treatment of maternal thyroid insufficiency on child neurodevelopment are being performed. Several trials on the use of fatty acid supplements during pregnancy have been conducted, but yet no consensus has been reached about the effects on pregnancy and neurodevelopmental outcomes.

Adequate assessment of behavioural and cognitive problems remain a major challenge in child psychiatric epidemiology. To enhance research on biological pathways, phenotypic refinement is warranted. Psychiatric disorders are often not specific. Diagnoses can include many different malfunctions, that not seldom overlap. Revision of taxonomy of psychiatric disorders will remain an ongoing process. As child psychiatric disorders are, by nature, subject to developmental changes the study neurodevelopmental trajectories could improve classification. In addition, the use of multiple informants are used to gain precision in measurement.

The impact of risk factors on the population level varies over time. For example, severe iodine deficiency or Rubella infection of the mother during pregnancy is known to have profound effects on the developing brain. However, as these exposures became very rare after the introduction of iodized salt or vaccines, they currently have limited impact on public health. Historically, individuals had to overcome food scarcity and therefore the impact of obesity was of minor importance for health on the population level given the limited prevalence. In the current century, rates of obesity are rising alarmingly in many parts of the world and are likely to remain of major health concern so for the foreseeable future. Although studies on the detrimental effects of adult obesity are emerging, more research is warranted to map the potential adverse effects of maternal obesity during pregnancy. Maternal obesity not only markedly increases the risk of pregnancy complications, including preeclampsia, and delivery complications but also impact the in utero environment and, thus, on foetal development. Of particular note is the link between maternal obesity and altered brain development and behaviour of the child. In the most unfavourable case, these altered brain functioning itself can promote obesity and lead to a vicious circle of obesity in succeeding generations (12). Recent studies observed fetal epigenetic patterns and neurological circuits are being changed in pregnancies of obese mothers (54, 55). Besides, children of obese mothers are at increased risk for adverse metabolic outcomes including insulin resistance, hypertension and dyslipidaemia, what can also have implications for brain functioning. Children born to obese women were more likely to show symptoms of attention-deficit hyperactivity disorder (ADHD), inattention and difficulty with regulating emotionality. (59, 60). In the current cohort, Brion and coworkers (56) described an association between

maternal overweight and more behavioural problems of the child. However, this association could not be replicated in te British Avon Longitudinal Study of Parents and Children. This indicates that the association initially observed is likely to reflect confounding by socioeconomic or postnatal factors rather that a direct intrauterine effect of overweight.

To explore the effects of maternal obesity on child development, large cohorts with good recording of maternal obesity prior to and during pregnancy with considerable follow up time are needed. A major challenge for human studies would be to distinguish between shared environmental factors and direct intrauterine effects. An integrated approach of genetic, epigenetic, neuroimaging, metabolic and behavioural could shine light on etiopathological mechanisms.

CONCLUSION

From conception onwards a complex interplay between genetic and environmental factors may result in altered trajectories of typical brain development.

In the current thesis, we assessed the association of common prenatal exposures with foetal growth and childhood behaviour and cognition. Although causality cannot be ascertained, our results indicated that maternal subtle thyroid dysfunction and intake of fatty acids influence foetal growth, cognition and behaviour. Alterations in DNA methylation and foetal growth are suggested to underlie the effects of prenatal exposures on child neurodevelopmental outcomes.

The effects of prenatal influences on children's cognition and behaviour in this thesis are modest, as one could expect from common exposures. The clinical heterogeneity of neurodevelopmental disorders is likely to reflect the multiplicity etiological pathways and of moderating and mediating factors involved in symptom expression. However, due to the large number of children at risk, the impact of potential modifiable prenatal risk factors on child neurodevelopmental disorders at the population level, should not be underestimated (57).

Pregnancy and the period of planning pregnancy could be used as a 'window of opportunity´ to reduce the risk of neurodevelopmental problems (58). For women and their partners, pregnancy can be an motivator to reduce unhealthy lifestyles for a healthy child. Ultimately, this will interrupt the potential vicious circle of potential harmful foetal adaptations to an adverse prenatal environment, which could in turn, increase lifestyle and behaviour related problems in succeeding generations.

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Chapter 13

Summary / Samenvatting

SUMMARY

In just 270 days of rapid cell division and tissue differentiation that follow each other iteratively, the zygote transforms into a term newborn. This makes the prenatal phase a critical period, in particular for the complex central nerve system.

In recent years, research after a new understanding of the relationship between the prenatal period and later psychiatric disorder has gained momentum. Primary outcomes of interest are neurodevelopmental disorders, a group of conditions with onset in the developmental period that produce impairments of personal, social, and academic arenas. Previous population-based research repeatedly related weight at birth, seen as a reflection of the intrauterine development, to neurodevelopmental outcomes. Prenatal influences that impair foetal growth could, on the long term, also contribute to disrupted neurodevelopment.

The objective of the current thesis was to extend existing knowledge on the influence of the prenatal environment on foetal growth and children's behaviour and cognition. The studies in this thesis are conducted in the Generation R Study, a prospective population-based cohort study from foetal life until young adulthood in Rotterdam. This creates an unique setting to assess the effects of the prenatal environment on growth and development. In **PART II**, we studied the associations of prenatal exposures on foetal growth. We suggested that maternal hypothyroxinaemia during pregnancy was related to increased growth of the foetal and infant head as compared to children of mothers with normal thyroid functioning (**chapter 2**). The observed growth difference showed a consistent dose–response pattern in early pregnancy with more severe hypothyroxinaemia associated with more severe deviations in head growth than mild hypothyroxinaemia. The difference in head size continued after birth. This study showed that hypothyroxinaemia was not due to lower iodine concentrations suggesting that thyroid problems in this iodine sufficient area seem to have a different origin. We found no indication that a larger head in the first trimester is related to cognitive functioning. Possibly, structural changes not expressed by changes in head size may underlie this cognitive delay.

We related maternal pregnancy plasma n-3 and n-6 fatty acid concentrations to foetal growth velocity and pregnancy outcomes (**chapter 3**). A higher n-3: n-6 fatty acid ratio in mid-pregnancy is associated with a higher foetal growth velocity and better pregnancy outcomes. As described in **chapter 4**, a higher maternal mid-pregnancy TFA-C18:1 plasma concentration was associated with a lower birth weight and placental weight and a higher risk of pre-eclampsia. Although the intake of TFA in our population decreased during the inclusion period, the association with adverse pregnancy outcomes persisted even at lower maternal TFA-C18:1 plasma concentrations.

PART III describes contains two studies on prenatal influences and their association with children's cognition and behaviour. Children of mothers with low urinary iodine concentrations showed higher scores on the problem scales of inhibition and working memory (**chapter 5**). Maternal dietary intake and thyroid hormones did not significantly modify these associations.

Possible explanations for the association between seasonality of birth and child IQ were tested in **chapter 6**. We found spring birth to be associated with more than 1 point lower non-verbal IQ. Adjustment for the different possible mechanisms led to a substantial reduction of this association, to which maternal socio-demographic factors and IQ were prominent contributing factors.

The studies presented in **PART IV** addressed the relationship between DNA methylation and child development. **Chapter 7** describes alterations in DNA methylation of *IGF2DMR* and *H19*, two genes implicated in early growth, to be associated with child birth weight.

We explored the association of DNA methylation profiles at birth and ADHD symptoms of the child at 6 years of age. Lower DNA methylation levels of the 7 genes assessed were associated with more ADHD symptoms (**chapter 8**).

Determinants of maternal one carbon metabolism in early pregnancy and the DNA methylation profiles of the newborn were studied in **chapter 9**. We observed that in particular the maternal *MTHFR* C677T genotype is a determinant of DNA methylation profiles assessed. After adjustment for confounding factors including maternal socio-demographic factors and pregnancy characteristics, the association between maternal folate deficiency and lower DNA methylation in newborns disappeared.

In **PART V** we focussed on the relationship between foetal growth and children's cognition and behaviour. A study of the relationship between birth weight and attention problems at age 6 years is presented in **chapter 10**. This study describes that a higher birth weight was related to less attention problems. From a birth weight of about 3.6 kg or more, however, a higher birth weight did not reduce the risk of attention problems any further. In children of obese mothers, high birth weight may increase the risk of attention problems.

Chapter 11 describes that gestational duration and birth weight, were, independent and with different patterns, of predictive value for childhood non-verbal IQ. Gestational duration showed, even in the a-term-period, a linear association with non-verbal IQ while the association between birth weight and non-verbal IQ showed a curvilinear relation.

In the general discussion, I discussed the main findings in this thesis and addressed methodological issues involved in the studies (**chapter 12**). Some implications of our findings are put forward and recommendations for future research are provided.

SAMENVATTING

In slechts 270 dagen van een iteratief proces van snelle celdeling en weefsel differentiatie transformeert de zygote in een voldragen pasgeborenen. Dit maakt de prenatale fase een kritieke periode, met name voor het ingewikkelde centrale zenuwstelsel.

In de afgelopen jaren heeft onderzoek naar nieuwe kennis van de relatie tussen de prenatale periode en latere psychiatrische stoornissen een impuls gekregen. De belangrijkste uitkomsten zijn neurologische stoornissen; een groep van aandoeningen ontstaan tijdens de vroege ontwikkeling met gevolgen voor het functioneren op persoonlijke, sociale en academische gebieden. Eerder populatieonderzoek toonde herhaaldelijk een verband tussen geboortegewicht, gezien als een weerspiegeling van de intra-uteriene ontwikkeling, en neuro-ontwikkelingsuitkomsten. Prenatale invloeden die foetale groei beïnvloeden kunnen op lange termijn ook bijdragen aan een verstoring van de neuro-ontwikkeling.

Het doel van dit proefschrift was om de bestaande kennis uit te breiden over de invloed van de prenatale omgeving op de foetale groei en gedrag en cognitie van kinderen.

De studies in dit proefschrift werden verricht binnen de Generation R Studie, een groot bevolkingsonderzoek in Rotterdam waarin kinderen en hun ouders worden gevolgd vanaf de zwangerschap. Deze opzet biedt een unieke kans om de effecten van prenatale omgevingsfactoren op de groei en ontwikkeling te onderzoeken.

In **deel 2** bestudeerden wij het verband tussen blootstelling aan prenatale omgevingsfactoren en foetale groei. We suggereerden dat hypothyroxinemie van de moeder tijdens de zwangerschap verband houdt met een toegenomen hoofdgroei van de foetus en de zuigeling in vergelijking met kinderen van moeders met een normaal functionerende schildklier (**hoofdstuk 2**). Het waargenomen verschil in groei tijdens de vroege zwangerschap vertoonde een consistent dosis-respons patroon waarbij ernstige hypothyroxinemie was geassocieerd met grotere afwijkingen in de hoofdgroei dan milde hypothyroxinemie. Het verschil in de grootte van het hoofd werd voortgezet na de geboorte. Deze studie toonde eveneens aan dat hypothyroxinemie niet te verklaren was door lagere jodiumconcentraties in de maternale urine, wat suggereert dat schildklierproblemen in deze jodiumsufficiënte omgeving een andere oorzaak hebben. We vonden geen aanwijzingen dat een groter hoofd in het eerste trimester gerelateerd is aan cognitief functioneren op de kinderleeftijd. Mogelijk liggen structurele veranderingen, die niet tot veranderingen in de grootte van het hoofd leiden, ten grondslag aan cognitieve vertraging.

We relateerden maternale plasma n-3 en n-6 vetzuur concentraties tijdens de zwangerschap aan foetale groeisnelheid en zwangerschapsuitkomsten (**hoofdstuk 3**). Een hogere n-3: n-6-vetzuren was geassocieerd met een hogere foetale groeisnelheid en betere zwangerschapsuitkomsten. Zoals beschreven in **hoofdstuk 4**, was een hogere mid-zwangerschap TFA-C18: 1 plasmaconcentratie geassocieerd met een lager geboortegewicht en placentagewicht en een hoger risico op pre-eclampsie. Hoewel de inname van transvetzuren in onze bevolking daalde tijdens de inclusieperiode van de studie, was de

associatie met ongunstige zwangerschapsuitkomsten ook aanwezig bij lagere maternale TFA-C18: 1 plasma concentraties.

Deel 3 bevat twee studies over prenatale invloeden en hun associatie met cognitie en gedrag van kinderen. Kinderen van moeders met lage urine jodium concentraties hadden hogere scores op de probleem schalen van inhibitie en werkgeheugen (**hoofdstuk 5**). Wanneer maternale dieet en schildklierfunctie warden meegenomen in de analyses veranderde deze associatie niet. Mogelijke verklaringen voor het verband tussen de seizoen van geboorte en het IQ van het kind werden getest in **hoofdstuk 6**. Een geboorte in de lente was geassocieerd met een meer dan 1 punt lager non-verbaal IQ. Correctie voor de verschillende mogelijke mechanismen in de analyse leidde tot een aanzienlijke reductie van dit verband, waarbij maternale socio-demografische factoren en IQ de meest prominent bijdragende factoren waren.

De studies beschreven in **deel 4** gaan in op de relatie tussen DNA methylatie en de ontwikkeling van het kind. **Hoofdstuk 7** beschrijft een associatie tussen veranderingen in DNA methylering van *IGF2DMR* en *H19*, twee genen geassocieerd met vroege groei, en geboortegewicht van het kind. We onderzochten de associatie van DNA methylatie-profielen bij de geboorte en ADHD-symptomen van het kind op 6 jarige leeftijd. Een lagere DNA methylatie niveau van 7 onderzochte genen was geassocieerd met meer ADHD symptomen (**hoofdstuk 8**). Determinanten van het maternale one-carbon metabolisme in vroege zwangerschap en DNA methylatie profielen van de pasgeborene werden bestudeerd in **hoofdstuk 9**. We zagen dat met name het maternale *MTHFR* C677T genotype een determinant was van DNA methylatie-profielen. Correctie door vertekenende factoren, waaronder maternale socio-demografische factoren en zwangerschapskenmerken, deden de associatie tussen maternale folaatdeficiëntie en lagere DNA methylatie bij pasgeborenen verdwijnen.

In **deel 5** hebben we ons gericht op de relatie tussen de groei van de foetus en cognitie en gedrag van kinderen. Een onderzoek naar de relatie tussen geboortegewicht en aandachtsproblemen op de leeftijd van 6 jaar wordt gepresenteerd in **hoofdstuk 10**. Deze studie beschrijft dat een hoger geboortegewicht was gerelateerd aan minder aandachtsproblemen. Vanaf een geboortegewicht van ongeveer 3,6 kg of meer, geeft een hoger geboortegewicht geen verdere afname van het risico op aandachtsproblemen. Bij kinderen van obese moeders, kan een hoog geboortegewicht het risico op aandachts-problemen doen toenemen. **Hoofdstuk 11** beschrijft dat zwangerschapsduur en geboortegewicht, onafhankelijk en met verschillende patronen, van voorspellende waarde waren voor non-verbaal IQ op de kinderleeftijd. Zwangerschapsduur bleek, zelfs binnen de aterme periode, linear geassocieerd met non-verbaal IQ terwijl de associatie tussen geboortegewicht en non-verbale IQ een curvilinear verband volgt.

In de algemene discussie, bediscussieerde ik de belangrijkste bevindingen in dit proefschrift en besprak de methodologische vraagstukken bij de uitgevoerde studies (**hoofdstuk 12**). Enkele implicaties van onze bevindingen worden naar voren gebracht en aanbevelingen voor toekomstig onderzoek worden verstrekt.

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