EARLY HUMAN GROWTH

Periconception Epidemiological and Epigenetic Studies

Marieke I. Bouwland – Both

Early Human Growth: Periconception Epidemiological and Epigenetic Studies

PhD thesis, Erasmus University Rotterdam, The Netherlands

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Early Human Growth

Periconception Epidemiological and Epigenetic Studies

Vroege Humane Groei

Periconceptie Epidemiologische en Epigenetische Studies

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Voor mijn moeder

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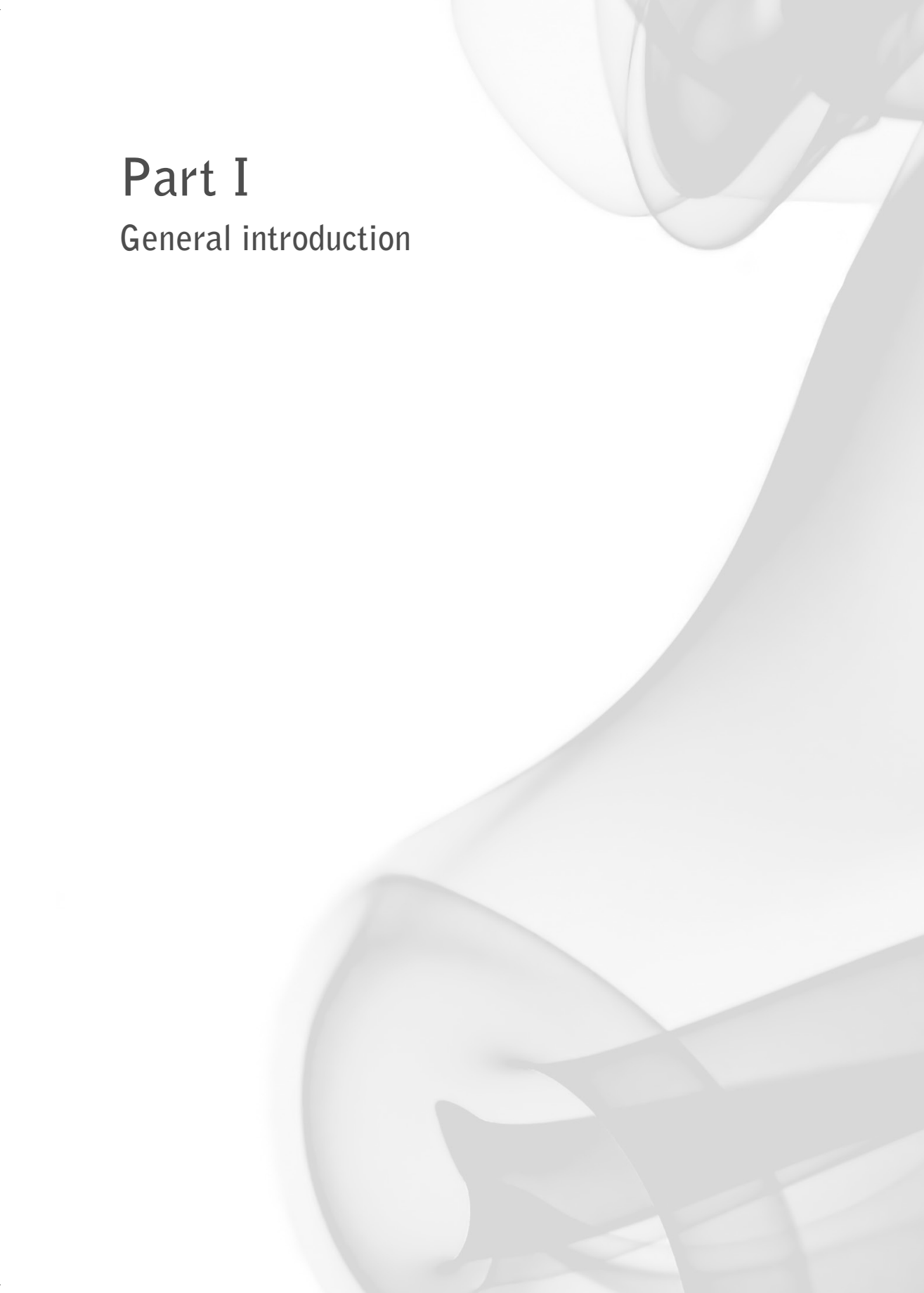
Part I

General introduction



Adverse birth outcomes, such as intrauterine growth restriction, preterm birth and congenital malformations, are major contributors to short and long term morbidity and mortality.[1] From the late eighties of the last century, evidence is accumulating that the course and outcome of pregnancy is not only important for the health of the mother and child, but can also be considered a predictor of future health and disease.[2] A mismatch between pre- and postnatal life can increase the vulnerability of the development of non-communicable diseases.[3,4] This insight led to the Development Origins of Health and Disease (DOHaD) hypothesis, which states that prenatal insults and especially a suboptimal intrauterine environment can result in endocrine and metabolic adaptations in the fetus (reprogramming). Although these adaptations are beneficial to the fetus at first, this eventually could lead to increased risks of non-communicable diseases in adulthood.

Most pregnancy complications originate in the periconceptional period and first trimester growth restriction has been related to an increased risk of adverse birth outcomes, including being born SGA.[5-7] The periconceptional period has been defined based on biological mechanisms as a time span of 14 weeks before conception up to ten weeks after conception.[8] Until recently, epidemiological research has largely neglected the periconceptional period by focusing on fetal size and growth trajectories in the second half of pregnancy and on pregnancy outcome. These outcomes however, are largely influenced by first trimester size and growth trajectories making this an especially vulnerable period in life. Therefore, future research and clinical care will be gradually shifted towards the periconceptional period.[8]

Adequate embryonic and fetal growth and placentation depend on an optimal intrauterine environment, which is determined amongst others by environmental maternal conditions and exposures. The deleterious effects of tobacco smoking have been well established by numerous studies.[7,9,10] Maternal tobacco smoking increases the risk of subfertility, congenital malformations and compromises fetal and infant weight and growth.[11-14] Another important and modifiable environmental exposure is maternal nutrition. Malnutrition indicates over- and under-nourishment in macronutrients, or a relative poor intake of vitamins and minerals compared to the excessive intake of macronutrients.[15] Deficiencies in the essential B-vitamin folate have been linked to the occurrence of neural tube defects and altered fetal growth trajectories.[16-18] All women planning pregnancy are recommended to use 0.4–0.5 mg folic acid per day, which contributes to the prevention of neural tube defects.[19] In addition to micronutrients, the identification of beneficial or harmful dietary patterns have gained interest in the last decades. Periconceptional dietary patterns have been associated with fetal growth, congenital malformations and perinatal outcome.[20-23] However, knowledge on the effects of maternal nutrition on fertility and first trimester pregnancy outcome remains scarce.[21,24,25]

In addition, the prenatal environment is also determined by the metabolic, endocrine, immunological and vascular state of the pregnant woman.[26] Early placentation, which includes trophoblast invasion, spiral artery remodeling and angiogenesis is of great importance for normal fetal growth and development.[27] Defects in early placentation processes are implicated in the pathogenesis of major adverse birth outcomes, including being born small-for-gestational age (SGA) and pre-eclampsia.[28] Several pathways are implicated in placenta development. Both the vascular endothelial growth factor (VEGF) and the plasminogen activator (PA) pathway have been

associated with pregnancy outcome.[29] The influence on fetal growth and subsequent postnatal growth remains to be established.

In recent years, epigenetics have been postulated to be one of the mechanisms underlying the effects of a suboptimal intrauterine environment.[30] Epigenetics is the study of changes in gene function that cannot be explained by changes in DNA sequence.[31] Modifications in DNA methylation are one of the best understood epigenetics mechanisms which predominantly, but not exclusively, occur at cytosine's of CpG dinucleotides.[32] The establishment and maintenance of DNA methylations requires methyl donors from the one carbon pathway, such as folate.[8,33] A shortage or excess of methyl donors or polymorphisms in the genes involved in this pathway, such as methylenetetrahydrofolate reductase (*MTHFR*), can result in variations of DNA methylation.[34]

Each cell has its own epigenetic signature, eventually resulting in the phenotype of the cell and tissue. Epigenetic modifications are relatively stable in somatic cells. However, during development, or some disease situations, genome-wide epigenetic reprogramming occurs. The reprogramming during discrete developmental stages is needed for the resetting of methylation patterns, the erasure of acquired epimutations and to restore the pluripotency of the cell.[32,35] Therefore, the epigenome is particularly susceptible for derangements during developmental stages when adverse prenatal environmental exposures can permanently alter methylation patterns in the offspring.[36] 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.[33]

The *IGF2/H19* imprinted region is one of the best studied imprinted loci. This imprinted domain at human chromosome 11p15.5 contains the maternally imprinted gene Insulin-like Growth Factor-II (*IGF2*) and the paternally imprinted gene *H19*.[37] 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.[38] 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.[39]

OBJECTIVES

This thesis addresses the following main objectives:

1. The relationship between periconception maternal nutrition and early and late fetal growth trajectories and birth outcome.
2. The influence of late first trimester maternal angiogenic factors, fibrinolytic factors and folate concentrations on fetal and postnatal infant growth.
3. Gene-specific DNA methylation in newborns and associations with prenatal parental conditions and exposures, and birth outcomes.

CORE STUDY MATERIAL

Most of the objectives of this thesis were addressed in the Generation R Study. The objective with regards to the epigenetic profile and the risk of developing a neural tube defect (NTD) has been addressed in the Dutch Spina Bifida Study and the Texas Neural Tube Defect Project (**Chapter 5**).

Generation R

The Generation R Study is a prospective population-based cohort study from early pregnancy onwards in Rotterdam, the Netherlands. The Generation R Study was designed to identify early environmental and (epi)genetic determinants of growth, development and health in fetal life and childhood. Eligible mothers were those who were resident in the study area and delivered between April 2002 and January 2006. Enrolment was aimed in the first trimester, but was possible until the birth of the child. Detailed measurements were planned in early pregnancy (<18 weeks of gestation), mid-pregnancy (18–25 weeks gestation) and late pregnancy (>25 weeks gestation) and included fetal ultrasound measurements, physical examinations, collection of biological samples and self-administered questionnaires. In total, 9.778 women were included, of whom 8880 (91%) were included during pregnancy.[40,41]

Dutch spina bifida study

Between August 1999 and December 2001, a case-control triad study was conducted in which mothers of a child with a non-syndromatic meningo(myelo)cèle, i.e., spina bifida, were enrolled in collaboration with the Dutch Spina Bifida Teams of the University Medical Centers of Nijmegen, Utrecht, Groningen, Rotterdam, Leiden, Amsterdam, and the regional hospitals in Tilburg and Zwolle in The Netherlands. Dutch Caucasian women and their children between 1 and 3 years of age were eligible to participate. The spina bifida was diagnosed by a neuro-pediatrician at birth. Control subjects were recruited from acquaintances and nurseries in Nijmegen and surroundings in the Netherlands. The original study population comprised of 132 cases with spina bifida and 236 controls.[42] Participants visited the outpatient clinic of the University Medical Center Radboud in Nijmegen, the Netherlands. Assessments included physical examinations, collection of biological samples and self-administered questionnaires.[43]

Texas Neural Tube Defect Project

Between 1995 and 2000, a case-control study was conducted in a Mexican American population derived from 14 counties along the Texas-Mexico border. Cases were terminations (spontaneous or selective abortions), live births, or stillbirths with any diagnosis of NTD, defined as spina bifida or anencephaly. They were identified through hospitals, birth centers, ultrasound centers, abortion centers, prenatal clinics, genetics clinics, and birth attendants (midwives and non-hospital physicians). Controls were non-malformed live births that occurred in the same counties during that time period. Women were interviewed at home and biological samples were collected. In total, 225 cases and 378 controls were eligible to participate of which 184 cases (82%) and 225 controls (60%) completed the interview.[44,45]

OUTLINE

In **Part II** we address the first and second objective are addressed. In **Chapter 2**, we address the first objective, namely the relationship between periconception maternal nutrition and early and late fetal growth trajectories and birth outcome. **Chapter 3** and **4** addresses the second objective and focusses on the association between biomarkers of placentation in maternal and umbilical cord blood and fetal and childhood growth. In **Part III** we address the third objective. **Chapter 5, 6** and **7** concentrates on gene-specific DNA methylation in newborns and associations with prenatal parental conditions and exposures, and birth outcomes. In **Part III** we provide an overall discussion of the main findings of this thesis, including recommendations for future research and implications for clinical practice (**Chapter 8**) and provide a summary of the results described in this thesis (**Chapter 9**).

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Part II

Epidemiological studies

Chapter 2

Maternal dietary patterns and early fetal growth

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ABSTRACT

Objective | This study aims to identify periconceptional maternal dietary patterns associated with crown-rump length (CRL), estimated fetal weight (EFW) and birth weight.

Study design | From a population-based prospective birth cohort study in Rotterdam, the Netherlands, 847 pregnant Dutch women were selected. Women were included between 2001 and 2005. Information on nutritional intake was collected by a semi quantitative food frequency questionnaire. For extracting dietary patterns, principal component factor analysis was used. Fetal growth was assessed using ultrasound measurements. Information on birth outcomes was retrieved from medical records. Multivariate regression analyses were used. The main outcome measures included crown-to-rump length, estimated fetal weight in second and third trimester and birth weight.

Results | An 'energy-rich dietary pattern' was identified, characterized by high intakes of bread, margarine and nuts. A significant association was shown between a high adherence to this dietary pattern (difference, mm: 2.15, 95% confidence interval 0.79; 3.50) and CRL (P-trend = 0.015). No association was revealed between increasing adherence to this dietary pattern and EFW in second or third trimester, or birth weight.

Conclusion | This study suggests that increasing adherence to an energy-rich dietary pattern is associated with increased CRL in the first trimester.

INTRODUCTION

Embryonic and fetal growth and placentation are determined by several pathways and molecular biological processes, in which multiple genetic and environmental factors interact. The first trimester is a sensitive period of pregnancy. Despite associations between adverse exposures during early pregnancy and the occurrence of pregnancy complications and adverse birth outcomes, this period is largely neglected in antenatal care.[1-4] Ultrasound measurements in the first trimester are mainly used to estimate crown-rump length (CRL) for an accurate determination of gestational age. Discrepancies between menstrual and ultrasound estimations usually lead to gestational age adjustment as it is interpreted as earlier/delayed ovulation or implantation.[5] These discrepancies can also be the result of differences in embryonic growth.[6] Chromosomal abnormalities can affect embryonic growth[7] and it has been shown that maternal characteristics, such as age, smoking and the use of a folic acid supplement may also affect embryonic growth.[1]

Another important exposure during pregnancy is nutrition, interest in which has increased in the last decade. The Mediterranean dietary pattern (high intakes of vegetables, fruits, nuts, fish, olive oil and moderate intakes of alcohol) has been associated with better embryo quality, increased fetal growth and reduced risk of adverse birth outcomes.[8-11] Important characteristics of this pattern are its high content of methyl donors for the one-carbon pathway. This pathway plays a vital role in biological processes implicated in growth and programming, especially periconceptional. So far no information is available on the influence of the quality and the quantity of specific periconceptional maternal dietary patterns on first trimester growth, and on the biomarkers of the one-carbon pathway. Therefore, in a population-based prospective birth cohort, we aimed to study (1) first-trimester maternal dietary patterns; (2) the influence of the dietary patterns on the biomarkers of the one-carbon pathway; and (3) associations with growth in each trimester of pregnancy and birth weight.

MATERIALS AND METHODS

Design and study population

This study was embedded in the Generation R Study, a population-based prospective cohort from early pregnancy onwards (Rotterdam, the Netherlands) and approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, the Netherlands.[12] Written informed consent was obtained from all mothers for both maternal and child data. Eligible women were those who were resident in the study area and who delivered between April 2002 and January 2006.

As dietary habits are culturally determined,[13] only Dutch women were selected for this study. Furthermore, the Food Frequency questionnaire that was used was not validated in non-Caucasian women. A total of 2243 prenatally enrolled Dutch women with a singleton pregnancy and a CRL measurement were included (**Figure 1**). Only women with a CRL measurement within the recommended gestational age range (10^{+0} weeks to 13^{+6} weeks) were selected. Mothers with

an unknown first day of the last menstrual period (LMP) or mothers with no regular menstrual cycle of 28 ± 4 days were excluded (n = 1179) to minimize confounding of gestational age. The LMP was obtained from the referring letter and confirmed at enrolment. Additional information on regularity and cycle duration was obtained. Of the remaining 1064 women, 961 filled out a food frequency questionnaire (FFQ). If mothers participated in Generation R with two or more pregnancies, one sibling was randomly selected to avoid bias due to paired data (n = 87). After exclusion of women who underwent any form of fertility treatment (n = 10) or who reported drug abuse (n = 17), 847 women were eligible for present study.

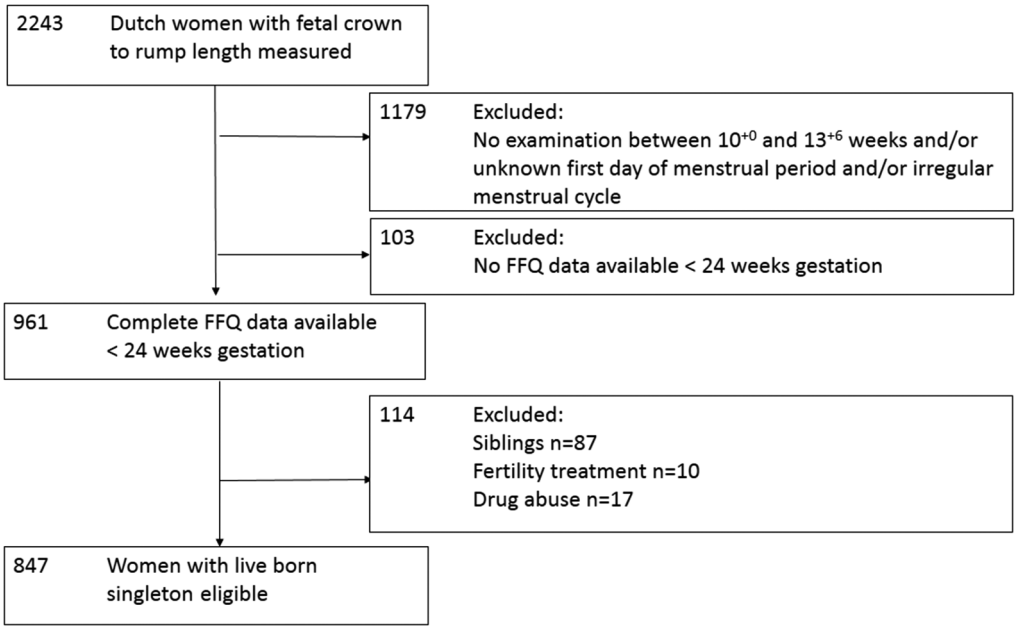

Figure 1 | Flow chart.

Fetal ultrasound measurements and birth outcomes

Standard ultrasound planes and intraclass correlation coefficients for first-trimester measurements were used as described previously.[14,15] Fetal growth measurements were performed using standardized ultrasound procedures in second trimester (median 20.3 weeks, 90% range 19.1–22.0) and third trimester (median 30.1 weeks, 90% range 29.0–31.9). Estimated fetal weight (EFW) was calculated using Hadlock's formula.[16] Ultrasound examination was performed using an Aloka model SSD-1700 (Tokyo, Japan) or the ATL-Philips Model HDI 5000 (Seattle, WA, USA). Gestational age-adjusted standard deviation scores (SDS), based on reference growth curves from the entire study population, were constructed for CRL and EFW.[1,14] Information on date of birth; birth anthropometrics and gender of the child were obtained from community midwife and

hospital registries. Gestational age and sex-adjusted SDS for birth weight were constructed using standards from Usher and McLean.[17]

Dietary assessment

At enrolment (median 12.3 weeks, 90% range 11.0–13.7), nutritional intake of the previous 3 months was assessed using a modified, validated semi quantitative FFQ, consisting of 293 food items and structured according to meal patterns.[18] Questions included consumption frequency, portion size, preparation method and food additions. Portion sizes were estimated using household measures and photographs.[19] To calculate average daily nutritional values the Dutch food composition Table 2006 was used.[20] The original food items were reduced to 20 predefined food groups (listed in **Table 1**) based on origin, culinary usage and nutrient profiles. This was followed by principal component factor analysis (PCA) applied for energy intake unadjusted food groups of the women to construct overall dietary patterns. The PCA aims to explain the largest proportion of variation in food groups in terms of a few linear functions called principal components. A dietary pattern consists of a selection of the initial food groups, each with its own coefficient, defining the observed correlation of the food group with the dietary pattern. The three most prevalent dietary patterns with the highest explained variance were selected.[21] A woman was given scores for each of the dietary patterns. According to their personal score the women were stratified into three equally sized groups and labelled as having a low; intermediate or high adherence to the dietary pattern. The dietary patterns were analyzed in a continuous and categorical measure. This approach was chosen to explore both linearity and nonlinearity of the association.

Biomarkers of the one-carbon pathway

Venous blood serum and plasma samples were drawn and stored at -80°C and at the same time the CRL was measured and the FFQ was handed out.[22] Biomarkers of the one-carbon pathway, folate and total homocysteine in plasma and vitamin B12 in serum, were assessed. Between-run coefficients of variation for the biomarkers are listed in **Supplement Table S1**. All three biomarkers were available in 81.3% of the women (n = 689/847). No significant difference in nutritional intake was observed between women with and without all three biomarker concentrations available (P-value = 0.54).

Statistical analyses

The analyses were performed using multiple linear regression models with the identified dietary pattern as regressor (as a continuous and categorical measure) and CRL (SDS) as response of outcome. This model was compared with a different approach to extract dietary patterns, namely multiple linear regression analyses where each food group was included separately as a continuous measure to predict CRL. In additional analyses, multiple linear regression analyses were performed to assess associations between the dietary pattern and SDS of EFW in second/third trimester and birth weight. The CRL SDS was added to the latter model to assess whether the effect on CRL mediates the effect on EFW and birth weight.

The final multiple regression analyses take into account potential confounders, which were selected on previous literature and determined a priori. From self-administered questionnaires, data were available on maternal age, education, marital status, parity, smoking, folic acid supplement use, nausea/vomiting/fever during first trimester, comorbidity (chronic hypertension and/or heart disease and/or diabetes and/or high cholesterol and/or thyroid disease and/or systemic lupus erythematosus and/or multiple sclerosis), repeated miscarriages (two or more miscarriages), sexually transmitted diseases and the season in which the FFQ was filled out. Education was assessed by the highest completed education and classified as (1) low (none/primary); (2) medium (secondary); (3) high (college/university). 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 (preconceptional or postconceptional start); (2) no folic acid supplement use. At enrolment maternal weight and height were measured to calculate body mass index (BMI, kg/m^2). Information on fertility treatment was obtained from community midwives and obstetricians. Blood pressure was measured using the validated Omron 907 automated digital oscillometric sphygmomanometer (OMRON Healthcare Europe B.V., Hoofddorp, the Netherlands). The mean value of two blood pressure readings over a 60-second interval was documented. Covariates were selected as confounders by testing whether the potential confounder changed the dietary pattern as a continuous measure with at least 10% in the exploratory analyses. By using this approach, maternal age, maternal BMI, maternal folic acid supplement use, duration of the last menstrual cycle and fetal gender were included in the final analyses. Maternal and paternal height, paternal BMI, maternal educational level, parity, maternal smoking, mean diastolic blood pressure and mean systolic blood pressure at intake were included by default. All variables were added to the model simultaneously. We have tested for potential interactions with the dietary pattern. No significant interaction term was observed in our study population. Therefore, no interaction terms were added to the model.

Missing data on maternal BMI (0.4%), paternal height (9.3%), paternal BMI (9.7%), maternal educational level (0.5%), parity (0.1%), maternal smoking (7.0%), the use of folic acid supplement (14.9%) and maternal diastolic (0.7%) and systolic (0.7%) blood pressure were completed on the Dutch population with a CRL measurement (n = 2243) using the Markov-Chain-Monte-Carlo multiple imputation technique.[23] Details of the multiple imputation model are provided in the **Supplement Table S2**. For all analyses, results including imputed missing data are presented. All analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Three major factor were identified in 847 women of which the correlation coefficients are shown in **Table 1**. These three principal components explained the highest percentage of variance of all the principal components and together, they explained 29.8% of the variance.[21] The first component was labelled the Mediterranean dietary pattern, explaining 12.3% of the variance of dietary intake

of the total study group. It comprised high intakes of vegetables, legumes, pasta/rice, dairy, fish/shellfish, vegetable oils, alcohol, non-sweetened non-alcoholic beverages and low intakes of processed meat (all $r \geq 0.20$ and P-value <0.05). The second component was labeled the energy-rich dietary pattern, explaining 9.9% of the total variance and comprising high intakes of bread/breakfast cereals, margarine, nuts, snacks/sweets and non-sweetened nonalcoholic beverages and low intakes of sweetened, nonalcoholic beverages (all $r \geq 0.20$ and P-value <0.05). The third component was labelled the Western dietary pattern, explaining 7.6% of the total variance and comprising high intakes of potatoes, pasta/rice, dairy, fresh meat, processed meat, margarine and alcohol and low intakes of nuts, fish/shellfish (all $r \geq 0.20$ and P-value <0.05). The Mediterranean and Western dietary patterns showed no significant association with CRL. For that reason we continue by describing the analysis of the energy-rich dietary pattern.

Table 1 | Relationships between food groups and the identified dietary patterns.

			Mediterranean dietary pattern (12.3%)	Energy-rich dietary pattern (9.9%)	Western dietary pattern (7.6%)
Food group	**Median**[1]	**90% range**	**ρ**	**ρ**	**ρ**
Potatoes	48.0	7.0–131.9	-0.07*	0.02	0.32*
Vegetables	142.5	62.5–260.6	0.76*	0.05*	-0.02
Legumes	6.4	0.0–21.9	0.35*	0.10*	-0.14*
Fruit	169.6	25.6–412.8	0.02	-0.01	-0.06*
Nuts	18.4	1.6–53.4	0.14*	0.68*	-0.33*
Eggs	10.7	0.0–28.6	0.11*	0.02	-0.05*
Bread, breakfast cereals	145.4	52.8–242.9	0.06*	0.80*	0.09*
Pasta, rice	46.0	6.6–114.7	0.68*	0.07*	0.29*
Dairy	466.0	122.3–950.6	0.24*	0.16*	0.22*
Fresh meat	53.2	4.6–106.5	0.17*	-0.15*	0.74*
Processed meat	24.6	0.2–65.2	-0.27*	0.14*	0.67*
Fish, shellfish	10.4	0.0–32.2	0.50*	-0.07*	-0.20*
Vegetable oils	7.8	0.8–23.0	0.49*	0.10*	-0.01
Margarine	15.7	0.0–48.4	-0.12*	0.59*	0.23*
Butter	0.0	0.0–17.8	-0.03*	-0.01	-0.02
Condiments, starches, sauces	29.5	9.8–63.7	-0.01	0.00	-0.04*
Snacks, sweets	89.4	27.3–211.5	-0.08*	0.26*	0.17*
Alcoholic beverages	0.0	0.0–42.9	0.28*	0.00	0.25*
Non-sweetened, non-alcoholic beverages	1107.1	259.3–2142.9	0.26*	0.28*	0.04*
Sweetened, non-alcoholic beverages	322.5	31.9–1215.8	-0.01	-0.24*	0.09*

[1] Median daily intake in grams; ρ: Spearman's Rank correlation coefficient; *p-value<0.05.

Table 2 | Maternal and fetal characteristics.

	Excluded Dutch women with CRL measurement	Study group		Energy-rich dietary pattern			
				Low adherence	Intermediate adherence	High adherence	
	n = 1,396	n = 847	P-value[1]	n = 283	n = 282	n = 282	P-value[2]
MATERNAL							
Age (years)*	30.8 (4.2)	31.7 (4.0)	<0.001	31.1 (4.1)	31.8 (4.1)	32.2 (3.7)	0.004
Maternal BMI†	23.5	23.1	NS	23.3	23.5	22.8	NS
	(19.0–30.7)	(19.6–32.3)		(19.7–32.5)	(19.3–32.2)	(19.5–28.1)	
<19.9 (%)	10.7	8.9	NS	6.8	11.4	8.5	0.032
20–24.9 (%)	58.5	58.9		61.2	52.7	63.5	
25–29.9 (%)	21.0	23.5		21.4	28.5	20.9	
30–34.9 (%)	6.2	5.9		8.5	4.3	5.0	
>35 (%)	3.7	2.8		2.1	3.2	2.1	
Education (%)			0.001				NS
Low	4.1	2.3		3.2	1.1	2.5	
Medium	39.7	33.9		38.2	34.5	29.1	
High	56.2	63.8		58.6	64.4	68.4	
Household income (%)			0.016				0.045
<1200 euro	5.3	3.3		5.0	2.7	2.4	
1200–2200 euro	20.8	16.8		17.9	12.2	20.4	
>2200 euro	73.9	79.9		77.1	85.1	77.3	
Parity (%)			NS				NS
0	59.3	62.1		66.1	60.1	59.9	
≥1	40.7	37.9		33.9	39.9	40.1	
Smoking (%)			NS				0.001
Yes	16.2	12.6		18.7	8.7	10.3	
Until pregnancy recognition	10.4	10.4		13.0	8.0	10.2	
No	73.5	77.0		68.3	83.3	79.5	
Folic acid supplement use (%)			0.017				0.04
Adequate	57.8	63.4		56.5	68.8	64.6	
Inadequate	31.9	29.5		33.3	26.3	29.1	
No	10.3	7.1		10.2	4.9	6.3	
Daily nausea (%)	32.0	28.2	NS	26.7	30.0	27.8	NS
Daily vomiting (%)	5.3	3.6	NS	4.2	3.4	3.1	NS
Pre-eclamspia (%)	2.1	2.1	NS	1.6	3.5	1.2	NS

Table 2 | Maternal and fetal characteristics *(Continued)*.

	Excluded Dutch women with CRL measurement	Study group		Energy-rich dietary pattern			
				Low adherence	Intermediate adherence	High adherence	
	n = 1,396	n = 847	P-value[1]	n = 283	n = 282	n = 282	P-value[2]
FETAL							
Male gender (%)	50.3	49.6	NS	50.4	48.2	50.4	NS
Gestational age at birth (weeks)[†]	39.9 (36.3–42.1)	40.1 (36.7–42.0)	0.031	40.0 (35.8–42.1)	40.3 (37.3–42.0)	40.1 (36.9–42.1)	NS
Small for gestational age (<5th birth centile) (%)	NA	4.5	NA	6.2	3.8	3.5	NS

Data on the group of Dutch Generation. R participants with CRL measurement, but excluded from the study group (n = 1,396) and the study group (n = 847) are presented. Values are presented as *mean (SD); [†]median (90% range); NS: not significant; [1]t- and chi-square tests are used to test overall differences between the total group of Dutch Generation R participants (without the study group) and the study group [2]ANOVA and chi-square tests were used to test overall differences between maternal and fetal characteristics and the Energy-rich dietary pattern. Gestational age was based on LMP. NA: not available, as these women had not reliable gestational age based on LMP, SGA based on LMP could not be determined.

Maternal and fetal characteristics are shown in **Table 2**. Women with high adherence to the energy-rich dietary pattern were older. Furthermore, differences were observed between the adherence categories and BMI, household income, smoking habits and the use of a folic acid supplement. Nonresponse analyses showed that excluded mothers were younger, lower educated, had a lower income, were less frequently folic acid supplement users and gave birth earlier. Maternal and fetal characteristics for the Mediterranean and Western dietary patterns are shown in **Supplement Table S3.**

The biomarker concentrations and nutrient intakes are shown in **Table 3**. Lower homocysteine levels were observed in women with a high adherence to the energy-rich dietary pattern. High adherence to the energy-rich dietary pattern was associated with a higher energy intake. These women showed a relatively high total fat intake, but also a high protein and carbohydrate intake. Most energy was derived from fats and less from carbohydrates and proteins. Furthermore, higher intakes of saturated fats, monounsaturated and polyunsaturated fats, vegetable proteins, linoleic acid and fibers were observed. The biomarker concentrations and nutrient intakes for the Mediterranean and Western dietary patterns are shown in **Supplement Table S4**.

Table 3 | Biomarker concentrations and nutrient intakes.

			Energy–rich dietary pattern			
		Total (n = 847)	Low adherence (n = 283)	Intermediate adherence (n = 282)	High adherence (n = 282)	Linear trend analyses
	ρ	Median (90% range)	Median (90% range)	Median (90% range)	Median (90% range)	
BIOMARKER CONCENTRATIONS						
Folate (nmol/L), plasma	0.07	20.5 (7.65–35.2)	20.7 (6.7–33.5)	20.3 (8.7–35.2)	21.0 (8.3–36.5)	NS
Folic acid supplement use	0.09	24.2 (11.1–37.0)	24.6 (8.5–37.4)	23.0 (11.1–35.3)	24.6 (12.5–37.4)	NS
No supplement use	-0.06	16.3 (6.1–30.7)	17.0 (5.9–32.7)	16.4 (5.9–29.1)	15.0 (6.5–30.0)	NS
tHcy (µmol/L), plasma	-0.15	7.0 (5.0–10.2)	7.2 (4.9–10.5)	7.0 (5.0–10.6)	6.7 (5.0–9.7)	0.006
Folic acid supplement use	-0.13	7.3 (5.0–9.4)	7.0 (4.9–10.0)	6.8 (4.9–9.6)	6.6 (5.0–9.0)	NS
No supplement use	-0.08	6.8 (5.4–12.1)	7.4 (4.9–12.1)	7.4 (5.4–13.4)	7.1 (5.3–11.6)	NS
Vitamin B12 (pmol/L), serum	-0.04	174.0 (90.0–368.0)	191.0 (90.1–349.7)	171.0 (89.1–334.9)	174.5 (90.5–401.8)	NS
Folic acid supplement use	0.04	175.0 (90.2–379.8)	189.0 (86.5–327.6)	171.0 (89.0–427.4)	187.0 (100.2–396.0)	NS
No supplement use	-0.22	173.0 (88.6–380.2)	206.0 (91.1–435.7)	171.0 (88.4–358.6)	161.0 (78.8–441.2)	NS
ENERGY/MACRONUTRIENTS						
Energy (KJ/day)	0.58	8847 (5564–12726)	7171 (4772–10978)	8771 (6370–11685)	10446 (7373–13473)	<0.001
Fat (% of energy)	0.26	36.3 (27.3–44.3)	34.6 (25.3–43.6)	35.7 (27.7–42.8)	38.3 (29.4–46.1)	<0.001
Total fat (g/day)*	0.65	83.3 (51.0–126.9)	35.7 (39.9–99.7)	82.8 (55.4–116.4)	104.8 (70.0–139.3)	<0.001
Saturated fats (g/day)*	0.50	30.6 (18.4–48.5)	25.1 (15.0–41.8)	30.3 (19.9–45.1)	37.1 (23.8–55.1)	NS
Mono-unsaturated fats (g/day)*	0.58	30.2 (17.5–46.6)	24.5 (14.4–37.5)	29.6 (19.4–43.4)	37.1 (25.6–50.7)	NS
Poly-unsaturated fats (g/day)*	0.72	19.1 (9.6–32.7)	13.6 (8.6–21.9)	19.0 (11.5–27.6)	26.6 (17.6–39.5)	<0.001

Table 3 | Biomarker concentrations and nutrient intakes *(Continued)*.

			Energy–rich dietary pattern			
		Total (n = 847)	Low adherence (n = 283)	Intermediate adherence (n = 282)	High adherence (n = 282)	Linear trend analyses
	ρ	Median (90% range)	Median (90% range)	Median (90% range)	Median (90% range)	
ENERGY/MACRONUTRIENTS						
Linoleic acid (g/day)*	0.73	15.5 (7.2–27.0)	10.7 (6.3–18.0)	15.3 (8.6–22.5)	22.1 (13.9–33.2)	<0.001
Cholesterol (mg/day)*	0.17	170.9 (91.6–279.1)	159.2 (84.1–273.7)	171.2 (99.7–284.8)	181.1 (103.4–272.9)	<0.001
Protein (% of energy)	-0.15	14.9 (11.4–19.3)	15.2 (11.3–20.2)	15.0 (11.7–19.1)	14.4 (11.3–17.6)	<0.001
Total protein (g/day)*	0.47	79.1 (47.9–112.0)	66.8 (40.7–96.3)	79.1 (57.0–106.1)	88.4 (63.3–119.2)	NS
Vegetable protein (g/day)*	0.76	30.4 (16.5–46.6)	22.7 (14.5–32.5)	30.7 (22.2–40.3)	38.7 (28.6–52.6)	<0.001
Animal protein (g/day)*	0.17	47.4 (25.9–73.2)	44.5 (23.7–68.4)	47.9 (28.4–72.4)	49.4 (28.6–78.2)	<0.001
Carbohydrate (% of energy)	-0.16	48.3 (39.2–59.1)	49.7 (38.5–61.3)	48.5 (40.7–58.1)	47.0 (38.5–57.7)	0.001
Total carbohydrate (g/day)*	0.42	254.7 (149.6–397.6)	212.5 (115.4–359.9)	251.1 (170.3–381.0)	293.0 (192.6–412.8)	<0.001
Carbohydrates mono- and di-saccharides (g/day)*	0.19	139.8 (69.6–242.7)	124.7 (60.6–238.8)	137.6 (70.2–244.0)	154.2 (81.6–250.2)	<0.001
Carbohydrates polymers (g/day)*	0.63	113.5 (63.2–172.4)	84.3 (50.6–133.5)	116.6 (82.6–154.7)	135.2 (98.7–195.3)	<0.001
Fibers (g/day)*	0.62	23.3 (13.3–35.6)	17.8 (10.4–28.1)	23.5 (15.7–32.5)	28.3 (21.0–40.8)	<0.001

Venous blood serum and plasma samples were drawn at enrolment (median 12.4 weeks gestation, 90% range 11.0–13.7). Linear trend analyses reflect the change in biomarker concentration, energy or macronutrient in SD-score per unit (factor score) change, adjusted for gestational age at venous puncture. *additionally adjusted for energy intake; ρ Spearman's Rank correlation coefficient; NS: not significant.

The associations between maternal adherence to the energy-rich dietary pattern and CRL are presented in **Table 4**. In the univariate analyses, positive associations between CRL and high adherence (difference, mm = 2.15, 95% confidence interval (95% CI) 0.79; 3.50 and SD = 0.28, 95% CI 0.10; 0.47) to the energy-rich dietary pattern were observed. In the multivariable analyses these associations remained significant among women with high adherence (difference, mm = 1.62, 95% CI 0.52; 2.72 and SD = 0.23, 95% CI 0.08; 0.38) to the energy-rich dietary pattern.

Additional adjustment for energy resulted in significant associations among women with high adherence (difference, mm = 1.84, 95% CI 0.49; 3.18 and SD = 0.25, 95% CI 0.06; 0.43) to the energy-rich dietary pattern. These associations remained after multiple testing adjustments (three independent factors). Thereafter, we restricted the analyses to women with a gestational age based on LMP within 7 days of the gestational age based on CRL (n = 785). The results attenuated, but a significant association between the high adherence group and CRL remained in the univariate (difference in mm = 1.48, 95% CI 0.33; 2.63 and SD = 0.21, 95% CI 0.05; 0.38) and multivariate analyses (difference in mm = 1.04, 95% CI 0.10; 1.99 and SD = 0.15, 95% CI 0.01; 0.29). Finally, we compared the analyses, performed with PCA, with a multiple linear regression model where the food groups were included continuously to predict CRL. This model showed an association between CRL and the intake of fresh meat (difference in mm = 0.023, 95% CI 0.002; 0.044 and SD = 0.003, 95% CI 0.000; 0.006).

Table 4 | Associations between the degree of adherence to the Energy-rich dietary pattern and CRL.

	CRL in mm, effect size (95% CI)		CRL in SD-score, effect size (95% CI)	
Energy-rich dietary pattern	**Crude***	**Adjusted†**	**Crude•**	**Adjusted†**
Low adherence	Reference	Reference	Reference	Reference
Intermediate adherence	1.17 (-0.0; 2.34)	0.87 (-0.23; 1.96)	0.13 (-0.03; 0.29)	0.10 (-0.05; 0.25)
High adherence	2.15 (0.79; 3.50)[2]	1.62 (0.52; 2.72)[2]	0.28 (0.10; 0.47)[1]	0.23 (0.08; 0.38)[2]
Linear trend analyses**	0.64 (0.18; 1.09)[2]	0.56 (0.11; 1.01)[1]	0.09 (0.02; 0.15)[1]	0.08 (0.01; 0.14)[1]

Results from multiple linear regression analyses. Values are presented as regression coefficients (95% confidence interval), representing the change in CRL (mm or standard deviation score (SD-score)) compared to the reference category. **Linear trend analyses reflect the change in CRL (SD-score) as a continuous measure per unit (factor score) change; *Adjusted for gestational age, duration of last menstrual cycle; •Adjusted for duration of last menstrual cycle; †Additionally adjusted for maternal age, maternal and paternal BMI, maternal and paternal height, maternal educational level, fetal gender, parity, educational level, smoking, folic acid supplement use, mean diastolic blood pressure, mean systolic blood pressure; [1]P-value <0.05; [2]P-value <0.01.

The associations between maternal adherence to the energy-rich dietary pattern and EFW and birth weight are shown in **Table 5**. The energy-rich dietary pattern was not associated with EFW in second or third trimester or with birth weight. After addition of the SDS for CRL to the multivariable model, the effect estimates for the SDS for EFW in the second and third trimester and birth weight were attenuated.

Table 5 | Associations between the degree of adherence to the Energy-rich dietary pattern and EFW and birth weight.

	EFW second trimester	EFW third trimester	Birth weight
Energy-rich dietary pattern	**Effect size (SD-score), 95% CI**	**Effect size (SD-score), 95% CI**	**Effect size (SD-score), 95% CI**
Model 1			
Low adherence	Reference	Reference	Reference
Intermediate adherence	0.04 (-0.12; 0.20)	0.15 (-0.02; 0.32)	0.05 (-0.13; 0.23)
High adherence	0.14 (-0.02; 0.30)	0.14 (-0.03; 0.31)	0.15 (-0.03; 0.33)
Linear trend analyses	0.05 (-0.02; 0.12)	0.06 (-0.01; 0.12)	0.04 (-0.04; 0.11)
Model 2			
Low adherence	Reference	Reference	Reference
Intermediate adherence	-0.01 (-0.15; 0.12)	0.11 (-0.04; 0.26)	0.04 (-0.14; 0.22)
High adherence	0.01 (-0.12; 0.15)	0.03 (-0.12; 0.18)	0.10 (-0.08; 0.28)
Linear trend analyses	0.01 (-0.05; 0.06)	0.02 (-0.04; 0.08)	0.02 (-0.05; 0.09)

Results from multiple linear regression analyses. Values are presented as regression coefficients (95% confidence interval), representing the change in standard deviation score (SD-score) of EFW or birth weight compared to the reference category. Linear trend analyses reflect the change in EFW or birth weight in SD-score per unit (factor score) change. Model 1: Adjusted for duration of last menstrual cycle, maternal age, maternal and paternal BMI, maternal and paternal height, fetal gender, parity, educational level, smoking, folic acid supplement use and mean diastolic blood pressure, mean systolic blood pressure. Model 2: Additionally adjusted for the SD score of CRL.

DISCUSSION

In 847 Dutch women derived from a multiethnic population based birth cohort, increasing maternal adherence to an energy-rich dietary pattern in the periconceptional period is significantly associated with CRL. These associations are independent of several covariates.

The identified dietary pattern is determined by higher intakes of fats (especially unsaturated fats), but also by higher intakes of carbohydrates and proteins and is therefore labeled 'energy-rich'. It consists of higher intakes of bread and nuts, containing carbohydrates and vitamin B6. Nuts also contain high amounts of methionine. In the one-carbon pathway methionine is an important substrate. Vitamin B6 is a cofactor. In addition, methionine, choline, folate and vitamin B12 are essential one-carbon donors for this pathway. Deficiencies of cofactors and substrates result in hyperhomocysteinaemia and excessive release of reactive oxidative species. In 50%, homocysteine is trans-sulphurated, a vitamin B6-dependent conversion, which is stimulated by high methionine intake to prevent hyperhomocysteinaemia. Therefore, the observed lower plasma concentrations of total homocysteine in women with high adherence to the energy-rich dietary pattern is in accordance with high intakes of methionine and vitamin B6.[24]

A study in the same cohort comprising 1631 multiethnic women showed that maternal age, diastolic blood pressure, higher hematocrit levels, smoking and folic acid supplement use were associated with CRL at the end of the first trimester.[1] In agreement with our study, they found no

effects of maternal weight, height or BMI on CRL in this study. The effects of nutrition found in the present study are small, but comparable in effect size to these associations. The association between the energy-rich dietary pattern and CRL may be attributable to the first-trimester nourishment of the embryo by mainly energy-rich carbohydrate secretions from the endometrial glands.[25] Another explanation might be that fetal growth is being programmed in the first trimester. Moreover, up to 11 weeks of gestation the embryo develops in a stable nutritional environment. This may explain why the effects are only found on CRL and seem to disappear in the second trimester. Unfortunately, we did not observe an effect of adherence to the energy-rich dietary pattern and fetal growth from the second trimester onwards. Therefore, the clinical implications remain unclear. However, first-trimester growth restriction has been linked to an increased risk of adverse birth outcomes and growth acceleration in early child hood in the same cohort.[1] The lack of association of nutrition with growth from the second trimester onwards may be a result of our small study group. This is substantiated by the association of the dietary pattern with lower homocysteine levels. It has to be elucidated whether increased growth in the first trimester is a beneficial predictor for subsequent pregnancy course and outcome.

Although maternal nutrition in pregnancy has been studied extensively, this is the first study showing associations between maternal nutrition and early growth. Godfrey et al.[26] showed an inverse association between the energy intake in early pregnancy and placental weight (difference in grams = 38, 95% CI 5; 72) and birth weight (difference in grams = 134, 95% CI 11; 256). Maternal use of a Traditional dietary pattern in early pregnancy (high intakes of potatoes, meat, vegetables) reduced the risk of having a small-for-gestational-age (SGA) child by 14% (95% CI 0.75; 0.99).[27] In contrast, Knudsen et al.[28] showed that maternal adherence to a Western dietary pattern, based on red and processed meat and high-fat dairy, was associated with an increased risk of SGA (odds ratio = 0.74, 95% CI 0.64; 0.86 for women in the Health Conscious class compared with women in the Western Diet class). The Dutch Famine study demonstrated that exposure to famine during early pregnancy resulted in a risk for cardiovascular disease in later life, but independent of birthweight.[29] Whether our finding is beneficial for pregnancy course and outcome warrants further investigation.

Previous research within Generation R revealed that low adherence to a Mediterranean dietary pattern was associated with an increased risk of SGA (relative risk = 2.78, 95% CI 1.64; 4.76).[30] The Mediterranean dietary pattern identified in this study, however, was not associated with CRL. This difference between our study and that by Timmermans et al.[30] can have multiple causes. First, we used a different method to define the dietary patterns. Second, we have a smaller study population. The analyses were performed on this smaller group and therefore our finding might be explained by this selection. Third, we defined 20 food groups, whereas Timmermans et al.[30] defined 21 food groups. Last, we were also interested in the quantity of the used food groups. Therefore, we did not adjust the food groups for energy before we defined the dietary patterns. Inadequate dietary intake can indicate overnutrition and undernutrition as well as vitamin and mineral deficiency. Our study implies that both the quality and quantity of the dietary pattern matters in the first trimester of pregnancy. It must be noted that the Mediterranean diet is currently considered to constitute a healthier diet.

Methodological considerations

Some strengths and limitations have to be addressed. This study was embedded in a large cohort from whom a selection of Dutch women was studied. Detailed information on food consumption and potential confounders was prospectively collected. Selective participation in this study did occur, because mothers of lower socio-economic status were less represented in our study. This selection towards a healthy study population may have been harmful to the internal validity of our study, especially when the associations of the dietary pattern with CRL differ between the study population and the excluded mothers. This is difficult to ascertain because we do not know these associations, but they have to be taken into consideration.

The timing of pregnancy to assess gestational age at the moment of CRL measurement is very important in the analysis and interpretation of the results. We were not able to assess in this population-based cohort the exact timing of ovulation. However, to minimize misclassification of gestational age, we have restricted the analyses to women with a reliable first day of the LMP and a regular period of 28 ± 4 days. Moreover, we also adjusted the analyses for the duration of the menstrual cycle. We are aware however that residual confounding by gestational age cannot be excluded completely. The duration of the menstrual cycle can be confounded by recall bias[31] and other maternal characteristics, such as age and smoking.[32] Therefore, we repeated the analyses with a selection of women with a gestational age based on LMP with a 7-day range of the gestational age based on CRL (785/847) shown in **Supplement Table S5**. The association of women with a high adherence to the energy-rich dietary pattern and CRL remained significant. However, the effect attenuated and the linear trends were no longer significant.

A PCA was used for the dietary pattern analyses, which does not take into account previous knowledge. This approach has the advantage over a hypothesis-oriented approach that it takes into account the correlation structure of the food groups and does not focus on selected aspects of a diet.[21] The amount of variance explained by the dietary patterns is small but is comparable with previous studies in pregnant women.[33,34] Furthermore, the variance explained by dietary patterns depends heavily on the number of food groups used in the PCA analyses.[35] We used 20 predefined food groups, which allows more variance in the model than if we had used fewer food groups. However, it should be noted that PCA assumes approximate normality of the data, but can still produce a good projection of the data when it is not normally distributed. Balder et al.[36] examined the influence of analytical decisions on the stability of the dietary pattern in four European cohort studies. Sensitivity analyses were conducted with dichotomization of extremely skewed variables, but this transformation did not affect the food groups with significant factor loadings, the magnitude of the factor loadings or the explained variance, and so the order of the extracted patterns. Accordingly we also dichotomized variables with a high percentage (>75%) of nonusers and found no difference in results. This supports the maintenance of the PCA using the continuous data.

Nutritional studies are prone to some bias, including imprecise measurement. Maternal dietary intake was assessed once at intake using a modified version of the validated semi quantitative FFQ of Klipstein-Grobusch et al.[18] This FFQ was validated in an older white population. Several studies compared the results of dietary pattern analyses with the use of an FFQ, prospective

diaries and weighted dietary records and observed no differences.[33,35] Determination of dietary intake in our study was carried out at the end of the periconceptional period, covering the previous 3 months. For EFW and birth weight, it could be argued that diet can change throughout pregnancy. This may also explain the lack of association of our dietary pattern and growth from the second trimester onwards. Previous research, however, revealed no differences in nutritional intake during different time periods.[37,38] Furthermore, underreporting of nutritional intake could occur in women with high BMI.[39] We observed differences between the adherence categories and the different BMI groups. We tested underreporting by estimating the mean basal metabolic rate using the new Oxford equation for women aged 30–60 years: basal metabolic rate (mJoule/day) = 0.0407*weight (kg) + 2.9. The physical activity level[40] was then calculated: mean reported energy intake/mean basal metabolic rate.[41] To evaluate underreporting, a cut-off of 1.35 was used. In the total study group, the PAL was 1.56. In women with a BMI >25, the physical activity level was 1.34, suggesting underreporting. Restricting analyses to women with normal BMI did not change the effect estimates. Therefore analyses on the entire study population are displayed.

Finally, to minimize multiple testing, we applied PCA to reduce the information on nutrition in principal components leading to fewer parameters in our regression model. By taking all nutrients into account, we limited the possibility of selective testing. Moreover, associations between the dietary pattern and biomarker concentrations of the one-carbon pathway were examined, to validate the dietary patterns and minimize the possibility of chance-finding. In addition, the associations of women with a high adherence to the energy-rich dietary pattern with CRL remain after multiple testing adjustment.

CONCLUSION

This study suggests that increasing adherence to the energy-rich dietary pattern is significantly associated with CRL and second-trimester EFW. The relevance of these findings on pregnancy outcome warrants further investigation.

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Table S1 | Between-run coefficients of variation of the biomarkers.

	Between run coefficients						Analytic range
	First		Second		Third		
BIOMARKER CONCENTRATIONS	%		%		%		
Folate, plasma	8.9	5.6 nmol/L	2.5	16.6 nmol/L	1.5	33.6 nmol/L	1.8–45.3 nmol/L
tHcy, plasma	3.1	7.2 µmol/L	3.1	12.9 µmol/L	2.1	26.1 µmol/L	1–50 µmol/L
Vitamin B12, serum	3.6	142 pmol/L	7.5	308 pmol/L	3.1	633 pmol/L	44–1476 pmol/L

The biomarkers were analyzed using a microparticle-enhanced immunoassay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands).

Table S2 | Details of the multiple imputation modelling.

Sofware used	SPSS 17.0 for windows (SPSS Inc, Chicago, IL, USA)
Imputation method and keysettings	Fully conditional specification (Markov chain Monte Carlo method); Maximum iterations: 10
No of imputed data sets created	5
Variable included in the imputation procedure: (imputed and used as predictors of missing data)	– maternal BMI at intake – maternal diastolic and systolic blood pressure at intake – maternal educational level – maternal folic acid supplement use – maternal smoking – paternal height – paternal BMI – parity
Variables additionally added as predictors of missing data to increase plausibility of missing at random assumption:	– birthweight (SD-score) – estimated fetal weight in second and third trimester (SD-score) – fetal crown-to-rump length (SD-score) – fever during the first trimester – gender of the child – gestatational age at birth – gravidity – history of miscarriage – history of sexual transmitted disease – household income – hypertensive disorder during pregnancy – marital status – maternal age – maternal alcohol use during pregnancy – maternal BMI in before pregnancy and in second and third trimester – maternal C-reactive protein concentration (early pregnancy) – maternal calorie intake during the first trimester – maternal comorbidity – maternal folate concentration (early pregnancy) – maternal height – maternal homocysteine concentration (early pregnancy) – maternal vitamin B12 concentration (early pregnancy) – maternal weight before pregnancy, and in first/second/third trimester – nausea during the first trimester – paternal age – season of inclusion – vomitting during the first trimester

Table S2 | Details of the multiple imputation modelling *(Continued)*.

Treatment of none normally distributed variables	Logarithmic transformation
Treatment of binary/categorical variables	Logistic regression models
Statistical interaction included in the imputation models	None

Table S3 | Maternal and fetal characteristics.

	Mediterranean dietary pattern				Western dietary pattern			
	Low adherence n = 283	Intermediate adherence n = 282	High adherence n = 282	P-value	Low adherence n = 283	Intermediate adherence n = 282	High adherence n = 282	P-value
Age (years)*	30.9 (4.3)	31.8 (3.8)	32.4 (3.7)	<0.001	32.3 (4.0)	31.6 (3.8)	31.3 (4.1)	0.01
Maternal BMI†	23.8	23.0	22.9	NS	22.8	23.2	23.6	0.029
	(19.6–32.5)	(19.5–32.4)	(19.4–32.0)		(19.5–31.7)	(19.2–32.2)	(19.7–34.1)	
<19.9 (%)	9.2	8.2	9.2	NS	9.9	9.2	7.4	NS
20-24.9 (%)	51.6	63.8	61.3		62.9	56.7	57.1	
25-29.9 (%)	29.3	20.2	20.9		20.1	25.2	25.2	
30-34.9 (%)	7.4	4.6	5.7		5.3	6.0	6.4	
>35 (%)	2.5	3.2	2.8		1.8	2.8	3.9	
Education (%)				<0.001				NS
Low	3.9	1.1	1.8		2.9	1.4	2.5	
Medium	48.9	33.6	19.2		30.0	33.8	37.9	
High	47.2	65.4	79.0		67.1	64.8	59.6	
Household income (%)				0.04				NS
<1200 euro	3.7	3.2	3.1		3.2	1.6	5.2	
1200–2200 euro	22.4	16.3	12.1		17.3	15.7	17.5	
>2200 euro	73.9	80.6	84.8		79.5	82.7	77.4	
Parity (%)				NS				<0.001
0	64.3	61.3	60.5		71.0	62.4	52.7	
≥1	35.7	38.7	39.5		29.0	37.6	47.3	
Smoking (%)				NS				<0.001
Yes	13.7	12.6	11.4		8.2	9.1	20.2	
Until pregnancy recognition	10.6	8.8	11.8		8.2	11.3	11.6	
No	75.7	78.6	76.8		83.6	79.6	68.2	

Table S3 | Maternal and fetal characteristics *(Continued)*.

	Mediterranean dietary pattern			P-value	Western dietary pattern			P-value
	Low adherence n = 283	Intermediate adherence n = 282	High adherence n = 282		Low adherence n = 283	Intermediate adherence n = 282	High adherence n = 282	
MATERNAL								
Folic acid supplement use (%)				NS				NS
Adequate	62.6	61.5	66.1		65.0	68.0	57.1	
Inadequate	27.6	31.8	29.3		28.6	26.3	33.8	
No	9.9	6.7	4.6		6.4	5.7	9.2	
Daily nausea (%)	33.7	27.8	23.0	0.024	29.8	27.3	27.3	NS
Daily vomiting (%)	5.0	4.2	1.5	NS	3.9	3.0	3.8	NS
Pre-eclamspia (%)	1.6	2.4	2.3	NS	1.5	2.7	2.0	NS
FETAL								
Male gender (%)	48.4	51.1	49.5	NS	45.0	54.6	49.3	NS
Gestational age at birth (weeks)†	40.1	40.1	40.3	NS	40.1	40.3	40.1	NS
	(36.9–42.1)	(36.7–42.0)	(36.6–42.0)		(36.6–42.1)	(36.9–42.0)	(36.7–42.1)	
Small for gestational age (<5th birth centile) (%)	6.7	3.5	3.5	NS	4.9	3.2	5.4	NS

Data on the Mediterranean and Western dietary pattern are presented. Values are presented as *mean (SD), †median (90% range); NS: not significant; ANOVA and chi-square tests were used to test overall differences between maternal and fetal characteristics and the dietary patterns. Gestational age was based on LMP.

Table S4 | Biomarker concentrations and nutrient intakes.

	Mediterranean dietary pattern					Western dietary pattern				
	Total (n = 847)	Low adherence (n = 283)	Intermediate adherence (n = 282)	High adherence (n = 282)	Linear trend analyses	Total (n = 847)	Low adherence (n = 283)	Intermediate adherence (n = 282)	High adherence (n = 282)	Linear trend analyses
	ρ	Median (90% range)	Median (90% range)	Median (90% range)		ρ	Median (90% range)	Median (90% range)	Median (90% range)	
BIOMARKER CONCENTRATIONS										
Folate (nmol/L), plasma	0.06	20.2 (7.3–33.4)	19.9 (7.5–36.5)	21.8 (8.4–35.7)	NS	-0.11	22.4 (7.7–37.4)	19.5 (8.6–33.8)	19.6 (7.2–35.6)	0.003
Folic acid supplement use	0.02	24.0 (11.0–33.1)	23.8 (11.0–37.8)	25.0 (11.0–37.3)	NS	-0.09	25.1 (12.0–37.8)	22.4 (10.5–35.3)	24.3 (10.6–37.7)	NS
No supplement use	0.13	14.8 (6.0–30.5)	14.5 (5.4–28.1)	18.2 (7.8–32.6)	0.044	-0.07	17.1 (6.9–33.3)	16.7 (6.3–28.6)	14.5 (5.4–29.7)	NS
tHcy (µmol/L), plasma	-0.03	7.0 (5.1–10.1)	7.0 (4.9–11.5)	7.0 (5.0–9.5)	NS	0.04	7.0 (5.0–10.2)	6.8 (5.0–10.1)	7.1 (5.0–10.7)	NS
Folic acid supplement use	0.05	6.8 (5.0–9.4)	6.8 (4.9–10.0)	6.8 (5.0–9.3)	NS	-0.01	6.8 (5.0–9.6)	6.7 (4.9–9.9)	6.8 (5.0–9.1)	NS
No supplement use	-0.08	7.5 (5.5–15.0)	7.5 (5.1–12.7)	7.1 (5.0–9.4)	NS	0.06	7.4 (5.2–12.1)	7.1 (5.5–11.2)	7.4 (5.1–14.7)	NS
Vitamin B12 (pmol/L), serum	0.09	159.5 (86.0–325.1)	184.0 (97.5–384.0)	191.0 (91.3–410.6)	0.01	0.05	173.0 (90.0–323.8)	174.0 (85.6–332.0)	180.0 (92.6–407.6)	NS
Folic acid supplement use	0.11	160.5 (85.4–323.7)	186.0 (99.6–329.8)	192.0 (93.6–440.4)	0.02	0.01	177.0 (86.4–383.6)	180.0 (85.7–331.1)	171.0 (94.7–400.8)	NS
No supplement use	0.06	165.0 (84.3–375.3)	180.0 (92.4–411.4)	195.0 (81.6–420.8)	NS	0.09	174.0 (90.0–296.0)	166.0 (81.0–340.0)	192.0 (87.2–466.2)	NS

Table S4 | Biomarker concentrations and nutrient intakes *(Continued)*.

	Mediterranean dietary pattern					Western dietary pattern				
	Total (n = 847)	Low adherence (n = 283)	Intermediate adherence (n = 282)	High adherence (n = 282)	Linear trend analyses	Total (n = 847)	Low adherence (n = 283)	Intermediate adherence (n = 282)	High adherence (n = 282)	Linear trend analyses
	ρ	Median (90% range)	Median (90% range)	Median (90% range)		ρ	Median (90% range)	Median (90% range)	Median (90% range)	
ENERGY/MACRONUTRIENTS										
Energy (KJ/day)	0.17	8621 (5357–12742)	8603 (5736–12566)	9262 (6091–12956)	<0.001	0.31	8062 (5161–11774)	8679 (5535–11780)	9894 (6522–13364)	<0.001
Fat (% of energy)	-0.02	36.5 (25.7–45.5)	36.7 (27.8–44.3)	35.9 (27.6–43.3)	NS	0.05	36.1 (25.3–44.4)	36.1 (37.4–44.3)	36.5 (28.8–44.3)	NS
Total fat (g/day)*	0.13	81.6 (45.1–128.8)	80.8 (27.8–126.4)	85.5 (27.6–127.0)	NS	0.28	75.1 (42.9–122.2)	80.7 (52.5–123.4)	93.8 (62.0–134.7)	NS
Saturated fats (g/day)*	0.08	30.4 (16.3–50.5)	29.8 (18.9–50.2)	31.1 (18.9–47.0)	0.007	0.31	27.7 (15.6–45.7)	29.2 (18.8–45.2)	34.8 (22.8–54.6)	0.01
Mono-unsaturated fats (g/day)*	0.21	28.9 (15.7–45.6)	29.2 (18.1–46.5)	31.8 (21.1–47.2)	<0.001	0.28	27.6 (15.1–45.4)	28.8 (18.2–44.0)	34.1 (22.9–48.0)	NS
Poly-unsaturated fats (g/day)*	0.07	19.0 (8.8–32.1)	19.1 (9.7–34.5)	19.4 (10.0–32.8)	NS	0.13	18.1 (8.8–32.4)	19.1 (9.6–32.3)	20.6 (11.0–33.3)	0.025
Linoleic acid (g/day)*	0.06	15.2 (6.8–26.6)	15.5 (7.3–28.3)	15.8 (7.6–27.6)	NS	0.11	14.5 (6.5–27.5)	15.4 (7.3–26.6)	16.7 (8.3–27.7)	0.008
Cholesterol (mg/day)*	0.19	165.0 (87.7–270.4)	166.4 (91.9–260.3)	181.6 (99.1–301.5)	0.008	0.23	160.6 (84.9–293.8)	162.9 (88.2–248.6)	188.5) (115.2–295.5)	NS
Protein (% of energy)	0.35	13.8 (10.7–17.6)	15.1 (12.2–19.7)	15.7 (12.6–20.0)	<0.001	0.16	15.5 (10.9–18.3)	15.0 (12.0–19.6)	15.0 (11.2–20.2)	<0.001

Table S4 | Biomarker concentrations and nutrient intakes *(Continued)*.

	Mediterranean dietary pattern					Western dietary pattern				
	Total (n = 847)	Low adherence (n = 283)	Intermediate adherence (n = 282)	High adherence (n = 282)	Linear trend analyses	Total (n = 847)	Low adherence (n = 283)	Intermediate adherence (n = 282)	High adherence (n = 282)	Linear trend analyses
	ρ	Median (90% range)	Median (90% range)	Median (90% range)		ρ	Median (90% range)	Median (90% range)	Median (90% range)	
ENERGY/MACRONUTRIENTS										
Total protein (g/day)*	0.40	70.9 (41.4–99.0)	79.0 (52.9–108.9)	86.8 (57.8–118.5)	<0.001	0.40	70.5 (42.2–99.6)	77.2 (53.2–104.4)	86.4 (61.9–119.4)	<0.001
Vegetable protein (g/day)*	0.26	28.6 (15.8–44.5)	30.0 (16.4–45.4)	33.4 (18.1–49.0)	<0.001	-0.02	30.7 (16.5–49.1)	29.2 (16.2–44.0)	31.2 (17.1–46.7)	<0.001
Animal protein (g/day)*	0.36	42.1 (23.2–66.7)	48.6 (28.3–69.5)	53.2 (30.5–79.3)	<0.001	0.54	39.9 (19.9–62.7)	46.9 (29.6–68.1)	56.1 (39.9–80.5)	<0.001
Carbohydrate (% of energy)	-0.15	49.9 (40.0–60.9)	47.5 (39.1–58.0)	47.7 (38.6–57.4)	<0.001	-0.13	48.9 (40.2–61.1)	48.5 (39.8–58.1)	47.1 (38.5–58.2)	0.001
Total carbohydrate (g/day)*	0.08	247.7 (154.5–417.9)	248.2 (144.7–372.2)	270.4 (150.2–395.9)	<0.001	0.23	242.1 (147.2–362.7)	253.9 (144.5–373.2)	282.0 (157.5–420.4)	<0.001
Carbohydrates mono- and di-saccharides (g/day)*	0.0	145.2 (68.9–258.1)	135.9 (68.0–226.9)	141.4 (70.4–230.1)	<0.001	0.18	130.5 (68.9–205.0)	139.0 (68.6–230.0)	153.6 (73.5–268.8)	0.002
Carbohydrates polymers (g/day)*	0.18	107.6 (61.9–170.4)	110.0 (63.1–168.8)	121.2 (65.5–179.8)	NS	0.22	107.9 (60.5–163.8)	109.8 (63.7–162.3)	121.7 (66.5–189.6)	NS
Fibers (g/day)*	0.33	20.7 (11.6–33.1)	23.5 (13.6–33.9)	26.2 (14.1–40.6)	<0.001	-0.04	23.3 (13.2–37.4)	22.8 (12.4–34.4)	23.7 (13.7–35.7)	<0.001

Data on the Mediterranean and Western dietary pattern are presented. Venous blood serum and plasma samples were drawn at enrolment (median 12.4 weeks gestation, 90% range 11.0–13.7). Linear trend analyses reflect the change in biomarker concentration, energy or macronutrient in SD-score per unit (factor score) change, adjusted for gestational age at venous puncture; *additionally adjusted for energy intake. NS; not significant.

Table S5 | Associations between the degree of adherence to the Energy-rich dietary pattern and CRL.

	CRL in mm, effect size (95% CI)		CRL in SD-score, effect size (95% CI)	
ENERGY-RICH DIETARY PATTERN	**Crude***	**Adjusted†**	**Crude•**	**Adjusted†**
Low adherence	Reference	Reference	Reference	Reference
Intermediate adherence	0.78 (-0.21; 1.77)	0.59 (-0.34; 1.53)	0.09 (-0.05; 0.23)	0.07 (-0.07; 0.20)
High adherence	1.48 (0.33; 2.63)[1]	1.04 (0.10; 1.99)[1]	0.21 (0.05; 0.38)[1]	0.15 (0.01; 0.29)[1]
Linear trend analyses	0.24 (-0.15; 0.63)	0.22 (-0.17; 0.61)	0.03 (-0.03; 0.09)	0.03 (-0.03; 0.09)

Results from multiple linear regression analyses. Analyses are restricted to women with a gestational age based on LMP within 7 days of a gestational age based on CRL (n = 785). Values are presented as regression coefficients (95% confidence interval), representing the change in CRL (mm or standard deviation score (SD-score)) compared to the reference category. Linear trend analyses reflect the change in CRL (SD-score) per unit (factor score) change; *Adjusted for gestational age, duration of last menstrual cycle; •Adjusted for duration of last menstrual cycle; †Additionally adjusted for maternal age, maternal and paternal BMI, maternal and paternal height, fetal gender, parity, educational level, smoking, folic acid supplement use, mean diastolic blood pressure, mean systolic blood pressure; [1]P-value <0.05.

Chapter 3

Maternal biomarkers of placentation and early fetal growth

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ABSTRACT

Objective | Fetal growth is dependent on adequate development of the placenta. Impaired angiogenesis and vasculogenesis in early pregnancy compromises placental and embryonic development. The proteins soluble fms-like tyrosine kinase (sFlt)-1, placental growth factor (PlGF), and plasminogen activator inhibitor (PAI)-2, and the B vitamin folate are determinants of placental development. This study aims to identify associations between these maternal biomarkers and early fetal size.

Study design | From a prospective birth cohort study in The Netherlands, 1491 pregnant women were selected for this study. At a mean gestational age (GA) of 12.4 weeks (SD 0.8) maternal venous blood samples were obtained to determine the concentrations of sFlt-1, PlGF, PAI-2, and folate. Early fetal size was assessed with measurement of the crown-to-rump length (CRL) at a mean of 12.4 weeks' GA (SD 0.8). Analyses were performed using multivariable linear regression analyses with the biomarkers (continuous, quintiles) as regressors and CRL as main outcome measure.

Results | Linear trend analysis showed positive associations between maternal sFlt-1 (P-value <0.001), PlGF (P-value = 0.042), PAI-2 (P-value <0.001), and folate (P-value = 0.039) and CRL. These associations were independent of GA, maternal age, height, body mass index, ethnicity, fetal sex, parity, educational level, smoking, and folic acid supplement use (folate not adjusted).

Conclusion | sFlt-1, PlGF, PAI-2 2, and folate are positively associated with first-trimester fetal size.

INTRODUCTION

Fetal growth is dependent on adequate development of the placenta. Early development takes place in a relatively low oxygen environment, shielding the embryo against oxygen free radicals, which is vital for trophoblast differentiation and migration, and angiogenesis.[1] Impaired trophoblast invasion can lead to insufficient vascular remodeling of the spiral arteries. This can lead to reduced placenta perfusion and subsequent adverse pregnancy outcomes.[2]

The vascular endothelial growth factor (VEGF) pathway is essential for early development and includes placental growth factor (PlGF) and soluble fms-like tyrosine kinase (sFlt)-1.[3] PlGF is secreted by the cytotrophoblast and syncytiotrophoblast and stimulates endothelial proliferation, migration, and activation.[4] sFlt-1 is secreted by endothelial cells and syncytiotrophoblasts and inhibits PlGF.[5] Both factors are suggested to stimulate and regulate early placentation.[4] Newly formed vessels in the decidua are thought to serve as the first exchange apparatus for the embryo until the placenta becomes completely functional.[6] Inactivation of the VEGF receptor may compromise placental and embryonic development.[7]

Another important system in placental development is the plasminogen activator (PA) system. This pathway is responsible for intravascular blood clot degradation and extracellular proteolysis.[8,9] One important inhibitor of the PA system is PA inhibitor (PAI)-2 produced by the trophoblast. Previously, low levels of PAI-2 were suggested as a marker of decreased placental development.[10]

Lastly, an important B vitamin and growth factor is folate. Synthetic folic acid has shown to improve endothelial function and is associated with reduced uteroplacental resistance.[11-13] Williams et al.[14] suggested a vital role of folate during placental development, in particular trophoblast invasion and angiogenesis.

From this background, we hypothesized that sFlt-1, PlGF, PAI-2, and folate, as determinants of placental development, ensure an optimal environment for early fetal growth and development. We investigated associations between these biomarkers in maternal blood and early fetal size, measured as crown-to-rump length (CRL).

MATERIALS AND METHODS

Design and study population

This study was embedded in the Generation R Study, a prospective cohort study from early pregnancy onwards in Rotterdam, The Netherlands.[15] The study was approved by the medical ethics committee of the Erasmus University Medical Center, Rotterdam, The Netherlands. Written informed consent was obtained from all mothers for maternal and child data. From 2001 through 2005, 9778 women were included.

All prenatally enrolled Dutch women with a live born singleton (n = 4195) and with a CRL measurement were selected from the total study sample (**Figure 1**). Women with no CRL measurement within the recommended gestational age (GA) range (10^{+0} weeks to 13^{+6} weeks)

were excluded. Only mothers with a known first day of the last menstrual period (LMP) and a regular menstrual cycle of 28 ± 4 days were included. LMP was obtained of the referring letter from the community midwife or hospital and was verified at the inclusion moment. Additional information on regularity and cycle duration was obtained. If mothers participated in Generation R with ≥2 pregnancies, 1 sibling was randomly selected to avoid bias due to paired data (n = 130). After exclusion of women who underwent a fertility treatment (n = 10) or experienced a stillbirth or miscarriage (n = 21), 1491 women were eligible for the present study. Sensitivity analyses were carried out with exclusion of: (1) diabetics; and (2) women who later develop preeclampsia (PE). As this did not substantially change our results, these women were included in the analyses.

Biomarker assessment

In early pregnancy venous blood samples were drawn at the same moment as the CRL was measured (mean 12.4 weeks' GA, SD 0.8). The samples were stored at room temperature before being transported to the regional laboratory for processing and storage for future studies. Processing was planned to finish within 3 hours after venous puncture. The samples were centrifuged and thereafter stored at -80°C.[16] In plasma, sFlt-1, PlGF, and folate were analyzed using a microparticle-enhanced immunoassay on the Architect System (Abbott Diagnostics BV, Hoofddorp, The Netherlands) as described previously.[17,18] Between-run coefficients of variation and analytical ranges are listed in Supplement Table S1. Plasma PAI-2 was analyzed using enzyme linked immunosorbent assay with the same experimental setup as described previously.[17] The analytical and functional sensitivity were 11 pg/mL and 32 pg/mL, respectively. The mean PAI-2 was 88.4 pg/mL and the intra-assay variation, between-plates variation, and interassay variation were 3.4%, 2.9%, and 8.4%, respectively.

Fetal ultrasound measurements

Standard ultrasound planes for CRL measurement were used as described previously.[19] Intraclass correlation coefficients for intra-observer (0.998) and inter-observer (0.995) reproducibility of CRL measurements have been described before.[20] GA-adjusted SD scores (SDS) for CRL were constructed and were based on reference growth curves from the entire study population.[19,21]

Covariates

From self-administered questionnaires, data were extracted on maternal age, education, marital status, parity, smoking, folic acid use, nausea/vomiting/fever during the first trimester, and repeated (≥2) miscarriages. Ethnicity was defined according to the classification of Statistics Netherlands.[22] Education was assessed by the highest completed level of the mother and classified as: (1) low (none/primary); (2) medium (secondary); or (3) high (college/university). Maternal smoking habits were assessed for each trimester. Women, who reported any or no smoking during pregnancy were respectively classified as smokers and nonsmokers. Folic acid supplement use was assessed by asking women about the use and moment of initiation of a folic acid supplement. This information was used to categorize women into 3 groups of folic acid use: (1) adequate, defined as preconceptional initiation; (2) inadequate, defined as postconceptional

initiation; and (3) no use. At enrollment maternal weight and height were measured to calculate body mass index (BMI) (kg/m^2). Information on fertility treatment, pregnancy outcome, date of birth, birth anthropometrics, and the sex of the child were obtained from community midwives, obstetricians, and hospital registries.

Statistical analyses

Analyses were performed to correlate sFlt-1, PlGF, PAI-2, and folate with GA. Multivariable linear regression analyses were performed with the biomarker concentrations continuous and in quintiles as regressors and CRL (SDS) as main outcome measure. This approach was chosen to explore linearity and nonlinearity of the association. The lowest quintile of each biomarker was used as reference group. In the final analysis, we accounted for potential confounding variables, which were first selected on previous literature and determined a priori. Thereafter, covariates were selected as confounding variables if the effect estimates changed ≥10% in the exploratory analyses. By using this approach, maternal age, parity, smoking, and the use of a folic acid supplement (not for the analyses of folate) were included in the final analyses. Maternal ethnicity, maternal education, height, BMI, and fetal sex were included by default. Furthermore, as sFlt-1 and PlGF belong to the same pathway, analyses of sFlt-1 and PlGF were additionally adjusted for PlGF and sFlt-1, respectively. To assess the possibility of multicolinearity, we have calculated the variance inflation factor (VIF) and tolerance. If the VIF is <10 and/or the tolerance is >0.2, then there is no cause for concern.[23]

Missing data of the covariables ethnicity (3.8%), education (4.9%), parity (0.7%), smoking (10.0%), height (0.3%), BMI (0.3%), and use of a folic acid supplement (19.2%) were completed using the Markov chain Monte Carlo multiple imputation technique for the linear regression models. Five datasets were created. Subsequently multivariable regression analyses were performed separately on each completed data set and combined to one pooled estimate.[24] For all analyses, results including imputed missing data are presented. Nonresponse analyses were performed by comparing characteristics of mothers (age, education, parity, smoking, folic acid supplement use, BMI) and birth characteristics of the children of mothers who were included in the analyses to those who were excluded using t, Mann-Whitney U, and χ^2-tests. Furthermore, analyses were performed to examine correlations between the biomarkers and GA. All analyses were performed using software (SPSS, version 17.0; IBM Corp, Armonk, NY).

RESULTS

The maternal and fetal characteristics are presented in **Table 1**. Nonresponse analyses showed that women who had been excluded were less often Dutch (62.8%), were younger (mean = 29.5 years, SD 5.0), had lower education levels (9.4%), were more often multiparous (43.8%), and less frequently used a folic acid supplement (no use = 24.9%) (all P-value <0.05). Within the GA range (from 10^{+0} to 13^{+6} weeks' GA), we observed positive correlations between GA and the concentrations of PlGF (Spearman ρ = 0.29, P-value <0.001) and PAI-2 (Spearman ρ = 0.30, P-value <0.001), and a

small negative correlation with folate (Spearman ρ = -0.09, P-value = 0.002). Furthermore, positive correlations were observed between sFlt-1 and PlGF (Spearman ρ = 0.12, P-value <0.001), sFlt-1 and PAI-2 (Spearman ρ = 0.23, P-value <0.001), and PlGF and PAI-2 (Spearman ρ = 0.36, P-value <0.001). No correlation was observed between folate and the other biomarkers.

Table 1 | Baseline characteristics.

	n = 1,491
MATERNAL	
Age,† **years**	30.7 (4.7)
Gestational age at intake,† **weeks**	12.4 (0.8)
Height,† cm	168.6 (7.1)
Body mass index at intake,‡ kg/m^2	23.5 (4.6)
Ethnicity (%)	
Dutch/Caucasian	67.9
Surinamese	5.5
Turkish	6.1
Moroccan	3.8
Indonesian	3.2
Others	9.7
Missing	3.8
Education (%)	
Low	5.9
Medium	38.5
High	50.7
Missing	4.9
Gravidity (%)	
Primigravida	48.8
Multigravida	50.6
Missing	0.6
Parity (%)	
Nullipara	61.0
Multipara	38.3
Missing	0.7
Folic acid supplement use (%)	
Adequate (preconception initiation)	42.0
Inadequate (postconception initiation)	26.3
No	12.5
Missing	19.2

Table 1 | Baseline characteristics *(Continued)*.

	n = 1,491
MATERNAL	
Smoking (%)	
Yes	13.5
Until pregnancy recognition	9.0
No	67.5
Missing	10.0
Daily nausea (%)	30.0
Daily vomiting (%)	7.0
FETAL	
Crown-to-rump length,† mm	60.9 (11.4)
Males (%)	49.0
GA at birth,‡ weeks*	40.1 (2.0)
Birth weight,† grams	3455 (563)

*Based on first day of last menstruation; †mean (SD); ‡median (IQR).

The associations between maternal sFlt-1, PlGF, PAI-2, and folate levels and CRL (millimeters, SDS) are shown in **Table 2** and **Figure 2**. In all analysis the first quintile was taken as a reference category. In the crude analyses, a larger CRL was observed in women with sFlt-1 levels in quintile (Q)5 (difference SDS = 0.16; 95% confidence interval (95% CI) 0.00; 0.32). A significant linear trend was observed (change = 0.15 mm, 95% CI 0.03; 0.26 and SDS = 0.03, 95% CI 0.01; 0.04 per ng/mL change in sFlt-1). In the multivariable analyses, the association remained significant in Q5. After additional adjustment for PlGF levels, the associations between sFlt-1 and CRL remained in Q5, but slightly weakened (difference = 1.33 mm, 95% CI 0.09; 2.57 and SDS = 0.21, 95% CI 0.04; 0.39), with a significant linear trend (change = 0.18 mm, 95% CI 0.05; 0.32 and SDS = 0.03, 95% CI 0.01; 0.05 per ng/mL change in sFlt-1).

In the crude analyses, positive associations were observed between CRL and PlGF in Q3 (difference = 1.58 mm, 95% CI 0.48; 2.69 and SDS = 0.22, 95% CI 0.06; 0.38), Q4 (difference = 2.73 mm, 95% CI 1.60; 3.86 and SDS = 0.39, 95% CI 0.23; 0.56), and Q5 (difference = 2.65 mm, 95% CI 1.44; 3.87 and SDS = 0.34, 95% CI 0.17; 0.52). After adjustment for confounders in the multivariable analysis, all significant associations remained. After additional adjustment for sFlt-1, all associations between PlGF and CRL remained in Q3 (difference = 1.96 mm, 95% CI 0.75; 3.17 and SDS = 0.29, 95% CI 0.12; 0.46), Q4 (difference = 2.86 mm, 95% CI 1.63; 4.09 and SDS = 0.42, 95% CI 0.24; 0.59), and Q5 (difference = 3.18 mm, 95% CI 1.80; 4.56 and SDS = 0.42, 95% CI 0.22; 0.61).

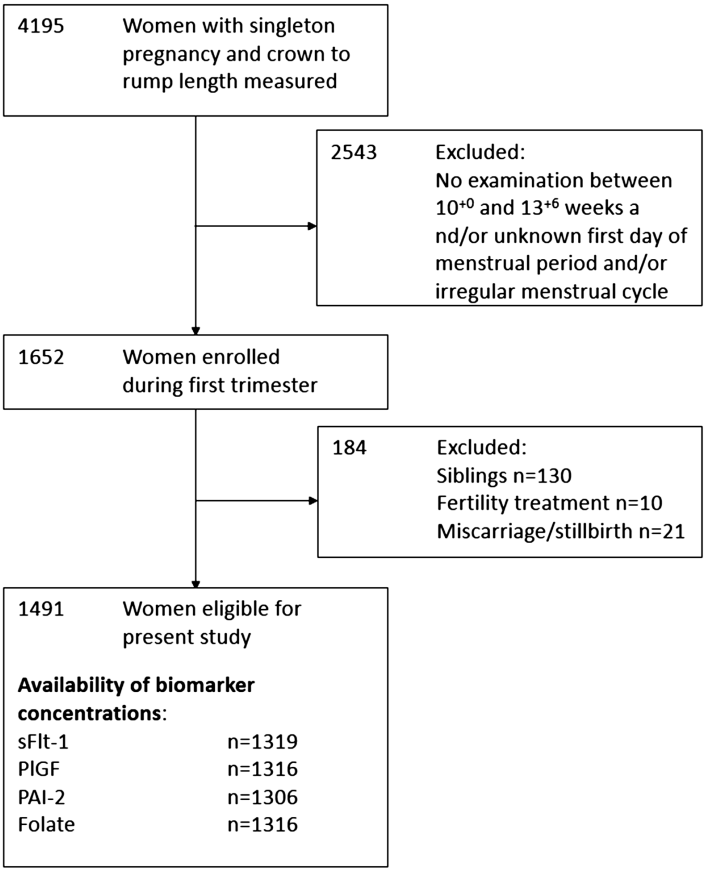

Figure 1 | Flow chart.

PAI, plasminogen activator inhibitor; PlGF, placental growth factor; sFlt, soluble fms-like tyrosine kinase.

In the crude analyses, women with PAI-2 levels in Q2 (difference = 2.52 mm, 95% CI 1.41; 3.62 and SDS = 0.38, 95% CI 0.22; 0.54), Q3 (difference = 1.66 mm, 95% CI 0.54; 2.77 and SDS = 0.25, 95% CI 0.09; 0.41), Q4 (difference 3.70 mm, 95% CI 2.56; 4.85 and SDS = 0.52, 95% CI 0.35; 0.68), and Q5 (difference = 3.23 mm, 95% CI 2.02; 4.43 and SDS = 0.45, 95% CI 0.27; 0.62) showed associations with CRL. The linear trend tests were significant (change = 0.06 mm, 95% CI 0.03; 0.09 and SDS = 0.01, 95% CI 0.00; 0.01 per ng/mL change in PAI-2). In the multivariable analyses, the associations remained significant.

The crude analyses showed significant associations between folate and CRL in Q3 (difference = 1.26 mm, 95% CI 0.16; 2.36) and Q5 (difference = 1.86 mm, 95% CI 0.75; 2.96 and SDS = 0.24, 95% CI 0.08; 0.40). Significant trends were observed between folate and CRL (change = 0.06 mm, 95% CI 0.02; 0.09 and SDS = 0.01, 95% CI 0.00; 0.01 per nmol/mL change in folate). In the multivariable analyses the associations remained significant in Q5. Last, to assess multicolinearity, we calculated the VIF and tolerance. In all the models, the VIF was <10 and the tolerance was >0.2.

Table 2 | Maternal sFlt-1, PlGF, PAI-2 and folate concentrations and early fetal size.

	Effect size for CRL (mm)		Effect size for CRL (SDS)	
Biomarkers	**Crude****	**Adjusted†**	**Crude****	**Adjusted†**
sFlt-1 (ng/mL)‡				
Q1 (≤3.29)	Reference	Reference	Reference	Reference
Q2 (3.3–4.35)	0.01 (-1.10; 1.11)	-0.11 (-1.32; 0.19)	-0.01 (-0.17; 0.15)	-0.02 (-0.20; 0.15)
Q3 (4.36–5.54)	0.03 (-0.96; 1.26)	-0.22 (-1.42; 1.10)	0.01 (-0.15; 0.17)	-0.01 (-0.20; 0.14)
Q4 (5.55–7.43)	1.00 (-0.10; 2.11)	0.85 (-0.67; 0.98)	0.15 (-0.01; 0.31)	0.13 (-0.05; 0.30)
Q5 (≥7.44)	0.95 (-0.16; 2.05)	**1.44 (0.20; 2.06)**[1]	**0.16 (0.00; 0.32)**[1]	**0.23 (0.05; 0.40)**[1]
Linear trend analyses*	**0.15 (0.03; 0.26)**[1]	**0.20 (0.07; 0.33)**[2]	**0.03 (0.01; 0.04)**[2]	**0.03 (0.02; 0.05)**[2]
PlGF (pg/mL)‡				
Q1 (≤24.0)	Reference	Reference	Reference	Reference
Q2 (24.01–30.4)	0.95 (-0.15; 2.06)	0.89 (-0.28; 2.07)	0.13 (-0.03; 0.29)	0.14 (-0.03; 0.31)
Q3 (30.41–37.6)	**1.58 (0.48; 2.69)**[2]	**1.92 (0.71; 3.14)**[2]	**0.22 (0.06; 0.38)**[2]	**0.29 (0.11; 0.46)**[2]
Q4 (37.61–47.9)	**2.73 (1.60; 3.86)**[2]	**2.89 (1.67; 4.12)**[2]	**0.39 (0.23; 0.56)**[2]	**0.43 (0.25; 0.60)**[2]
Q5 (≥47.91)	**2.65 (1.44; 3.87)**[2]	**3.19 (1.82; 4.56)**[2]	**0.34 (0.17; 0.52)**[2]	**0.42 (0.23; 0.62)**[2]
Linear trend analyses*	0.01 (-0.00; 0.03)	**0.02 (0.00; 0.04)**[1]	0.00 (0.00; 0.00)	**0.00 (0.00; 0.01)**[1]
PAI 2 (ng/mL)‡				
Q1 (≤25.6)	Reference	Reference	Reference	Reference
Q2 (25.61–31.21)	**2.52 (1.41; 3.62)**[2]	**3.58 (2.37; 4.79)**[2]	**0.38 (0.22; 0.54)**[2]	**0.53 (0.36; 0.70)**[2]
Q3 (31.22–36.82)	**1.66 (0.54; 2.77)**[2]	**2.43 (1.19; 3.67)**[2]	**0.25 (0.09; 0.41)**[2]	**0.37 (0.19; 0.55)**[2]
Q4 (36.83–45.32)	**3.70 (2.56; 4.85)**[2]	**4.50 (3.20; 5.81)**[2]	**0.52 (0.35; 0.68)**[2]	**0.64 (0.46; 0.83)**[2]
Q5 (≥45.33)	**3.23 (2.02; 4.43)**[2]	**4.06 (2.68; 5.44)**[2]	**0.45 (0.27; 0.62)**[2]	**0.57 (0.37; 0.76)**[2]
Linear trend analyses*	**0.06 (0.03; 0.09)**[2]	**0.08 (0.04; 0.11)**[2]	**0.01 (0.00; 0.01)**[2]	**0.01 (0.01; 0.02)**[2]
Folate (nmol/L)				
Q1 (≤11.5)	Reference	Reference	Reference	Reference
Q2 (11.51–16.8)	0.59 (-0.52; 1.70)	0.48 (-0.64; 1.60)	0.06 (-0.10; 0.22)	0.05 (-0.12; 0.21)
Q3 (16.81–22.1)	**1.26 (0.16; 2.36)**[1]	1.09 (-0.04; 2.22)	0.15 (-0.01; 0.30)	0.13 (-0.04; 0.29)
Q4 (22.11–28.2)	0.69 (-0.42; 1.79)	0.57 (-0.58; 1.72)	0.09 (-0.07; 0.25)	0.07 (-0.09; 0.24)
Q5 (≥28.21)	**1.86 (0.75; 2.96)**[2]	**1.66 (0.50; 2.81)**[2]	**0.24 (0.08; 0.40)**[2]	**0.22 (0.05; 0.38)**[1]
Linear trend analyses*	**0.06 (0.02; 0.09)**[2]	**0.05 (0.01; 0.09)**[1]	**0.01 (0.00; 0.01)**[1]	**0.01 (0.00; 0.01)**[1]

Results from multivariable linear regression analyses. Values are presented as regression coefficients (95% confidence interval), representing the change in CRL (mm or SDS) compared to the reference category. *Linear trend analyses reflect the change in CRL (SDS) as a continuous measure per unit (biomarker) change; **Adjusted for GA based on LMP at CRL measurement, GA based on LMP at venous puncture and duration of last menstrual cycle; †Additionally adjusted for maternal age, maternal height, maternal BMI, maternal ethnicity, fetal gender, parity, educational level, smoking; ‡Multivariable analyses also adjusted for folic acid supplement use; [1]P-value <0.05 [2]P-value <0.01. Abbreviations: CRL crown-to-rump length; Q, quintile; SDS, standard deviation score.

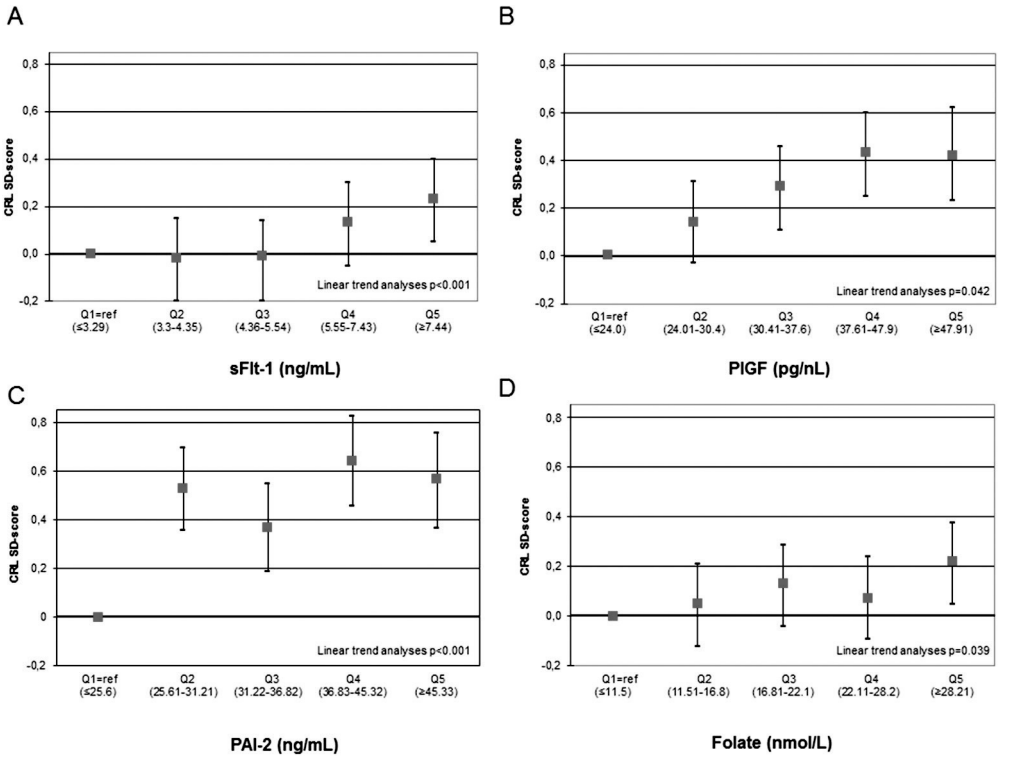

Figure 2 | Maternal sFlt-1, PlGF, PAI-2, and folate concentrations and early fetal size.

Values are regression coefficients (error bars indicate 95% confidence interval) and represent difference in crown-to-rump length (CRL) SD score (SDS), compared to quintile (Q)1, in A, sFlt-1, B, PlGF, C, PAI-2, and D, folate. Linear trend analyses reflect change in CRL (SDS) as continuous measure per unit (biomarker) change. All values are adjusted for gestational age at venous puncture (based on last menstrual period [LMP]), duration of LMP, maternal age, maternal height, maternal body mass index, maternal ethnicity, fetal sex, parity, educational level, smoking. Analyses of sFlt-1, PlGF, and PAI-2 were additionally adjusted for folic acid supplement use. PAI-2, plasminogen activator inhibitor 2; PlGF, placental growth factor; sFlt-1, soluble fms-like tyrosine kinase 1.

DISCUSSION

We demonstrate positive associations between maternal sFlt-1, PlGF, PAI-2, and folate levels in early pregnancy and increased early fetal size, measured as CRL.

This finding is in line with previous studies in relation to pregnancy complications and outcome.[25] Low PlGF in early pregnancy has been associated with an increased risk of PE and small-for-GA (SGA).[26] In addition, women who developed PE showed increased sFlt-1, but not earlier than in the second trimester.[27] In contrast, some evidence reveals that higher sFlt-1 and PlGF levels during the first trimester are associated with reduced risks of adverse birth outcomes.[28] In the same birth cohort, sFlt-1, PlGF, PAI-2, and folate were studied in early pregnancy in relation to pregnancy outcomes.[17,18] Higher placental and birth weight and a lower frequency of preterm

births was reported in women with high sFlt-1 (90th percentile, >9.85 ng/mL). Associations were also shown between low maternal PlGF (10th percentile, ≤21.50 pg/nL) and lower placental and birth weight, and a higher prevalence of prematurity, SGA, and PE. Moreover, low maternal PAI-2 (10th percentile, ≤23.61 ng/mL) was observed in pregnancies with lower placental and birth weight and a higher prevalence of SGA.[17] Other studies in the Generation R cohort[18] support our folate findings by showing associations between low maternal folate (lowest quintile, ≤9.2) and lower placental and birth weight and a positive association between folic acid supplement use and lower uteroplacental vascular resistance.[11]

To our knowledge, for the first time in a large birth cohort associations are shown between maternal sFlt-1, PlGF, PAI-2, and folate levels and early fetal size. Recently, Kasdaglis et al[29] estimated a linear relationship between PlGF and CRL (Spearman ρ = 0.36, P-value <0.001) and proposed to create reference curves for PlGF based on CRL rather than GA, assuming no variation in growth in the first trimester. Currently, CRL measurements are used for an accurate determination of GA. Discrepancies between menstrual and ultrasound estimations usually lead to GA adjustment as it is interpreted as earlier/delayed ovulation or implantation.[30] However, new evidence reveals discrepancies due to differences in embryonic growth.[31] Recently, Mook-Kanamori et al.[21] showed in the same birth cohort that maternal characteristics and lifestyle habits, such as maternal age (difference = 0.79 mm, 95% CI 0.41; 1.18), smoking (difference = -0.98 mm, 95% CI -1.79; -0.16), and no folic acid supplement use (difference = -1.33 mm, 95% CI -2.41; -0.24), were associated with CRL measured in early pregnancy (GA: mean (SD) 12.4 (0.8)). Moreover, early fetal growth restriction was also linked to an increased risk of adverse birth outcomes, suggesting that adverse outcomes originate in early pregnancy. Interestingly, the effects of the measured maternal biomarkers on CRL observed in the present study are comparable in effect size or even larger. Our data may imply that vascular development can influence early fetal development, which can have implications for subsequent development and outcome.

The trophoblast produces (anti) angiogenic and fibrinolytic factors[3,32] to regulate vasculogenesis and angiogenesis and therefore secures a low oxygen environment and nutritional supply to the developing fetus. Hemotrophic nutrition to the embryo is limited during the first trimester due to the absence of intervillous blood flow <11 weeks of gestation in normal pregnancies. Burton et al.[6] suggested that nutrient supply in early pregnancy is, at least in part, supported by secretions of decidual glands (histiotrophic nutrition). Little is known regarding these secretions, however, they contain a cocktail of growth factors, including VEGF. Therefore, the secretions have been suggested to play an important role in placental cell proliferation and differentiation in the first trimester. In addition, Burton and colleagues[6] proposed an important role for the yolk sac in nutrient exchange.

We hypothesize that the levels of angiogenic factors in maternal blood reflect different biological processes and stages of placentation and maternal adaptation during pregnancy. In early pregnancy, <11–12 weeks' GA, the partial pressure of oxygen in the intervillous space is relatively low, due to the occlusion of the spiral arteries by endovascular trophoblastic plugs.[2] After this period, the tension in the intervillous spaces gradually rises.[2]

Research has shown that low oxygen levels increase sFlt-1 levels in cytotrophoblasts cultured in vitro.[33] Thus, elevated maternal sFlt-1 levels in early pregnancy may potentially reflect the low and stable oxygen environment needed for early placental and embryonic development. Whereas elevated sFlt-1 levels in second and third trimester may reflect the response to fetal-placental hypoxia, which is associated with placental impairment.[34] This could explain the positive association between sFlt-1 and CRL, as the maternal sFlt-1 levels were studied at the end of the first trimester. Although sFlt-1 and PlGF are in the same pathway, we showed independent associations of both sFlt-1 and PlGF with CRL. To assess the possibility of multicolinearity in the latter models, we calculated the VIF and tolerance. In the analyses, the VIF was <10 and the tolerance was >0.2. Therefore, it is not likely that multicolinearity is an issue of concern.[23]

The PA system involves 2 PA's - tissue-type activator and urokinase-type activator (uPA) – and 2 plasminogen inhibitors, of which PAI-2 is an important inhibitor of uPA.[35] uPA has a well-established role in cell invasion and tissue remodeling through the degradation of the extracellular matrix.[9] PAI-2 is mainly produced by the trophoblast.[10] PAI-2 and uPA have been detected at early implantation sites, which support their role in the invasion of the decidua.[36] In addition, PAI-2 blocks tumor necrosis factor-α-mediated apoptosis independently of uPA, suggesting a role of PAI-2 in regulating cell death.[37] Furthermore, it has been suggested that during implantation the PA system is regulated through VEGF and as such implicated in implantation and trophoblast invasion.[8] An increase of uPA above physiological levels could lead to impaired placental development by hyperplastic proliferation of the muscular component of the wall of the spiral arteries.[38] This supports the positive association between PAI-2 and CRL and is consistent with previous findings of negative associations between PAI-2 and intrauterine growth restriction.[17]

During the first trimester of pregnancy, nutrition is covered by diffusion of nutrients from the trophoblast through the extra embryonic coelomic and amniotic cavities and fluids to the embryo (histotrophic nutrition). High methionine and low total homocysteine levels have been detected in both extra embryonic coelomic and amniotic fluid in the first trimester, supporting the role of the one-carbon metabolism in early growth and development.[39] Folate is essential in this metabolism as it provides one-carbon donors for the remethylation of total homocysteine into methionine, thereby reducing oxidative stress. Of interest is that folate deficiency has been associated with increased cytotrophoblast apoptosis thereby possibly influencing trophoblast invasion and placental development.[14,40] Thus, it seems that folate contributes to a stable oxygen environment needed for optimal trophoblast invasion and remodeling of the spiral arteries.[41] These findings support our observation of a positive association between folate and CRL.

Methodological considerations

This study was embedded in a large birth cohort from which a selection of women was studied. Selective participation in this study did occur, since mothers of lower socioeconomic status were less represented in our study. This selection toward the healthy study population may have been harmful to the internal validity of our study, especially when the associations of the biomarkers with CRL differ between the study population and the excluded mothers. This is difficult to ascertain as we do not know these associations, but has to be taken into consideration. The timing

of pregnancy to assess GA at the moment of CRL measurement is very important to consider in the analyses and interpretation of the results. In our cohort, we were not able to determine the exact timing of ovulation. To minimize confounding of GA, analyses were restricted to women with reliable LMP and regular menstrual period. However, misclassification of GA can still be an issue as we were unable to measure the exact timing of ovulation. Therefore, analyses were adjusted for the duration of the last menstrual cycle, which is highly associated with the timing of ovulation.[42] Moreover, analyses were repeated after exclusion of women with a GA based on LMP with >7-day range of the GA based on CRL (1380/1491) as shown in **Supplement Table S2**. The effect estimates were attenuated, however, most of the previously identified significant associations remained. Thereafter, we restricted the analyses to women who stopped using contraceptives >2 months before conception (1301/1491) as shown in **Supplement Table S3**. The effect estimates remained the same and in some cases the association was even stronger. Lastly, even though we adjusted for potential confounders, residual confounding should always be taken into account.

CONCLUSION

Maternal sFlt-1, PlGF, PAI-2 and folate levels in early pregnancy are suggested to influence early fetal size. These findings could help to elucidate underlying mechanisms of early embryonic and fetal growth. New techniques have emerged to visualize early embryonic growth and placental function, including first-trimester villous vascularization and yolk sac volume.[43,44] The measurements of the embryo and placenta by these techniques would give the opportunity to study normal and abnormal embryonic growth and development. Thereafter, the clinical utility of these biomarkers to assess early pregnancy health has to be investigated.

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Supplement table S1 | Between-run coefficients of variation of the biomarkers.

	Between run coefficients						Analytic range
	First		Second		Third		
Biomarker concentrations	%		%		%		
sFlt-1, plasma	2.8	5.5 ng/mL	2.3	34 ng/mL	–	–	0–150 ng/mL
PlGF, plasma	4.7	24 pg/mL	3.8	113 pg/mL	–	–	10–1500 pg/nL
Folate, plasma	8.9	5.6 nmol/L	2.5	16.6 nmol/L	1.5	33.6 nmol/L	1.8–45.3 nmol/L

The biomarkers were analyzed using a microparticle-enhanced immunoassay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands). The assays used to analyze sFlt-1 and PlGF were performed with pre-launch assays.

Supplement table S2 | Associations between sFlt-1, PlGF, PAI-2 and folate and CRL.

	Effect size for CRL in mm		Effect size for CRL SDS	
BIOMARKERS	**Crude****	**Adjusted†**	**Crude****	**Adjusted†**
sFlt-1 (ng/mL)‡				
Q1 (≤3.29)	Reference	Reference	Reference	Reference
Q2 (3.3–4.35)	-0.04 (-1.01; 0.92)	-0.19 (-1.25; 0.87)	-0.01 (-0.15; 0.13)	-0.01 (-0.17; 0.14)
Q3 (4.36–5.54)	-0.24 (-1.21; 0.72)	-0.39 (-1.44; 0.66)	-0.04 (-0.18; 0.11)	-0.05 (-0.20; 0.10)
Q4 (5.55–7.43)	0.93 (-0.04; 1.90)	0.65 (-0.45; 1.75)	0.14 (-0.01; 0.28)	0.10 (-0.05; 0.25)
Q5 (≥7.44)	0.96 (-0.01; 1.93)	**1.14 (0.05; 2.23)**[1]	**0.14 (0.00; 0.28)**[1]	**0.18 (0.02; 0.34)**[1]
Linear trend analyses*	**0.11 (0.01; 0.21)**[1]	**0.13 (0.01; 0.26)**[1]	**0.02 (0.00; 0.03)**[1]	**0.02 (0.00; 0.04)**[1]
PlGF (pg/mL)‡				
Q1 (≤24.0)	Reference	Reference	Reference	Reference
Q2 (24.01–30.4)	0.89 (-0.08; 1.85)	0.77 (-0.25; 1.79)	0.13 (-0.01; 0.27)	0.12 (-0.02; 0.26)
Q3 (30.41–37.6)	**1.75 (0.79; 2.72)**[2]	**1.88 (0.90; 2.85)**[2]	**0.26 (0.12; 0.40)**[2]	**0.29 (0.15; 0.43)**[2]
Q4 (37.61–47.9)	**2.39 (1.40; 3.38)**[2]	**2.15 (1.03; 3.28)**[2]	**0.35 (0.21; 0.49)**[2]	**0.33 (0.24; 0.42)**[2]
Q5 (≥47.91)	**2.42 (1.35; 3.48)**[2]	**2.61 (1.40; 3.81)**[2]	**0.34 (0.19; 0.49)**[2]	**0.38 (0.20; 0.55)**[2]
Linear trend analyses*	**0.02 (0.00; 0.03)**[1]	**0.02 (0.00; 0.04)**[1]	**0.00 (0.00; 0.00)**[1]	**0.00 (0.00; 0.01)**[1]
PAI 2 (ng/mL)‡				
Q1 (≤25.6)	Reference	Reference	Reference	Reference
Q2 (25.61–31.21)	**1.82 (0.86; 2.78)**[2]	**2.67 (1.62; 3.72)**[2]	**0.28 (0.14; 0.42)**[2]	**0.40 (0.25; 0.55)**[2]
Q3 (31.22–36.82)	**1.16 (0.18; 2.14)**[1]	**1.73 (0.64; 2.82)**[2]	**0.18 (0.04; 0.33)**[1]	**0.27 (0.11; 0.43)**[2]
Q4 (36.83–45.32)	**3.16 (2.16; 4.17)**[2]	**3.75 (2.60; 4.90)**[2]	**0.48 (0.34; 0.63)**[2]	**0.58 (0.42; 0.75)**[2]
Q5 (≥45.33)	**2.68 (1.62; 3.74)**[2]	**3.56 (2.35; 4.77)**[2]	**0.39 (0.24; 0.55)**[2]	**0.53 (0.35; 0.70)**[2]
Linear trend analyses*	**0.06 (0.04; 0.09)**[2]	**0.08 (0.05; 0.11)**[2]	**0.01 (0.01; 0.01)**[2]	**0.01 (0.01; 0.02)**[2]
Folate (nmol/L)				
Q1 (≤11.5)	Reference	Reference	Reference	Reference
Q2 (11.51–16.8)	0.30 (-0.68; 1.28)	0.33 (-0.66; 1.33)	0.06 (-0.08; 0.20)	0.06 (-0.08; 0.21)
Q3 (16.81–22.1)	0.97 (-0.01; 1.94)	0.99 (-0.02; 1.99)	0.13 (-0.01; 0.27)	0.13 (-0.01; 0.28)
Q4 (22.11–28.2)	0.62 (-0.35; 1.59)	0.68 (-0.34; 1.71)	0.09 (-0.05; 0.23)	0.10 (-0.05; 0.25)
Q5 (≥28.21)	**1.41 (0.42; 2.39)**[2]	**1.44 (0.40; 2.47)**[2]	**0.20 (0.06; 0.34)**[2]	**0.20 (0.06; 0.34)**[2]
Linear trend analyses*	**0.04 (0.01; 0.08)**[1]	**0.04 (0.01; 0.08)**[1]	**0.01 (0.00; 0.01)**[1]	**0.01 (0.00; 0.01)**[1]

Results from multivariable linear regression analyses. Analyses are restricted to women with a gestational age based on LMP within 7 days of a gestational age based on CRL (n = 1,380). Values are presented as regression coefficients (95% confidence interval), representing the change in CRL (in mm or SDS) compared to the reference category (Q1). *Linear trend analyses reflect the change in CRL (SDS) as a continuous measure per unit (biomarker) change. **Adjusted for gestational age based on LMP, gestational age based on LMP at venous puncture and duration of last menstrual cycle. †Additionally adjusted for maternal age, maternal height, maternal BMI, maternal ethnicity, fetal gender, parity, educational level, smoking. ‡Additionally adjusted for folic acid supplement use. [1]P-value <0.05 [2]P-value <0.01. Abbreviations: CRL crown-to-rump length; Q, quintile; SDS, standard deviation score.

Supplement table S3 | Associations between maternal sFlt-1, PlGF, PAI-2 and folate and CRL.

	Effect size for CRL in mm		Effect size for CRL SDS	
BIOMARKERS	**Crude****	**Adjusted†**	**Crude****	**Adjusted†**
sFlt-1 (ng/mL)‡				
Q1 (≤3.29)	Reference	Reference	Reference	Reference
Q2 (3.3–4.35)	0.43 (-0.75; 1.61)	0.53 (-0.76; 1.82)	0.04 (-0.13; 0.21)	0.05 (-0.13; 0.24)
Q3 (4.36–5.54)	0.00 (-1.16; 1.17)	-0.16 (-1.43; 1.11)	0.02 (-0.15; 0.19)	-0.02 (-0.20; 0.17)
Q4 (5.55–7.43)	1.02 (-0.16; 2.2)	0.92 (-0.36; 2.20)	0.16 (-0.01; 0.33)	0.14 (-0.05; 0.32)
Q5 (≥7.44)	1.09 (-0.08; 2.27)	**1.68 (0.36; 3.00)**[2]	**0.18 (0.01; 0.35)**[1]	**0.25 (0.06; 0.44)**[1]
Linear trend analyses*	**0.14 (0.02; 0.25)**[1]	**0.20 (0.07; 0.34)**[2]	**0.03 (0.01; 0.04)**[2]	**0.03 (0.01; 0.05)**[2]
PlGF (pg/mL)‡				
Q1 (≤24.0)	Reference	Reference	Reference	Reference
Q2 (24.01–30.4)	0.89 (-0.39; 2.06)	0.75 (-0.43; 1.93)	0.12 (-0.05; 0.29)	0.12 (-0.06; 0.30)
Q3 (30.41–37.6)	**1.34 (0.16; 2.51)**[1]	**1.71 (0.41; 3.01)**[1]	**0.19 (0.02; 0.36)**[1]	**0.26 (0.07; 0.45)**[2]
Q4 (37.61–47.9)	**2.75 (1.54; 3.96)**[2]	**2.75 (1.47; 4.03)**[2]	**0.40 (0.22; 0.57)**[2]	**0.41 (0.22; 0.59)**[2]
Q5 (≥47.91)	**2.82 (1.53; 4.11)**[2]	**3.19 (1.73; 4.65)**[2]	**0.37 (0.18; 0.55)**[2]	**0.43 (0.22; 0.64)**[2]
Linear trend analyses*	0.01 (-0.00; 0.03)	0.02 (-0.00; 0.04)	0.00 (0.00; 0.01)	0.00 (0.00; 0.01)
PAI 2 (ng/mL)‡				
Q1 (≤25.6)	Reference	Reference	Reference	Reference
Q2 (25.61–31.21)	**2.36 (1.18; 3.53)**[2]	**3.64 (2.35; 4.92)**[2]	**0.36 (0.19; 0.53)**[2]	**0.55 (0.37; 0.74)**[2]
Q3 (31.22–36.82)	**1.60 (0.41; 2.79)**[2]	**2.34 (1.02; 3.65)**[2]	**0.25 (0.08; 0.43)**[2]	**0.37 (0.18; 0.55)**[2]
Q4 (36.83–45.32)	**3.88 (2.66; 5.10)**[2]	**4.91 (3.52; 6.29)**[2]	**0.54 (0.36; 0.72)**[2]	**0.70 (0.50; 0.90)**[2]
Q5 (≥45.33)	**3.11 (1.81; 4.41)**[2]	**4.06 (2.59; 5.54)**[2]	**0.44 (0.25; 0.63)**[2]	**0.58 (0.37; 0.79)**[2]
Linear trend analyses*	**0.06 (0.02; 0.09)**[2]	**0.08 (0.05; 0.12)**[2]	**0.01 (0.00; 0.01)**[2]	**0.01 (0.01; 0.02)**[2]
Folate (nmol/L)				
Q1 (≤11.5)	Reference	Reference	Reference	Reference
Q2 (11.51–16.8)	0.36 (-0.82; 1.53)	0.30 (-0.89; 1.49)	0.03 (-0.15; 0.20)	0.02 (-0.15; 0.20)
Q3 (16.81–22.1)	**1.36 (0.18; 2.55)**[1]	1.21 (-0.01; 2.43)	0.16 (-0.01; 0.34)	0.15 (-0.03; 0.33)
Q4 (22.11–28.2)	0.44 (-0.74; 1.62)	0.31 (-0.90; 1.52)	0.05 (-0.12; 0.22)	0.04 (-0.14; 0.22)
Q5 (≥28.21)	**1.72 (0.56; 2.89)**[2]	**1.52 (0.29; 2.75)**[1]	**0.22 (0.06; 0.39)**[2]	**0.20 (0.02; 0.38)**[1]
Linear trend analyses*	**0.05 (0.01; 0.09)**[1]	**0.04 (0.00; 0.09)**[1]	**0.01 (0.00; 0.01)**[1]	0.01 (0.00; 0.01)

Results from multivariable linear regression analyses. Analyses are restricted to women who stopped using contraceptives >2 months before conception (n = 1,301). Values are presented as regression coefficients (95% confidence interval), representing the change in CRL (in mm or SDS) compared to the reference category (Q1). *Linear trend analyses reflect the change in CRL (SDS) as a continuous measure per unit (biomarker) change. **Adjusted for gestational age based on LMP, gestational age based on LMP at venous puncture and duration of last menstrual cycle. †Additionally adjusted for maternal age, maternal height, maternal BMI, maternal ethnicity, fetal gender, parity, educational level, smoking. ‡Additionally adjusted for folic acid supplement use. [1]P-value < 0.05. [2]P-value <0.01. Abbreviations: CRL crown-to-rump length; Q, quintile; SDS, standard deviation score.

Chapter 4

Biomarkers of placentation in maternal and umbilical cord blood and fetal and childhood growth

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ABSTRACT

Objective | Plasma concentrations of soluble fms-like tyrosine kinase 1 (sFlt-1) and placental growth factor (PlGF) have been suggested to affect pregnancy outcomes. However, the effects on fetal and childhood growth, in particular of fetal concentrations, remain largely unknown. This study aims to identify associations of both maternal and fetal sFlt-1 and PlGF with fetal and childhood growth patterns.

Study design | This study was performed in 5980 mothers and 4108 of their children followed from fetal life onwards in a population-based prospective cohort study. Blood samples were obtained from mothers in early and mid-pregnancy and from the umbilical vein at delivery. Fetal and childhood growth characteristics were measured repeatedly.

Results | Lower early pregnancy sFlt-1 and PlGF and a relatively small increase of PlGF from early to mid-pregnancy resulted in a lower fetal weight growth which remained present throughout childhood. Mothers with an increase in sFlt-1 from early to mid-pregnancy showed a lower fetal weight growth, but showed inconsistent results into childhood. Newborns with the highest sFlt-1 and the lowest PlGF concentrations in umbilical cord blood showed a lower fetal and childhood weight growth from 30 weeks gestation onwards up to the age of 6 years. Similar patterns were observed in relation to fetal and childhood length growth.

Conclusion | Children of mothers with a unfavorable angiogenic profile showed a lower fetal weight growth which tracked throughout childhood. Additionally, children with a high sFlt-1 and low PlGF at birth also showed a lower fetal and childhood weight growth.

INTRODUCTION

Impaired fetal growth is associated with neonatal morbidity and mortality[1] and a subsequent risk of developing cardiovascular disease in later life.[2,3] Early placental development is of great importance for normal fetal growth and development.[4] Placental development comprises both vasculogenesis and angiogenesis.[5,6] Within this processes, the vascular endothelial growth factor (VEGF) system is essential.[6] Placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) are included in the VEGF-system. PlGF is produced by endothelial cells, cyto- and syncytiotrophoblasts and binds to VEGF receptor 1 (VEGFR-1 or Flt-1), thereby promoting proliferation, migration and activation of endothelial cells. sFlt-1 is the soluble form of Flt-1 and is synthesized by the trophoblast of the placenta,[7] but also made in cells outside the placenta such as endothelial cells and monocytes.[8,9] sFlt-1 binds PlGF, thereby reducing the free circulating concentrations of PlGF.[10,11] Low maternal PlGF concentrations have been related to adverse maternal and fetal outcomes.[12,13] Results regarding the associations of sFlt-1 with fetal growth seem not consistent.[10] One of our previous studies focused on the maternal circulation and pregnancy outcome.[12] Only a few small studies evaluated the associations between maternal and fetal PlGF and sFlt-1 concentrations and intrauterine growth restriction.[14,15] Angiogenesis is not only essential for early placental development, but also crucial for organ growth and cardiovascular development in the embryo.[16] However, little is known about the impact of sFlt-1 and PlGF in the fetal circulation on the development of the cardiovascular system and subsequent fetal and childhood growth.

In the present study, we therefore examined associations of both maternal and umbilical cord PlGF and sFlt-1 concentrations with repeatedly measured fetal and childhood size measurements to estimate growth.

MATERIALS AND METHODS

Design and study population

This study was embedded in the Generation R Study, a population-based prospective cohort study from early pregnancy onwards in Rotterdam, the Netherlands.[17] The study has been approved by the Medical Ethics Committee of the Erasmus MC, University Medical Centre, Rotterdam, the Netherlands. Written informed consent was obtained from all mothers for both maternal and child data.[18]

In total, 8880 mothers were enrolled during their pregnancy. We excluded pregnancies not leading to singleton live births (n = 197), loss to follow-up (n = 45), pregnancies with a gestational age below 24 weeks (n = 10) and mothers without any fetal growth measurements (n = 3). For the growth analysis with the maternal sFlt-1 and PlGF concentrations we additionally excluded mothers without sFLt-1 or PlGF concentrations available in early pregnancy (n = 2645), leading to 5980 mothers available for analysis (**Supplement Figure S1**). For the growth analysis with umbilical cord data we excluded children without sFlt-1 or PlGF concentrations available from

umbilical cord blood (n = 4517), leading to 4108 children available for analysis (**Supplement Figure S1**).

Fetal and childhood measurements

Fetal ultrasound examinations were carried out in two dedicated research centers in early, mid- and late pregnancy. We established gestational age by using data from the first ultrasound examination.[20] In mid- and late pregnancy, fetal head circumference, abdominal circumference, and femur length (FL) were measured to the nearest millimeter using standardized ultrasound procedures.[21] Estimated fetal weight (EFW) was subsequently calculated by using formula of Hadlock.[22] Gestational-age-adjusted standard deviation scores (SDS) of all fetal growth characteristics were constructed based on data from the study group.[20] Information about date of birth, fetal gender, birth weight and length was obtained from medical records and hospital registries. Gestational-age-adjusted SDS for birth weight and length were constructed using North European growth Standards.[23]

Well-trained staff in the Community Health Centers obtained postnatal growth characteristics according to standard schedule and procedures at the ages of 3 months, 6 months, 12 months, 24 months, 36 months, 48 months and 72 months. SDS for childhood growth characteristics were obtained with Dutch growth reference Charts (Growth Analyzer 3.0; Dutch Growth Research Foundation, Rotterdam, Netherlands).

Angiogenic factors

In early and mid-pregnancy maternal venous blood plasma samples were drawn. Immediately following delivery, 30 mL cord blood from the umbilical vein was collected. sFlt-1 and PlGF were analyzed using a prototype of a microparticle-enhanced immunoassay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands). The between-run coefficients of variation and analytical ranges are listed in **Supplement Table S1**. Details of data collection and procedures have been described previously.[12,19]

Covariates

Information on maternal age, ethnicity, education, parity folic acid supplement use was obtained from self-administrated questionnaires at enrolment. Ethnicity was defined according to Statistics Netherlands.[17,24] Education was assessed by the highest completed education of the mother. Information on maternal smoking habits was obtained by questionnaires. Maternal weight and height were measured at intake to calculate body mass index (BMI, kg/m^2). Information on fertility treatment was obtained from community midwives and obstetricians. Maternal blood pressure was measured at enrolment. The mean value of two blood pressure readings over a 60-second interval was documented.[25] Information about the presence of preeclampsia was retrieved from medical records after delivery.[26] Information about breastfeeding was obtained by questionnaires in infancy.

Statistical analyses

First, we performed nonresponse analyses for the analysis in the mothers and children separately. Differences were tested using Student's *t* test, Mann-Whitney's U test and chi-square test. Second, correlations between sFlt-1 and PlGF in maternal and umbilical cord blood were determined with the use of Spearman's correlation coefficients. Third, early to mid-pregnancy changes in sFlt-1 and PlGF (difference in concentrations / gestational weeks interval) was referred to as delta sFlt-1 and delta PlGF, respectively. Subsequently, we explored the associations of maternal and umbilical cord sFlt-1 and PlGF concentrations with repeatedly measured fetal and childhood growth characteristics ((estimated fetal) weight and (femur) length) using unbalanced repeated measurement regression models with an unstructured covariance structure. These regression models take the correlation of multiple measurements within one subject into account and assesses both the time-independent and time-dependent effect of sFlt-1 and PlGF on fetal and childhood growth. Moreover, they have an optimal use of available measurements by allowing for incomplete outcome data.[27] For all regression analyses, maternal and umbilical cord biomarker concentrations, as well as maternal delta sFlt-1 and PlGF concentrations were categorized as quintiles and subsequently used as a categorical measure. Confounding variables were determined a priori and based on previous literature.[12,28] Potential confounders were then selected as a result of exploratory analysis and included in the analysis if the effect estimates changed >10%. By using this approach maternal age, folic acid supplement use, maternal height and BMI, systolic blood pressure, education, ethnicity, parity, smoking and fetal gender were included in the final analysis. Models focused on childhood growth outcomes were additionally adjusted for age at visit and breastfeeding. Missing data of the covariates were completed using the Markov Chain Monte Carlo multiple imputation technique.[29] The percentages of missing values within the population for analysis were lower than 12 %, except for folic acid supplement use (24.3%). The prevalence of preeclampsia was 2.1% for the analysis in the mothers. Since exclusion of these mothers did not materially change our results, they were included in the analysis. Associations were considered significant at $p<0.05$. The unbalanced repeated measurements regression analyses were performed with the Statistical Analysis System version 9.3 (SAS, Institute Ic. Gary NC, USA). The remaining analyses were performed using the Statistical Package of Social Sciences release 20.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Maternal and child characteristics are presented in **Table 1.** Fetal and childhood growth characteristics are presented in **Table 2**. Supplement Table S2 presents the correlations between maternal sFlt-1 and PlGF concentrations in early and mid-pregnancy and also the correlation between umbilical cord sFlt-1 and PlGF concentrations as well as the correlations between these biomarkers in the maternal and fetal (umbilical cord) circulation. **Supplement Table S3** and **S4** show that characteristics of those included in the analysis revealed that mothers were on average taller, had a lower BMI, were more often nulliparous and of western ethnicity, were

higher educated and gave birth to larger babies. **Figure 1A** shows that mothers with higher sFlt-1 concentrations in early pregnancy had a higher fetal weight growth from 20 weeks onwards, resulting in a significantly higher birth weight as compared to mothers in the reference group. Children of these mothers remained heavier until the age of 6 years, although this difference decreased with increasing age. **Figure 1C** shows that an increase in maternal sflt-1 concentrations from early to mid-pregnancy resulted in lower fetal weight growth from 30 weeks onwards. However, in contrast to children of mothers in the fourth and third quintile, children of mothers with the highest increase in sFlt-1 during pregnancy (fifth quintile) did not have a significantly lower weight growth from 6 months onwards as compared to the reference group. **Figure 1B** and **1D** show that mothers with lower PlGF concentrations in early pregnancy and the smallest increase in PlGF concentrations from early to mid-pregnancy had a lower fetal weight growth from 20 weeks onwards, resulting in a smaller newborn. Children of these mothers remained smaller until the age of 6 years, also this difference decreased with increasing age. Similar tendencies were observed for the association between maternal sFlt-1 and PlGF concentrations and fetal and childhood length growth (**Supplement Figure S2**). The individual data points and confidence intervals for the different quintiles regarding these associations of the repeated measurement analyses are given in Supplement Tables S5 and S6.

Table 1 | Baseline characteristics.

	Mothers for analysis n = 5980	**Children for analysis n = 4108**
MATERNAL CHARACTERISTICS		
Age at intake (years)	29.8 (5.1)	29.5 (5.2)
Gestational age at intake (weeks)	13.4 (10.6–17.4)	14.4 (10.9–22.8)
Height (cm)	167.5 (7.4)	167.7 (7.4)
Weight at intake (kg)	68.8 (13.1)	69.4 (13.1)
Body mass index at intake (kg/m^2)	23.5 (19.3–33.3)	23.8 (19.3–33.2)
Blood pressure		
Systolic	115.6 (12.3)	115.4 (12.3)
Diastolic	68.2 (9.6)	67.9 (9.5)
Parity at intake, %		
Nulliparous	43.2	44.0
Multipara	56.8	56.0
Education, %		
Primary/secondary school	41.7	43.8
Higher education	46.6	44.9
Missing	11.6	11.3

Table 1 | Baseline characteristics *(Continued)*.

	Mothers for analysis n = 5980	Children for analysis n = 4108
MATERNAL CHARACTERISTICS		
Race/Ethnicity, %		
Western	58.9	57.8
Non-Western	35.4	36.7
Missing	5.6	5.5
Smoking, %		
No	64.1	64.5
Yes, until pregnancy was known	8.4	7.8
Yes, continued	16.6	17.6
Missing	10.9	10.1
Folic acid supplement use, %		
No use	19.1	21.5
Start before eight weeks	24.3	23.9
Preconception start	33.0	30.3
Missing	23.7	24.3
Pre-eclampsia, %		
No	90.5	93.7
Yes	2.1	1.1
Missing	7.5	5.1
Early pregnancy biomarker concentrations		
Gestational age blood sampling (<18 weeks)	13.2 (10.5–17.2)	
sFlt-1 (ng/ml)	5.1 (2.2–11.9)	
PlGF (pg/ml)	43.1 (17.6–156.1)	
Mid-pregnancy biomarker concentrations (n = 5269)		
Gestational age blood sampling (18–25 weeks)	20.4 (19.1–22.4)	
sFlt-1 (ng/ml)	4.9 (1.8–14.2)	
PlGF (pg/ml)	199.7 (90.2–502.1)	
INFANT CHARACTERISTICS		
Umbilical cord blood biomarker concentrations		
Gestational age at blood sampling		40.1 (37.4–42.0)
sFlt-1 (ng/ml)		0.5 (0.2–2.2)
PlGF (pg/ml)		8.6 (2.0–15.7)
Gender, %		
Boy	50.6	51.3
Girl	49.4	48.7

Abbreviations: sFlt-1; soluble fms-like tyrosine kinase 1; PlGF; placental growth factor. Values represent mean (standard deviation), median (90% range) or percentages.

Table 2 | Fetal and childhood growth measurements.

	Mothers for analysis n = 5980	Children for analysis n = 4108
FETAL		
Mid-pregnancy measurements, %	96.8	95.7
Gestational age (weeks)	20.5 (19.1–22.6)	20.5 (18.9–22.8)
Estimated fetal weight (g)	377.3 (84.2)	382.9 (94.5)
Femur length (mm)	33.4 (3.3)	33.5 (3.6)
Late pregnancy measurements, %	96.6	98.7
Gestational age (weeks)	30.4 (29.0–32.2)	30.3 (28.8–32.3)
Estimated fetal weight (g)	1611.9 (250.6)	1613.6 (251.4)
Femur length (mm)	57.4 (3.0)	57.4 (52.9–62.4)
Birth measurements, %	99.5	99.9
Weight (g)	3420.1 (564.2)	3461.1 (503.6)
Length (cm)	50.2 (2.4)	50.2 (2.3)
CHILDHOOD		
Visit 5–10 months, %	71.6	70
Age (months)	6.2 (5.4–7.5)	6.2 (5.4–7.7)
Weight (kg)	7.9 (0.9)	7.9 (0.9)
Length (cm)	67.7 (2.6)	67.7 (2.7)
Visit 10–13 months, %	64.7	63.4
Age (months)	11.1 (10.2–12.3)	11.1 (10.2–12.4)
Weight (kg)	9.7 (1.1)	9.7 (1.1)
Length (cm)	74.4 (2.7)	74.5 (2.6)
Visit 23–35 months, %	62.5	63.4
Age (months)	25.0 (23.6–30.5)	25.1 (23.5–30.7)
Weight (kg)	13.1 (1.6)	13.1 (1.6)
Length (cm)	88.7 (3.7)	88.9 (3.7)
Visit 35–44 months, %	54.3	56.2
Age (months)	36.7 (35.6–39.8)	36.8 (35.6–39.8)
Weight (kg)	15.2 (1.9)	15.3 (1.9)
Length (cm)	97.4 (3.8)	97.4 (3.8)
Visit 72 months, %	70.4	71.6
Age (months)	72.5 (68.9–87.7)	73.2 (69.2–90.6)
Weight (kg)	23.2 (4.1)	23.6 (4.3)
Length (cm)	119.4 (5.9)	120.1 (6.1)

Values represent mean (standard deviation), median (90% range) or percentages.

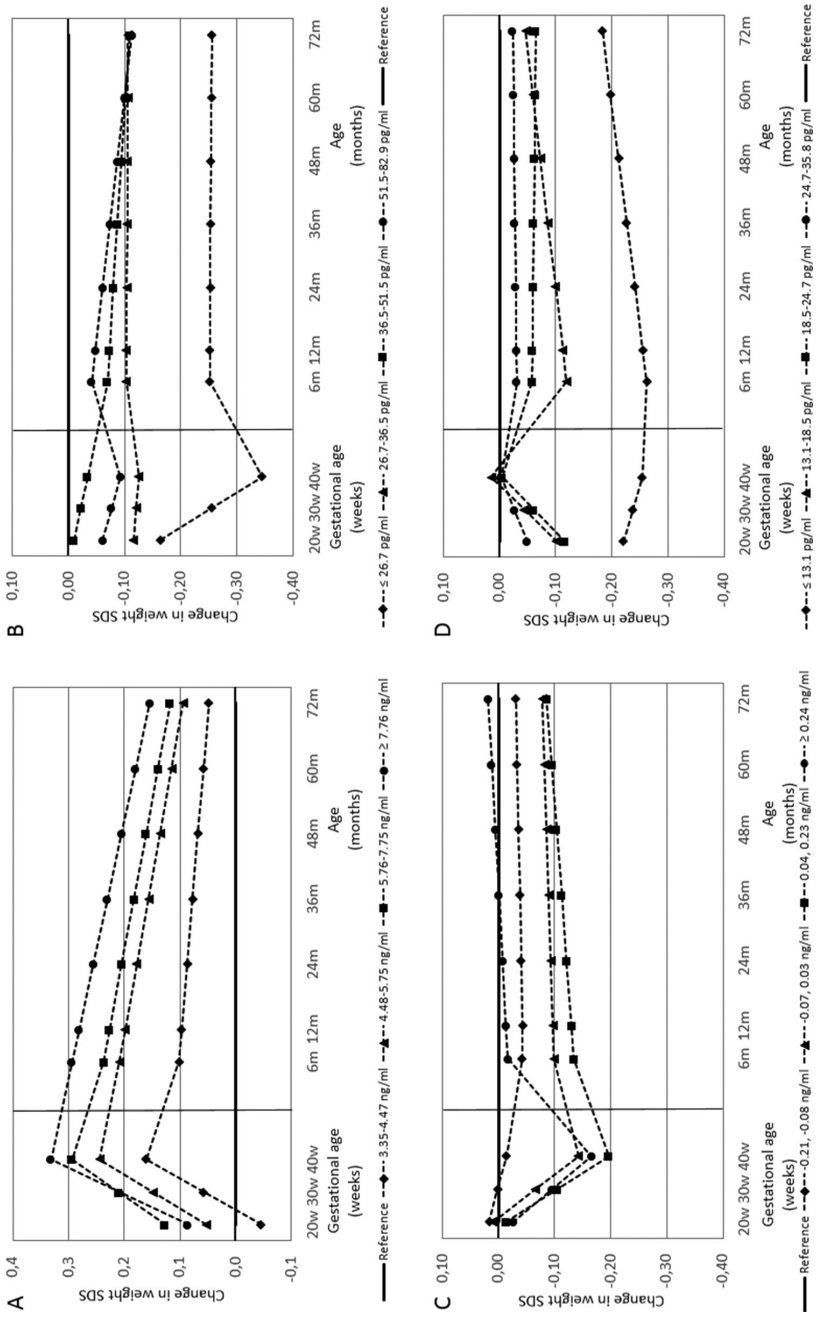

Figure 1 | Associations between maternal sFlt-1 and PlGF concentrations and repeatedly measured fetal and infant weight growth (n = 5980).

Fetal and childhood weight growth among early pregnancy sFlt-1 (1A) and PlGF (1B) and delta sFlt-1 (1C) and PlGF (1D) categories. Adjustments: gestational age (blood sampling), maternal age, height, BMI, parity, education, ethnicity, smoking, folic acid supplement use, systolic blood pressure and fetal gender. Childhood analysis additionally adjusted for breastfeeding.

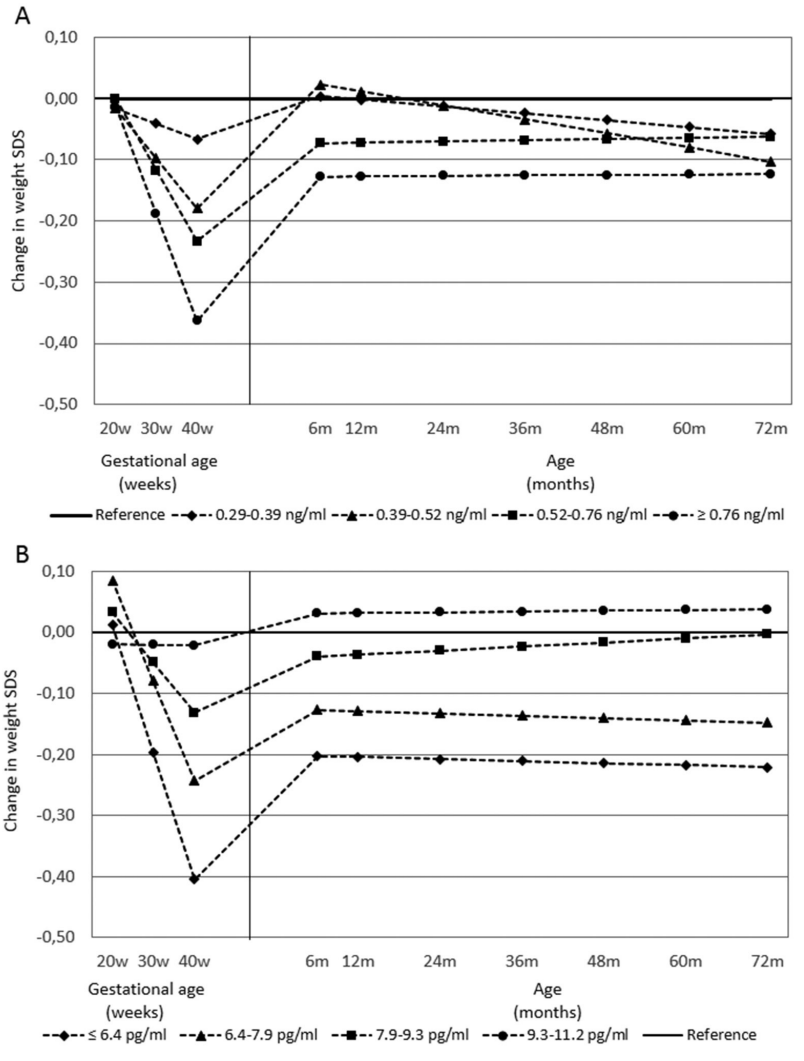

Figure 2 | Associations between umbilical cord sFlt-1 and PlGF concentrations and repeatedly measured fetal and infant weight growth (n = 4108).

Fetal and childhood weight growth among umbilical cord sFlt-1 (2A) and PlGF (2B) categories. Adjustments: gestational age (blood sampling), maternal age, height, BMI, parity, education, ethnicity, smoking, folic acid supplement use, systolic blood pressure and fetal gender. Childhood analysis additionally adjusted for breastfeeding.

Figure 2A shows that children with the highest sFlt-1 concentrations in umbilical cord blood had a lower weight growth from 30 weeks onwards, resulting in a smaller birth weight. These children remained smaller until the age of 6 years. **Figure 2B** shows that the lowest PlGF concentrations in umbilical cord blood was associated with a lower weight growth from 30 weeks gestation onwards,

resulting in a lower weight at birth and a lower weight at the age of 6 years. Similar tendencies were observed for the association between umbilical cord sFlt-1 and PlGF concentrations and fetal and childhood length growth (**Supplement Figure S3**). The individual data points and confidence interval for the different quintiles regarding these associations of the repeated measurement analyses are given in **Supplement Tables S7** and **S8**.

DISCUSSION

Findings from this large population-based prospective cohort study suggest that higher maternal sFlt-1 concentrations in early pregnancy resulted in a higher fetal weight growth and a subsequent heavier child at the age of 6 years. An increase in maternal sFlt-1 concentrations from early to mid-pregnancy, however, resulted in a lower fetal weight growth. We observed a lower fetal growth in mothers with lower PlGF concentrations in early pregnancy as well as in mothers with a relatively small increase in PlGF concentrations from early to mid-pregnancy, followed by smaller children at the age of 6 years. High sFlt-1 and low PlGF concentrations in umbilical cord blood were associated with lower weight growth from 30 weeks gestation onwards until the age of 6 years.

The associations between maternal angiogenic factors and birth weight have frequently been studied.[12,13,30-32] However, most studies have focused on pregnancy outcomes and complications. In line with our results, low maternal PlGF concentrations have consistently been associated with adverse birth outcomes.[12,13,31,33] However, studies on the association between maternal sFlt-1 concentrations and fetal growth have shown inconsistent results. A number of factors, including differences in study population and design, confounders, biological differences and differences in GA at blood sampling may have contributed to these conflicting results. In the same cohort as our current study, Coolman et al. showed that higher maternal sFlt-1 and PlGF concentrations in early pregnancy were associated with a higher birth weight.[1] Similar results were found by Smith and colleagues.[30] They observed a reduced risk of delivering an small for gestational age infant in mothers with higher sFlt-1 and PlGF concentrations at 10-14 weeks of gestation (odds ratio (OR) 0.92; *P*-value <0.05 and OR 0.93; *P*-value <0.05, respectively). Furthermore, Åsvold et al. found a 6-fold increased risk on having a small for gestational age newborn in women with low sFlt-1 in the first trimester and high sFlt-1 concentrations in the second trimester.[31]

Others, however, have shown a negative association between increased sFlt-1 and birth weight, but not earlier than the second trimester.[10] Hypoxia has been shown to increase sFlt-1.[34] Recent studies have therefore proposed that the positive relation between early pregnancy sFlt-1 concentrations and fetal weight reflects the low oxygen environment needed for early placental and embryonic development,[12,28] whereas in subsequent development may reflect the response to fetal-placental hypoxia which is associated with placental impairment.[4] This is in accordance with the adverse effect of an increase in sFlt-1 between early and mid-pregnancy on fetal growth as shown by our data and is substantiated by others.[31,35] Suboptimal placental growth and function due to an angiogenic imbalance is unable to secure oxygen and nutritional supply to the fetus consequently leading to developmental adaptations with a permanent influence on growth and

development in later life.[3,36] In line with this hypothesis we observed that differences in length and weight growth starting in pregnancy as a result of an unfavorable angiogenic prolife led to smaller children until the age of 6 years. We therefore propose that alterations in maternal sFlt-1 and PlGF may have long-term effects on childhood growth and development.

To our knowledge, only a few small studies examined the association between fetal sFlt-1 and PlGF concentrations and intrauterine growth restriction.[14,15] These studies did not take into account postnatal growth patterns. In line with these previous studies, we observed that low PlGF and high sFlt-1 concentrations in umbilical cord blood were associated with slower fetal growth rates resulting in children with a lower birth weight. Moreover, we also showed that these children remained smaller until the age of 6 years. We were not able to find a correlation between maternal and fetal sFlt-1 and PlGF concentrations. Also, maternal concentrations were much higher than the concentrations present in umbilical cord blood. This could be explained by the enhanced secretion of sFlt-1 and PlGF from the placenta into the maternal circulation rather than into the fetal circulation as both villous and extra-villous trophoblasts contain very high levels of sFlt-1 and are in direct contact with the maternal circulation.[37] This supports the idea that angiogenic factors in the fetal circulation are mainly produced by the fetus itself rather than having a placental origin. Additionally, previous studies have shown that sFlt-1 and PlGF concentrations are also detectable in males and non-pregnant females as sFlt-1 and PlGF are both expressed on cells different from those of the placenta[38] and after birth still contribute to organ growth and repair.[39] However, an angiogenic imbalance may result in an angiogenic switch which could cause vascular effects in the fetus during pregnancy and subsequently affect fetal and childhood growth patterns. With regard to the latter, this may in the long term be important for the well-established association of fetal growth restriction with the long-term risk of vascular disease in adulthood.[40] However, future studies are needed to gain a further understanding of the effects of angiogenic factors on early and late growth patterns in children and possible cardiovascular consequences in later life.

Methodological considerations

This study was conducted within a large birth cohort with the availability of a large number of prospectively collected data. Fetal growth was assessed by actually measuring fetal growth characteristics in mid- and late pregnancy instead of using only birth outcomes as proxy for fetal growth. To our knowledge, this is the first study that has investigated the associations of sFlt-1 and PlGF in umbilical cord blood with repeatedly measured fetal and childhood growth characteristics. However, some limitations should be considered. The Generation R Study is characterized by a relatively highly educated and healthy study population compared to the population in the study area.[17] Our estimates can therefore be too conservative and underestimate the true effect measures. Moreover, 67% of the mothers had early pregnancy blood samples available and 46% of the newborns had umbilical cord blood samples available. Nonresponse analyses for both populations for analysis showed a similar pattern. Characteristics of those included in the analysis showed that mothers were on average taller, had a lower BMI, were more often nulliparous and of western ethnicity, were higher educated and gave birth to larger babies as compared to those not included in the study. This nonresponse would lead to biased effect estimates if associations

would differ between those included and not included in the analyses. This is difficult to ascertain, but seems unlikely.[41] The nonresponse at baseline and at follow-up might have led to a selection towards a more healthy population, which might affect the generalizability of our results. We used a multiple imputation model in this study for missing values in covariates. This reduces selection bias due to random missing in the covariates. Last, we were able to control for a large number of potential confounders. However, residual confounding cannot be ruled out completely due to the observational design of the study.

CONCLUSION

An angiogenic profile that is characterized by both low maternal sFlt-1 and PlGF concentrations in early pregnancy, as well as a subsequent relatively small increase in PlGF towards mid-pregnancy is associated with reduced fetal and childhood growth patterns. Higher sFlt-1 and lower PlGF concentrations in umbilical cord blood also seem to impair fetal and childhood weight growth.

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Table S1 | Between-run coefficients of variation of angiogenic factors.

	Between run coefficients				Analytic range
	First		Second		
ANGIOGENIC FACTORS	%		%		
sFlt-1, plasma	2.8	5.5 ng/mL	2.3	34 ng/mL	0–150 ng/mL
PlGF, plasma	4.7	24 pg/mL	3.8	113 pg/mL	10-1500 pg/nL

Abbreviations: sFlt-1; soluble fms-like tyrosine kinase 1; PlGF; placental growth factor. The angiogenic factors were analyzed using a microparticle-enhanced immunoassay on the Architect System (Abbott Diagnostics B.V., Hoofddorp, the Netherlands). The assays used to analyze sFlt-1 and PlGF were performed with pre-launch assays.

Table S2 | Spearman correlation coefficients between maternal and umbilical cord sFlt-1 and PlGF concentrations.

			Mother				Child	
			<18 weeks		18–25 weeks		Birth	
			sFlt-1 (ng/ml)	**PlGF** (pg/ml)	**sFlt-1** (ng/ml)	**PlGF** (pg/ml)	**sFlt-1** (ng/ml)	**PlGF** (pg/ml)
Mother	**<18 weeks**	**sFlt-1** (ng/ml)	1					
		N						
		PlGF (pg/ml)	0.127**	1				
		N	5918					
	18–25 weeks	sFlt-1 (ng/ml)	0.747**	0.059**	1			
		N	5233	5245				
		PlGF (pg/ml)	0.139**	0.447**	0.130**	1		
		N	5235	5247	5267			
Child	**Birth**	sFLt-1 (ng/ml)	0.023	0.03	0.034	0.049*	1	
		N	2861	2874	2572	2574		
		PlGF (pg/ml)	-0.024	0.002	-0.005	0.041	0.001	1
		N	2579	2589	2300	2302	2570	

* P-value <0.05; **P-value <0.01

Table S3 | Nonresponse analysis of mothers included versus excluded from the study.

	Included n = 5980	Excluded n = 2900	P-value*
MATERNAL CHARACTERISTICS			
Age at intake (years)	29.4 (5.8)	29.8 (5.1)	0.006
Gestational age at intake (weeks)	13.4 (10.6–17.4)	19.9 (11.9–30.5)	<0.001
Height (cm)	167.5 (7.4)	166.3 (7.4)	<0.001
Weight at intake (kg)	68.8 (13.1)	71.1 (13.7)	<0.001
Body mass index at intake (kg/m^2)	23.5 (19.3–33.3)	24.8 (19.7–34.8)	<0.001
Body mass index before pregnancy (kg/m^2)	22.6 (18.7–32.1)	22.8 (18.5–33.2)	0.01
Blood pressure at intake (mmHg)			
Systolic	115.5 (12.3)	114.9 (12.0)	0.03
Diastolic	68.2 (9.6)	67.3 (9.6)	<0.001
Nulliparous, %	56.8	50.5	<0.001
Education, %			<0.001
Primary/secondary school	47.2	56.2	
Higher education	52.8	43.8	
Race/ethnicity, %			<0.001
Western	62.5	48.7	
Non-Western	37.5	51.3	
Smoking, %			0.002
No	72.0	74.4	
Yes, until pregnancy known	9.4	7.0	
Yes, continued	18.6	18.6	
Folic acid supplement use, %			<0.001
No use	25.0	39.5	
Start before eight weeks	31.9	29.4	
Preconception start	43.2	31.1	
Pre-eclampsia, %	2.2	2.4	0.69
Male gender, %	50.6	50.1	0.65
Mid pregnancy measurements			
Estimated fetal weight (g)	377.3 (84.2)	395.6 (119.9)	<0.001
Femur length (mm)	33.4 (3.3)	33.9 (4.5)	<0.001
Late pregnancy measurements			
Estimated fetal weight (g)	1611.9 (250.6)	1624.6 (299.7)	0.06
Femur length (mm)	57.4 (3.0)	57.6 (3.4)	0.002
Birth outcomes			
Weight (g)	3420.1 (564.2)	3357.8 (581.1)	<0.001
Length (cm)	50.2 (2.4)	50.1 (2.4)	0.03

Values are means (SD), median (90% range) or percentages. *Differences between women included versus excluded from the study were tested by using Student's *t*-test or Mann-Whitney U-test for continuous variables and Chi-square test for categorical variables.

Table S4 | Nonresponse analysis of children included versus excluded from the study.

	Included n = 4108	Excluded n = 4772	P-value*
MATERNAL CHARACTERISTICS			
Age at intake (years)	29.5 (5.2)	29.8 (5.4)	0.06
Gestational age at intake (weeks)	14.4 (10.9–22.8)	14.5 (10.9–23.8)	0.001
Height (cm)	167.7 (7.4)	166.7 (7.4)	<0.001
Weight at intake (kg)	69.4 (13.1)	69.6 (13.6)	0.48
Body mass index at intake (kg/m^2)	23.8 (19.3–33.2)	24.0 (19.5–34.2)	0.001
Body mass index before pregnancy (kg/m^2)	22.6 (18.6–32.2)	22.7 (18.7–32.8)	0.03
Blood pressure (mmHg)			
Systolic	115.4 (12.3)	115.3 (12.2)	0.98
Diastolic	67.9 (9.5)	67.9 (9.7)	0.88
Nulliparous, %	56.0	53.7	0.03
Education, %			0.002
Primary/secondary school	49.4	50.6	
Higher education	50.6	49.4	
Race/ethnicity, %			<0.001
Western	61.2	55.5	
Non-Western	38.8	44.5	
Smoking, %			0.11
No	71.7	73.6	
Yes, until pregnancy known	8.7	8.7	
Yes, continued	19.6	17.7	
Folic acid supplement use, %			0.27
No use	28.4	30.3	
Start before eight weeks	31.5	30.7	
Preconception start	40.0	39.0	
Pre-eclampsia, %	1.2	3.3	<0.001
Male gender, %	51.3	49.7	0.13
Mid pregnancy measurements			
Estimated fetal weight (g)	382.9 (95.5)	382.4 (98.2)	0.81
Femur length (mm)	33.5 (3.6)	33.6 (3.7)	0.39
Late pregnancy measurements			
Estimated fetal weight (g)	1613.6 (251.4)	1617.8 (280.2)	0.47
Femur length (mm)	57.4 (3.0)	57.5 (3.2)	0.23
Birth outcomes			
Weight (g)	3461.1 (503.6)	3346.2 (619.1)	<0.001
Length (cm)	50.2 (2.3)	50.1 (2.5)	0.02

Values are means (SD), median (90% range) or percentages. *Differences between women included versus excluded from the study were tested by using Student's *t*-test or Mann-Whitney U-test for continuous variables and Chi-square test for categorical variables.

Table S5 | Estimates of associations between maternal sFlt-1 and PlGF concentrations and fetal and childhood weight growth (n = 5980).

	Change in weight SDS									
	Fetal			Childhood						
	20 weeks gestation	30 weeks gestation	40 weeks gestation	6 months	12 months	24 months	36 months	48 months	60 months	72 months
	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)
Early pregnancy sFlt–1 (ng/ml)										
Q1 (≤0.34)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Q2 (0.35–4.47)	-0.05 (-0.12; 0.03)	0.06 (0.00; 0.12)	0.16 (0.08; 0.24)	0.10 (0.03; 0.18)	0.10 (0.02; 0.17)	0.09 (0.02; 0.16)	0.08 (0.01; 0.15)	0.07 (0.00; 0.14)	0.06 (-0.02; 0.13)	0.05 (-0.03; 0.13)
Q3 (4.48–5.75)	0.05 (-0.02; 0.03)	0.15 (0.09; 0.21)	0.24 (0.17; 0.32)	0.21 (0.13; 0.28)	0.20 (0.13; 0.27)	0.18 (0.11; 0.25)	0.16 (0.09; 0.23)	0.14 (0.06; 0.21)	0.12 (0.04; 0.19)	0.09 (0.01; 0.17)
Q4 (5.76–7.75)	0.13 (0.05; 0.20	0.21 (0.15; 0.27)	0.29 (0.22; 0.37)	0.24 (0.16; 0.31)	0.23 (0.16; 0.31)	0.20 (0.13; 0.28)	0.18 (0.11; 0.25)	0.16 (0.09; 0.23)	0.14 (0.04; 0.21)	0.12 (0.04; 0.20)
Q5 (≥7.76)	0.09 (0.01; 0.16)	0.21 (0.15; 0.27)	0.33 (0.25; 0.41)	0.29 (0.22; 0.37)	0.28 (0.21; 0.36)	0.26 (0.18; 0.33)	0.23 (0.16; 0.30)	0.20 (0.13; 0.28)	0.18 (0.10; 0.26)	0.15 (0.07; 0.24)
Early pregnancy PlGF (pg/ml)										
Q1 (≤26.7)	-0.16 (-0.26; -0.07)	-0.25 (-0.34; -0.17)	-0.34 (-0.44; -0.25)	-0.25 (-0.35; -0.15)	-0.25 (-0.35; -0.15)	-0.25 (-0.35; -0.15)	-0.25 (-0.35; -0.15)	-0.25 (-0.35; -0.15)	-0.25 (-0.36; -0.15)	-0.26 (-0.36; -0.15)
Q2 (26.7–36.5)	-0.12 (-0.21; -0.03)	-0.12 (-0.20; -0.04)	-0.13 (-0.22; -0.04)	-0.10 (-0.20; -0.01)	-0.10 (-0.20; -0.01)	-0.10 (-0.19; -0.01)	-0.10 (-0.19; -0.01)	-0.11 (-0.20; -0.01)	-0.11 (-0.20; -0.01)	-0.10 (-0.20; -0.01)
Q3 (36.5–51.5)	-0.01 (-0.10; 0.08)	-0.02 (-0.16; 0.01)	-0.03 (-0.12; 0.05)	-0.07 (-0.16; 0.02)	-0.07 (-0.16; 0.01)	-0.08 (-0.16; 0.00)	-0.09 (-0.17; 0.00)	-0.09 (-0.18; -0.01)	-0.10 (-0.19; -0.02)	-0.11 (-0.20; -0.02)
Q4 (51.5–82.9)	-0.06 (-0.14; 0.02)	-0.08 (-0.14; -0.01)	-0.09 (-0.17; -0.01)	-0.04 (-0.12; 0.04)	-0.05 (-0.13; 0.03)	-0.06 (-0.14; 0.02)	-0.07 (-0.15; 0.00)	-0.09 (-0.16; -0.01)	-0.10 (-0.18; -0.02)	-0.11 (-0.20; -0.03)
Q5 (≥82.9)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference

Table S5 | Estimates of associations between maternal sFlt-1 and PlGF concentrations and fetal and childhood weight growth (n = 5980) *(Continued)*.

	Change in weight SDS									
	Fetal			Childhood						
	20 weeks gestation	30 weeks gestation	40 weeks gestation	6 months	12 months	24 months	36 months	48 months	60 months	72 months
	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)
Delta sFlt–1 (ng/ml)										
Q1 (≤-0.22)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Q2 (-0.21, -0.08)	0.02 (-0.07; 0.10)	0.00 (-0.06; 0.07)	-0.01 (-0.10; 0.07)	-0.04 (-0.12; 0.04)	-0.04 (-0.12; 0.04)	-0.04 (-0.12; 0.04)	-0.04 (-0.11; 0.04)	-0.04 (-0.11; 0.04)	-0.03 (-0.11; 0.05)	-0.03 (-0.12; 0.06)
Q3 (-0.07, 0.03)	0.01 (-0.07; 0.09)	-0.07 (-0.13; 0.00)	-0.14 (-0.23; -0.06)	-0.10 (-0.18; -0.02)	-0.10 (-0.18; -0.02)	-0.09 (-0.17; -0.02)	-0.09 (-0.17; -0.01)	-0.09 (-0.16; -0.01)	-0.08 (-0.16; 0.00)	-0.08 (-0.17; 0.01)
Q4 (0.04, 0.23)	-0.01 (-0.10; 0.07)	-0.11 (-0.17; -0.04)	-0.20 (-0.28; -0.11)	-0.13 (-0.22; -0.05)	-0.13 (-0.21; -0.05)	-0.12 (-0.20; -0.05)	-0.11 (-0.19; -0.04)	-0.10 (-0.18; -0.03)	-0.09 (-0.18; -0.01)	-0.09 (-0.17; 0.00)
Q5 (≥0.24)	-0.03 (-0.11; 0.06)	-0.10 (-0.16; -0.03)	-0.17 (-0.25; -0.08)	-0.02 (-0.10; 0.07)	-0.01 (-0.09; 0.07)	-0.01 (-0.08; 0.07)	0.00 (-0.08; 0.08)	0.01 (-0.07; 0.08)	0.01 (-0.07; 0.09)	0.02 (-0.07; 0.11)
Delta PlGF (pg/ml)										
Q1 (≤13.1)	-0.22 (-0.30; -0.14)	-0.24 (-0.30; -0.17)	-0.25 (-0.34; -0.17)	-0.26 (-0.34; -0.18)	-0.26 (-0.33; -0.18)	-0.24 (-0.32; -0.16)	-0.23 (-0.30; -0.15)	-0.21 (-0.29; -0.13)	-0.20 (-0.28; -0.12)	-0.18 (-0.27; -0.10)
Q2 (13.1–18.5)	-0.10 (-0.19; -0.02)	-0.04 (-0.11; 0.02)	0.02 (-0.07; 0.10)	-0.12 (-0.20; -0.04)	-0.11 (-0.19; -0.03)	-0.10 (-0.18; -0.02)	-0.09 (-0.16; -0.01)	-0.07 (-0.15; 0.00)	-0.06 (-0.14; 0.02)	-0.05 (-0.13; 0.04)
Q3 (18.5–24.7)	-0.12 (-0.20; -0.03)	-0.06 (-0.13; 0.00)	0.00 (-0.09; 0.08)	-0.06 (-0.14; 0.02)	-0.06 (-0.14; 0.02)	-0.06 (-0.13; 0.02)	-0.06 (-0.14; 0.01)	-0.06 (-0.14; 0.01)	-0.06 (-0.14; 0.02)	-0.06 (-0.15; 0.02)
Q4 (24.7–35.8)	-0.05 (-0.13; 0.03)	-0.03 (-0.09; 0.04)	0.00 (-0.08; 0.08)	-0.03 (-0.11; 0.05)	-0.03 (-0.11; 0.05)	-0.03 (-0.10; 0.05)	-0.03 (-0.10; 0.05)	-0.03 (-0.10; 0.05)	-0.02 (-0.11; 0.06)	-0.02 (-0.11; 0.06)
Q5 (≥35.8)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference

Fetal and childhood weight growth among early pregnancy and delta sFlt-1 and PlGF categories, based on repeated measurement regression models. Adjustments: gestational age (blood sampling), maternal age, height, BMI, parity, education, ethnicity, smoking, folic acid supplement use, systolic blood pressure and fetal gender. Childhood analysis additionally adjusted for breastfeeding.

Table S6 | Estimates of associations between maternal sFlt-1 and PlGF concentrations and fetal and childhood length growth (n = 5980).

	Change in length SDS									
	Fetal			Childhood						
	20 weeks gestation	30 weeks gestation	40 weeks gestation	6 months	12 months	24 months	36 months	48 months	60 months	72 months
	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)
Early pregnancy sFlt-1 (ng/ml)										
Q1 (≤0.34)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Q2 (0.35–4.47)	-0.03 (-0.11; 0.05)	-0.01 (-0.07; 0.12)	0.00 (-0.09; 0.10)	0.11 (0.03; 0.18)	0.10 (0.03; 0.17)	0.09 (0.03; 0.16)	0.09 (0.02; 0.15)	0.08 (0.01; 0.15)	0.07 (0.00; 0.14)	0.06 (-0.02; 0.14)
Q3 (4.48–5.75)	0.05 (-0.03; 0.13)	0.06 (-0.01; 0.12)	0.07 (-0.03; 0.16)	0.17 (0.10; 0.24)	0.16 (0.09; 0.23)	0.15 (0.08; 0.22)	0.14 (0.07; 0.20)	0.12 (0.05; 0.19)	0.11 (0.03; 0.18)	0.09 (0.01; 0.17)
Q4 (5.76–7.75)	0.09 (0.01; 0.17)	0.11 (0.05; 0.17)	0.13 (0.04; 0.23)	0.23 (0.15; 0.30)	0.22 (0.14; 0.29)	0.20 (0.13; 0.27)	0.18 (0.11; 0.25)	0.16 (0.09; 0.23)	0.14 (0.07; 0.22)	0.12 (0.05; 0.20)
Q5 (≥7.76)	0.05 (-0.04; 0.13)	0.13 (0.06; 0.18	0.21 (0.11; 0.30)	0.28 (0.21; 0.36)	0.27 (0.19; 0.34)	0.24 (0.17; 0.31)	0.21 (0.14; 0.28)	0.19 (0.12; 0.26)	0.16 (0.08; 0.24)	0.13 (0.05; 0.21)
Early pregnancy PlGF (pg/ml)										
Q1 (≤26.7)	-0.25 (-0.5; -0.15)	-0.30 (-0.39; -0.22)	-0.35 (-0.47; -0.24)	-0.23 (-0.33; -0.13)	-0.22 (-0.32; -0.12)	-0.20 (-0.29; -0.10)	-0.17 (-0.27; -0.08)	-0.15 (-0.25; -0.05)	-0.13 (-0.23; -0.03)	-0.11 (-0.21; 0.00)
Q2 (26.7–36.5)	-0.16 (-0.25; -0.06)	-0.19 (-0.27; -0.11)	-0.23 (-0.33; -0.12)	-0.12 (-0.21; -0.03)	-0.11 (-0.20; -0.02)	-0.08 (-0.17; 0.01)	-0.06 (-0.15; 0.03)	-0.04 (-0.13; 0.05)	-0.01 (-0.11; 0.08)	0.01 (-0.09; 0.10)
Q3 (36.5–51.5)	-0.07 (-0.16; 0.02)	-0.13 (-0.20; -0.06)	-0.19 (-0.29; -0.09)	-0.06 (-0.15; 0.02)	-0.06 (-0.14; 0.03)	-0.05 (-0.13; 0.03)	-0.04 (-0.12; 0.04)	-0.03 (-0.11; 0.05)	-0.02 (-0.10; 0.07)	-0.01 (-0.10; 0.08)
Q4 (51.5–82.9)	-0.05 (-0.14; 0.03)	-0.11 (-0.18; -0.05)	-0.17 (-0.27; -0.08)	-0.07 (-0.15; 0.01)	-0.07 (-0.14; 0.01)	-0.06 (-0.31; 0.02)	-0.05 (-0.13; 0.02)	-0.05 (-0.12; 0.03)	-0.04 (-0.12; 0.04)	-0.04 (-0.12; 0.05)
Q5 (≥82.9)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference

Table S6 | Estimates of associations between maternal sFlt-1 and PlGF concentrations and fetal and childhood length growth (n = 5980) *(Continued)*.

	Change in length SDS									
	Fetal			Childhood						
	20 weeks gestation	30 weeks gestation	40 weeks gestation	6 months	12 months	24 months	36 months	48 months	60 months	72 months
	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)
Delta sFlt-1 (ng/ml)										
Q1 (≤ -0.22)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Q2 (-0.21, -0.08)	0.02 (-0.07; 0.10)	0.01 (-0.05; 0.08)	0.01 (-0.09; 0.11)	-0.07 (-0.15; 0.01)	-0.07 (-0.14; 0.01)	-0.05 (-0.13; 0.02)	-0.04 (-0.11; 0.03)	-0.03 (-0.10; 0.05)	-0.02 (-0.09; 0.06)	0.00 (-0.09; 0.08)
Q3 (-0.07, 0.03)	0.02 (-0.06; 0.11)	-0.03 (-0.10; 0.04)	-0.08 (-0.18; 0.02)	-0.12 (-0.20; -0.04)	-0.11 (-0.19; -0.04)	-0.10 (-0.18; -0.03)	-0.09 (-0.16; -0.01)	-0.07 (-0.15; 0.00)	-0.06 (-0.14; 0.02)	-0.05 (-0.13; 0.04)
Q4 (0.04, 0.23)	0.01 (-0.08; 0.09)	-0.06 (-0.12; 0.01)	-0.12 (-0.22; -0.02)	-0.15 (-0.23; -0.07)	-0.14 (-0.22; -0.06)	-0.12 (-0.20; -0.05)	-0.11 (-0.18; -0.04)	-0.09 (-0.17; -0.02)	-0.08 (-0.16; 0.00)	-0.06 (-0.15; 0.02)
Q5 (≥0.24)	-0.01 (-0.1; 0.07)	-0.05 (-0.1; 0.02)	-0.09 (-0.2; 0.01)	-0.07 (-0.15; 0.01)	-0.06 (-0.14; 0.02)	-0.04 (-0.12; 0.03)	-0.02 (-0.10; 0.05)	0.00 (-0.08; 0.07)	0.01 (-0.07; 0.09)	0.03 (-0.05; 0.12)
Delta PlGF (pg/ml)										
Q1 (≤ 13.1)	-0.14 (-0.22; -0.05)	-0.13 (-0.20; -0.06)	-0.12 (-0.22; -0.02)	-0.23 (-0.31; -0.16)	-0.22 (-0.30; -0.14)	-0.19 (-0.26; -0.11)	-0.16 (-0.23; -0.08)	-0.13 (-0.20; -0.05)	-0.10 (-0.18; -0.02)	-0.07 (-0.15; 0.02)
Q2 (13.1-18.5)	-0.03 (-0.11; 0.06)	0.00 (-0.07; 0.07)	0.02 (-0.08; 0.12)	-0.08 (-0.16; 0.00)	-0.07 (-0.15; 0.00)	-0.05 (-0.13; 0.02)	-0.03 (-0.11; 0.04)	-0.02 (-0.09; 0.06)	0.00 (-0.08; 0.08)	0.02 (-0.6; 0.11)
Q3 (18.5-24.7)	-0.04 (-0.13; 0.04)	-0.02 (-0.09; 0.04)	0.00 (-0.10; 0.10)	-0.05 (-0.13; 0.02)	-0.05 (-0.12; 0.03)	-0.04 (-0.11; 0.03)	-0.03 (-0.10; 0.04)	-0.02 (-0.09; 0.06)	-0.01 (-0.09; 0.07)	0.00 (-0.08; 0.08)
Q4 (24.7-35.8)	-0.02 (-0.10; 0.07)	0.02 (-0.05; 0.08)	0.05 (-0.04; 0.15)	-0.02 (-0.10; 0.06)	-0.01 (-0.09; 0.06)	0.00 (-0.08; 0.07)	0.00 (-0.07; 0.08)	0.01 (-0.06; 0.09)	0.02 (-0.06; 0.10)	0.03 (-0.05; 0.11)
Q5 (≥35.8)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference

Fetal and childhood length growth among early pregnancy and delta sFlt-1 and PlGF categories, based on repeated measurement regression models. Adjustments: gestational age (blood sampling), maternal age, height, BMI, parity, education, ethnicity, smoking, folic acid supplement use, systolic blood pressure and fetal gender. Childhood analysis additionally adjusted for breastfeeding.

Table S7 | Estimates of associations between umbilical cord sFlt-1 and PlGF concentrations and fetal and childhood weight growth (n = 4108).

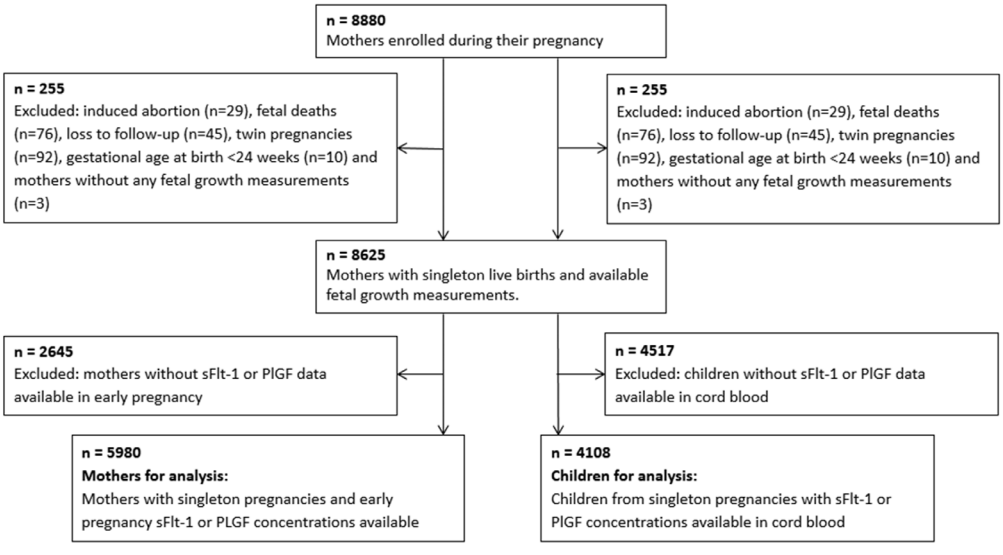
	Change in weight SDS									
	Fetal			Childhood						
	20 weeks gestation	30 weeks gestation	40 weeks gestation	6 months	12 months	24 months	36 months	48 months	60 months	72 months
	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)
sFlt-1 (ng/ml)										
Q1 (≤0.29)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Q2 (0.29–0.39)	-0.02 (-0.12; 0.09)	-0.04 (-0.11; 0.03)	-0.07 (-0.15; 0.02)	0.00 (-0.08; 0.09)	0.00 (-0.08; 0.08)	-0.01 -0.09; 0.07)	-0.02 (-0.10; 0.06)	-0.04 (-0.12; 0.05)	-0.05 (-0.13; 0.04)	-0.06 (-0.15; 0.040
Q3 (0.39–0.52)	-0.02 (-0.12; 0.09)	-0.10 (-0.17; -0.02)	-0.18 (-0.27; -0.09)	0.02 (-0.06; 0.11)	0.01 (-0.07; 0.09)	-0.01 (-0.09; 0.07)	-0.03 (-0.11; 0.05)	-0.06 (-0.14; 0.03)	-0.08 (-0.17; 0.01)	-0.10 (-0.20; -0.01)
Q4 (0.52–0.76)	0.00 (-0.10; 0.10)	-0.12 (-0.19; -0.04)	-0.23 (-0.32; -0.15)	-0.07 (-0.16; 0.01)	-0.07 (-0.15; 0.01)	-0.07 (-0.15; 0.01)	-0.07 (-0.15, 0.01)	-0.07 (-0.15; 0.02)	-0.06 (-0.15; 0.02)	-0.06 (-0.16; 0.03)
Q5 (≥0.76)	-0.01 (-0.12; 0.09)	-0.19 (-0.26; -0.11)	-0.36 (-0.45; -0.27)	-0.13 -0.21; -0.04)	-0.13 (-0.21; -0.04)	-0.13 (-0.21; -0.05)	-0.13 (-0.21; -0.05)	-0.12 (-0.21; -0.04)	-0.12 (-0.21; -0.04)	-0.12 (-0.22; -0.03)
PlGF (pg/ml)										
Q1 (≤6.4)	0.01 (-0.10; 0.12)	-0.20 (-0.28; -0.11)	-0.40 (-0.50; -0.31)	-0.20 (-0.30; -0.11)	-0.20 (-0.30; -0.11)	-0.21 (-0.30; -0.12)	-0.21 (-0.30; -0.12)	-0.21 (-0.30; -0.12)	-0.22 (-0.30; -0.12)	-0.22 (-0.33; -0.12)
Q2 (6.4–7.9)	0.09 (-0.03; 0.20)	-0.08 (-0.16; 0.00)	-0.24 (-0.34; -0.15)	-0.13 (-0.22; -0.03)	-0.13 (-0.22; -0.04)	-0.13 (-0.22; -0.04)	-0.14 (-0.22; -0.05)	-0.14 (-0.23; -0.05)	-0.14 (-0.24; -0.05)	-0.15 (-0.25; -0.04)
Q3 (7.9–9.3)	0.03 (-0.08; 0.14)	-0.05 (-0.13; 0.03)	-0.13 (-0.23; -0.04)	-0.04 (-0.13; 0.06)	-0.04 (-0.13; 0.06)	-0.03 (-0.13; 0.06)	-0.02 (-0.11; 0.07)	-0.02 (-0.11; 0.07)	-0.01 (-0.11; 0.09)	0.00 (-0.11; 0.10)
Q4 (9.3–11.2)	-0.02 (-0.13; 0.09)	-0.02 (-0.10; 0.06)	-0.02 (-0.12; 0.07)	0.03 (-0.06; 0.13)	0.03 (-0.06; 0.12)	0.03 (-0.05; 0.12)	0.03 (-0.05; 0.12)	0.04 (-0.05; 0.13)	0.04 (-0.06; 0.13)	0.04 (-0.06; 0.14)
Q5 (≥11.2)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference

Fetal and childhood weight growth among umbilical cord sFlt-1 and PlGF categories, based on repeated measurement regression models. Adjustments: gestational age (blood sampling), maternal age, height, BMI, parity, education, ethnicity, smoking, folic acid supplement use, systolic blood pressure and fetal gender. Childhood analysis additionally adjusted for breastfeeding.

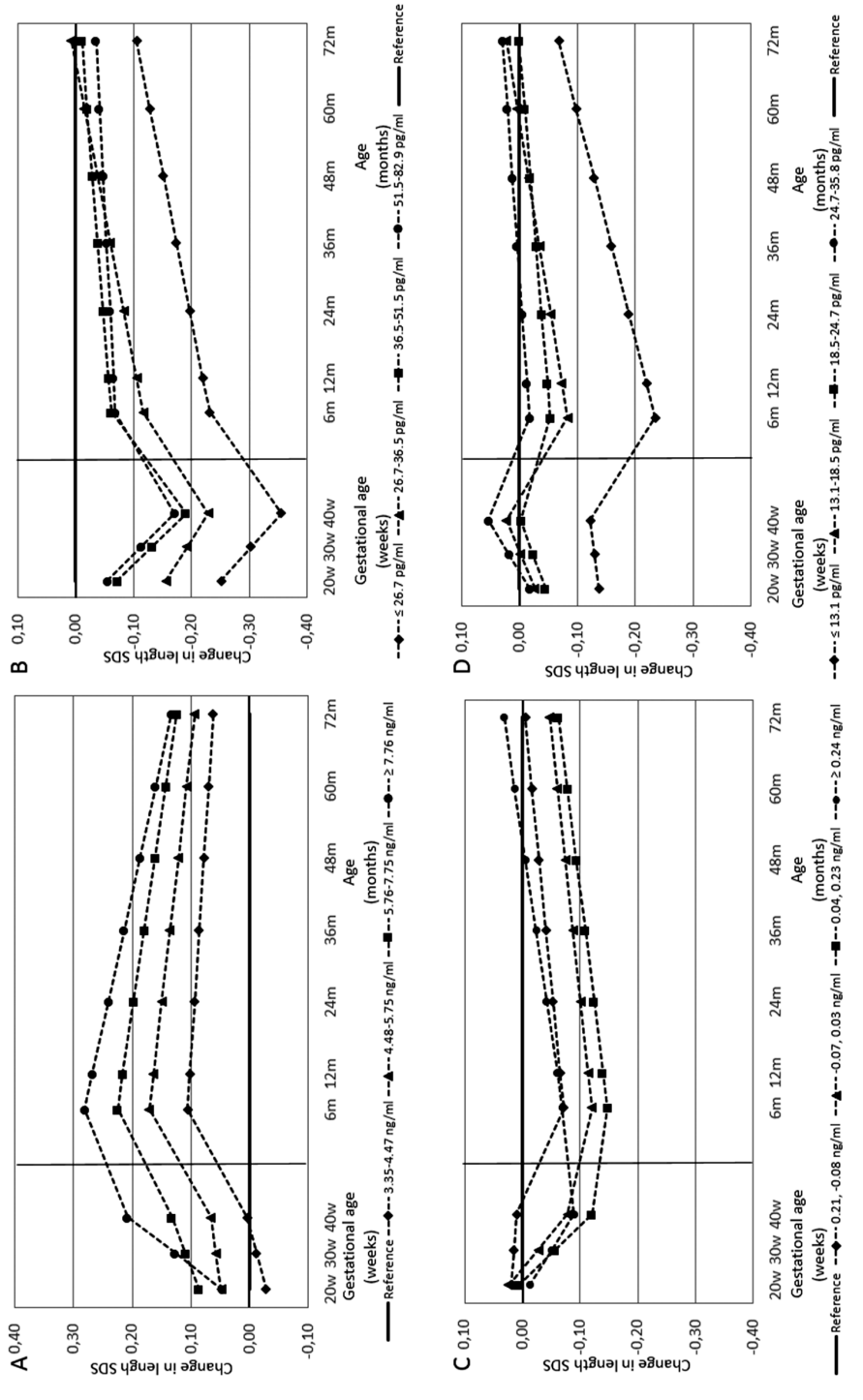
Table S8 | Estimates of associations between umbilical cord sFlt-1 and PlGF concentrations and fetal and childhood length growth (n = 4108).

	Change in length SDS									
	Fetal			Childhood						
	20 weeks gestation	30 weeks gestation	40 weeks gestation	6 months	12 months	24 months	36 months	48 months	60 months	72 months
	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)	Effect estimate (95% CI)
sFlt-1 (ng/ml)										
Q1 (≤0.29)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Q2 (0.29–0.39)	-0.04 (-0.15; 0.06)	-0.07 (-0.14; 0.01)	-0.09 (-0.20; 0.01)	-0.02 (-0.10; 0.06)	-0.03 (-0.10; 0.05)	-0.03 (-0.11; 0.04)	-0.04 (-0.11; 0.04)	-0.04 (-0.12; 0.04)	-0.05 (-0.13; 0.04)	-0.05 (-0.15; 0.04)
Q3 (0.39–0.52)	0.03 (-0.07; 0.13)	-0.04 (-0.11; 0.04)	-0.10 (-0.21; 0.00)	-0.05 (-0.13; 0.03)	-0.06 (-0.13; 0.02)	-0.07 (-0.14; 0.01)	-0.08 (-0.15; 0.00)	-0.09 (-0.17; -0.01)	-0.10 (-0.18; -0.01)	-0.11 (-0.20; -0.02)
Q4 (0.52–0.76)	0.04 (-0.06; 0.14)	-0.03 (-0.11; 0.04)	-0.11 (-0.22; 0.00)	-0.02 (-0.10; 0.06)	-0.02 (-0.10; 0.05)	-0.04 (-0.11; 0.04)	-0.05 (-0.13; 0.03)	-0.06 (-0.14; 0.02)	-0.07 (-0.16; 0.01)	-0.09 (-0.18; 0.00)
Q5 (≥0.76)	0.04 (-0.06; 0.14)	-0.09 (-0.17; -0.02)	-0.22 (-0.33; -0.12)	-0.12 (-0.20; -0.04)	-0.12 (-0.20; -0.04)	-0.12 (-0.20; -0.04)	-0.12 (-0.19; -0.04)	-0.11 (-0.20; -0.03)	-0.11 (-0.20; -0.03)	-0.11 (-0.20; -0.02)
PlGF (pg/ml)										
Q1 (≤6.4)	-0.05 (-0.6; 0.06)	-0.10 (-0.18; -0.01)	-0.14 (-0.26; -0.02)	-0.22 (-0.31; -0.13)	-0.22 (-0.31; -0.13)	-0.23 (-0.31; -0.14)	-0.23 (-0.35; -0.14)	-0.23 (-0.32; -0.15)	-0.24 (-0.33; -0.15)	-0.24 (-0.34; -0.14)
Q2 (6.4–7.9)	0.01 (-0.10; 0.12)	-0.04 (-0.12; 0.04)	-0.09 (-0.20; 0.02)	-0.12 (-0.21; -0.03)	-0.12 (-0.21; -0.03)	-0.12 (-0.21; -0.04)	-0.12 (-0.21; -0.04)	-0.12 (-0.21; -0.03)	-0.12 (-0.21; -0.03)	-0.12 (-0.22; -0.02)
Q3 (7.9–9.3)	-0.02 (-0.13; 0.09)	0.00 (-0.08; 0.08)	0.02 (-0.09; 0.14)	-0.06 (-0.15; 0.03)	-0.05 (-0.14; 0.03)	-0.05 (-0.13; 0.04)	-0.05 (-0.13; 0.04)	-0.04 (-0.13; 0.05)	-0.04 (-0.13; 0.05)	-0.03 (-0.13; 0.06)
Q4 (9.3–11.2)	-0.03 (-0.14; 0.08)	0.03 (-0.06; 0.11)	0.08 (-0.04; 0.19)	-0.04 (-0.12; 0.05)	-0.04 (-0.12; 0.05)	-0.04 (-0.12; 0.05)	-0.04 (-0.12; 0.05)	-0.04 (-0.12; 0.05)	-0.04 (-0.13; 0.05)	-0.04 (-0.13; 0.06)
Q5 (≥11.2)	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference

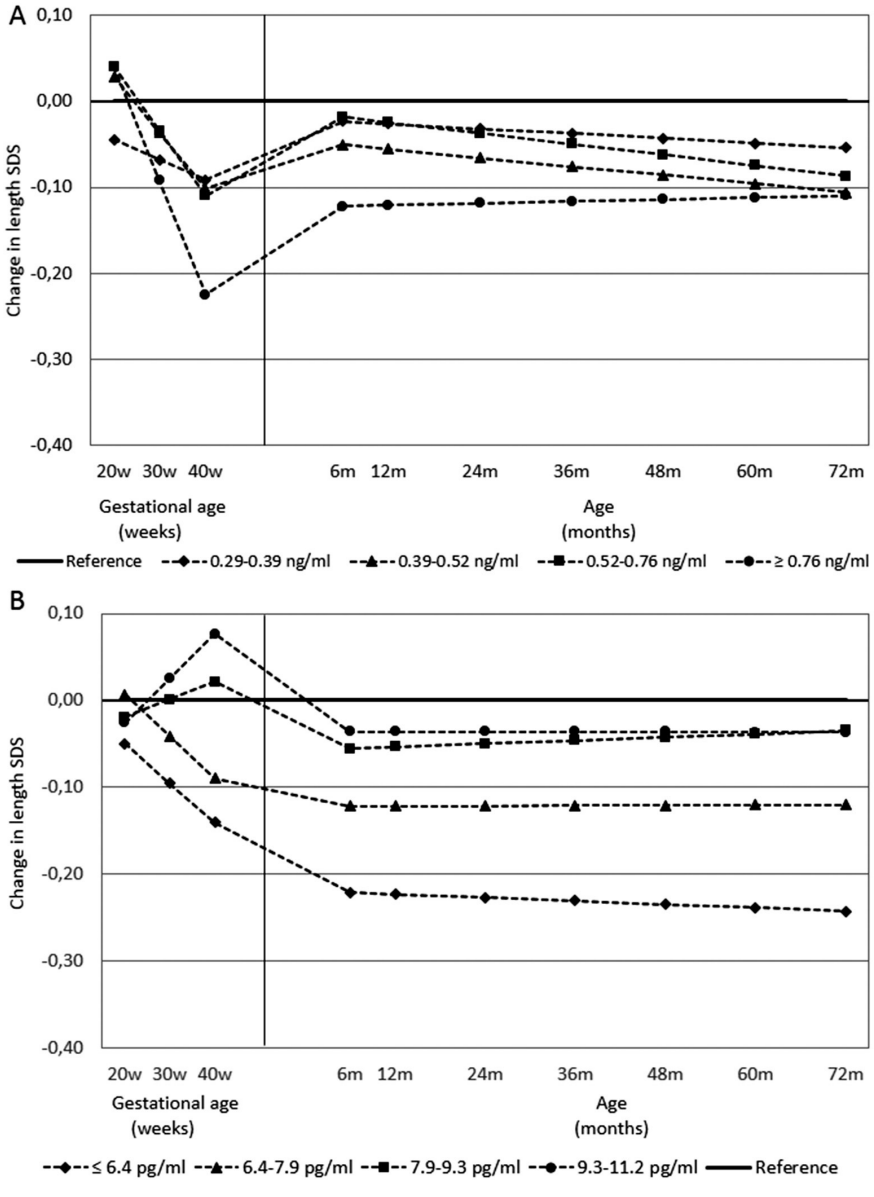
Fetal and childhood length growth among umbilical cord sFlt-1 and PlGF categories, based on repeated measurement regression models. Adjustments: gestational age (blood sampling), maternal age, height, BMI, parity, education, ethnicity, smoking, folic acid supplement use, systolic blood pressure and fetal gender. Childhood analysis additionally adjusted for breastfeeding.



Supplement Figure S1 | Flow chart.



Supplement Figure S2 | Associations between maternal sFlt-1 and PlGF concentrations and repeatedly measured fetal and infant length growth (n = 5980).
Fetal and childhood length growth among early pregnancy sFlt-1 (1A) and PlGF (1B) and delta sFlt-1 (1C) and PlGF (1D) categories. Adjustments: gestational age (blood sampling), maternal age, height, BMI, parity, education, ethnicity, smoking, folic acid supplement use, systolic blood pressure and fetal gender. Childhood analysis additionally adjusted for breastfeeding.



Supplement Figure S3 | Associations between umbilical cord sFlt-1 and PlGF concentrations and repeatedly measured fetal and infant length growth (n = 4108).

Fetal and childhood length growth among umbilical cord sFlt-1 (2A) and PlGF (2B) categories. Adjustments: gestational age (blood sampling), maternal age, height, BMI, parity, education, ethnicity, smoking, folic acid supplement use, systolic blood pressure and fetal gender. Childhood analysis additionally adjusted for breastfeeding.

Part III

Epigenetic studies

Chapter 5

DNA methylation profiles in children with a neural tube defect

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ABSTRACT

Objective | Folate deficiency is implicated in the causation of neural tube defects (NTDs). The preventive effect of periconceptional folic acid supplement use is partially explained by the treatment of a deranged folate-dependent one carbon metabolism, which provides methyl groups for DNA-methylation as an epigenetic mechanism. Here, we hypothesize that variations in DNA methylation of genes implicated in the development of NTDs and embryonic growth are part of the underlying mechanism.

Study design | In 48 children with a neural tube defect and 62 controls from a Dutch case-control study and 34 children with a neural tube defect and 78 controls from a Texan case-control study, we measured the DNA-methylation levels of imprinted candidate genes (*IGF2-DMR*, *H19*, *KCNQ1OT1*) and non-imprinted genes (the *LEKR/CCNL* gene region associated with birth weight, and *MTHFR* and *VANGL1* associated with NTD). We used the MassARRAY EpiTYPER assay from Sequenom for the assessment of DNA-methylation. Linear mixed model analysis was used to estimate associations between DNA-methylation levels of the genes and a neural tube defect.

Results | In the Dutch study group, but not in the Texan study group we found a significant association between the risk of having an NTD and DNA methylation levels of *MTHFR* (absolute decrease in methylation of 20.33% in cases, P-value = 0.001), and *LEKR/CCNL* (absolute increase in methylation: 1.36% in cases, P-value = 0.048), and a borderline significant association for VANGL (absolute increase in methylation: 0.17% in cases, P-value = 0.063). Only the association between *MTHFR* and NTD-risk remained significant after multiple testing correction. The associations in the Dutch study were not replicated in the Texan study.

Conclusion | This study suggests a small but significant association of *MTHFR* methylation levels with NTD, which could support a potential functional link between 1C-metabolism and NTDs. However, this association could not be replicated and should be investigated in other studies.

INTRODUCTION

Neural tube defects (NTDs) are congenital malformations accompanied with low birth weight, which result from a failure of the neural tube to close during the fourth week of embryogenesis. The most common phenotypes of NTDs are spina bifida and anencephaly.[1] Interactions between environmental exposures and subtle variations in genes are thought to be involved in the pathogenesis of NTD. A maternal folate shortage during the sensitive period of neural tube development contributes to the development of NTD, and the occurrence of the disease can be reduced by periconceptional maternal folic acid supplement use.[2] However, the mechanisms underlying this relationship are not completely understood.

Epigenetic alterations can cause changes in gene expression that are not directly related to the DNA sequence itself, of which DNA methylation is one of the best understood epigenetic mechanisms.[2] DNA-methylation of genes is rather stable throughout life, except for example in the periconception period. It is an important mechanism in epigenetic programming of tissues, organ development and functioning. To establish DNA-methylation, DNA methyltransferases use methyl donors such as folate derived from the one carbon metabolism (1-C metabolism) essential during the periconceptional period.[3] Therefore, a reduced availability of methyl donors can lead to DNA hypomethylation with consequences for epigenetic programming. Several substrates like folate and cofactors such as vitamin B12 are implicated in 1-C metabolism. The 1C-metabolism has repeatedly been associated with DNA-methylation.[4,5] Maternal vitamin B12 deficiency has been associated with an increased risk for NTD,[6-10] linking maternal 1-C metabolism to closure of the neural tube in the developing child.

Higher DNA-methylation levels of *IGF2 DMR* have been reported in children of mothers taking periconceptional folic acid in supplements.[11] High maternal homocysteine levels are associated with NTDs in the offspring[12] and with genome-wide hypo DNA-methylation in umbilical cord blood cells.[13,14] In addition, vitamin B12 has also been suggested to influence DNA-methylation in umbilical cord blood cells.[15] Moreover, genetic variants in key genes that are involved in the 1C-metabolism are related to changes in DNA methylation. Common variants of the *MTHFR* gene (c.677C>T and c.1298A>C) can result in a reduction of enzymatic activity,[16,17] which together with a low folate and or vitamin B12 status can result in elevated homocysteine levels and global DNA hypomethylation.[18-20]

These *MTHFR* polymorphisms have been well studied in associations with NTD.[16,17] Animal studies suggest that DNA methylation is implicated in neural tube closure,[21,22] indicating that changes in DNA methylation could influence the risk for NTD. Recently, it was shown by Chang et al. that maternal serum folate was lower in NTD cases compared to controls, and that the maternal serum folate levels were correlated with methylated cytosines in the brain of NTD fetuses.[23] A previous study reported hypomethylation of the DNA-repair gene MGMT in brain tissue of NTD cases.[24] Global hypomethylation was also found to be association with NTD in brain tissue as was LINE1 hypomethylation.[25,26] From this background, we hypothesize that derangements in DNA-methylation of (non)imprinted genes implicated in embryonic and neural tube development contribute to NTD risk. In two cohorts of NTD cases and controls, we studied associations between

DNA-methylation levels in fetuses and very young children using a candidate gene approach of imprinted candidate genes (*IGF2*, *H19*, and *KCNQ1OT1*) and non-imprinted *LEKR/CCNL* gene region involved in embryonic development and *MTHFR*, and *VANGL1* that are associated with NTDs.

MATERIALS AND METHODS

Design and study population

Dutch case-control study. Between August 1999 and December 2001, a case-control triad study was conducted in which mothers of a child with a non-syndromatic meningo(myelo)cele, i.e., spina bifida, were enrolled in collaboration with the Dutch Spina Bifida Teams of the University Medical Centers of Nijmegen, Utrecht, Groningen, Rotterdam, Leiden, Amsterdam, and the regional hospitals in Tilburg and Zwolle in The Netherlands. Dutch Caucasian women and their children between 1 and 3 years of age were eligible to participate. The spina bifida was diagnosed by a neuropediatrician at birth. Control subjects were recruited from acquaintances and nurseries in Nijmegen and surroundings in the Netherlands as described in detail previously.[6,27] Written informed consent was obtained from the parents of the children. The informed consent and study protocol was approved by the Institutional Review Board of the University Medical Center Nijmegen, the Netherlands.

The original study population comprised 63 mothers and 70 children with spina bifida and 102 control mothers and 85 children. In the present study we selected 48 case and 62 control children with available high quality DNA derived from peripheral white blood cells. For standardized research data collection parents and their children visited the outpatient clinic of the University Medical Center Nijmegen, the Netherlands. Mothers filled out questionnaires concerning demographic data and lifestyle factors in the periconceptional period (3 months before until 3 months after conception) of the pregnancy and at the moment of blood sampling.[3] Maternal body weight and length were measured to calculate the body mass index (BMI, kg/m^2). During the visit 2 ml of maternal venous blood and 1 ml of venous blood of the child were drawn to measure concentrations of red blood cell (RBC) and serum folate, serum vitamin B12 and plasma total homocysteine (tHcy) and handled as described previously.[6] Maternal blood samples were drawn after an overnight fast. Children's white blood cell genomic DNA was extracted from whole blood according to standard procedures.[28]

Texan case-control study. Study subjects were recruited from a Mexican American population from the 14 counties along the Texas-Mexico border region that terminated their pregnancies or delivered between 1995 and 2000. Cases were terminations (spontaneous or selective abortions), live births, or stillbirths with any diagnosis of NTD, defined as spina bifida or anencephaly. They were identified through hospitals, birth centers, ultrasound centers, abortion centers, prenatal clinics, genetics clinics, and birth attendants (midwives and non-hospital physicians). Controls were non-malformed live births that occurred in the same counties during that time period. Subjects were approached about study enrollment either prenatally or in the hospital at the

time of delivery or pregnancy termination. Interviews were scheduled approximately 1 month postpartum. Before the interview, written informed consent was obtained from both parents of the subject in the parents preferred language. The protocol, consent forms, and questionnaire were approved by the Texas Department of Health institutional review board for the protection of human subjects.[29] The study protocol was reviewed and approved by the Texas A&M University System Health Science Center Internal Review Board (IRB) and the Department of State Health Services (formerly Texas Department of Health) IRB. Participant identification and data collection have been described in detail elsewhere.[9,30,31]

In the present study 34 cases and 78 control children were included. Women were interviewed at home using a standard instrument modeled after the Centers for Disease Control and Prevention's 1993 mother questionnaire for birth defects risk factor surveillance. With this instrument, information was obtained on maternal age at conception, years of schooling, maternal folic acid supplement use and smoking habits during the periconceptional period (from 3 months before to 3 months after conception). BMI was calculated from self-reported pre-pregnancy height and weight. During the interview, maternal blood samples were drawn to measure concentrations of red blood cell (RBC) and serum folate, and serum vitamin B12 and handled as described previously.[9]

Genomic DNA was extracted from 5 2.5 mm dried blood spots (Guthrie paper) using the Quickgene SP DNA tissue kit (Fujifilm) according to the manufacturers protocol. Isolated DNA was stored at -20°C.

Quantitative Assessment of DNA-methylation

The amplicons for *IGF2-DMR*, *H19* were described previously.[32] Amplicons for KCNQOT1, *MTHFR*, *VANGL1* and *LEKR/CCNL* were designed using the EpiDesigner tool (Sequenom, Inc, San Diego, USA). The three imprinted genes are *IGF2-DMR*, *H19* and KCNQOT1, which are involved in embryonic growth and development. The three non-imprinted genes are *MTHFR*, *VANGL1* and *LEKR/CCNL*. *MTHFR* is involved in 1-C metabolism and the 677C>T and 1298A>C polymorphisms in this gene as well as mutations in *VANGL1* are associated with the risk of NTD.[16,17,33-35] We also selected the candidate gene-region *LEKR/CCNL*. A polymorphism in the same LD-block as the CpG-island in this region is associated with birth weight, a marker often used for prenatal growth.[36] Amplicons for *IGF2-DMR* and *H19* were taken from the study by Heijmans et al.[37] Supplement Table S1 shows the location, length and primers of the amplicons. First, the amplicons were tested on a standard curve constructed from DNA with low and high methylation (EpigenDx, Worcester, MA, USA) in steps of 10% methylation difference. Only amplicons with a good distribution of the methylation percentages were used for the measurements of the samples. In supplement figure S1 the locations of the CpG-islands studied with respect to the genes are depicted.

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). This was followed by PCR amplification, fragmentation after reverse transcription and analysis on a mass spectrometer (Sequenom, Inc, San Diego, USA). This generated mass signal patterns that were translated into quantitative DNA-methylation levels of different CpG sites of earlier mentioned genes in all tissues

by MassARRAY EpiTYPER Analyzer software (v1.0, build 1.0.6.88 Sequenom, Inc, San Diego, USA).[38] Fragments containing one or more CpG sites were called CpG units. Measurements were done in triplicate on DNA from the same bisulfite-treatment batch on different PCR-plates. On every bisulphite plate, standard DNA with low methylation, 25%, 50%, 75% and high methylation was included. This standard DNA was used to check the technical steps of the experiment.

Data cleaning

During quality control CpG units with a too low or too high mass or CpG units with overlapping RNA fragments were excluded from further analysis. Furthermore, two out of three of the replicate measurements per CpG unit had to be successful, and the SD of the duplicates or triplicates had to be ≤0.10. Outliers per CpG (>3SD) were excluded from further analysis, CpG units with interference of SNP's were also excluded. Bisulfite conversion was assessed using the MassArray R package,[39] which uses fragments containing a TpG and a cytosine to assess the conversion. This showed >99% bisulfite conversion.

Statistical analyses

To test for differences in maternal or child characteristics between cases and controls, χ^2-, *t*- and Mann-Whitney U tests were used. The mean methylation fractions presented in the tables are based on raw data. ANOVA was used to detect possible batch effects of bisulphite-plate and PCR plate. For analysis of differences in total methylation per amplicon between cases and controls, linear mixed models were applied. Linear mixed models were chosen as these models allow for the analyses of multiple CpG dinucleotides in one test, account for correlation between CpG dinucleotides, incorporate relevant adjustments within the models and have the ability to accommodate missing data. The REML likelihood method was used for the model fitting. DNA methylation was treated as a continuous variable. All models account for the correlation between CpG dinucleotides, and bisulphate plate number. The final analysis takes into account potential confounding variables, including infant age and maternal education (Dutch study) and maternal education (Texas study) which were all entered as fixed effects. Person identifier was entered as random effect.

Next, a possible mediating effect of DNA-methylation in the association between maternal biomarker concentrations and NTD risk was tested. As extended information was available of the Dutch population, these analyses were only performed within this study. First, we created a standard deviation score (SDS) for maternal biomarkers levels as the absolute differences were minor. We assessed the association between serum folate, vitamin B12 and total homocysteine (tHcy) and the occurrence of NTD using multivariable logistic regression models. The mean *MTHFR* methylation was added to the latter model to assess possible mediation.

All tests were performed using the data measured in triplets and all reported P-values are 2-sided. Effect sizes for NTD were expressed in relation to the SD in the controls. To account for multiple testing the Benjamini-Hochberg FDR correction was used. All analyses were performed using SPSS software, version 17.0 (IBM SPSS Inc. Chicago, IL, USA).

RESULTS

The two cohorts in this study are a Dutch case-control study with surviving spina bifida cases, and a Texan study with both alive and deceased spina bifida and anencephaly cases. Next to the difference in cases of both studies the main difference is the age of the children at investigation. The Dutch cases and controls were investigated at the standardized age of approximately 15 months, while the Texan study groups were investigated after pregnancy termination or birth at the time of the Guthrie test.

The characteristics of the cases and controls are shown in **Table 1**. Dutch case mothers showed a higher BMI (+2.4 kg/m^2, P-value = 0.011), a lower maternal education (20% less high education, P-value = 0.021) and lower serum vitamin B12 levels (286 pmol/L, P-value = 0.007) compared to control mothers. Children with NTD had a slightly higher age at blood collection (+0.5 y, P-value <0.001), and lower birth weight (2230 g, P-value <0.001) and shorter gestational age at birth (21.1 week, P-value = 0.001) compared to controls. Whereas case mothers of the Texan study only had a significantly higher BMI compared to control mothers (+1.8 kg/m^2, P-value = 0.035). Characteristics of the Texan children were not available for analysis.

The mean absolute DNA-methylation in the child according to case-control status is shown in **Table 2** and **Figure 1**. The mean methylation presented in **Figure 1** is stratified by CpG unit, study and case-control status. Effects and P-values in **Table 2** are corrected for CpG, bisulfite treatment plate, maternal education (Dutch and Texan study samples) and age of the child (in the Dutch study only). In the Dutch study, the absolute methylation of *MTHFR* was 0.33% lower in case children compared to controls (P-value = 0.001). Methylation of *VANGL1* was 0.17% (P-value = 0.063) higher and of *LEKR/CCNL* 1.36% higher (P-value = 0.048) in spina bifida cases as compared to controls. After Benjamini-Hochberg correction for multiple testing only the association with *MTHFR* remained significant.

In the Texan study of all six studied genes only *LEKR/CCNL* showed a borderline significant association with NTD risk (effect = 20.15, P-value = 0.08), however, in the opposite direction from the results in the Dutch study group. So, we could not replicate the associations found in the Dutch study.

The associations between maternal biomarker concentrations and the risk of spina bifida were tested. These analyses were restricted to the Dutch population as these data were not available for the Texas population. We found that for each standard deviation increase of maternal vitamin B12 levels, a reduced risk (adjusted odds ratio (aOR) = 0.992, 95% CI 0.99; 1.00, P-value = 0.050) for NTD was observed. Subsequently, the model was additionally adjusted for *MTHFR* methylation, to test for a mediation effect. The odds ratio was attenuated and no longer significant (aOR = 0.995, 95% CI 0.99; 1.00, P-value = 0.27) (**Table 3**). However, *MTHFR* methylation itself was not significant in the model (P-value = 0.77, data not shown).

Table 1 | Baseline characteristics for the two study groups.

	Dutch study group			Texan study group		
Characteristics	**NTD children (n = 48)**	**Control children (n = 62)**	**P-value**	**NTD children (n = 40)**	**Control children (n = 79)**	**P-value**
MATERNAL						
Age at delivery (y)*	**30.5 (3.9)**	**31.5 (3.3)**	**NS**	**24.8 (6.0)**	**24.8 (6.1)**	**NS**
BMI (kg/m^2)†	**25.4 (7.5)**	**23.0 (4.2)**	**0.011**	**26.5 (8.1)**	**24.7 (7.4)**	**0.035**
Education: high, n (%)	**6 (12.8)**	**20 (33.3)**	**0.021**	–	–	–
Education (years of school)†	–	–	–	**11 (5)**	**12 (4)**	**NS**
Periconceptional folic acid supplement use: yes, n (%)	**31 (66.0)**	**38 (63.3)**	**NS**	**3 (7.5)**	**2 (2.6)**	**NS**
Periconceptional smoking: yes, n (%)	**12 (25.5)**	**15 (25.0)**	**NS**	**9 (22.5)**	**10 (12.8)**	**NS**
tHcy, plasma (µmol/L)†	**10.6 (3.3)**	**11.1 (4.7)**	**NS**	-	-	-
Folate, serum (nmol/L)†	**16.3 (15.9)**	**13.4 (13.2)**	**NS**	**25.4 (23.6)**	**22.9 (31.3)**	**NS**
Folate, RBC (nmol/L)†	**780 (665)**	**595 (552)**	**NS**	**738 (616)**	**824 (555)**	**NS**
Vitamin B12, serum (pmol/L)†	**227 (152)**	**313 (184)**	**0.007**	**323 (140)**	**387 (162)**	**NS**
CHILD						
Age at the study moment (y)†	**1.7 (1.5)**	**1.2 (0.3)**	**<0.001**	–	–	–
Male sex, n (%)	**16 (34.0)**	**24 (40.0)**	**NS**	–	–	–
Birth weight (g)*	**3113 (552)**	**3343 (313)**	**<0.001**	–	–	–
Gestational age at birth (weeks)†	**38.9 (2.2)**	**40.0 (1.3)**	**0.001**	–	–	–
tHcy, plasma (µmol/L)†	**6.3 (3.1)**	**7.1 (1.3)**	**NS**	–	–	–
Folate, RBC (nmol/L)†	**1086 (752)**	**1228 (813)**	**NS**	–	–	–
Folate, serum (nmol/L)†	**31.5 (28.3)**	**31.0 (47.6)**	**NS**	–	–	–
Vitamin B12, serum (pmol/L)†	**503 (335)**	**471 (282)**	**NS**	–	–	–

Data is presented of the Dutch study group (n = 110) and the Texan study group (n = 119). Values are presented as *mean (SD) or †median (IQR). Chi-square, *t*- and Mann-Whitney U tests were used to test differences between NTD and control children.

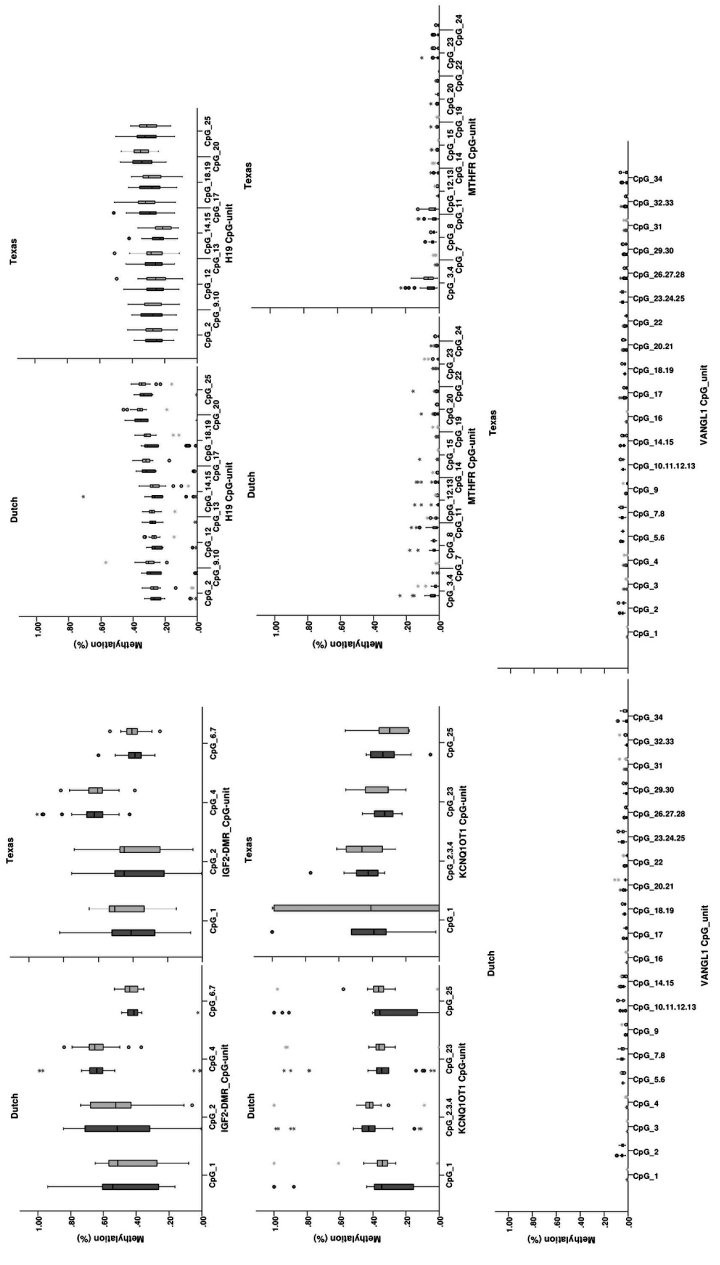

Figure 1 | Methylation levels per locus stratified by study and case-control status.
Box and whisker plots of methylation values (y-axis) of all individuals are shown for each CpG unit (x-axis) for NTD case and control children separately.

Table 2 | Methylation in NTD and control children in the Dutch and Texan study groups.

	N cases	N controls	Control methylation % (SD)	Absolute difference	P-value
DUTCH CHILDREN					
IGF2-DMR	43	35	51.2 (22.1)	+0.96	0.494
H19	43	27	26.1 (11.9)	+0.62	0.333
KCNQ1OT1	41	54	38.7 (20.9)	-0.21	0.886
LEKR/CCNL	46	61	2.6 (2.4)	+1.36	0.048
MTHFR	47	61	1.2 (2.5)	-0.33	0.001#
VANGL1	47	59	2.0 (2.9)	+0.17	0.063
TEXAN CHILDREN					
IGF2-DMR	32	67	46.1 (17.9)	+0.69	0.498
H19	24	50	28.2 (9.4)	-0.27	0.616
KCNQ1OT1	11	22	38.4 (18.0)	-0.49	0.890
LEKR/CCNL	22	48	2.5 (1.1)	-0.15	0.084
MTHFR	22	48	1.2 (2.3)	-0.07	0.470
VANGL1	33	78	1.9 (2.3)	+0.07	0.149

Linear Mixed Model analysis. Analyses were corrected for CpGs, bisulfite treatment plate, age of the child (Dutch children only), and maternal education. # P<0.05 after multiple testing correction by Benjamini-Hochberg FDR

Table 3 | Maternal biomarker concentrations and NTD risk in offspring in the Dutch study.

	NTD		
	aOR	**95% CI**	**P-value**
MODEL 1: adjusted for age of the child at blood sampling, maternal educational level, and folic acid supplement use at the moment of blood sampling			
Maternal folate SDS, serum (nmol/L)	0.785	0.118–5.221	0.802
Maternal tHcy SDS, plasma (µmol/L)	1.133	0.495–2.591	0.767
Maternal vitamin B12 SDS, serum (pmol/L)	0.380	0.145–0.999	0.050
MODEL 2: additionally adjusted for mean *MTHFR* methylation			
Maternal folate SDS, serum (nmol/L)	1.115	0.130–9.536	0.921
Maternal tHcy SDS, plasma (µmol/L)	1.158	0.499–2.686	0.733
Maternal vitamin B12 SDS, serum (pmol/L)	0.535	0.177–1.619	0.268

Result from multivariable logistic regression analyses. aOR, adjusted odds ratio.

DISCUSSION

In this combined study of a Dutch and a Texan case-control study, we found in the Dutch study group an association of DNA methylation levels of the *MTHFR* CpG-island with NTD in children. In addition, our results suggest mediation of *MTHFR* methylation in the association between maternal vitamin B12 and spina bifida in offspring. This association was not observed in the Texan study group. We did not find significant associations for the imprinted *IGF2DMR*, *H19*, and *KCNQ1OT1* genes or for the non-imprinted genes *VANGL1* and *LEKR/CCNL* after correction for multiple testing.

The role of the folate dependent 1-C metabolism in the etiology of NTD has been consistently shown. Maternal hyperhomocysteinemia as a sensitive marker of a deranged 1-C metabolism has been associated with an increased risk of NTD in the offspring.[12,40,41] One of the proposed underlying mechanisms of this association is the induction of alterations in DNA-methylation of genes implicated in embryonic and neural tube development. DNA methyltransferases (DNMTs) use methyl donors from the 1-C metabolism to establish and maintain DNA-methylation.[42] Disruption of DNMT3b in animal models, which is responsible for de novo methylation, resulted in NTD,[22] which supports DNA methylation as possible mechanism in the causation of NTD. A key gene within the 1C-metabolism is *MTHFR*. Common variants of the *MTHFR* gene have been shown to be also associated with NTD risk.[9,20] The combined heterozygozity of these two polymorphisms resulted in reduced activity of the enzyme and was associated with NTD.[17] In the present study, we found a small difference but significantly lower *MTHFR* DNA-methylation levels in Dutch children with spina bifida. Lower DNA-methylation levels could indicate higher activity of the gene, which would suggest an opposite effect from the study by Van den Put et al., where lower activity of the gene was associated with higher NTD risk.[17] Because we conducted a cross-sectional study we cannot distinguish whether the observed association is a cause or outcome of NTD. The samples in the Dutch study were drawn around 15 months after birth. Therefore, we cannot exclude an environmental or disease-driven effect on the DNA-methylation levels of *MTHFR* in these children.

The Dutch NTD have previously been associated with higher zinc, glutamine, lower glucose, and creatinine concentrations in amniotic fluid of the children,[43,44] and with higher glucose, lower zinc, myo-inositol and vitamin B12 concentrations in the mothers.[6,27,45] The lower maternal B12 concentrations in cases could lead to a derangement of 1C-metabolism, which could have effected DNA-methylation levels of *MTHFR* in the children. This could indicate that the lower *MTHFR* DNA-methylation level is implicated in the causative pathway of the development of NTDs. Nevertheless, *MTHFR* methylation was not significant in the mediation model. This could be due to the relatively small sample size of our study, so additional studies are warranted to confirm these results.

While we do not observe any association of *H19* with NTDs in blood of these subjects, a recent study in brain tissue of Chinese NTD cases and controls showed a significant decrease in *H19-DMR*1 DNA-methylation in cases.[46] One of the reasons that we find different results for *H19* DNA-methylation in association with NTDs is that we studied a different region 5′ of the *H19* gene. Liu et al. studied a region located around 2 kb upstream of the gene, while the region we studied was located 300–700 bp 5′ of the gene. Methylation at different regions at a locus could show uncorrelated results.

Methodological considerations

Some strengths and limitations of this study have to be addressed. Our study is the first to investigate DNA-methylation levels in peripheral white blood cells in relation to the occurrence of NTD in children. Previous studies on DNA-methylation levels in NTD studied DNA-methylation in brain tissue, but neither candidate gene nor global DNA-methylation in white blood cells.[24-26] In the present study we were not able to study brain tissue of NTD cases and controls, and therefore we are not sure if DNA methylation status of white blood cells is an appropriate surrogate for that of brain tissues during development. In particular, there is a possibility that the differences in DNA-methylation could be attributed to the cellular heterogeneity in leukocytes. Nonetheless, the ENCODE project showed recently that CpGs in promoters and upstream regulatory regions display less variation between cell types and tissues compared to CpGs in gene bodies and intergenic regions.[47] In the present study we have mainly focused on CpGs in CpG-islands in the promoter and upstream regions of the genes. Therefore, we expect that the levels we measure in whole blood are similar to those in brain tissue, or at least that the levels show moderate to good correlation. It remains unclear to what extend our results in white blood cells are comparable to brain tissue. However, it is impossible to perform these studies on human brain tissue in similar cases and controls. Although it is difficult to generalize our results, they might give us a clue to what genes are epigenetically involved in the development of NTDs.

Another strength of our study is that with the EpiTYPER method we have covered a great part of the CpG-island in the promoter or regulatory region of each gene, so we have conducted a very thorough search for the candidate genes we selected. Furthermore, we have compared data for two independent studies for the association of DNA-methylation with NTD, although we could not replicate our findings.

One of the limitations is the relative small control group for both the Dutch and the Texas study. For the most optimal power one would need 3 controls for each case and we only had one to two controls per case. However, based on previous studies we assume the effect of small DNA-methylation differences to be relatively large.[5] So we expect to identify true significant associations with our sample size and case:control ratio. The blood of the Texan study was obtained at the time of the Guthrie test, shortly after birth or after pregnancy termination while the blood of the Dutch study was obtained 15 months after birth. Therefore, due to this time difference we could not combine the Dutch and the Texas study samples, because we assume the 15 months difference in age to have an effect on the DNA-methylation levels of the children. Also, the Dutch cases are all spina bifida cases surviving one year of age, while the Texas samples include both spina bifida and anencephaly cases of which not all were survivors. For genetic variation it is well known that a different ethnic background could influence the linkage disequilibrium and therefore the association with outcomes. This is not known for epigenetic variations, which we could not study in our study populations.

A technical limitation of this study is the sensitivity of the method we have used to detect DNA-methylation levels. It is known for the MassARRAY technique that this is around 5%, while part of our results are lower than this. Therefore, additional studies using a different technique are needed to validate the observed association of *MTHFR* DNA-methylation with the risk for NTDs.

Lastly, we performed a candidate gene approach to investigate the effect of DNA-methylation in association with NTD, however, the evidence based selected genes may not be the main genes involved in NTD. So, we might have missed DNA-methylation differences between cases and control in other more important genes. To circumvent this, a hypothesis-free epigenome-wide association approach should be followed. However, this requires ample amounts of DNA, and because we studied DNA of children we did not obtain enough DNA from the small amounts of blood to perform these experiments in the present study.

CONCLUSION

This study was the first to investigate DNA-methylation levels of several imprinted and non-imprinted candidate genes in WBC in association with NTD. We observed a small but significant association of *MTHFR* methylation levels with NTD, which could support a potential functional link between 1C-metabolism and NTDs. This link should be investigated in other studies and more in depth in animal studies for example in order to establish causality of lower *MTHFR* DNA-methylation in NTD.

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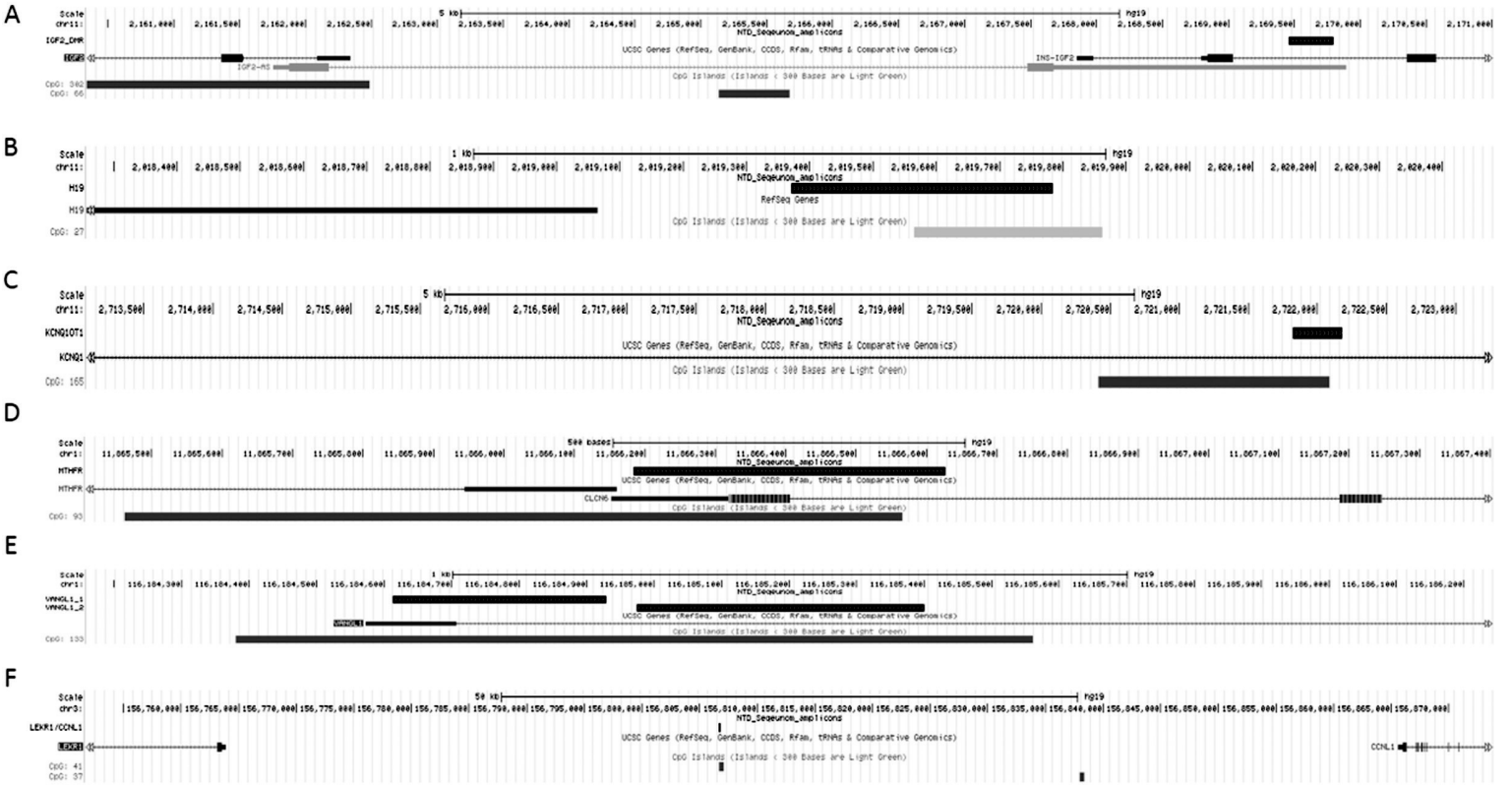
Supplement table S1 | Details of measured amplicons and PCR primers.

Gene	Genomic location[1]	Number of CpG units assessed	Primer sequence[2]	Source
IGF2-DMR	Chr 11: 2169458-2169796	3 CpG units (4 CpG sites)	F: TGGATAGGAGATTGAGGAGAAA R: AAACCCCAACAAAAACCACT	Heijmans 2007 [**33**]
H19	Chr 11: 2019371-2019784	10 CpG units (13 CpG sites)	F: GGGTTTGGGAGAGTTTGTGAGGT R: ATACCTACTACTCCCTACCTACCAAC	Heijmans, 2007 [**33**]
KCNQ1OT1	Chr11: 2721823-2722182	4 CpG units (6 CpG sites)	F: GTTAGGGAAGTTTTAGGGTGTGAAT R: TTCTAAAACCCCCACTACTATACCT	Designed using the Epidesigner tool[3]
MTHFR	Chr 1: 11866184-11866627	12 CpG units (14 CpG sites)	F: GTTTGTAGTTATTTTTGGTTTTAGTTTT R: TAACCTAAATTCTCCCTCAAATTCC	Designed using the Epidesigner tool[3]
VANGL1, primerset #1	Chr 1: 116184613-116184929	8 CpG units (15 CpG sites)	F: GAGAAGAGTGGAGTTAGAGGAAGTATTATT R: ACTCTACCTCTCCAAAAACCCAAC	Designed using the Epidesigner tool[3]
VANGL1, primerset #2	Chr 1: 116184974-116185401	12 CpG units (20 CpG sites)	F: AGTAGGGATATTTTGGGTAGAGATT R: CACCCCACTCCTTAAAATCC	Designed using the Epidesigner tool[3]
Region of *LEKR1* and *CCNL1*	Chr 3: 156806655-156806812	2 CpG units (4CpG sites)	F: GTAAGGTTTTTGGGAAAGTTGTTTT R: CTCTAAAACCCTCCCCTACCTC	Designed using the Epidesigner tool[3]

[1] Genome built: GRch 37.67 [2] Forward and reverse primer that will amplify the bisulphite converted genomic DNA. Primers were delivered with standard Sequenom MassCleave tags. 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 [3] Sequenom Inc, San Diego, USA



Supplement Figure 1 | Location of the Sequenom amplicons.

Location of the Sequenom amplicons tested with respect to IGF2 (A), H19 (B), KCNQ1OT1 (C), MTHFR (D), VANGL1 (E), LEKR1-CCNL1 (F). Amplicons are depicted in black bars with arrows, genes are depicted in blue, CpG-islands are depicted in green. Figures are created in the UCSC Genome Browser (build hg19).

Chapter 6

DNA methylation profiles in newborn and fetal and infant growth

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ABSTRACT

Objective | Changes in epigenetic programming of embryonic growth genes during pregnancy seem to affect fetal growth. Therefore, in a population-based prospective birth cohort in the Netherlands, we examined associations between fetal and infant growth and DNA methylation of *IGF2DMR*, *H19* and *MTHFR*.

Study design | For this study, we selected 69 case children born small-for-gestational age (SGA, birth weight <-2SDS) and 471 control children. Fetal 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.

Results | 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 (β = 21.07, 95% CI 21.93; 20.21, P-value = 0.015), but not with *H19* methylation. A weight gain in the first three months after birth was associated with lower *IGF2DMR* DNA methylation (β = 20.53, 95% CI 20.91; 20.16, P-value = 0.005). Genetic variants in the *IGF2*/*H19* locus were associated with *IGF2DMR* DNA methylation (P-value <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.

Conclusion | *IGF2DMR* and *H19* DNA methylation is associated with fetal 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 fetal 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] (Heijmans, Tobi et al. 2008) 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 fetal growth and subsequent early infant growth are partly due to alterations in DNA methylation of genes implicated in fetal 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 fetal 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

This study was embedded in the Generation R Study, a population-based cohort study from early fetal life onwards in Rotterdam, the Netherlands.[14] The study has been approved by the Medical Ethical Committee of the Erasmus Medical Center in Rotterdam (MEC 198.782/2001/31). Written consent was obtained from all participating mothers for both maternal and child data. Mothers were enrolled during pregnancy, between 2001 and 2005. In total, 8880 mothers were enrolled during pregnancy.

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 fetal growth genes. Therefore, 92 children were included with ADHD based on parent interview Diagnostic Inventory of Screening Children or Child Behavior 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.

Fetal and infant growth

Fetal ultrasound measurements were performed to assess gestational age and to estimate fetal 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 bi-parietal diameter (BPD) for pregnancy dating thereafter (gestational age from 12 weeks and 5 days onwards, BPD >23 mm). Fetal 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 fetal 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, four SNPs in the *IGF2/H19* locus were identified that were associated with birth weight in Caucasian children.[23] 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 **Supplement 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 analyses

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 fetal 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 (>3SD) 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 fetal 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-value <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 and DNA methylation levels of *IGF2DMR* and *H19*. SGA was associated with lower *IGF2DMR* DNA methylation (β = -1.07, 95% CI -1.93; -0.21, P-value = 0.015). Expressed relative to the standard deviation, this difference corresponds with a standardized effect size 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 fetal 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 (β = -0.46, 95% CI -0.86; -0.07, P-value = 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 (β = 0.35, 95% CI 0.03; 0.68, P-value = 0.034) in children born AGA, corresponding with a standardized difference of -0.05 SDS.

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: β = 1.20, 95% CI 0.71; 1.69, P-value = 2.0 x 10^{-6}, rs2067051, C-allele: β = -0.44, 95% CI – 0.86; -0.02, P-value = 0.041 and rs2251375, A-allele: β = 0.48, 95% CI 0.04; 0.93, P-value = 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 (β = 127 grams, 95% CI 40; 215, P-value = 0.004), but the H19 SNPs did not (rs2067051: β = -35 grams, 95% CI -109; 39, P-value = 0.349; rs2251375: β = 18 grams ,95% CI -60; 97, P-value = 0.643; rs4929984: β = -21 grams, 95% CI -96; 53, P-value = 0.572). Next, we explored the possibility of mediation of DNA methylation *IGF2DMR* DNA methylation. Our results suggest partial mediation of *IGF2DMR*

Table 1 | Maternal and infant characteristics.

	All infants n = 540	Controls n = 471	SGA n = 69	P-value[1]
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 fetal 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 fetal 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)**		
	bèta[1]	**95% CI**	**P-value**	**bèta**[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 fetal 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).

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 associated with an increased risk of SGA (adjusted odds ratio (aOR) = 1.41, 95% CI 1.24; 1.61, P-value = 1.9×10^{-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-value = 0.001).

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 3 | Associations between fetal and infant growth parameters and DNA methylation.

	Crude1	P-value	Adjusted2	P-value	Crude1	P-value	Adjusted2	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 fetal 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 fetal gender.

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

		***IGF2DMR* methylation (n = 499)**			***H19* methylation (n = 510)**		
Genetic variant	**Gene**	**bèta[1]**	**95% CI**	**P-value**	**bèta[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 fetal 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 fetal 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 fetal side of the placenta in relation to fetal 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.[29] has found no association between *IGF2DMR* methylation and SGA (difference SGA and AGA -0.2%), 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 DMR*0 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 *IGF2DMR* 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.

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 $\rho \sim 0.5$).[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.

CONCLUSION

Our analyses suggest that fetal 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 fetal development and health over the lifespan and ultimately suggest new ways to prevent human disease.

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Supplement table S1 | Details of measured amplicons and PCR primers.

Gene	Genomic location[1]	Number of CpG units assessed	Primer sequence2	Source
Insulin-like growth factor 2 (IGF2)	Chr 11: 2169458-2169796	3 CpG units (4 CpG sites)	F: TGGATAGGAGATTGAGGAGAAA R: AAACCCCAACAAAAACCACT	Heijmans 2007
H19	Chr 11: 2019371-2019784	10 CpG units (13 CpG sites)	F: GGGTTTGGGAGAGTTTGTGAGGT R: ATACCTACTACTCCCTACCTACCAAC	Heijmans, 2007
Methylenetetra-hydrofolate reductase (MTHFR)	Chr 1: 11866184-11866627	12 CpG units (14 CpG sites)	F: GTTTGTAGTTATTTTTGGTTTTAGTTTT R: TAACCTAAATTCTCCCTCAAATTCC	Designed using the Epidesigner tool[3]

[1]Genome built: GRch 37.67. [2]Forward and reverse primer that will amplify the bisulphite converted genomic DNA. According to the 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. [3]Sequenom, Inc, San Diego, USA

Supplement table S2 | Details quality control.

Locus/unit	Reason for exclusion	Success rate (if included)
IGF2 DMR		
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%

Supplement table S2 | Details quality control *(Continued)*.

Locus/unit	Reason for exclusion	Success rate (if included)
H19		
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 due to >25% missing	
H19_25		94.7%
MTHFR		
MTHFR_01	Excluded due to >25% missing	
MTHFR_02	Excluded due to low mass, duplicate MTHFR_05 and silent signals	
MTHFR_03.04		92.0%
MTHFR_05	Excluded due to low mass, duplicate MTHFR_02 and silent signals	
MTHFR_06	Excluded due to duplicate MTHFR_09 and silent signals	
MTHFR_07		94.6%
MTHFR_08		94.6%
MTHFR_09	Excluded due to duplicate MTHFR_06 and silent signals	
MTHFR_10	Excluded due to silent signal	
MTHFR_11		94.6%
MTHFR_12.13		94.8%
MTHFR_14		94.6%
MTHFR_15		94.8%
MTHFR_16-18	Excluded due to high mass	
MTHFR_19		94.6%
MTHFR_20		94.8%
MTHFR_21	Excluded due to low mass and silent signal	
MTHFR_22		94.8%
MTHFR_23		94.1%
MTHFR_24		94.8%

Supplement table S3 | Details of genetic variants.

rs number	Gene	Minor allele	Major allele	MAF	HWE P-value
rs3741205	*IGF2*	C*	A	0.25	0.706
rs2067051	*H19*	T	C*	0.48	0.451
rs2251375	*H19*	A*	C	0.33	0.573
rs4929984	*H19*	A*	C	0.50	0.477
rs1801131	*MTHFR*	T*	C	0.30	0.482
rs1801133	*MTHFR*	A*	C	0.31	0.472

MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium; *effect allele

Supplement Table S4 | DNA methylation and newborn growth parameters, with exclusion of ADHD cases.

	***IGF2* DMR (n = 412)**			***H19* (n = 382)**		
	bèta	**95% CI**	**P-value**	**bèta**	**95% CI**	**P-value**
MODEL: adjusted for correlations between CpG sites, bisulphite batch, maternal characteristics (age, educational level, parity, BMI, folic acid supplement use, smoking and the occurrence of preeclampsia) and fetal gender						
Small-for-gestational age (<-2 SDS)	**-1.10**	**-2.00; -0.19**	**0.018**	-0.30	-1.01; 0.40	0.395
Birth weight SDS	0.06	-0.22; 0.34	0.688	0.04	-0.20; 0.27	0.761
Δ weight 2nd trimester – birth	0.13	-0.64; 0.38	0.606	-0.20	-0.55; 0.15	0.270
Δ weight birth-3 months	**-0.46**	**-0.86; -0.07**	**0.022**	0.22	-0.08; 0.52	0.153
Δ weight birth-6 months	-0.16	-0.50; 0.18	0.367	0.11	-0.15; 0.37	0.407

Results from linear mixed model analyses with DNA methylation as dependent variable and the fetal growth parameters as independent variables. ADHD cases were excluded for these analyses. Analyses were performed with square-root transformed methylation data and values are presented as regression coefficients (95% confidence interval).

Supplement Table S5 | DNA methylation and newborn growth parameters, with exclusion of LGA cases.

	IGF2 DMR (n = 491)			*H19* (n = 502)		
	bèta	**95% CI**	**P-value**	**bèta**	**95% CI**	**P-value**
MODEL: adjusted for correlations between CpG sites, bisulphite batch, maternal characteristics (age, educational level, parity, BMI, folic acid supplement use, smoking and the occurrence of preeclampsia) and fetal gender						
Small-for-gestational age (<-2 SDS)	**-1.07**	**-1.93; -0.22**	**0.014**	-0.28	-0.95; 0.39	0.411
Birth weight SDS	0.08	-0.19; 0.34	0.567	0.07	-0.15; 0.28	0.539
Δ weight 2nd trimester – birth	-0.08	-0.60; 0.44	0.762	-0.18	-0.54; 0.19	0.346
Δ weight birth-3 months	**-0.50**	**-0.90; 0.10**	**0.013**	0.21	-0.10; 0.51	0.179
Δ weight birth-6 months	-0.17	-0.51; 0.17	0.316	0.10	-0.15; 0.36	0.428

Results from linear mixed model analyses with DNA methylation as dependent variable and the fetal growth parameters as independent variables. LGA cases (>2SDS) were excluded for these analyses. Analyses were performed with square-root transformed methylation data and values are presented as regression coefficients (95% confidence interval).

Chapter 7

Parental smoking and DNA methylation profiles in newborn

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ABSTRACT

Objective | Deleterious effects of prenatal tobacco smoking on fetal growth and newborns weight are well-established. One of the proposed underlying mechanisms are epigenetic alterations.

Study design | We selected 506 newborns from a population-based prospective birth cohort in the Netherlands. Parental tobacco smoking was assessed using self-reporting questionnaires. Information on birth outcomes was obtained from medical records. *IGF2DMR* and *H19* DNA methylation was measured in newborns umbilical cord white blood cells. Associations were assessed between prenatal parental tobacco smoking and DNA methylation using linear mixed models and adjusted for potential confounders.

Results | Continued maternal smoking during pregnancy was associated with *IGF2DMR* methylation (β = -1.03, 95% CI -1.76; -0.30), with a dose-dependent association (P-trend = 0.030). Lower *H19* methylation was observed in mothers who continued smoking <5 cigarettes/day (β = -0.96, 95% CI -1.78; -0.14), without a dose-dependent association. Stronger effects of maternal smoking on *IGF2DMR* methylation were observed among females (β = -1.38, 95% CI -2.63; -0.14) than among males (β = -0.72, 95% CI -1.68; 0.24). Among non-smoking mothers, paternal tobacco smoking was not associated with *IGF2DMR* or *H19* methylation. Mediation analyses indicated that the association between maternal smoking and SGA is mediated through *IGF2DMR* methylation (β = -0.095, 95% CI -0.249; -0.018).

Conclusion | Maternal smoking is associated with lower *IGF2DMR* methylation in newborns. Additionally, epigenetic alterations could be one of the mechanisms through which smoking affects fetal growth.

INTRODUCTION

Impaired fetal growth and low birth weight increase the risk of short and long-term morbidity and mortality.[1,2] A suboptimal prenatal environment contributes to fetal endocrine and metabolic adaptations with permanent effects.[2,3] An important modifiable and adverse exposure during pregnancy is tobacco smoking by parents-to-be. The deleterious effects of maternal tobacco smoking on fetal growth and newborns weight are well-established.[4] The underlying mechanisms are not fully known, but include vasoconstriction, reduced placental and fetal perfusion and hypoxia.[5] Accumulating data suggest that derangements in epigenetic fetal and placental programming may underlie the association between prenatal tobacco smoking and impaired fetal growth.[6]

Epigenetic alterations are changes in gene expression potential that are not directly related to the DNA sequence itself. DNA methylation modifications are one of the best understood epigenetic mechanisms.[7] During pregnancy cells undergo major epigenetic reprogramming making them more susceptible for dysregulation during this period.[7] Both animal and human studies have shown that changes in the intrauterine environment can lead to aberrant epigenetic profiles.[8-10] Few studies have shown both global and gene specific differences in DNA methylation in different tissues in children prenatally exposed to tobacco smoking.[11,12] In umbilical cord blood, global DNA methylation was inversely correlated with cotinine levels, a metabolite of nicotine and an indicator of tobacco smoking.[13] *IGF2* and *H19* are maternally and paternally imprinted genes located next to each other, playing an important role during fetal growth and development.[14-16] Moreover, the *IGF2/H19* locus is a well-studied region that is known to be regulated through epigenetic mechanisms and sensitive to environmental exposures.[9]

Information on paternal smoking can be used to elucidate whether an effect of maternal smoking on DNA methylation is due to direct intrauterine mechanisms. If the effect of paternal tobacco smoking is smaller than the effect of maternal tobacco smoking, this suggests that the effect is more likely due to direct intrauterine effects of maternal smoking. Since mother and father often share the same environment, this is less likely due to unmeasured environmental factors. Little is known about the influence of paternal tobacco smoking during pregnancy on gene specific DNA methylation.

From this background, we aim to study whether parental tobacco smoking during pregnancy is associated with DNA methylation of *IGF2DMR* and *H19* in peripheral white cord blood cells of newborns. Furthermore, we examined the possibility of mediation of DNA methylation in the association between smoking and newborns born small-for gestational age (SGA).

MATERIALS AND METHODS

Design and study population

This study was embedded in the Generation R Study Rotterdam, the Netherlands, a population-based prospective cohort study from early pregnancy onwards.[17] The study has been approved by

the Medical Ethical Committee of the Erasmus Medical Center in Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained from all participating mothers for both maternal and child data. Mothers were enrolled during pregnancy, between 2001 and 2006. In total, 8880 mothers were enrolled during pregnancy.

For the present study, analyses were restricted to Dutch newborns, which was based on the country of birth of the parents and grandparents (n = 4,882).[17] In addition, newborns were selected with DNA extracted from umbilical cord white blood cells available (n = 3,127). DNA methylation measurements were limited to 540 newborns due to logistic reasons. The current study is part of a project, in which we investigate the hypothesis that both SGA and children with ADHD have a shared causality in DNA methylation of especially imprinted fetal growth genes. The sampling strategy was to oversample the children born SGA or diagnosed with ADHD to improve the power of the analyses. Therefore, all infants born with a gestational-age and sex-adjusted birth weight below -2 SDS (n = 69) were selected for analysis. Also, 92 children were included with ADHD based on parent interview Diagnostic Inventory of Screening Children or Child Behavior 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. Control infants were born with a gestational-age and sex-adjusted birth weight above -2 SDS. Of the 540 newborns, data on parental smoking habits was available in 93.7% (n = 506). The total population for analysis was 506 children (65 newborns born SGA, 88 cases of ADHD and 441 control newborns).

Paternal tobacco smoking habits during pregnancy

Information regarding maternal tobacco smoking was obtained by self-administered questionnaires sent in each trimester of pregnancy. Active maternal tobacco smoking at enrolment was assessed by asking the mother whether she smoked during pregnancy (no tobacco smoking; first trimester only tobacco smoking; continued tobacco smoking during pregnancy). This questionnaire was sent to all mothers, regardless of their gestational age at enrolment. In the second and third questionnaires, mothers were asked whether they smoked in the previous 2 months (no; yes). Mothers who reported that they did not smoke or only smoked during the first trimester, but still reported smoking in the second or third questionnaire were classified as continuous smokers. Among the mothers who continuously smoked during pregnancy, the number of cigarettes were assessed and classified as 1) less than 5 cigarettes a day and 2) 5 or more cigarettes a day. Dose-response analyses for continuous smokers were based on the third trimester questionnaires. Paternal tobacco smoking was assessed at enrolment by asking the mother whether the father smoked during pregnancy (no; yes; do not know). Similar information was provided by the father in a subset of the participants (n = 433). Agreement between the two assessments was good (sensitivity = 92%, specificity = 95%). As more data was available from the mother questionnaire compared to the father questionnaire (6.7% missing versus 19.8% missing, respectively), data obtained from the mother's questionnaire was used.

Assessment of DNA methylation

Genomic DNA was isolated from cord blood samples at birth as previously described.[18] We assessed DNA methylation of two imprinted loci, namely *IGF2DMR* and *H19DMR*. These loci were chosen based on their previously shown features of epigenetic regulation and their susceptibility for to environmental exposures.[19] 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 (MassARRAY EpiTYPER, 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 loci by MassARRAY EpiTYPER Analyzer software (v1.0, build 1.0.6.88 Sequenom, Inc, San Diego, USA).[20,21] Fragments containing one or more CpG sites were called CpG units. PCR and subsequent steps were performed 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 with interference of single nucleotide polymorphisms (CEU) 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**.

Covariates

From self-administered questionnaires, data was available on maternal age and maternal educational level, parity 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. Folic acid supplement use was categorized into 1) folic acid supplement use (pre- or postconception 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). Information concerning date of birth, offspring sex and birth weight was obtained from community midwives and hospital registries.

Statistical analyses

Differences in maternal or newborns characteristics between maternal tobacco smoking categories were tested using ANOVA and chi-square tests. Thereafter, linear mixed models were used to examine the associations between maternal or paternal tobacco smoking (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 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 (>3SDS) 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, maternal and paternal tobacco smoking were both entered as a fixed effects in separate models. In the adjusted analyses, potential confounders were additionally entered to the model at the same time as fixed effects. Since maternal and paternal smoking are correlated, investigating paternal smoking among all mothers would overestimate the effect of paternal smoking if direct intrauterine mechanisms were present. Therefore, the association between paternal smoking and DNA methylation was examined among mothers who did not smoke during pregnancy. Prenatal exposures have shown to alter *IGF2DMR* methylation differently according to sex.[9,22] Therefore, the association between maternal tobacco smoking and DNA methylation was assessed in both boys and girls. The analyses were also repeated with exclusion of the ADHD (n = 88) cases. Two newborns were classified as both ADHD and SGA.

Additionally, we performed a mediation analyses to investigate whether DNA methylation of either *IGF2DMR* or *H19* mediates the association between maternal tobacco smoking and the risk of a newborns being born SGA. The direct and indirect effects between the dependent (SGA) and independent variable (maternal tobacco smoking) as well as the mediator (DNA methylation) were tested (**Figure 1**) using a bootstrap approach (1,000 samples) described by Preacher and Hayes.[23]

Missing data of potential confounders (maternal educational level (0.6%), maternal BMI (0.2%) and maternal folic acid supplement use (13.2%)) were completed using the Markov-Ten imputed datasets were created. The linear mixed model analyses were performed using the data measured in triplicates including imputed missing data. Overall, the effect estimates using the imputed dataset were slightly smaller compared to the original dataset. All analyses were performed using the Statistical Package for the Social Sciences version 21.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

The median (90% range) DNA methylation levels of *IGF2DMR* and *H19* were 53.2% (44.3–61.3) and 30.1% (25.6–34.1), respectively. Maternal and newborns characteristics are presented in **Table 1**. This study was carried out in a subset of the original Generation R cohort. Therefore, we compared characteristics of the included and excluded mothers and newborns. Included mothers were younger (30.3 years versus 31.1 years in excluded mothers, P-value<0.001), smoked more often during pregnancy (32.9% versus 26.0% in excluded mothers, P-value<0.001) and were more often single (10.9% versus 8.4% in excluded mothers, P-value = 0.02). Included newborns were more often male (58.7% versus 49.7% in excluded newborns, P-value<0.001). However, the included and excluded participants were similar in terms of maternal BMI, maternal educational level,

parity, folic acid supplement use, alcohol use and APGAR score after 1 and 5 minutes (all P>0.05). Of the included mothers, 9.3% (n = 47) reported only tobacco smoking in the first trimester and 24.7% (n = 125) continued tobacco smoking during pregnancy. Mothers who continued tobacco smoking were younger, lower educated and less often used a folic acid supplement during the periconception period than mothers who reported to have never smoked during pregnancy. Paternal tobacco smoking was more frequent in families with mothers who continued smoking cigarettes (72.8% in continued smoking versus 37.4% in non-smoking mothers). Median (90% range) birth weights of children from non-smoking, first trimester only and continued smoking mothers were 3500 (2536–4383), 3410 (2154–4219) and 3195 (2348–4081) grams, respectively.

The associations between parental tobacco smoking and DNA methylation of *IGF2DMR* and *H19* are shown in **Table 2**. Although *IGF2DMR* methylation of mothers who only smoked during the first trimester showed a tendency to be lower compared to mothers who did not smoke, no significant differences were observed. However, continued smoking during pregnancy was associated with lower *IGF2DMR* methylation (crude model: β = -1.14, 95% CI -1.81; -0. mothers who smoked during the first trimester 47, P-value = 0.001, adjusted model: β = -1.03, 95% CI -1.76; -0.30, P-value = 0.006). Expressed as relative to the standard deviation, the difference found in the adjusted model corresponds with a standardized effect size in DNA methylation of -0.13 standard deviation score (SDS). Of the mothers who continued smoking, 77.6% (n = 97) provided information concerning the amount of cigarettes they smoked, of which 42.3% (n = 41) reported to have smoked less than 5 cigarettes per day and 57.7% (n = 56) reported to have smoked 5 or more cigarettes per day. No significant association was found between the amount of cigarettes and *IGF2DMR* methylation. However, a dose-dependent association of the number of cigarettes smoked was assessed in mothers who continued smoking (P-value for trend = 0.030). Although no association was observed between mothers who continued smoking and *H19* methylation, a significant inverse association was revealed in mothers smoking up to 5 cigarettes per day (crude model: -0.90, 95% CI -1.70; -0.11, P-value = 0.026, adjusted model: β = -0.96, 95% CI -1.78; -0.14, P-value = 0.021). Expressed relative to the standard deviation, the difference found in the adjusted model corresponds with a standardized effect size in DNA methylation of -0.13 SDS. No significant associations were observed in mothers who reported to have smoked 5 or more cigarettes per day with *H19* methylation. Among mothers who did not smoke during pregnancy, information on paternal tobacco smoking during pregnancy was provided by 99.4% of the mothers (n = 332/334). Of the fathers who smoked, 45.6% (n = 57/125) smoked less than 5 cigarettes per day and 53.6% (n=67/125) smoked 5 or more cigarettes per day. No association was observed between paternal smoking and *IGF2DMR* or *H19* methylation.

Table 1 | Baseline characteristics.

Characteristic	Maternal tobacco smoking during pregnancy					P-value[3]
	No (n = 334)	First trimester only (n=47)	Continued			
			All (n =125)	<5 cigarettes per day (n = 41)	≥5 cigarettes per day (n = 56)	
MATERNAL						
Age at intake (years) [1,2]	31.5 (22.5–38.1)	30.5 (20.1–38.3)	28.5 (19.0–37.7)	29.4 (20.0–37.5)	28.4 (18.4–39.9)	*<0.001*
Body mass index at intake (kg/m^2) [1,2]	23.1 (19.5–32.2)	23.2 (19.6–33.1)	23.7 (18.2–32.5)	22.8 (18.2–33.6)	24.8 (18.0–33.9)	NS
Education, n (%)						<0.001
Primary education	6 (1.8)	4 (8.5)	12 (9.6)	0 (0)	10 (17.9)	
Secondary education	134 (40.1)	21 (44.7)	78 (62.4)	23 (56.1)	34 (60.7)	
Higher education	193 (57.8)	22 (46.8)	33 (26.4)	17 (41.5)	11 (19.6)	
Missing	1 (0.3)	0 (0.0)	2 (1.6)	1 (2.4)	1 (1.8)	
Parity (%)						NS
0	220 (65.9)	34 (72.3)	82 (65.6)	28 (68.3)	35 (62.5)	
≥1	114 (34.1)	13 (27.7)	43 (34.4)	13 (31.7)	21 (37.5)	
Folic acid supplement use during pregnancy, n (%)						<0.001
Start preconception	188 (56.3)	18 (38.3)	29 (23.2)	11 (26.8)	15 (26.8)	
Start postconception	84 (25.1)	19 (40.4)	36 (28.8)	13 (31.7)	13 (23.2)	
No	25 (7.5)	7 (14.9)	33 (26.4)	10 (24.4)	14 (25)	
Missing	37 (11.1)	3 (6.4)	27 (21.6)	7 (17.1)	14 (25)	
Paternal smoking, n (%)	125 (37.4)	28 (59.6)	91 (72.8)	14 (34.1)	9 (16.1)	*<0.001*
<5 cigarettes per day	57 (17.1)	9 (19.1)	17 (13.6)	7 (17.1)	17 (30.4)	
≥5 cigarettes per day	67 (20.1)	18 (38.3)	73 (58.4)	4 (9.8)	39 (69.6)	
INFANTS						
Boys, n (%)	195 (58.4)	21 (44.7)	81 (64.8)	26 (63.4)	34 (60.7)	NS
Birth weight [1,2]	3500 (2536–4383)	3410 (2154–4219)	3195 (2348–4081)	3175 (2663–4252)	3210 (2334–4150)	*<0.001*
Gestational age at birth [1,2]	40.3 (37.6–42.0)	40.3 (36.5–41.9)	40.1 (37.1–42.5)	40.6 (37.0–42.9)	40.2 (36.9–42.3)	NS

Values are presented as [1]median (90% range) or as number (%). [2]Missings; age at intake (n = 0), body mass index at intake (n = 1), gender (n = 0) birth weight (n = 0), gestational age at birth (n = 0). [3]ANOVA and chi-square tests are used to test differences between the different smoking categories.

Table 2 | Parental tobacco smoking habits and DNA methylation.

	IGF2DMR methylation			*H19* methylation		
	bèta1	**95% CI**	**P-value**	**bèta**1	**95% CI**	**P-value**
MODEL 1: adjusted for correlations between CpG sites, bisulphite batch, gestational age at birth						
MATERNAL TOBACCO SMOKING						
No (n = 334)		Reference			Reference	
First trimester only (n = 47)	-0.68	-1.67; 0.31	0.178	0.61	-0.13; 1.35	0.108
Continued smoking, all (n = 125)	-1.14	-1.81; -0.47	0.001	-0.24	-0.75; 0.26	0.348
<5 cigarettes per day (n = 41)	-1.06	-2.13; -0.00	0.050	-0.90	-1.70; -0.11	0.026
≥5 cigarettes per day (n = 56)	-1.12	-2.06; -0.18	0.019	-0.13	-0.82; 0.56	0.703
P for trend		0.006			0.298	
PATERNAL TOBACCO SMOKING						
No (n = 207)		Reference			Reference	
Yes (n = 125)	-0.24	-0.96; 0.48	0.519	-0.10	-0.61; 0.41	0.707
<5 cigarettes per day (n = 57)	-0.26	-1.22; 0.70	0.597	-0.19	-0.87; 0.50	0.592
≥5 cigarettes per day (n = 67)	-0.16	-1.06; 0.73	0.719	-0.04	-0.68; 0.60	0.893
P for trend		0.643			0.795	
MODEL 2: model 1 + maternal characteristics (age, educational level, parity, BMI, periconception folic acid supplement use) and fetal gender						
MATERNAL TOBACCO SMOKING						
No (n = 334)		Reference			Reference	
First trimester only (n = 47)	-0.70	-1.71; 0.31	0.175	0.56	-0.20; 1.32	0.146
Continued smoking, all (n = 125)	-1.03	-1.76; -0.30	0.006	-0.37	-0.94; 0.20	0.195
<5 cigarettes per day (n = 41)	-1.01	-1.89; 0.10	0.069	-0.96	-1.78; -0.14	0.021
≥5 cigarettes per day (n = 56)	-0.89	-2.10; 0.08	0.079	-0.20	-0.93; 0.54	0.602
P for trend		0.030			0.242	
PATERNAL TOBACCO SMOKING						
No (n = 207)		Reference			Reference	
Yes (n = 125)	-0.17	-0.91; 0.58	0.663	-0.23	-0.75; 0.30	0.393
<5 cigarettes per day (n = 57)	-0.20	-1.18; 0.77	0.681	-0.23	-0.92; 0.45	0.502
≥5 cigarettes per day (n = 67)	-0.07	-1.01; 0.88	0.887	-0.25	-0.91; 0.42	0.463
P for trend		0.809			0.395	

Results from linear mixed model analyses with maternal and parental 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.

Table 3 shows the association between maternal tobacco smoking and DNA methylation, stratified by gender. Among girls, mothers who smoked during the first trimester showed an association with *IGF2DMR* methylation (crude model: β = -1.40, 95% CI -2.79; -0.01, P-value = 0.048), although this association did not remain in the adjusted model (β = -1.25, 95% CI -2.71; 0.21, P-value = 0.093). This effect was not observed among boys. In addition, among girls first trimester smoking showed a positive association with *H19* methylation, but only in the crude model (crude model: β = 1.04, 95% CI 0.04; 2.03, P-value = 0.041, adjusted model: β = 1.00, 95% CI -0.05; 2.03, P-value = 0.063). The effect of continued maternal tobacco smoking on *IGF2DMR* methylation was also stronger among girls (crude model: β = -1.46, 95% CI -2.59; -0.33, P-value = 0.011, adjusted model: β = -1.38, 95% CI -2.63; -0.14, P-value = 0.029) than among boys (crude model: β = -0.89, 95% CI -1.73; -0.06, P-value = 0.037, adjusted model: β = -0.72, 95% CI -1.68; 0.24, P-value = 0.142).

Table 3 | Maternal tobacco smoking and DNA methylation, stratified by gender.

	***IGF2DMR* methylation**			***H19* methylation**		
	bèta[1]	**95% CI**	**P-value**	**bèta**[1]	**95% CI**	**P-value**
MODEL 1: adjusted for correlations between CpG sites, bisulphite batch, gestational age at birth						
BOYS						
No (n = 195)		Reference			Reference	
First trimester only (n = 21)	0.12	-1.33; 1.57	0.870	0.18	-0.95; 1.31	0.754
Continued smoking, all (n = 81)	-0.89	-1.73; -0.06	0.037	-0.41	-1.07; 0.25	0.222
P for trend		0.044			0.249	
GIRLS						
No (n = 139)		Reference			Reference	
First trimester only (n = 26)	-1.40	-2.79; -0.01	0.048	1.04	0.04; 2.03	0.041
Continued smoking, all (n = 44)	-1.46	-2.59; -0.33	0.011	-0.10	-0.90; 0.71	0.816
P for trend		0.005			0.817	
MODEL 2: model 1 + maternal characteristics (age, educational level, parity, BMI, periconception folic acid supplement use)						
BOYS						
No (n = 195)		reference			Reference	
First trimester only (n = 21)	0.15	-1.32; 1.61	0.845	0.14	-1.00; 1.28	0.810
Continued smoking, all (n = 81)	-0.72	-1.68; 0.24	0.142	-0.69	1.45; 0.08	0.078
P for trend		0.166			0.098	
GIRLS						
No (n = 139)		Reference			Reference	
First trimester only (n = 26)	-1.25	-2.71; 0.21	0.093	1.00	-0.05; 2.03	0.063
Continued smoking, all (n = 44)	-1.38	-2.63; -0.14	0.029	-0.05	-0.93; 0.83	0.910
P for trend		0.018			0.813	

Results from linear mixed model analyses with maternal and parental tobacco smoking as independent variable and DNA methylation as dependent variable, stratified by gender. [1]Analyses were performed with square-root transformed methylation data and values are presented as regression coefficients (95% confidence interval).

Next, the associations between maternal tobacco smoking, the risk of a newborns being born SGA and gene specific DNA methylation were analyzed using mediation analyses. We hypothesized that epigenetic alterations mediate the association between maternal prenatal tobacco smoking and impaired fetal growth (**Figure 1**). The indirect effect, defined as the product of the coefficient for the effect of maternal tobacco smoking on DNA methylation (path a) and the coefficient for the effect of DNA methylation on the risk of a newborns being born SGA (path b), was calculated (**Figure 2**). This effect was significant (β = -0.095, 95% CI -0.249; -0.018), indicating that the effect of maternal smoking on the risk of a newborns being born SGA is mediated through *IGF2DMR* methylation. The remaining direct effect of smoking on SGA (c') could partially be explained by the slightly younger age and lower educational level of the smoking mother. No use of a folic acid supplement, higher BMI of the mother, nulliparity and male gender of the newborn did not significantly explain this effect.

Last, the analyses were repeated with the exclusion of the attention deficit hyperactivity disorder (ADHD) cases. The results are depicted in **table S3**. After exclusion of the ADHD cases, the effect estimates did not change substantially.

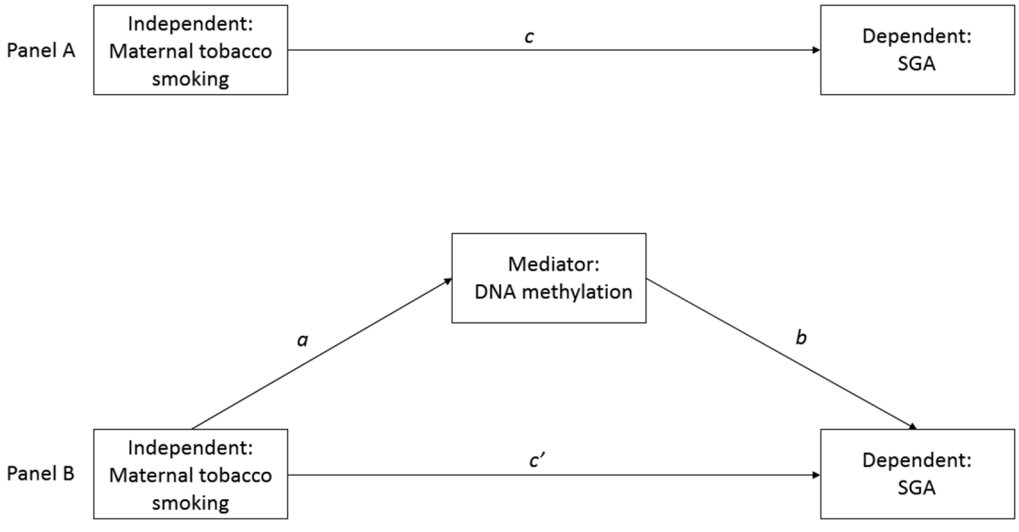

Figure 1 | Graphical representation of proposed mediation effect.

Panel A illustrates the total effect (path c). Panel B represents the mediation design. The direct effect is depicted as path c'. The indirect effect (ab) is defined as the product of path a and path b.

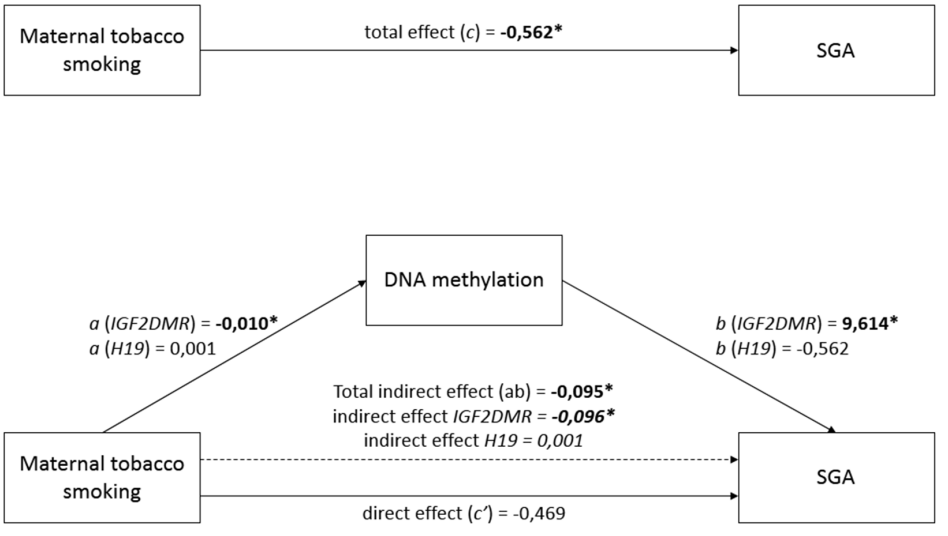

Figure 2 | Mediation analyses.
Values represent unstandardized beta's. *P <0.05

DISCUSSION

In 506 newborn, derived from a population-based birth cohort, we examined whether parental tobacco smoking is associated with *IGF2DMR* and *H19* methylation in umbilical white cord blood cells. After adjustment for confounders, continued maternal prenatal tobacco smoking was associated, in a dose-dependent manner, with lower *IGF2DMR* methylation in newborn. This association was stronger in females than in males. Lower *H19* methylation was also observed in mothers who continued to smoke <5 cigarettes/day. Paternal smoking was not associated with *IGF2DMR* or *H19* methylation. Furthermore, the association between maternal smoking and being born SGA may be partially mediated through *IGF2DMR* methylation.

Prenatal maternal tobacco smoking has been recognized as an important and modifiable adverse exposure and has repeatedly been associated with disease risks.[4,5,25] Several studies have investigated associations between maternal prenatal smoking and global and gene-specific DNA methylation with largely inconclusive results.[6,26] Epigenome-wide methylation analyses revealed methylation changes at birth in newborns whose mother had smoked during pregnancy.[27] Others investigated DNA methylation differences at the *IGF2/H19* locus in relation to prenatal exposure to maternal tobacco smoking, but found higher *IGF2DMR* methylation levels in male infants born to smokers compared to non-reported an inverse correlation between global DNA methylation and cotinine levels, a metabolite of nicotine, without stratification for gender which is consistent with our results.[13] In our study, the association between maternal tobacco smoking

and IGF2DMR methylation was more profound among girls from first trimester onwards. Thus, maternal smoking may affect *IGF2DMR* methylation already from early pregnancy onwards. This finding was unexpected as it contradicts previous research which demonstrated that males may be more susceptible to detrimental exposures than females.[22,28] The first days after conception are an important short period of reprogramming of the epigenome. Therefore, a limitation of our study is that we were not able to study difference in preconception and postconception smoking on the methyletion of the IGF2/H19 locus. Therefore, additional research is warranted.

Other adverse exposures, such as prenatal exposure to famine, have been previously linked to lower *IGF2DMR* methylation (-5.2% *IGF2DMR* methylation when periconception exposure to famine) whereas advantageous exposures, such as folic acid supplement use, have been associated with increased methylation (+4.5% *IGF2DMR* methylation).[8,9] In a previous study performed in the same population, we showed that SGA was associated with -1.13% lower *IGF2DMR* methylation.[29]

In contrast to previous studies, we have focused on the hypotheses that epigenetic alterations mediate the association between maternal tobacco smoking and adverse birth outcomes. Our study supports the notion that DNA methylation could be one of the mechanisms through which tobacco smoking affects fetal growth. As nicotine crosses the placenta, it can directly harm the fetus.[30] Cigarette smoke has been shown to contain high levels of reactive oxygen species (ROS), causing oxidative stress.[31] DNA ROS damage can interfere with the binding of DNA methyltransferases (DNMTs) to the DNA, resulting in DNA hypomethylation.[32] Tobacco smoking can also indirectly damage the fetus through the effects on the placenta. Nicotine leads to vasoconstriction in pregnancy and thus impaired vascular function.[33] Vasoconstriction limits blood flow and the provision of nutrients to the fetus, which could be compared to the effects of exposure to famine previously linked to epigenetic effects.[9]

Mothers who have been exposed to passive cigarette smoking have increased risks of adverse birth outcomes.[34,35] Strength of our study is that we were able to investigate direct intrauterine effects of maternal smoking by taking into account the effect of both parents. The effect of paternal smoking was not statistically significant and smaller than the effect of maternal smoking. This suggests that the observed effect is likely due to direct effects of maternal smoking and not to unmeasured environmental factors as mother and father are likely to share their environment. However, further studies are needed to further confirm this finding.

DNA methylation is believed to be relatively stable throughout life, but these patterns are remodeled dramatically during fetal and early postnatal life.[7] Therefore, the epigenome is susceptible for derangements during developmental stages when adverse prenatal environmental exposures can permanently alter methylation in the offspring. The periconception period is of particular importance, as epigenetic reprogramming takes mostly place during this period.[7] Our findings support the notion that changes in DNA methylation at the *IGF2DMR* are being established during the periconception period, as the methylation levels of mothers who quit smoking during the first trimester showed a tendency to be lower than in non-smokers. The non-significance may also be explained by small numbers of mothers who quit smoking during the first trimester. However, a larger effect was observed in mothers who continued to smoke during pregnancy, indicating that these effects are not limited to the periconception period.

Methodological considerations

This study was embedded in a large cohort from which a selection of Dutch newborn were studied. Selective participation did occur to some degree, as participating mothers were younger, more often single and smokers and newborns were more often male. This selection could have affected the validity and generalizability of the study, especially when the associations would differ between the study population without and with the excluded newborns. This is difficult to ascertain, as the associations in the excluded group are unknown.

The study was based on a prospective data collection starting in early pregnancy. Detailed information was available on parental tobacco smoking habits during pregnancy, which enabled studying both trimester specific and dose-response associations. Although the use of questionnaires seems to be a valid method misclassification could have occurred, mostly leading to underreporting of tobacco smoking and therefore an underestimation of our estimates.[36] Also, from previous research it is known that PCR bias can occur with quantitative DNA methylation measurements methods.[37] To address this potential problem, we created standard curves constructed from DNA with low and high methylation (EpigenDx, Worchester, USA) in steps of 10% methylation difference on both amplicons. CpGs showing irregularities were excluded from the analyses. Lastly, although analyses were corrected for potential confounders, the possibility of residual confounding cannot be excluded.

This study showed modest changes in DNA methylation in mothers who continued to smoke during pregnancy. Replication of these results is warranted. Unfortunately, we were not able to assess whether the DNA methylation variations also result in changes in expression and long-term functional effects. DNA methylation was measured in umbilical cord white blood cells and not in other tissues. It could be argued that DNA methylation patterns differ across various tissues.[38,39] However, DNA methylation of imprinted genes such as *IGF2DMR/H19* is believed to be largely comparable in different tissues. For example DNA methylation patterns of *IGF2DMR* in blood and in buccal cells and colon tissue showed moderate correlations.[38,40] We emphasize that it remains important to establish correlations between DNA methylation in peripheral tissues, e.g. blood, and tissues that are directly involved in the disease. However, in human epidemiological studies it is commonly accepted to use blood or buccal samples due to relatively poor accessibility of other tissues.

CONCLUSION

Our study shows an inverse association of maternal prenatal tobacco smoking and its dose and duration with *IGF2DMR* methylation in newborns. Moreover, *IGF2DMR* methylation may mediate the association between maternal tobacco smoking and SGA, supporting the notion that epigenetic alterations could be one of the mechanisms through which smoking affects fetal growth. The understanding how epigenetic control depends on early exposure may shed light on the link between fetal development and health over the lifespan. Our research emphasizes the importance to quit smoking preferably in the preconceptional period, although our data also shows that the advantageous effects of quitting smoking may not be limited to this period.

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Supplement table S1 | 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: TGGATAGGAGATTGAGGAGAAA R: AAACCCCAACAAAAACCACT	Heijmans, 2007
H19	Chr 11: 2019371-2019784	10 CpG units (13 CpG sites)	F: GGGTTTGGGAGAGTTTGTGAGGT R: ATACCTACTACTCCCTACCTACCAAC	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.

Supplement table S2 | Details quality control.

Locus/unit	Reason for exclusion	Success rate (if included)
IGF2 DMR		
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 due to >25% missing	
H19_25		94.7%

Supplement table S3 | Parental tobacco smoking habits and DNA methylation, with exclusion of ADHD cases.

	IGF2DMR methylation			*H19* methylation		
	bèta1	**95% CI**	**P-value**	**bèta**1	**95% CI**	**P-value**
MODEL 1: adjusted for correlations between CpG sites, bisulphite batch, gestational age at birth						
MATERNAL TOBACCO SMOKING						
No (n = 277)		Reference			Reference	
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	0.003	-0.43	-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	
MODEL 2: model 1 + maternal characteristics (age, educational level, parity, BMI, periconceptional folic acid supplement use) and fetal gender						
MATERNAL TOBACCO SMOKING						
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
P for trend		0.044			0.184	
PATERNAL TOBACCO SMOKING						
No (n = 176)		reference			reference	
Yes (n = 100)	-0.19	-1.01; 0.63	0.646	0.05	-0.51; 0.62	0.860
<5 cigarettes per day (n = 48)	-0.23	-1.29; 0.83	0.670	0.14	-0.59; 0.86	0.712
≥5 cigarettes per day (n = 51)	-0.07	-1.14; 1.00	0.895	-0.07	-0.82; 0.67	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.

Part IV

Chapter 8

General discussion

INTRODUCTION

Perinatal health is increasingly being recognized as an important determinant for adult health and disease.[1] Specifically, attention is shifting towards the periconception period.[2] The high embryonic growth and development rate makes this period extremely vulnerable for poor periconceptional exposures having permanent effects (**Figure 1**).[3,4] Changes in DNA methylation have been postulated to be one of the mechanisms underlying the associations between a suboptimal intrauterine environment and fetal growth restriction.[5]

As described earlier, the objectives of the present thesis were to gain more insight into the following three topics:

1. The relationship between periconception maternal nutrition and early and late fetal growth trajectories and birth outcome.
2. The influence of late first trimester maternal angiogenic factors, fibrinolytic factors and folate concentrations on fetal and postnatal infant growth.
3. Gene-specific DNA methylation in newborns and associations with prenatal parental conditions and exposures, and birth outcomes.
4. In this chapter, we discuss the methodological considerations. Thereafter, we focus on our main findings, implications and directions for future research and end with a general conclusion.

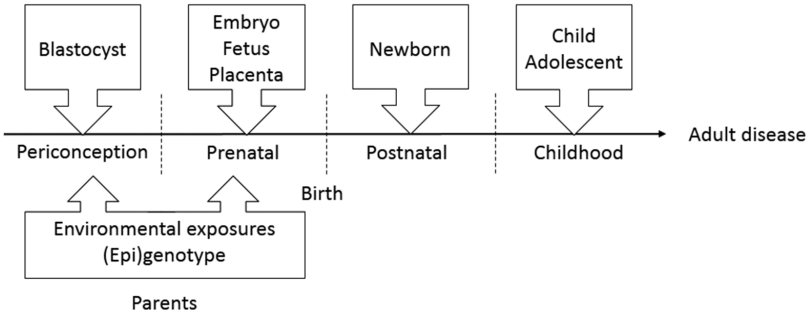

Figure 1 | Windows of susceptibility.

METHODOLOGICAL CONSIDERATIONS

The studies described in this thesis have mainly been conducted in the Generation R Study, except for one study (**Chapter 5**), which was embedded in the Dutch Spina Bifida Study and the Texas Neural Tube Defect Project. The Generation R Study is a population-based prospective birth cohort study from first trimester onwards in Rotterdam, the Netherlands.[6,7] The Dutch Spina Bifida Study is a case-control study conducted in the Netherlands.[8] The Texas Neural Tube Defect Project is a case-control study performed in a Mexican American population derived from 14 counties along

the Texas-Mexico border.[9,10] Both observational prospective studies and case-control studies have strengths and limitations. To what extent the results described in this thesis could be influenced will be discussed in the next paragraphs.

Selection bias

Selection bias can occur when an association between a determinant and outcome is different in those who have participated in the study and who did not participate in the study but were eligible.[11,12] This can be the result from non-response at baseline or loss to follow up. Within the Generation R Study, non-Dutch women of a lower social economic status are less represented.[7] This has led to a more homogeneous population, which may have diminished the variability of the outcome and power of the studies. In prospective cohort studies biased estimates usually occur as a result from loss to follow up instead of non-response at baseline.[12] In the studies performed with data from the Generation R Study, information was used from prenatal questionnaires collected during pregnancy. As there was little loss to follow up during pregnancy, bias as a result of loss to follow up is likely to be minimal. Within case-control studies, selection bias can occur when cases and controls are selected on criteria related to the exposure of interest.[11] In our study, participation in both case-control studies was most likely not related to the exposure of interest, DNA methylation, as participants were unaware of the hypothesis underlying our study.

Information bias

Information bias can arise from measurement error. There are two main types of information bias, namely recall bias and misclassification. Recall bias is not likely to occur in the Generation R Study, as data was collected prospectively. Information on maternal nutritional intake was collected with the use of food frequency questionnaires (FFQ) at the end of the periconception period, covering the previous three months (**Chapter 2**). Assessment of nutritional intake can be complicated by recall bias. Previous research did not find differences between the use of FFQ, prospective diaries and weighted dietary records in the assessment of dietary intake.[13] Case-control studies are sensitive to recall bias as a result of the retrospective data collection.[11] This can result in either an underestimation of exaggeration of an effect estimate. However, our determinant of interest was DNA methylation, which was measured in cases and controls in the same manner.

Some of the variables of interest in this thesis, including tobacco smoking and nutritional intake, have been collected by self-reported questionnaires. Therefore, socially acceptable answers could have been given and misclassification may have occurred. The information was collected with the mothers being unaware of the research questions and data on exposures were mainly collected before assessment of the outcome. Therefore, the possibility of differential misclassification is limited. Underreporting of nutritional intake is likely to occur in obese women. Therefore, BMI was taken into account in the analyses. In addition, to limit the possibility of non-differential misclassification, both ultrasound technicians and laboratory staff were blinded to the exposure or disease status of the participant.

Confounding bias

A confounding variable is a variable that is associated with both the dependent and the independent variable. When a confounding variable is not taken into account, a spurious relationship between determinant and outcome can be observed. Confounding factors were chosen based on previous literature or if a variable significantly influenced both determinant and outcome. Within the Generation R Study, a wide range of potential confounding factors is available. However, in observational studies residual confounding should be taken into account. In the studies described in this thesis, residual confounding is possible due to unmeasured variables such as medication use or physical activity. The Texas Neural Tube Defect Project did not have a wide range of potential confounding factors available. For example, the sex of the child and their birth weight was unknown to us. Therefore, potential confounding factors could have been missed, which could have influenced our results.

MAIN FINDINGS, IMPLICATIONS & RECOMMENDATIONS

The main findings described in this thesis are summarized in **Table 1**. We discuss the main findings, implications of the results and recommendations for future research for each study aim separately.

Objective 1: Maternal nutrition

During pregnancy, the mother is the environment of the developing embryo and fetus. The research described in **Chapter 2** has revealed a maternal dietary pattern, consumed in the periconception period, that was rich in bread, nuts and butter. The dietary pattern was labeled 'Energy-rich' as it was rich of (unsaturated) fats and carbohydrates. We showed that increasing adherence to this Energy-rich dietary pattern increases the crown-to-rump length (CRL) measured at the end of the first trimester. Thus, maternal diet can have detrimental or beneficial effects on the developing embryo and fetus as early as the periconception period.

Optimal fetal growth is required to ensure a healthy pregnancy outcome. Therefore, the developing embryo and fetus are dependent on the maternal nutrient supply.[14] In the second half of pregnancy, the fetus is provided with nutrients through the placenta, also known as haemotrophic nutrition. However, prior to the establishment of placental blood flow at the end of the first trimester, the embryo already receives nutrition from secretions from uterine glands, known as histiotrophic nutrition.[15] Therefore, an unhealthy lifestyle can affect the embryo in the first trimester. A multitude of maternal characteristics and life style factors have been linked to embryonic size and growth, which together with our studies emphasized the importance of preconception and early pregnancy care.[3,16] Therefore, preconception and early pregnancy recommendations include taking a folic acid supplement in the preconception period and to stop smoking, but it should also incorporate nutritional advice. However, more detailed first trimester ultrasound studies using 3D measurements are needed to assess early periconception adaptations in detail.

Table 1 | Overview main findings.

Exposure	Fetal growth	Adverse birth outcomes	Postnatal growth	Congenital malformations	Newborn DNA methylation
PARENTAL LIFESTYLE FACTORS					
Maternal					
Periconception dietary pattern					
Mediterranean	No effect				
Energy-rich	↑ CRL				
Tobacco smoking					*IGF2DMR* ↓, *H19* ↓
Paternal					
Tobacco smoking					No effect
BIOMARKERS RELATED TO PLACENTATION					
sFlt-1 ↑					
First trimester maternal blood	↑ CRL, ↑ EFW		↑ weight, ↑ length		
umbilical cord blood	↓ EFW		↓ weight, ↓ length		
PlGF ↑					
First trimester maternal blood	↑ CRL, ↑ EFW		↑ weight, ↑ length		
umbilical cord blood	↑ EFW		↑ weight, ↑ length		
PAI-2 ↑					
First trimester maternal blood	↑ CRL				
Folate ↑					
First trimester maternal blood	↑ CRL				
DNA METHYLATION					
Newborn at birth					
IGF2DMR ↓		↑ SGA	↑ weight	No effect	
H19 ↓		No effect	No effect	No effect	
Child at 1.5 years					
MTHFR ↓				↑ NTD	

CRL, crown-to-rump length; *IGF2DMR*, insulin-like growth factor-2 differentially methylated region; *MTHFR*, methylenetetrahydrofolate reductase; NTD, neural tube deefct;PAI-2, plasminogen activator inhibitor-2; PlGF, placental growth factor; SGA, small-for-gestational age; sFlt-1, soluble fms-like tyrosine kinase-1

First trimester growth restriction has been shown to be associated with an increased risk of being born premature and SGA and even increased postnatal growth rates, known risk factors for cardiovascular disease in adult life.[3,4,17,18] More importantly, a recent study has associated a smaller CRL at the end of the first trimester with an adverse cardiovascular risk profile at the age of 6 years.[19] Therefore, an impaired early environment can have detrimental effects before and just after the mother knows she is pregnant. Longer follow-up studies are needed to demonstrate whether adaptations as early as the periconception period can have longer lasting effects.

Objective 2: Biomarkers related to placentation

An imbalance in several maternal biomarker concentrations, such as sFlt-1, PlGF, PAI-2 and folate, have been associated with low birth weight and newborns who have been born small-for-gestational age (SGA).[20,21] We have shown that children of mothers with low early pregnancy sFlt-1, PlGF, PAI-2 and folate are small as early as at the end of the first trimester (**Chapter 3**). In **Chapter 4**, we demonstrated that children of mothers with low early pregnancy sFlt-1 and PlGF and a subsequent increase of sFlt-1 and relative small increase of PlGF remain small throughout pregnancy. This difference persisted until the age of 6 years. Furthermore, an angiogenic profile that is characterized by higher sFlt-1 and lower PlGF concentrations in umbilical cord blood seems to impair fetal and childhood weight growth.

As stated above, optimal fetal nutrition is needed for a healthy pregnancy. However, fetal nutrient supply is not solely dependent on maternal nutrition, but many factors contribute to the nutritional resources of the fetus.[14] The ability of the placenta to transport nutrients is also of great importance.[22] Inadequate placental development can therefore have detrimental effect on fetal growth and pregnancy outcome.[23] Early pregnancy biochemical measurements that are, amongst others, related to placentation may be predictive of subsequent fetal and childhood growth. Identifying women whom are at risk for adverse birth outcomes in the periconception period may have potential for screening and early intervention trials. However, the underlying mechanisms through which fetal and infant growth is affected have to be elucidated. Burton and colleagues have demonstrated that the nourishment of the developing embryo is dependent on carbohydrate-rich proteinaceous secretions from decidual glands (histiotrophic nutrition).[15] Little is known regarding the content of these secretions, however, they are thought to contain a cocktail of growth factors. Future research could focus on the biochemical content of these secretions and explore possible pathways of histiotrophic nutrition and its effect on subsequent development. In addition, it would be interesting to investigate the effect of early pregnancy biochemical measurements related to placentation and the possible cardiovascular consequences later in life.

Objective 3: DNA methylation in the newborn and child

In recent years, derangements in epigenetic fetal and placental programming have been postulated to be one of the mechanisms underlying the effects of a suboptimal intrauterine environment on fetal and placental growth and development.[5] The epigenetic study in children with a neural tube defect (NTD) revealed an association between methylation of the *MTHFR* gene and the risk of having a child with a NTD (**Chapter 5**). In this thesis, we observed an inverse

association between maternal tobacco smoking, the dose and duration with *IGF2DMR* methylation in newborns (**Chapter 7**). In addition, we have shown lower *IGF2DMR* methylation in children born SGA (**Chapter 6**). It also appeared that the known association between maternal tobacco smoking and the risk of being born SGA is mediated through *IGF2DMR* methylation (**Chapter 7**).

DNA methylation is re-programmed during early developmental stages.[24] After fertilization, epigenetic marks are erased and re-established in order to allow tissue-specific methylation patterns. During gametogenesis, gender-specific imprints are established according to the sex of the individual. Therefore, the periconception period is considered to be a vulnerable period in life, when adverse exposures can have long-lasting effects. Although our findings support the notion that changes in DNA methylation at the *IGF2DMR* are being established during this period, the effects were not limited to the periconception period. Moreover, in this thesis we have focused on the periconception period and on perinatal outcome. It would be interesting to investigate exposures in different time windows and the long term effects of alterations of the epigenome.

In recent years, epigenetic alterations have been postulated to be the basis of fetal programming.[25] We hypothesized that epigenetic alterations mediate the association between maternal prenatal tobacco smoking and impaired fetal growth (**Figure 2**). Panel A illustrates the total effect of maternal tobacco smoking on the risk of being born SGA. In panel B, the mediation design is illustrated. The direct effect is represented as path c'. The indirect effect is the product of the coefficient for the effect of maternal tobacco smoking on DNA methylation (path a) and the coefficient for the effect of DNA methylation on the risk of a newborn being born SGA (path b). In this thesis, we provided evidence that epigenetic reprogramming underlies the association between maternal tobacco smoking and size at birth. The understanding how epigenetic control depends on early exposure may shed light on the link between fetal development and health over the lifespan. Our research emphasizes the importance to quit smoking preferably in the preconception period, although our data also shows that the advantageous effects of quitting smoking may not be limited to this period. It would be interesting to investigate other prenatal exposures, including maternal dietary patterns.

Epigenetic mechanisms are thought to contribute to the development of a thrifty phenotype.[26] Dramatic shifts in DNA methylation are expected to be lethal or associated with disease, whereas small epigenetic alterations (epigenetic fine-tuning) accumulatively affect metabolic pathways. Therefore, the adaptive response to a detrimental intrauterine environment is likely to be subtle epigenetic modifications. The differences in DNA methylation found in the epigenetic studies described in this thesis are modest, as expected. Future studies are needed to replicate our findings. However, the detection of these subtle effects are a challenge with the current measurement techniques and a 'golden standard' remains to be established.

In this thesis, we have chosen for a candidate gene approach. In recent years, new technologies to measure DNA methylation have been developed, giving rise to the first epigenome-wide association studies (EWAS).[27] These developments could lead to similar successes that have been achieved by genetics. Also, longitudinal DNA methylation measurements will help to gain more insight on time-specific effects and enable us to distinguish between cause and effect. However, biological, technical and methodological challenges have to be overcome in order to make EWAS a success.[28]

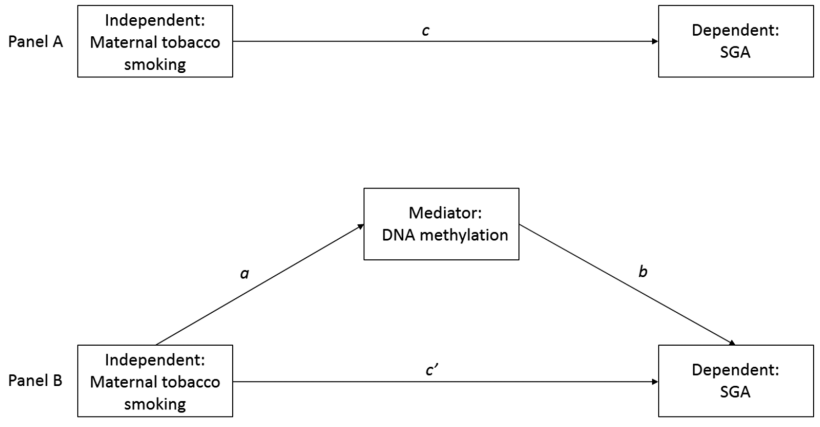

Figure 2 | Graphical representation of proposed mediation effect.

GENERAL CONCLUSION

The present thesis demonstrates that maternal nutrition as well as early biomarker concentrations reflecting placentation influence prolific growth of the fetus. Changes in DNA methylation, caused by maternal smoking, can lead to an increased risk of adverse birth outcomes, including the risk of being born small for gestational age and the risk of a neural tube defect, and altered postnatal weight growth. These observations emphasize the importance of periconception care and are suggestive of links between a suboptimal intrauterine environment by epigenetic reprogramming. Epigenetic epidemiology is a challenging field of research, which can help to gain insight in the underlying mechanisms of a suboptimal intrauterine environment. Future studies in larger sample sets will help to unravel to what extent beneficial or harmful exposures affect health as a consequence of altered DNA methylation at the studied loci.

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Chapter 9

Summary / Samenvatting

SUMMARY

In **Part 1**, we have provided the background and hypothesis for the studies presented in this thesis. Adverse birth outcomes, such as intrauterine growth restriction, preterm birth and congenital malformations, are major contributors to short and long term morbidity and mortality. Evidence is accumulating that the course and outcome of pregnancy is not only important for the health of the mother and child, but can also be considered a predictor of future adult health and disease. Most pregnancy complications are thought to originate in the periconception period. First trimester growth restriction has been related to an increased risk of adverse birth outcomes, including being born small-for-gestational age (SGA). Adequate embryonic and fetal growth and placentation depend on an optimal intrauterine environment, which is determined by environmental maternal conditions and exposures and the metabolic, endocrine, immunological and vascular state of the pregnant woman. Changes in DNA methylation have been postulated to be one of the mechanisms underlying the effects of a suboptimal intrauterine environment on pregnancy course and outcome. Therefore, the aim of the present thesis were to gain more insight into the following three objectives:

1. The relationship between periconception maternal nutrition and early and late fetal growth trajectories and birth outcome
2. The influence of late first trimester maternal angiogenic factors, fibrinolytic factors and folate concentrations on fetal and postnatal infant growth
3. Gene-specific DNA methylation in newborns and associations with prenatal parental conditions and exposures, and birth outcomes

The majority of studies described in this thesis were embedded in the Generation R study, a population-based cohort study from first trimester onwards in Rotterdam, the Netherlands. The objective addressed in **Chapter 5** was embedded in two case-control studies, namely the Dutch Spina Bifida Study and the Texas Neural Tube Defect Project. The Dutch Spina Bifida Study is a case-control study conducted in the Netherlands. The Texas Neural Tube Defect Project is a case-control study performed in a Mexican American population derived from 14 counties along the Texas-Mexico border.

Part II focusses on the first and second objective of this thesis. In **Chapter 2** we examined the effect of maternal dietary patterns on fetal growth and birth outcome. We found a dietary pattern rich of bread, nuts and butter. The dietary pattern was labeled 'Energy-rich' as it was rich of (unsaturated) fats and carbohydrates. We showed that increasing adherence to this Energy-rich dietary pattern increases the crown-to-rump length (CRL) measured at the end of the first trimester.

The second objective was addressed in **Chapter 3** and **Chapter 4**. We studied the influence of maternal biomarkers involved in placentation and fetal and postnatal growth. We have shown that children of mothers with low early pregnancy sFlt-1, PlGF, PAI-2 and folate are small as early as the first trimester (**Chapter 3**). Furthermore, we have demonstrated that children of mothers with low early pregnancy sFlt-1 and PlGF and a subsequent increase of sFlt-1 and relative small

increase of PlGF remain small throughout pregnancy. This difference persisted until the age of 6 years. Furthermore, an angiogenic profile that is characterized by higher sFlt-1 and lower PlGF concentrations in umbilical cord blood seems to impair fetal and childhood weight growth (**Chapter 4**). Early pregnancy biochemical measurements that are, amongst others, related to placentation may be predictive of subsequent fetal and childhood growth. Identifying women who are at risk for adverse birth outcomes in the periconception period may have potential for screening and early intervention trials.

In **Part III**, we have focused on the third objective of this thesis, which was to assess associations between gene-specific DNA methylation with parental exposures and birth outcomes. The epigenetic study in children with a neural tube defect (NTD) revealed an association between methylation of the *MTHFR* gene and the risk of having a child with a NTD (**Chapter 5**). Furthermore, we showed lower *IGF2DMR* methylation in children born SGA (**Chapter 6**). We found an inverse association between maternal tobacco smoking, the dose and duration with *IGF2DMR* methylation in newborns (**Chapter 7**). It also appeared that the known association between maternal tobacco smoking and the risk of being born SGA is mediated through *IGF2DMR* methylation (**Chapter 7**). Therefore, we provided evidence that epigenetic reprogramming underlies the association between maternal tobacco smoking and size at birth. The understanding how epigenetic control depends on early exposure may shed light on the link between fetal development and health over the lifespan.

In **Part IV**, we summarize the main findings of the studies in this thesis and discusses the methodological considerations and interpretation of the findings. In addition, suggestions for future research are proposed (**Chapter 8**).

In conclusion, findings from this thesis suggest that maternal nutrition as well as early biomarker concentrations reflecting placentation can influence growth of the fetus and infant. Changes in DNA methylation, caused by maternal smoking, are associated with an increased risk of adverse birth outcomes, including the risk of being born small for gestational age and the risk of a neural tube defect, and altered postnatal weight growth. These observations emphasize the importance of periconception care and are suggestive of links between a suboptimal intrauterine environment by epigenetic reprogramming.

SAMENVATTING

In **Deel 1** wordt de achtergrond en hypothese beschreven van de studies die in dit proefschrift worden uiteengezet. Nadelige zwangerschapsuitkomsten, zoals intra-uteriene groei vertraging, vroeggeboorte en congenitale afwijkingen, leveren een belangrijke bijdrage aan de morbiditeit en mortaliteit rond de geboorte, op de korte maar ook op de lange termijn. Er zijn steeds meer aanwijzingen dat het verloop en de uitkomst van de zwangerschap niet alleen van belang zijn voor de gezondheid van moeder en kind, maar dat het ook kan worden beschouwd als een voorspeller voor de gezondheid op volwassen leeftijd. Van de meeste complicaties in de zwangerschap wordt gedacht dat ze ontstaan in de periconceptie periode. Groei vertraging in het eerste trimester wordt gerelateerd aan een verhoogd risico op nadelige geboorte uitkomsten, inclusief een te laag geboortegewicht voor de zwangerschapsduur. Embryonale en foetale groei en placentatie zijn afhankelijk van een optimaal intra-uteriene omgeving, welke wordt bepaald door maternale erfelijke en omgevingsfactoren en de metabole, endocriene, immunologische en vasculaire status van de zwangere vrouw. Er wordt verondersteld dat veranderingen in DNA methylering ten grondslag liggen aan het effect dat een suboptimale intra-uteriene omgeving heeft op het verloop en de uitkomst van de zwangerschap. Daarom was het doel van dit proefschrift om meer inzicht te krijgen in de volgende drie onderwerpen:

1. De relatie tussen maternale voeding tijdens de periconceptie periode en vroeg en late foetale groei en geboorte uitkomsten
2. De invloed van angiogene factoren, fibrinolytische factoren en folaat concentraties in het late eerste trimester op foetale en postnatale groei
3. Gen specifieke DNA methylering in pasgeborenen en associaties met prenatale omstandigheden en blootstellingen van beide ouders, en met geboorte uitkomsten

De meeste studies in dit proefschrift werden uitgevoerd binnen het Generation R onderzoek, een cohort studie gebaseerd op de algemene populatie in Rotterdam, Nederland, waarbij deelnemers werden gevolgd vanaf het einde van het eerste trimester van de zwangerschap. Het onderzoek dat in **Hoofdstuk 5** wordt beschreven is uitgevoerd in twee patiënt-controle studies, namelijk de Nederlandse Spina Bifida studie en de Texas Neurale Buis Defect studie. De Nederlandse Spina Bifida studie is een patiënt-controle studie die is uitgevoerd in heel Nederland. De Texas Neurale Buis Defect studie is een patiënt-controle studie die is uitgevoerd in een Mexicaanse-Amerikaanse populatie afkomstig uit 14 provincies langs de grens tussen Texas en Mexico.

In **Deel 2** wordt het eerste en tweede onderwerp van dit proefschrift bestudeerd en beschreven. In **Hoofdstuk 2** hebben we het effect van maternale voedingspatronen op de foetale groei en geboorte uitkomsten onderzocht. Wij vonden een voedingspatroon wat rijk was aan brood, noten en boter. Het voedingspatroon werd bestempeld als 'Energierijk', omdat het rijk was aan (onverzadigde) vetten en koolhydraten. Wij lieten zien dat wanneer er meer van dit Energie rijk voedingspatroon werd geconsumeerd, dit gepaard ging met een grotere kop-romp lengte (CRL) gemeten aan het einde van het eerste trimester van de zwangerschap.

Het tweede onderwerp wordt in **Hoofdstuk 3** en **Hoofdstuk 4** uiteengezet. Wij bestudeerden de invloed van maternale biomarkers die zijn betrokken bij placentatie en foetale en postnatale groei. We laten zien dat kinderen van moeders met een laag sFlt-1, PlGF, PAI-2 en folaat in de vroege zwangerschap al in het eerste trimester klein zijn (**Hoofdstuk 3**). Daarnaast laten we zien dat kinderen van moeders met een laag sFlt-1 en PlGF in de vroege zwangerschap en wanneer dit gevolgd werd door toename van sFlt-1 en een relatief kleine toename van PlGF later in de zwangerschap klein blijven tijdens de zwangerschap. Dit verschil in grootte bleef bestaan tot de leeftijd van 6 jaar. Bovendien lijkt een angiogeen profiel gekenmerkt door hoog sFlt-1 en laag PlGF in het navelstrengbloed de foetale en postnatale groei in gewicht nadelig te beïnvloeden (**Hoofdstuk 4**). Biochemische metingen vroeg in de zwangerschap, die onder meer betrokken zijn bij placentatie, kunnen mogelijk bijdragen aan het voorspellen van de foetale groei en groei in de kindertijd. Het periconceptioneel identificeren van vrouwen die een verhoogd risico hebben op nadelige geboorte uitkomsten zou daarbij kunnen bijdragen aan een verbetering van de vroege prenatale screening en biedt mogelijkheden in de toekomst voor vroege interventie trials.

In **Deel 3** gaan we in op het derde onderwerp van dit proefschrift, namelijk het bestuderen van associaties tussen gen specifieke DNA methylering met blootstellingen van de ouders en geboorte uitkomsten. De epigenetische studie in kinderen met een neurale buis defect (NBD) liet een associatie zien tussen methylering van het *MTHFR* gen en het risico op het krijgen van een kind met een NBD (**Hoofdstuk 5**). Daarnaast vonden we lagere methylering van *IGF2DMR* bij kinderen die waren geboren met een te laag geboortegewicht voor de zwangerschapsduur (**Hoofdstuk 6**). We vonden een associatie met de dosis en duur van het maternale roken en lagere *IGF2DMR* methylering in pasgeborenen (**Hoofdstuk 7**). Daarnaast lijkt het erop dat de bekende associatie tussen maternaal roken en het risico op het krijgen van een kind dat een te laag geboortegewicht heeft voor de zwangerschapsduur onder andere wordt gemedieerd door *IGF2DMR* methylering (**Hoofdstuk 7**). Dit ondersteunt de gedachte dat epigenetische re-programmering ten grondslag ligt aan de associatie tussen het maternale roken en de grootte van het kind bij geboorte. Door meer zicht te krijgen in de wijze waarop blootstellingen vroeg in de zwangerschap invloed hebben op epigenetische mechanismen kan meer inzicht worden verkregen in de relatie tussen foetale ontwikkeling en gezondheid op latere leeftijd.

In **Deel 4** worden de belangrijkste bevindingen uit dit proefschrift samengevat en worden de methodologische beperkingen en interpretatie van de bevindingen bediscussieerd. Daarnaast worden suggesties gedaan voor toekomstig onderzoek (**Hoofdstuk 8**).

Concluderend stellen wij dat de bevindingen in dit proefschrift suggereren dat maternale voeding evenals biomarkers in de vroege zwangerschap, die betrokken zijn bij de placentatie, de groei van de foetus en het kind beïnvloeden. Veranderingen in DNA methylering, veroorzaakt door het maternale roken, zijn geassocieerd met een verhoogd risico op nadelige geboorte uitkomsten, inclusief het risico op een te laag geboortegewicht voor de zwangerschapsduur en het risico op een neurale buis defect en veranderingen in postnatale gewichtsgroei. Onze observaties benadrukken het grote belang van periconceptie zorg en suggereren een verband tussen een suboptimale intra-uteriene omgeving en epigenetische re-programmering.

Addendum

List of abbreviations
Author's affiliations
Publication list
Curriculum Vitae
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Dankwoord

LIST OF ABBREVIATIONS

AC	abdominal circumference
ADHD	attention deficit hyperactivity disorder
AGA	appropriate for gestational age
BMI	body mass index
BPD	bi-parietal diameter
BWS	Beckwith-Wiedemann syndrome
CBCL	child behavior checklist
CI	confidence interval
CpG	CG dinucleotide
CRL	crown to rump length
DISC	diagnostic inventory of screening children
DMR	differentially methylated region
DNA	desoxyribonucleïnezuur
DNMT	DNA methyltransferase
ECM	extra cellular matrix
EFW	estimated fetal weight
FFQ	food frequency questionnaire
FL	femur length
GA	gestational age
HC	head circumference
ICC	Intra class correlation
IGF2	insulin-like growth factor 2
IUGR	intra-uterine growth retardation
LD	linkage disequilibrium
LMP	last menstrual period
MEC	medical ethical committee
MTHFR	methylenetetrahydrofolate reductase
NTD	neural tube defect
NS	not significant
OR	odds ratio
PA	plasminogen activator
PAL	physical activity level
PAI-2	plasminogen activator inhibitor type 2
PCA	principle component analysis
PE	pre-eclampsia
PlGF	placental growth factor
pO_2	partial pressure of oxygen
Q	quintile
QC	quality control

RNA	ribonucleic acid
SD	standard deviation
SDS	standard deviation score
sFlt	soluble fms-like tyrosine kinase-1
SGA	small for gestational age
SNP	single nucleotide polymorphism
SPSS	statistical package social sciences
tHcy	total homocysteine
TNF-α	tumor necrosis factor alpha
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor

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PUBLICATION LIST

1. **Bouwland-Both MI**, Steegers-Theunissen RPM, Vujkovic M, Lesaffre EMEH, Mook-Kanamori DO, Hofman A, Lindemans J, Russcher H, Jaddoe V.W.V., Steegers E.A.P.
   A periconceptional energy-rich dietary pattern is associated with early fetal growth: the Generation R Study.
   BJOG 2013; 120(4): 435-445

2. **Bouwland-Both MI,** Steegers EAP, Lindemans J, Russcher H, Hofman A, Geurts-Moespot AJ, Sweep FCGJ, Jaddoe VWV, Steegers-Theunissen RPM.
   Maternal soluble fms-like tyrosine kinase-1, placental growth factor, plasminogen activator inhibitor-2, and folate concentrations and early fetal size: the Generation R Study.
   AJOG 2013; 209(2):121.e1-11

3. Stolk L, **Bouwland-Both MI**, van Mil NH, Verbiest MMPJ, Eilers PHC, Zhu H, Suarez L, Uitterlinden AG, Steegers-Theunissen RPM.
   Epigenetic profiles in children with a neural tube defect; a case-control study in two populations.
   PLoS One 2013; 8(11):e78462

4. **Bouwland-Both MI**, van Mil NH, Stolk L, Eilers PHC, Verbiest MMPJ, Heijmans BT, Tiemeier H, Hofman A, Steegers EAP, Jaddoe VWV, Steegers-Theunissen RPM.
   DNA Methylation of *IGF2DMR* and *H19* Is Associated with Fetal and Infant Growth: The Generation R Study.
   PLoS One 2013; 12(8): e81731

5. Bergen NE, **Bouwland-Both MI**, Steegers-Theunissen RPM, Hofman A, Russcher H, Lindemans J, Jaddoe VW, Steegers EAP.
   Maternal and fetal angiogenic factors and childhood growth: a prospective cohort study.
   Submitted

6. **Bouwland-Both MI**, van Mil NH, Tolhoek CP, Stolk L, Eilers PHC, Verbiest MMPJ, Heijmans BT, Uitterlinden AG, Hofman A, van IJzendoorn MH, Duijts L, de Jongste JC, Tiemeier H, Steegers EAP, Jaddoe VWV, Steegers-Theunissen RPM.
   Periconception parental tobacco smoking, gene-specific DNA methylation and newborn size: a prospective cohort study.
   Submitted

Other publications

7. **Both MI**, Overvest MA, Wildhagen MF, Golding J, Wildschut HIJ.
   The association of daily physical activity and birth outcome: a population-based cohort study.
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9. Böhmer AC, Mangold E, Tessmann P, Mossey PA, Steegers-Theunissen RP, Lindemans J, **Bouwland-Both MI**, Rubini M, Franceschelli P, Aiello V, Peterlin B, Molloy AM, Nöthen MM, Knapp M, Ludwig KU.
   Analysis of susceptibility loci for nonsyndromic orofacial clefting in a European trio sample.
   Am J Med Genet A 2013; 161(10): 2545-9

10. Van Mil NH, Steegers-Theunissen RP, **Bouwland-Both MI,** Verbiest MM, Rijlaarsdam J, Hofman A, Steegers EA, Heijmans BT, Jaddoe VW, Verhulst FC, Stolk L, Eilers PH, Uitterlinden AG, Tiemeier H.
    DNA methylation profiles at birth and child ADHD symptoms.
    J Psychiatr Res 2014; 49: 51-9

11. Van Mil NH, **Bouwland-Both MI**, Stolk L, Verbiest MMPJ, Hofman A, Jaddoe VWV, Verhulst FC, Eilers PHC, Uitterlinden AG, Steegers EAP, Tiemeier H, Steegers-Theunissen RPM.
    Determinants of Maternal Pregnancy One Carbon Metabolism and Newborn DNA Methylation Profiles
    Submitted

ABOUT THE AUTHOR

Marieke Bouwland – Both werd geboren op 25 augustus 1984, te Eindhoven. Zij is de dochter van Pieter Both en Joke Both – van der Heide. Na het behalen van het VWO diploma aan het Christiaan Huygens College te Eindhoven, begon zij in 2002 met de studie Gezondheidswetenschappen aan de Erasmus Universiteit Rotterdam. In 2003 verruilde zij deze studie voor de studie geneeskunde aan dezelfde universiteit. Tijdens haar studie deed zij haar wetenschappelijke stage op de afdeling verloskunde en vrouwenziekten onder leiding van Dr. Hajo Wildschut naar het effect van fysieke activiteit in de zwangerschap op zwangerschapsuitkomsten. In december 2009 behaalde zij haar artsexamen om vervolgens te starten met haar promotieonderzoek wat beschreven is in dit proefschrift onder leiding van Prof. Dr. Régine Steegers-Theunissen en Prof. Dr. Vincent Jaddoe. Dit onderzoek was een samenwerking tussen de afdelingen Verloskunde en Gynaecologie en Generation R en werd financieel mogelijk gemaakt door de Bo Hjelt stichting. Op 1 maart 2013 startte zij met haar opleiding tot huisarts aan de Erasmus Universiteit Rotterdam. Zij is getrouwd met Narada Bouwland en de trotse moeder van Isabel (2011) en Joachim (2013).

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Research school:	NIHES
PhD Period:	2009 – 2014
Promoters:	Prof. dr. R.P.M. Steegers – Theunissen
	Prof. dr. V.W.V. Jaddoe

	Year	Workload (ECTS)
1. PhD Training		
Master of Health Sciences, specialization in Clinical Epidemiology, NIHES, Erasmus University Rotterdam, The Netherlands	2010–2013	
General courses		
Principles of research in medicine		0.7
Study design		4.3
Classical methods for data-analyses		5.7
Clinical epidemiology		5.7
Methodologic topic in epidemiologic research		1.4
Modern statistical methods		4.3
Clinical decision analyses		0.7
Methods of public health research		0.7
Topics in meta-analyses		0.7
Pharmaco-epidemiology		0.7
Genome wide association analysis		1.4
Principles of genetic epidemiology		0.7
Genomics in molecular medicine		1.4
Markers of prognostic research		0.7
The practice of epidemiologic analysis		0.7
Advanced courses		
Courses for the quantitative researcher		1.4
Repeated measurements in clinical studies		1.4
Advances in genomics research		0.4
Introduction to clinical and public health genomics		1.9

	Year	Workload (ECTS)
General academic skills		
Working with SPSS for windows	2010	0.15
(Inter)national Conferences – presentations		
Bo Hjelt Foundation Board meeting, Rotterdam, the Netherlands – oral presentation	2010	0.7
Society for Gynecologic Investigation, 57th Annual Scientific Meeting, Florida, USA – poster presentation	2010	0.7
Wladimiroff research meeting, Rotterdam, the Netherlands – oral presentation	2011	0.7
2nd European Congress on Preconception Care and Health, Rotterdam, the Netherlands – three oral presentations	2012	1.4
Bo Hjelt Foundation Board meeting, Rotterdam, the Netherlands – oral presentation	2012	0.7
DOHaD satellite meeting, Rotterdam, the Netherlands – oral and poster presentation	2012	1.4
Generation R research meeting, Rotterdam, the Netherlands – oral presentation	2012	0.7
Wladimiroff research meeting, Rotterdam, the Netherlands – oral presentation	2012	0.7
Obstetrics and gynecology research meeting, Rotterdam, the Netherlands – two oral presentations	2012	1.4
Seminars and workshops		
Attending seminars of the department of Obstetrics and Gynecology	2009–2013	1.0
Attending the Generation R research meetings	2009–2013	1.0
Attending seminars at the department of Epidemiology, Erasmus MC	2010–2013	1.0
2. Teaching activities		
Supervising Master's thesis: Cathleen Tolhoek, Medical student, Erasmus University, Rotterdam. Project title: Prenatal maternal characterics and gene-specific DNA methylation in newborn	2012	2.0

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