Decoding Placental Development

Using three-dimensional ultrasound and (cell-free) DNA methylation



Maaike Marjolein van Vliet

Stellingen behorende bij het proefschrift

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Using three-dimensional ultrasound and (cell-free) DNA methylation

1. Het preconceptioneel starten met foliumzuur supplementen en een hogere foliumzuur-concentratie zijn geassocieerd met een groter 1e trimester placenta volume *(dit proef-schrift)*

2. De veranderingen in maternaal cfDNA methylatie die ontstaan als gevolg van zwanger-schap zijn complexer dan enkel te verklaren door het extra vrijkomende placentaire cfDNA *(dit proefschrift)*

3. Voor het detecteren van gehypermethyleerde placenta-specifieke markers in cfDNA is het van belang dat deze gehypomethyleerd zijn in bloedcellen *(dit proefschrift)*

4. Bij vrouwen die worden gediagnosticeerd met vroege pre-eclampsie is de totale hoeveel-heid cfDNA verhoogd terwijl de placentaire fractie in cfDNA verlaagd is *(dit proefschrift)*

5. De meeste methylatie verschillen gevonden in het cfDNA van vrouwen die zijn gediagnos-ticeerd met vroege pre-eclampsie, kunnen niet worden verklaard door de veranderingen in placentaire DNA methylatie *(dit proefschrift)*

6. Een hoge inname van vitamine supplementen tijdens de zwangerschap is niet per definitie beter, en kan potentieel schadelijk zijn

7. De beschikbaarheid en het gebruiksgemak van biomarkers is cruciaal voor een succesvol-le implementatie in de klinische praktijk

8. Het vergroten van de hooiberg in genoomwijde studies leidt niet per definitie tot het vinden van meer spelden

9. Het legaal en veilig beschikbaar stellen van abortus vermindert de gezondheidsrisico's en sterfte bij vrouwen zonder dat het aantal abortussen exponentieel toeneemt

10. Onvoldoende financiering van promovendi is veel voorkomend en draagt bij aan een verminderde mentale gezondheid

11. Aannemen doen ze in de bouw

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Decoding Placental Development

Using three-dimensional ultrasound and (cell-free) DNA methylation

Ontrafelen van placenta ontwikkeling

Door middel van driedimensionale echografie en (celvrij) DNA methylatie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr.ir. A.J. Schuit

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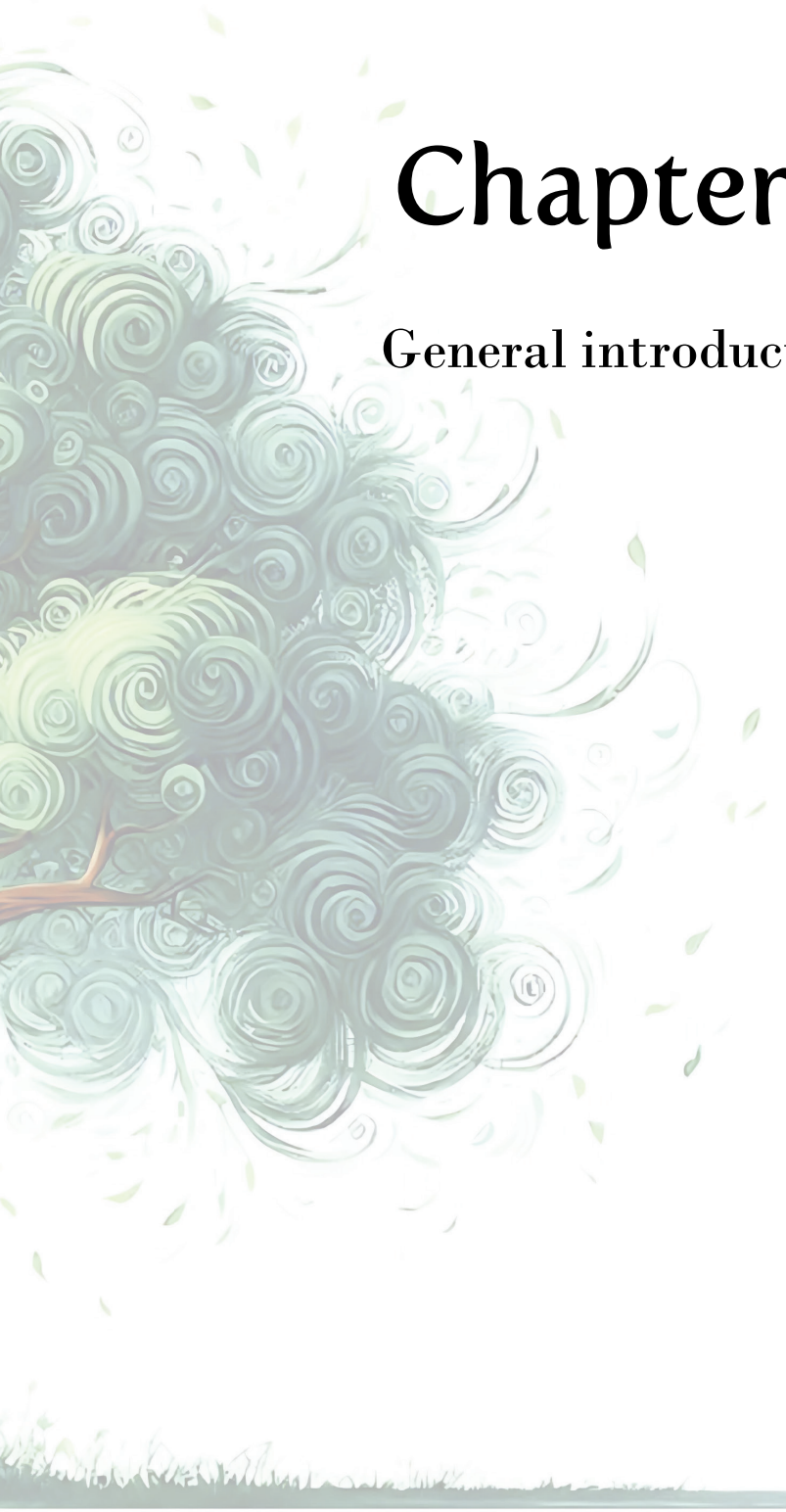
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Chapter I

General introduction

The placenta is a crucial organ during fetal development, and placental dysfunction is involved in the pathogenesis of major obstetric complications such as preeclampsia and fetal growth restriction. These complications are a leading cause of both maternal and neonatal morbidity and mortality, and have negative health implications later in life (1). This emphasizes the need to study the (patho)physiological development of the placenta; however, this is challenged by the inaccessibility of the placenta during pregnancy.

Placental growth and development

The development of the placenta starts early during pregnancy (2). The blastocyst contains the inner cell mass that will develop into the embryo, and contains an outer layer of trophectoderm that will form the later placenta. The trophectoderm invades the uterine endometrium (decidua) around 6 days after conception. This is followed by the trophectoderm starting to differentiate into early syncytiotrophoblast cells (Figure 1A). At approximately 12 days after conception, extra villous trophoblasts begin invading and remodelling the maternal spiral arteries to facilitate future blood flow from mother to the developing fetus via the intervillous space of the placenta. Chorionic villi are formed, covered in an inner layer of cytotrophoblast and an outer layer of syncytiotrophoblast, remaining immature until 11-12 weeks of gestation (Figure 1B). Till that time, the remodelled spiral arteries are mostly blocked by trophoblast plugs allowing the embryo to develop in a relatively low-oxygen environment, while extensive remodelling of the maternal microvasculature takes place. During this period, maternal-fetal exchange is independent of direct blood flow through the intervillous space of the placenta and occurs primarily via diffusion. Towards the end of the first trimester, these trophoblast plugs gradually dissolve, allowing large amounts of maternal blood to enter the intervillous space of the placenta for the provision of nutrients to the fetus and exchange of oxygen and waste products. In the second and third trimester, the chorionic villi mature and the cytotrophoblast layer becomes discontinuous and largely diminishes, leaving a layer of syncytiotrophoblast and further facilitating maternal-fetal exchange (2).

Factors influencing placental development

Placental development is a complex process that can be influenced by a range of parental and environmental factors. Contributing genetic and epigenetic factors delivered by the sperm cell may affect the expression of genes critical for placental formation and growth (3, 4). DNA methylation, which involves covalent binding of methyl groups to cytosines at cytosine-phosphate-guanine (CpG) sites in the genome, is an epigenetic mechanism that can impact gene expression without changing the DNA sequence. In general, DNA methylation of promoter regions often results in gene silencing, thus playing an important role in programming genomic activity (5). DNA methylation is involved in cell differentiation and genomic imprinting (5) and

plays a crucial role in embryonic and placental development (6, 7). Genomic imprinting is a process where genes are expressed in a parent-of-origin-specific manner, where DNA methylation at either the promoter or a nearby regulatory element silences one allele (either maternal or paternal), ensuring that only one allele is expressed (5). Paternally expressed imprinted genes are particularly expressed in the placenta and largely stimulate placental growth (3, 4). Previous studies have described differences in DNA methylation in sperm cells associated with paternal aging, which can possibly influence placental growth and development (8). Maternal influences include (epi)genetic factors carried by the oocyte, and nutritional, metabolic, hormonal and inflammatory components as part of the maternal homeostasis, which can impact the intrauterine environment and subsequently the developing fetus and placenta (9).

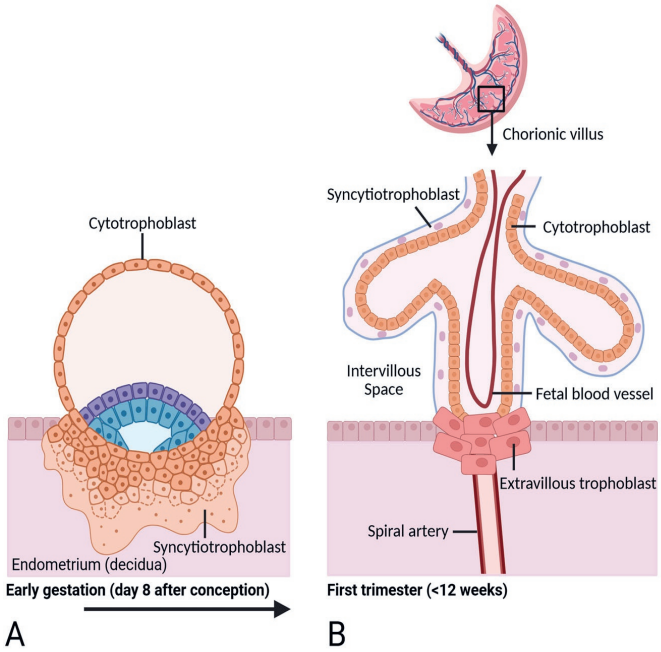


Figure 1: Schematic overview of the early development of the placenta. A) The blastocyst contains an outer layer of trophectoderm which will form the later placenta. The invasive trophoblasts invade the decidua, followed by the formation of early syncytiotrophoblasts infiltrating the endometrium. **B)** Chorionic villi are formed throughout the first trimester, containing an inner layer of cytotrophoblast and an outer layer of syncytiotrophoblasts. Maternal spiral arteries undergo extensive remodeling, but remain largely blocked by trophoblasts plugs until 11-12 weeks of gestation. Figure is created with BioRender.com.

Parental lifestyle factors such as diet can also affect epigenetic processes. For example, B-vitamins, particularly folate, vitamin B6 and vitamin B12, are retrieved from diet and are part of one-carbon metabolism. This one-carbon metabolism provides methyl groups required for DNA methylation processes, and compelling evidence indicates that differences in one-carbon

moieties during pregnancy can cause lasting DNA methylation differences in the offspring (10-12). As such, epigenetic mechanisms are proposed as underlying mechanisms explaining the impact of parental exposures on placental and fetal development, and long-term health outcomes in the offspring (13, 14).

Placental DNA methylation in health and disease

Differences in placental DNA methylation have been found in relation to gestational age (15-17), as well as in association with major obstetric complications, including preeclampsia and fetal growth restriction (18-20). Therefore, studying placental DNA methylation could increase our understanding of normal placental development and the possible role of DNA methylation in the pathophysiology of adverse obstetric outcomes. Moreover, identification of placental DNA methylation associated with obstetric complications may be used to develop novel disease-specific biomarkers. Prediction of disease by biomarkers already within the first trimester would enable timely targeted preventive interventions for identified high-risk pregnancies (1).

Placenta research in this thesis

The overall inaccessibility of the placenta during pregnancy complicates the study of placental development including placental DNA methylation in health and disease. The need to obtain physical placental tissue during pregnancy, which would require invasive procedures such as chorionic villus sampling, hampers the clinical applicability of placental DNA methylation markers as potential biomarkers for the (early) identification of high-risk pregnancies. Therefore, research using noninvasive methods is warranted to investigate which factors influence placental development and to better understand underlying mechanisms in (ab)normal placental development. These insights could lead to identification of novel targets supporting (optimal) placental growth, to predict and prevent placental-originated complications, and ultimately improve maternal and fetal health outcomes. This thesis describes the use of two noninvasive approaches as proxies for studying placental development or placental DNA methylation already during (early) pregnancy.

Ultrasound: measuring placental (vascular) volume

The first approach to study the in vivo placenta involves the use of ultrasound. Placental growth can be measured using three-dimensional (3D) ultrasound techniques that enable assessment of the total placental volume (PV) in the first trimester. The placental volume can be determined from 3D ultrasound volumes using the Virtual Organ Computer-aided AnaLysis (VOCAL) tool. A rotational step of 15° is used to divide the ultrasound volumes in twelve segments of the trophoblast which are manually measured (21). Figure 2A-B shows how the placental volume

can be deduced by subtracting the volume of the gestational sac (Figure 2B) from the total pregnancy volume (Figure 2A). The utero-placental vascular volume (uPVV) can be measured by combining 3D ultrasound with power doppler for visualization of blood vessels (22). To measure the uPVV, the 3D power doppler volumes of the utero-placental vasculature can be depicted as a hologram in a Virtual Reality system in which the uterine and fetal vascular volumes can subsequently be removed, enabling the measurement of the uPVV (Figure 2C-D).

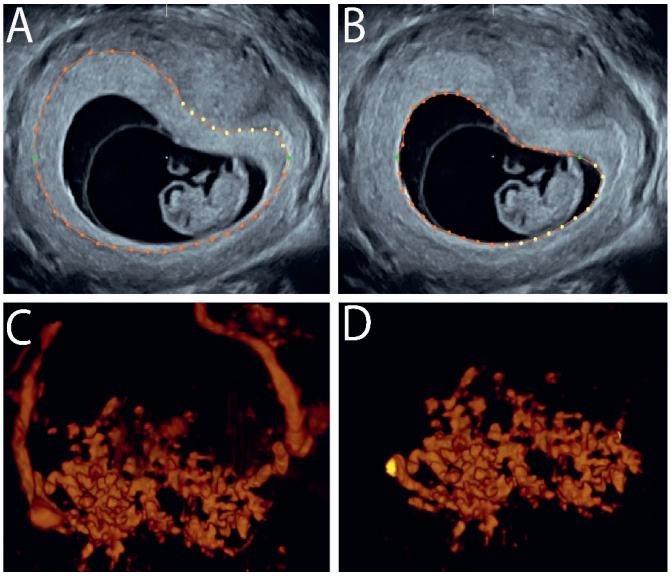


Figure 2: Measuring placental volume (PV) and utero-placental vascular volume (uPVV) using 3D ultrasound volumes. The PV is calculated by the total pregnancy volume **(Figure A)** minus the gestational sac volume **(Figure B)**. The uPVV is the total obtained utero-placental vascular volume **(Figure C)**, minus the uterine and fetal vascular volumes. The remaining uPVV is shown in **Figure D**.

DNA methylation: placental tissue and cell-free DNA

The second approach involves the study of DNA methylation in placental tissues and cell-free DNA (cfDNA) within maternal plasma. While placental tissues can only be obtained postpartum or after invasive procedures, cfDNA fragments originating from the placenta can be found in the maternal bloodstream during pregnancy (Figure 3). Currently, cfDNA retrieved from maternal plasma is widely used for noninvasive prenatal testing (NIPT) which is universally used to screen for fetal chromosomal abnormalities (23).

cfDNA is mainly derived from dying (e.g. apoptotic) cells and therefore reflects cell turnover from different tissues or cell types within the maternal body. Most cfDNA fragments in maternal plasma originate from maternal tissues, especially hematopoietic cell types, while the placental-derived fraction is approximately 10% at the end of the first trimester (24, 25).

Methylation profiles in cfDNA reflect a mix of DNA methylation data of all cfDNA fragments. The relatively low proportion of placental-originated cfDNA fragments in the total cfDNA is a challenge when using maternal cfDNA as a proxy for placental DNA. However, cfDNA fragments maintain the epigenetic features of their tissue of origin and different cell types have different DNA methylation patterns. Therefore, methylation markers can serve as unique barcode to identify their tissue of origin and can aid in distinguishing between maternal and placental DNA fragments (Figure 3) (26).

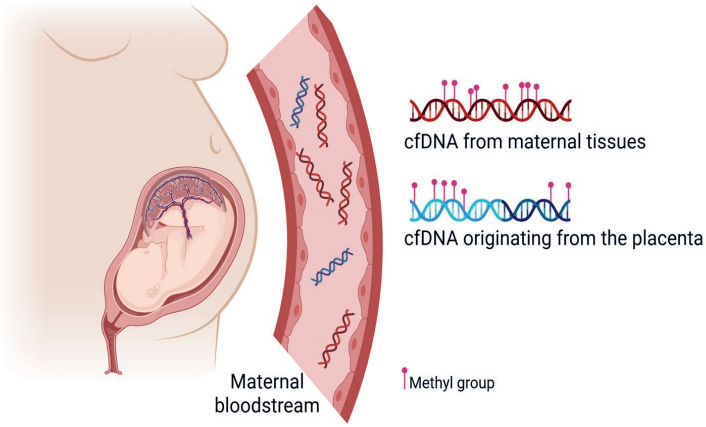


Figure 3: Cell-free DNA fragments originating from maternal tissues and from the placenta can be found in maternal plasma during pregnancy and maintain epigenetic features of their tissue of origin. Figure is created with BioRender.com.

Next to the presence of placental-originated cfDNA, pregnancy-induced changes in composition and turnover of maternal tissues such as immune cells (27), could also impact the cfDNA methylome. The contribution of maternal tissues to cfDNA could therefore not only be an interfering factor when using cfDNA as a proxy for placental DNA, but might also reflect important processes in the maternal body in relation to pregnancy.

Studying the (placental) cfDNA methylome has the potential for cfDNA applications beyond current practice, including the development of noninvasive biomarkers (28-30). Studies investigating genome-wide methylation of cfDNA during pregnancy are limited and generally technologically challenging. Only recently have the first studies investigating genome-wide cfDNA methylation in maternal plasma been performed (31-34). Excitingly, recent studies support the use of cfDNA methylation markers for the early prediction of pregnancies that will develop preeclampsia (35, 36). This emphasizes the need to further explore the epigenetic landscape of (placental) cfDNA already during early pregnancy.

(cf)DNA methylation techniques: MeD-seq

Different techniques are available to study DNA methylation genome-wide, all of which have their own disadvantages and advantages. Most techniques are limited to a subset of CpGs and/or rely on bisulfite conversion, which significantly degrades DNA (37). This can be particularly challenging for cfDNA methylation analyses, because cfDNA concentrations are already low and cfDNA by itself is already fragmented. Although some of these methods have been adapted to handle low input cfDNA levels, the number of CpG sites detected drops dramatically compared to the standard protocols. Therefore, other methodologies have also been developed, such as (cf)MeDIP-seq (38). However, the required immunoprecipitation step for (cf)MeDIP-seq leads to a higher background signal due to aspecific binding, and the assay is biased towards CpG-poor regions and incompatible with single CpG site resolution. In this thesis, we therefore investigated the applicability of Methylated DNA sequencing (MeD-seq). MeD-seq omits bisulfite conversion by using a methylation-dependent restriction enzyme (LpnPI), and is well compatible with low (cf)DNA levels. In addition, MeD-seq allows for the methylation profiling of >50% of all potentially methylated CpGs, detecting DNA methylation at >99% of all CpG islands and promoters. This is a more extensive genome-wide coverage than most other techniques and could therefore lead to the identification of novel placental (cf)DNA markers (39, 40).

Hypothesis and overall aim

By using these two noninvasive approaches to study placental development and/or placental DNA methylation, we can investigate their associations with parental exposures, and with timing in pregnancy and (adverse) pregnancy outcomes. We hypothesize that 1) parental exposures and characteristics can affect placental growth and development, partly through placental DNA methylation, and 2) (ab)normal placental DNA methylation profiles in relation to gestational age and placental-related complications can be found in both placental DNA and cfDNA obtained from maternal plasma during pregnancy using MeD-seq.

By investigating associations between parental exposures and placental DNA methylation and early placental growth, as well as investigating the applicability of using MeD-seq to study genome-wide cfDNA methylation in both health and disease during pregnancy, the overall aim of this thesis is to enhance our understanding of placental development and of dynamics in maternal cfDNA methylation. This will increase our insights into the (patho)physiology of placental development and dynamics in the maternal cfDNA methylome, which could lead to the identification of novel targets supporting (optimal) placental growth and the development of biomarkers for the (early) prediction of placental-related complications such as

preeclampsia. Ultimately, providing opportunities to prevent placental-originated complications and to improve maternal and fetal health outcomes.

The main objectives of this thesis are summarized as follows (Figure 4):

Part I:

I. To investigate the associations between one-carbon moieties and placental DNA methylation (Chapter 2) and with early placental growth (Chapter 3)
II. To investigate the association between paternal age and early placental growth (Chapter 4)

Part II

III. To explore the possibility of detecting placental DNA markers in maternal cfDNA using MeD-seq (Chapter 5)
IV. To investigate cfDNA methylation dynamics and their placental and maternal origins across gestation in uncomplicated pregnancies and in pregnancies complicated by preeclampsia (Chapter 6-8)

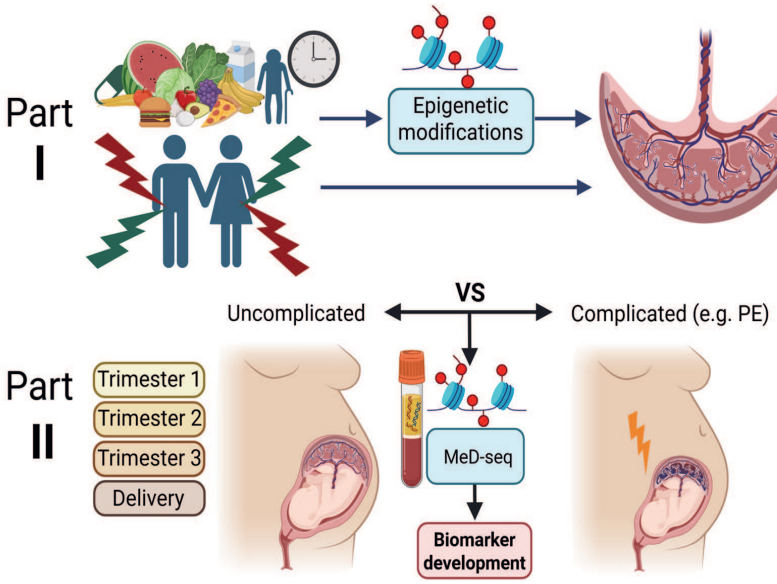


Figure 4: **Hypothesis and parts of this thesis.** PE = preeclampsia. Figure is created with BioRender.com.

Study design and setting

The studies described in this thesis are conducted at the Department of Obstetrics and Gynecology in collaboration with the Department of Developmental Biology, of the Erasmus MC, University Medical Centre, Rotterdam, the Netherlands.

The Rotterdam Periconception Cohort

The Rotterdam Periconception Cohort (Predict study) is an ongoing prospective cohort study conducted at the Erasmus MC, University Medical Center in Rotterdam (41, 42). The Predict study started in November 2010 and recruits couples before and during pregnancy. In the described studies in this thesis, we focused only on the pregnant participants. Women and their male partners were eligible if they had a singleton pregnancy and gestational age at inclusion was <10 weeks, if they were at least 18 years old and if they were able to speak and read the Dutch language.

Figure 5 provides a schematic overview of the study procedures during pregnancy as part of the Predict study. Anthropometric data, blood pressure and blood samples were collected at study entry. Extensive questionnaires were used to obtain data on maternal characteristics with regard to medical and obstetric history, as well as lifestyle factors. Women underwent 3D transvaginal ultrasound scans at 7, 9 and 11 weeks of gestation to measure (among others) the PV, while the uPVV was measured only in patients who participated between January 2017 and March 2018. At 22–24 and 30–32 weeks of gestation, fetal growth was assessed by fetal biometry. Data regarding pregnancy outcomes were retrieved from medical records and questionnaires. Between November 2022 and October 2024, a subcohort was embedded within the Predict study. Women participating in this subcohort underwent additional blood draws for cfDNA analyses in each trimester and at delivery, and their placental tissues were collected postpartum if the delivery occurred at the Erasmus MC. Additionally, between July 2024 and November 2024, women who were admitted to the Erasmus MC because of early-onset preeclampsia (EOPE) (diagnosis <34 weeks of gestation) were recruited as a separate case cohort. These women underwent a blood draw for cfDNA analysis at study inclusion. Similar to our first trimester subcohort, placental tissues were collected postpartum if the delivery took place at the Erasmus MC, and data regarding pregnancy outcomes were retrieved through medical records and questionnaires (Figure 5).

First and second trimester placental tissues

To obtain placental tissue biopsies from uncomplicated first- and second trimester pregnancies, we recruited participants at a Dutch abortion clinic. Participation of these women was anonymized. Women aged ≥18 years with a singleton pregnancy with no known birth defects and a gestational age between 9+0–12+0 (first trimester) or 16+0–17+6 (second trimester) were eligible for participation. Only leftover placental tissues were collected after the surgical procedures.

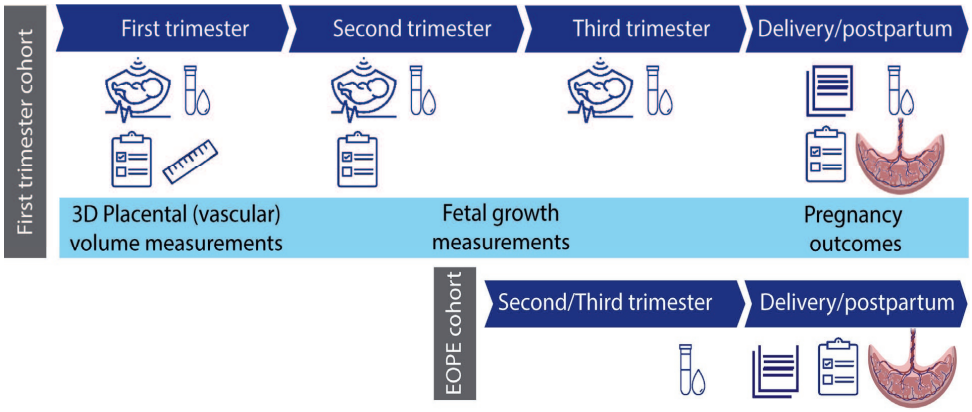


Figure 5: Schematic overview of study procedures during pregnancy in the Rotterdam Periconception Cohort. EOPE = early-onset preeclampsia

NIPT

Lastly, we retrospectively collected residual plasma samples collected for NIPT from women who later in pregnancy developed early-onset preeclampsia and matched controls. These samples will serve as a reference set for the prospective first trimester cohort embedded within the Predict study.

Outline of this thesis

The first part of this thesis focuses on the associations between parental characteristics and placental DNA methylation or placental growth (Chapters 2, 3 and 4). **Chapter 2** summarizes the current literature on the relationship between one-carbon moieties and DNA methylation in the human placenta. **Chapter 3** describes the associations between maternal folate and vitamin B12 concentrations, and the timing of folic acid supplement initiation with first trimester placental growth. **Chapter 4** describes the associations between paternal age and first trimester placental (vascular) growth. The second part of this thesis focuses on assessing DNA methylation in placental tissues and cfDNA obtained from maternal plasma using MeD-seq (Chapters 5, 6, 7 and 8). **Chapter 5** describes the results of our pilot study, in which we show the possibility of detecting placental DNA methylation markers in maternal cfDNA using MeD-seq, besides a methylation signature induced by immune cells. **Chapter 6** provides a detailed description of the study protocol for our follow-up (cf)DNA methylation studies. **Chapter 7** investigates methylation differences in placental tissues and cell types and in maternal cfDNA in relation to the timing of gestation in uncomplicated pregnancies. **Chapter 8** investigates differences in cfDNA methylation in relation to the occurrence of early-onset preeclampsia. Lastly, **Chapter 9** provides a general discussion including future perspectives, and **Chapter 10** provides a summary of the results of this thesis.

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Part I

Associations between parental characteristics and placental DNA methylation or early placental growth


2

Chapter 2

The one-carbon metabolism as an underlying pathway for placental DNA methylation – a systematic review

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Abstract

Epigenetic modifications, including DNA methylation, are proposed mechanisms explaining the impact of parental exposures to fetal development and lifelong health. Micronutrients including folate, choline and vitamin B12 provide methyl groups for one-carbon metabolism and subsequent DNA methylation processes. Placental DNA methylation changes in response to one-carbon moieties hold potential targets to improve obstetrical care. We conducted a systematic review on the associations between one-carbon metabolism and human placental DNA methylation. We included 22 studies. Findings from clinical studies with minimal ErasmusAGE quality score 5/10 (n=15) and in vitro studies (n=3) are summarized for different one-carbon moieties. Next, results are discussed per study approach: 1. global DNA methylation (n=9), 2. genome-wide analyses (n=4), and 3. gene-specific (n=14). Generally, one-carbon moieties were not associated with global DNA methylation, although conflicting outcomes were reported specifically for choline. Using genome-wide approaches, few differentially methylated sites associated with S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) or dietary patterns. Most studies taking a gene-specific approach indicated site-specific relationships depending on studied moiety and genomic region, specifically in genes involved in growth and development including *LEP*, *NR3C1*, *CRH* and *PlGF*; however, overlap between studies was low. Therefore, we recommend to further investigate the impact of an optimized one-carbon metabolism on DNA methylation and lifelong health.

Introduction

Fetal exposures within the intrauterine environment can affect health outcomes of the offspring during the life course. This is known as the developmental origins of health and disease (DOHaD) paradigm (1, 2). Epigenetic modifications can affect gene expression and consequently cell function without changing the DNA sequence and are proposed underlying mechanisms connecting parental exposures to gamete maturation, embryonic and fetal development, and long-term health outcomes in the offspring (1, 3).

The best characterized epigenetic mechanism is DNA methylation. DNA methylation is regulated by DNA methyltransferases (DNMTs), which transfer methyl groups predominantly to the C-5 position of a cytosine at a cytosine-phosphate-guanine (CpG) site (4, 5). Methylation of CpGs in regulatory genomic regions like promoters typically leads to gene silencing while non-genic, repetitive DNA sequences, such as transposable elements (e.g. LINE-1 and Alu elements), are often heavily methylated to maintain genomic stability and can serve as markers for global methylation (4-6).

During the periconception period, starting 14 weeks before conception till 10 weeks after conception, significant epigenetic modifications with potential effects along the life course take place (7). Most DNA methylation marks present in parental gametes are removed during the first cell divisions in the zygote and blastocyst stages, followed by the establishment of de novo DNA methylation most prominent after implantation. In utero, proper de novo DNA methylation is essential for key biological processes involved in regulation of gene expression during development and differentiation (4-8). Therefore, the periconception and pregnancy period is a vulnerable time span for epigenetic disruptions.

One-carbon metabolism provides methyl groups required amongst others for DNA methylation. Herein, methionine is converted to the major methyl donor S-adenosylmethionine (SAM), which in turn is converted to S-adenosylhomocysteine (SAH) thereby donating its methyl group. Other essential substrates of one-carbon metabolism, here referred to as one-carbon moieties, include methyl donors such as folate and choline, and co-factors including vitamin B2, B6 and B12, that are all mostly derived from diet (Figure 1) (9).

Evidence from predominantly animal studies indicates that differences in one-carbon metabolism during pregnancy can cause lasting DNA methylation changes in the offspring (7, 10-17). A famous example is that a methyl-supplemented diet fed to pregnant dams of the Agouti mouse increases DNA methylation of a regulating locus that results in a lower expression

of the Agouti gene in their offspring, resulting in changed adiposity and coat color (18, 19). In humans conceived during the Dutch Hunger Winter, an increased risk for obesity was observed and methylation differences in whole blood were found related to genes involved in growth and metabolism including lower methylation of insulin-like growth factor 2 (*IGF2*) (20). Additionally, offspring of women who used periconceptional folic acid supplements had a higher methylation of *IGF2* in blood (15). This illustrates that nutrition, particularly moieties of one-carbon metabolism, plays a crucial role in early life epigenetic programming with potential lifelong health consequences (7).

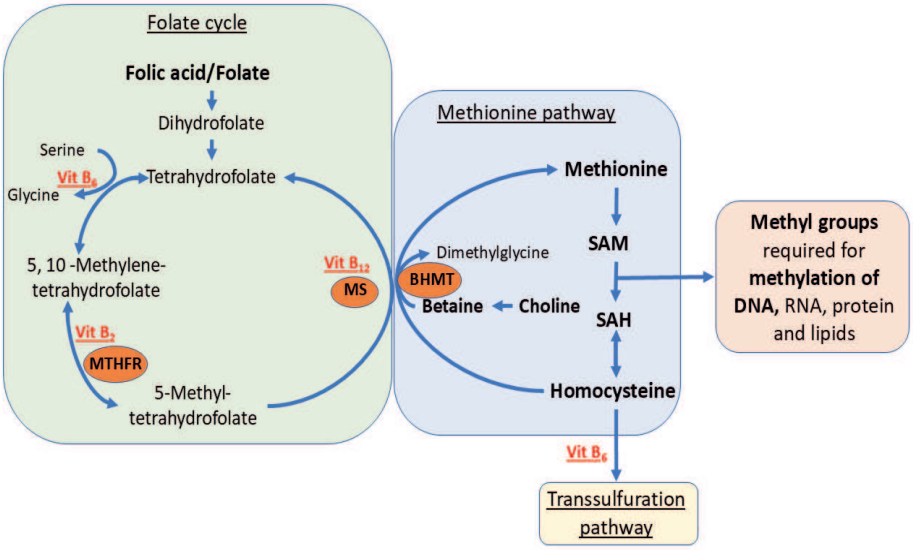


Figure 1: Simplified overview of one-carbon metabolism. Dietary methionine is converted to homocysteine via SAM and SAH. SAM is a major methyl donor for biological processes, including DNA methylation. The remethylation of homocysteine to methionine depends on other moieties including vitamin B12 as cofactor and 5-methyl-tetrahydrofolate as substrate. MTHFR = methylenetetrahydrofolate reductase, BHMT = betaine-homocysteine S-methyltransferase, MS = methionine synthase, SAM = S-adenosylmethionine, SAH = S-adenosylhomocysteine

The placenta is an interesting target organ for DNA methylation studies investigating the relationship between maternal exposures including nutrition, one-carbon metabolism, and foetal programming as it forms the unique and indispensable interface between the mother and the developing embryo and fetus (21). The changes observed in placental DNA methylation are suggested to reflect alterations in placental functioning and as such impact fetal programming. The treatment of these adverse variations could provide targets for future preventative or therapeutic interventions (Figure 2) (22). Additionally, we suggest that the presence of placental-originated cell free DNA (cfDNA) in maternal blood will become an opportunity to noninvasively assess placental DNA methylation profiles during early pregnancy,

whereas the noninvasive collection of other fetal tissues is not possible. This emphasizes the potential of the placenta as a proxy for fetal health conditions (23).

Previous reviews about the impact of one-carbon metabolism in reproduction have mainly focussed on DNA methylation in tissues other than placenta including umbilical cord blood, focussed on animal studies and/or were limited to one specific one-carbon moiety (7, 10-14, 24-26). Additionally, epigenome-wide association studies have specifically emerged over the last years (27). Therefore, this systematic review aims to provide an overview of the current knowledge obtained from human studies investigating the impact of one-carbon metabolism on placental DNA methylation. Epigenetic derangements in response to impaired one-carbon metabolism could hold potential targets for future therapeutic or preventative interventions.

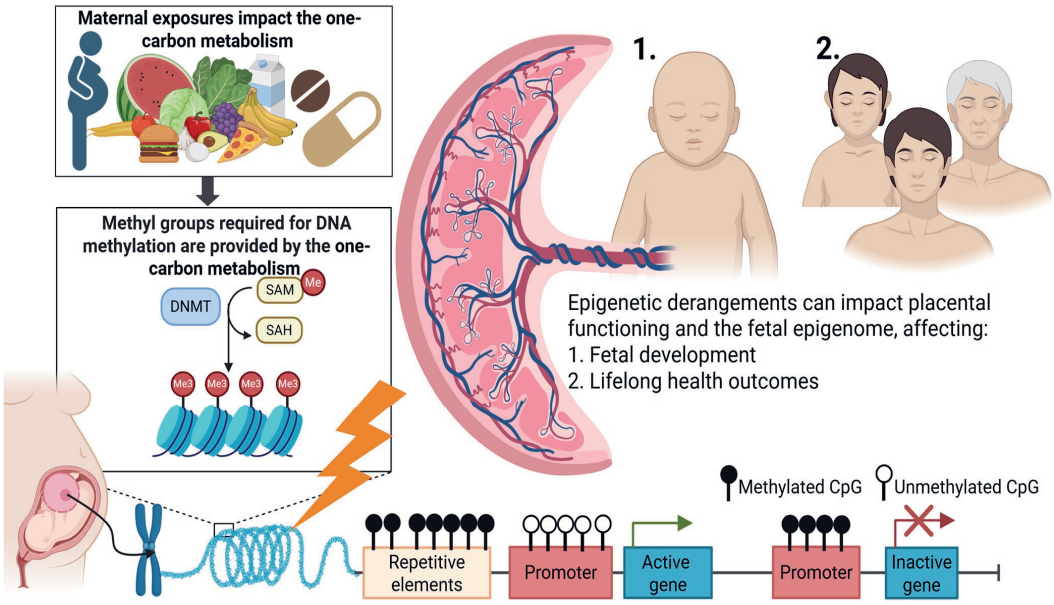


Figure 2: One-carbon metabolism as an underlying pathway affecting placental DNA methylation and lifelong health outcomes of the offspring. Maternal exposures such as nutrition and the use of vitamin supplements, are involved in one-carbon metabolism, which provides methyl groups for DNA methylation processes (left). DNA methyltransferases (DNMTs) transfer methyl groups to the cytosine-phosphate-guanine sides (CpGs). Derangement of one-carbon metabolism are proposed mechanisms affecting DNA methylation processes (bottom). The placenta is the intermediate between the maternal environment and embryonic and fetal growth and development. Epigenetic derangements can affect 1. directly the functioning of the placenta and subsequently fetal growth and development, and 2. the fetal epigenome with potential consequences for fetal growth and development, and 1 and 2 can both have consequences for lifelong health outcomes in the offspring.

Methods

Protocol and registration

The protocol for this systematic review was registered to the PROSPERO registry (PROSPERO 2023 CRD42023393358). The PRISMA Guidelines for systematic reviews and meta-analysis protocols were followed (28).

Search strategy

Medline (on the Ovid platform), Embase, Web of Science Core Collection, Cochrane Central Register of Controlled Trials and Google Scholar were searched until July 12, 2023. An expert research librarian was involved in setting up the search strategy. The search strategy included but was not limited to terms related to one-carbon metabolism like folate, homocysteine, and nutrition combined with placental related terms and key words related to epigenetics including DNA methylation. The full search is provided in Table S1.

Study selection

Clinical studies were eligible if they studied one-carbon moieties during the preconception period and/or during pregnancy including birth and studied DNA methylation in human placental tissues. Since in practice, one-carbon moieties are consumed by foods and vitamin supplements, we also included articles investigating associations between dietary patterns reflecting the intake of one-carbon moieties or intake of multivitamin supplements. In vitro studies were eligible if they studied one-carbon moieties and DNA methylation in a human in vitro placental model. Review articles, conference abstracts and articles not available in English language were excluded. MMV and SS performed the title and abstract screening and the full-text review of remaining articles.

Quality assessment of included studies

We used the ErasmusAGE quality score system for systematic reviews to assess the quality of included clinical studies (29). Quality of included articles was assessed by MMV and SS independently. All articles were scored based on five items and each item was scored zero, one or two points, leading to a score between 0-10 for each article. These items include study design (cross-sectional study=0, longitudinal study=1, intervention study=2), study size (<50 patients=0, 50-100 patients=1, >100 patients=2), method of measuring exposure and method of measuring outcome (both: no appropriate measure=0, moderate quality of measure=1, adequate measure=2), and adjustments for confounders (no adjustment=0, adjustment for key

confounders=1, adjustment for additional covariates or extra confounders=2) (Table S2) (29). We discussed all articles with an ErasmusAGE quality score ≥5.

Data extraction

Data extraction was done using a predefined form. Collected data included: study design, country of origin, year of publication, study population including sample size, method of measuring (exposure to) one-carbon moieties, timing of exposure (e.g. pre- and periconceptional, pregnancy), which part of placental tissue was studied (e.g. fetal or maternal side), focus of DNA methylation (global, genome-wide or gene-specific approach) and specified methylation technique. Lastly the main outcomes of the study related to DNA methylation were collected.

Results

Study selection

The literature search resulted in 1,490 unique records after duplicate removal. After title and abstract screening, 1,435 articles were excluded and a total of 55 full-text articles were assessed for eligibility of which 22 were included for the current review (Figure 3). The reasons for final exclusion included no exposure to or measurements of one-carbon moieties (n=15), no placental tissue studied (n=7), no DNA methylation as outcome (n=5) and other (n=6) (Figure 3).

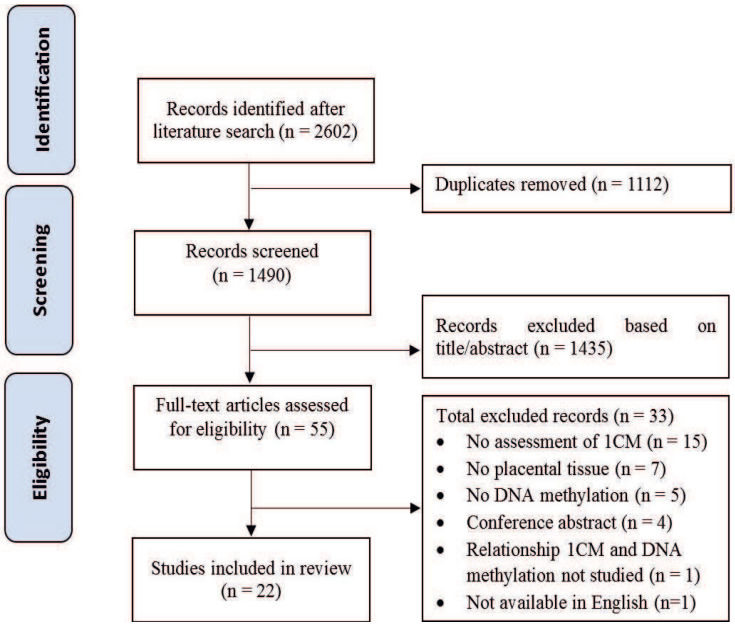


Figure 3: Flowchart of included and excluded articles. 1CM = one-carbon moieties.

Study characteristics

The study characteristics and main outcomes of included studies are provided in Table 1. The included clinical studies were observational (n=16) or clinical intervention studies (n=3). Two solely in vitro studies were included (30, 31) and one observational study combined clinical and in vitro work (32). All included in vitro studies used human placental cell lines.

One-carbon moieties that were studied in the clinical studies were folate/folic acid (n=9), vitamin B12 (n=9), homocysteine (n=4), choline (n=2), SAM and SAH (n=3), combination supplements (n=4) and dietary patterns reflecting differences in intake of one-carbon moieties (n=1). Two in vitro studies investigated the effect of folate and one studied the effect of choline on DNA methylation (Table 1). Results are discussed below for the different included moieties.

Most clinical studies used a gene-specific approach (n=14/20). Four used a genome-wide approach and global DNA methylation was investigated in nine studies. Some studies used multiple approaches in the same cohort. Table 2 summarizes the studies that investigated the associations for all three approaches in clinical studies with an ErasmusAGE quality score of at least 5 points. Included clinical studies scored between 3 and 8 points (median 5) (Table S2).

Metabolic determinants of one-carbon metabolism and placental DNA methylation outcomes

Folate/folic acid

Dietary intake / Supplement use

One study (ErasmusAGE score 6) assessed dietary folate intake during pregnancy by Food Frequency Questionnaires (FFQ) and found no association with methylation of partially- or highly methylated domains after controlling for the False Discovery Rate (FDR) (33).

Liu *et al*. (ErasmusAGE score 7) showed lower methylation of 5 out of 9 investigated sites within *FAM198A* in women who started using folic acid supplements before pregnancy in male offspring (p-values between 0.026 — 0.050) but not in female offspring, indicating a sex-specific impact on DNA methylation. No associations were found between use of folic acid supplements and methylation of two *ANKRD20B* sites (34). *FAM198A* and *ANKRD20B* sites were investigated, after these were formerly identified as top-ranked differentially methylated positions between three small for gestational age (SGA) children and three appropriate growth for gestational age (AGA) controls. Loke *et al*. (ErasmusAGE score 6) showed lower methylation of the differentially methylated region (DMR) of the *H19* promoter in women using folic acid supplements in first

trimester compared to no use of folic acid supplement, but found no methylation differences in the *IGF2/H19* imprinted control region (ICR) or two *IGF2* DMRs (35).

Blood

Maternal

In a multivariate model, folate levels associated positively with methylation of 9 out of 13 investigated CpGs at the transcription start site of *LEP* (range: β=0.16—0.26, p=0.003—0.03) but not in univariate analyses (ErasmusAGE score 5) (36). In another study, folate was positively associated with methylation of the *MMP-9* promoter (r=0.58, p<0.001) (37) (both ErasmusAGE score 5).

Fetal (umbilical cord)

Van Otterdijk *et al*. (ErasmusAGE score 5) investigated average methylation of CpG islands, defined as genomic regions between 200-500 base pairs with >50% CG content, the 0-2 kb and 2-4 kb up- and downstream regions next to CpG-islands, referred to as 'shores' and 'shelves' respectively, and CpGs outside these regions referred to as 'open sea'. Additionally, they took a genome-wide approach investigating single CpGs. The mother-infant dyads in their study were either homozygous for the wild-type *MTHFR677* genotype or for the *MTHFR* C677T variant. The *MTHFR* C667T polymorphism leads to reduced methylenetetrahydrofolate reductase (MTHFR) activity which is involved in one-carbon metabolism, leading to higher homocysteine and lower SAM concentrations (Figure 1). In the total study population, red blood cell (RBC) folate was positively associated with methylation of 'shelve' regions and CpG islands. In mother-infant dyads homozygous for the *MTHFR* C677T variant, a positive association was also found between RBC folate and methylation of 'open sea' and 'shore' regions. No associations between individual CpGs and RBC folate remained significant after correcting for multiple testing (38).

Other

Rahat *et al*. (2022) (ErasmusAGE score 5) found no association between folate levels within placental villi and global DNA methylation or LINE-1 methylation (32).

In vitro studies

Investigation of DNA methylation in JEG-3, a placental choricocarcinoma-derived cell line and in HTR-8/SVneo cell line, a model of extra-villous trophoblast, in response to folic acid treatment showed no changes in LINE-1 methylation (32). In contrast, global methylation decreased in both cell lines in a dose-dependent manner with the largest effect in HTR-8/SVneo

Table 1: Study characteristics and main findings of included studies ordered according to quality score.

Author (year) —Country	Study design	Population and sample size	Exposure and timing	DNA methylation technique	Placental tissue	Main outcomes	Quality Score
Devi (2017) — India (39)	Randomized controlled trial	Healthy pregnant women with low serum vitamin B12 (<200 pmol/L) in first trimester (n=66)	Group 1: 500mL milk + 10mg vitamin B12/day (n=23). Group 2: 500mL milk + placebo (n=20). Group 3: placebo (n=23). Late first trimester till delivery.	Global methylation: LINE-1 methylation — MethyLight Gene-specific: *VEGF* promoter — MS-HRM	Fetal side	Use of milk with or without vitamin B12 did not change *VEGF* promoter or LINE-1 methylation.	8
Dou (2022) — USA (46)	(Subgroup of) two prospective cohorts	Couples at increased risk for offspring with ASD. N=70 (MARBLES cohort) + N=88 (EARLI cohort)	Maternal multivitamin use in first month of pregnancy	Global methylation: Mean array methylation — MARBLES: 450K array EARLI: EPIC array Genome-wide: individual CpGs — MARBLES: 450K array, EARLI: EPIC array	Fetal side (MARBLES) Central full thickness biopsy (EARLI)	* Negative association between mean array methylation and vitamin use: -0.60% (CI -1.08 — -0.13; MARBLES); -0.52% (CI -1.04 — 0.01; EARLI). * No associations with individual CpGs after FDR correction. * Using a less stringent threshold (of p<0.01): 9,216 (MARBLES) and 3,442 (EARLI) differentially methylated CpGs of which ±95% were hypomethylated in women using vitamins (average -4%). * Identified CpGs were enriched in neuronal developmental pathways.	8
Lecorguille (2022) — France (44)	Prospective cohort	Healthy pregnant women (n=573)	1. Dietary intake in year before pregnancy reflecting differences in one-carbon moieties 2. Vitamin supplement use three months before and during pregnancy	Global methylation: LINE-1 and Alu repetitive elements methylation. — Pyrosequencing Genome-wide: DMRs and individual CpGs — 450K array	Fetal side, central	* No associations between dietary patterns and individual CpGs or global methylation. * *Varied and balanced* diet associated with a DMR related to *NPDC1* (p=0.02). * *Vegetarian tendency* associated negatively with two DMRs related to *DLL1* ($p=5.3x10^{-7}$, $p=7.0x10^{-9}$) and *FAR1* ($p=3.7x10^{-9}$, $p=7.8x10^{-3}$). * *Bread and starchy food* associated negatively with *AS3MT* (p=0.02) and *GSDMD* (p=0.004) and positively with *SLC25A46* (p=0.01) and *ZNF175* (p=0.006) DMRs. * Vitamin use associated with Alu repetitive elements methylation (β=0.40, p=0.005) compared to no vitamins. * No associations for vitamin use only before *or* during pregnancy or with LINE-1 methylation.	7

Castillo-Castrejon (2021) — Guatemale & Pakistan (45)	Randomized controlled trial	Preconcepti-onal women. Q1/Q4 based on birth length baby both n=24 in Guatemale and n=48 in Pakistan.	Group 1: Daily SQLNS ≥3 months before conception until delivery Group 2: No supplement use.	Gene-specific: P2 promoter region of *IGF1* — Pyrosequencing	Four representative biopsies	SQLNS use did not change *IGF1* promoter methylation.	6
Jiang (2012) — USA (41)	Randomized controlled trial	Healthy women in third trimester (n=24)	Group 1: 480mg choline/d (n=12). Group 2: 930mg choline/d (n=12). 12 weeks trial, supplements till delivery	Global methylation: — LC-MS/MS Gene-specific: *CRH*, *GNAS-AS1*, *LEP* and *IL10* promoters; the 5= untranslated exon 1F (and flanking regions) of *NR3C1*, DMR0 of *IGF2* — Base-specific cleavage and MS	Full-thickness biopsies from centre of each quadrant	* Higher choline intake (930mg/d) increased global DNA methylation (4.4 ±0.2% VS 3.6 ± 0.2%, p=0.02). * Higher choline intake (930mg/d) increased methylation of *CRH* (p=0.05) and *NR3C1* (p=0.002). Higher methylation was found for 4/15 *NR3C1* CpGs and no individual *CRH* CpG reached significance. * Higher choline intake (930mg/d) decreased methylation of 1/11 *GNAS-AS1* CpG (p=0.02), average *GNAS-AS1* methylation was not changed. * Higher choline intake did not change methylation of DMR0 of *IGF2* or promoter regions of *IL10* or *LEP*.	6
Liu (2019) — China (34)	Case-controls from prospective cohort	34 SGA cases, 62 AGA controls	Preconceptional folic acid supplement use	Gene-specific: two *ANKRD20B* CpGs, nine *FAM198A* CpGs; two top-ranked variable positions between n=3 SGA and n=3 AGA (450K array) — Pyrosequencing	Fetal side, central	* In male offspring, lower methylation of 5/9 sites within *FAM198A* associated with preconceptional folic acid supplement use (p=0.047, p=0.050, p=0.039, p=0.026, p=0.043). * No associations in female offspring or for *ANKRD20B*.	6
Loke (2013) — Australia (35)	(Subgroup of) prospective twin cohort	Monozygotic (n=34) and dizygotic (n=33) twin pairs	1. Folic acid supplements <12 weeks of gestation 2. Serum vitamin B12 and homocysteine at 28 weeks of gestation	Gene-specific: Four *IGF2/H19* DMRs —EpiTYPER MassARRAY	Unclear	* Folic acid supplement use associated with lower methylation (-4%) of *H19* promoter DMR. * No associations between folic acid supplement use and methylation of *IGF2/H19* ICR, DMR0 and DMR2 of *IGF2*. * No associations between vitamin B12 or homocysteine and methylation of four *IGF2/H19* DMRs.	6

Author (year) —Country	Study design	Population and sample size	Exposure and timing	DNA methylation technique	Placental tissue	Main outcomes	Quality Score
Nakanishi (2021) — Japan (42)	Prospective cohort	Healthy pregnant women (n=124)	Maternal plasma choline levels in first trimester, third trimester and at term, choline levels in cord blood.	Global methylation: LINE-1 methylation — Pyrosequencing Gene-specific: CpGs sites in *MEST*, *MEG*, *H19*, *PPARA*, *PPARG*, *ADIPOQ*, *LEP*, *NADH*, *NDUFb6*, *NR3C1*, *RXRA*, *HSD11ß2* — Pyrosequencing	Full-thickness biopsy, central	* Negative associations between maternal choline and *PPARG1A*, *NR3C1*, *HSD11ß2*, *PPARA* and *RXRA* methylation at all three time points (range: r=-0.188 — r=-0.452) and between cord blood choline and *NR3C1* (r=-0.20, p=0.033) and *PPARA* (r=-0.223, p=0.017) methylation. * Maternal choline in first trimester associated negatively with *AdipoQ* methylation (r=-0.307, p=0.001) and third trimester choline associated negatively with *AdipoQ* (r=-0.201, p=0.030) and *NDUFB6* (r=-0.214, p=0.021) methylation. * No associations with *MEG3*, *H19*, *MEST* and *LEP*. * LINE-1 methylation associated negatively with choline in first and third trimester (r= -0.205, p=0.022; r=-0.207, p=0.026) and in cord blood (r=-0.248, p=0.008).	6
Schmidt (2016) — USA (33)	Case-controls from prospective cohort	Couples at increased risk for offspring with ASD. ASD (n=24) and controls (n=23)	Food folate intake during pregnancy and vitamin use in first month of pregnancy	Global methylation: predefined partially- or highly methylated domains — MethylC-seq	Fetal side, midway cord insertion and periphery	No associations between folate intake (after FDR correction) or vitamin use and methylation of predefined partially- or highly methylated domains.	6
Heil (2019) — The Netherlands (43)	Case-control study	Pregnancies with PE (early onset n=11, late onset n=11), FGR (n=20), PTB (n=15) and controls (n=25)	SAM and SAH concentrations in placental tissue	Gene-specific: One CpG in the 5' flanking region of the *PIGF* gene —EpiTYPER MassARRAY	Fetal side, four biopsies 3 cm around cord insertion, removal 2 mm of top layer	SAM and SAM:SAH ratio were positively associated with *PIGF* methylation (r=0.31, p=0.006; r=0.24, p=0.04, respectively).	5
Khot (2017) — India (40)	Case-control study	Term (n=69) and preterm (n=71) deliveries in healthy women	Plasma vitamin B12, folate and homocysteine just after delivery	Gene-specific: *MTR* promoter — Pyrosequencing	Random biopsies from decidual side	* Vitamin B12 associated negatively with methylation of 3/7 investigated CpGs in the *MTR* promoter (r =-0.208, p=0.049; r =-0.248, p=0.017; r=-0.298, p=0.004). * Women delivering preterm had lower plasma folate and higher homocysteine compared to term controls. Mean *MTR* promoter methylation was 0.5% higher (p< 0.01) in preterm births. 5/7 studied CpGs had higher methylation in preterm placentas only after vaginal deliveries. * No associations between homocysteine or folate and DNA methylation were investigated.	5

Moeini (2022) — Iran (37)	Case-control study	Term (n=25) and preterm (n=25) births in healthy women	Serum folate and vitamin B12, timing unclear	Gene-specific: *MMP-9* promoter —Epitect Methyl-II PCR assay kit	Unclear	Folate and vitamin B12 associated with *MMP-9* promoter methylation (r=0.58, p<0.001; r=0.68, p <0.001, respectively).	5
van Otterdijk (2023) — USA (38)	Subgroup of cohort study	Mother-infants dyads homozygous for the C or T allele of *MTHFR* C677T polymorphism (both n=45)	Red blood cell folate, vitamin B12, SAM, SAH in cord blood	Global methylation: Average methylation of the following regions: CpG islands, shores, shelves and open sea — 450K array Genome-wide: individual CpGs — 450K array	Fetal side, near umbilical cord	* RBC folate correlated positively with methylation of CpG islands and ' shelves' for the total study population and in ' open sea' and ' shores' only for the TT genotype. * No significant associations between individual CpGs and RBC folate after multiple testing correction. * SAM:SAH ratio correlated positively with methylation of ' open sea' and ' shore' regions and with individual loci located in *RAD54L2*, *ETS2*, *ZNF836* and *ABAT*. * No significant associations between SAM or SAH and region-specific DNA methylation patterns. * SAM significantly associated with two loci located in *LOXL3* and *GNL1* gene bodies, and SAH with one CpG in a *ZC3H12D* CpG island. * No associations between vitamin B12 and regional methylation or methylation of individual loci.	5
Rahat (2022)* — India (32)	Case-control study	Uncomplicated first/second trimester terminations (both n=30), term and PE cases (both n=30)	Folate levels in placental villi	Global methylation: —ELISA based kit and LINE-1 methylation — MS-HRM	Placenta villi	No significant associations between folate levels in placental villi and global methylation.	5
Sletner (2021) — Norway (36)	(Subgroup of) prospective cohort	Healthy pregnant women, ethnic south Asian (N=40) or European (n=40)	Serum folate and vitamin B12 at enrollment (<20 weeks of gestation)	Gene-specific: 13 CpGs in the transcription start site of *LEP* — Pyrosequencing	Full thickness biopsy, central	* Folate associated with methylation of 9/13 CpGs in a multivariate model (range: β=0.16—0.26, p=0.003—p=0.03) but not in univariate models. * No associations between vitamin B12 and methylation of 13 *LEP* CpGs in univariate analyses, negative associations with 2/13 CpGs in multivariate models (β=-0.8, p=0.02; β=-0.02, p=0.02).	5
Ge (2015) — China (59)	Case-control study	Pregnancies with PE (n=127) and controls (n=132)	Homocysteine in maternal plasma on admission	Gene-specific: *MTHFR* promoter — Methylation specific PCR	Central biopsy, one from either side	* *MTHFR* methylation indices were higher in PE (26.8% VS 15.2%, p<0.05) and homocysteine was higher in PE (p<0.05). * No association between homocysteine and methylation was investigated.	4

Author (year) —Country	Study design	Population and sample size	Exposure and timing	DNA methylation technique	Placental tissue	Main outcomes	Quality Score
Pinunuri (2020) — Chile (47)	Case-controls from prospective cohort	Term (n=16) and preterm (32-36 weeks, n=23) deliveries in healthy women	Folate and vitamin B12 in umbilical cord blood	Gene-specific: CpG island upstream of transcription initiation site *FOLR1* — MS-HRM	Chorionic and basal plates biopsies near cord insertion	* Cord blood folate associated with *FOLR1* methylation in the chorionic plate (R=0.665, p≤0.05) but not in the basal plate. * There were no associations between cord blood vitamin B12 and *FOLR1* methylation.	4
Tserga (2017) — USA (60)	(Subgroup of) prospective cohort	Healthy Caucasian women homozygous for C or T allele *MTHFR* C677T polymorphism in mother and child (n=48)	1. Folic acid supplement use started before VS during pregnancy 2. Red blood cell folate, SAM, SAH, vitamin B12 in cord blood	Gene-specific: *H19ICR* — Pyrosequencing	Fetal side near cord insertion	* No associations between preconceptional start of FA, red blood cell folate, vitamin B12 or SAM levels in cord blood and *H19ICR* methylation. * *H19ICR* methylation associated with SAM:SAH ratio (R=0.38) and negatively with log(SAH) (R=-0.40) in cord blood.	4
Zhu (2019) — USA (61)	(Subgroup of) prospective cohort	Couples at increased risk for offspring with ASD. ASD cases (n=20) and controls (n=21)	Vitamin use in first month of pregnancy	Gene-specific: *CYP2E1* and *IRS2* DMRs (two ASD-associated DMRs) — Pyrosequencing Genome-wide: DMRs —WGBS	Fetal side	* Vitamin use associated with 376 DMRs. Genes were enriched for neuron fate commitment, central nervous system development, regulation of transcription and of phosphatidylinositol 3-kinase activity functions. * Vitamin use associated negatively with *IRS2* DMR methylation (p=0.039) and positively but not significantly with *CYP2E1* (p=0.118).	4
Kulkarni (2011) — India (62)	Case-control study	Pregnancies with term PE (n=30), preterm PE (n=27) and controls (n=30)	Vitamin B12 in maternal plasma at delivery, homocysteine in maternal plasma in subset (16 controls, 28 term PE, 19 preterm PE)	Global DNA methylation — Methylamp™ Global DNA Methylation Quantification Kit	Five different villi biopsies	* Vitamin B12 was higher in preterm PE (261 ng/mL) and term PE (191 ng/mL) compared to controls (145 ng/mL) and homocysteine was also higher in preterm PE (17.59 uM) and term PE (14.43 uM) compared to controls (11.01 uM). * Mean global methylation was higher in term PE (0.68%) and preterm PE (0.72%) compared to controls (0.53%), p <0.05. * No association between vitamin B12 or homocysteine and DNA methylation was studied.	3

In vitro studies using human placental cell lines							
Jiang (2016) — USA (30)	In vitro study	BeWo cell line	Incubation for 96 hours with 1mM choline chloride	Global methylation — ELISA based kit	NA	Choline did not change global DNA methylation.	NA
Rahat (2017) — India (31)	In vitro study	JEG-3 and HTR-8/SVneo cell lines, first trimester cytotrophoblasts	Incubation for 48 hours with folic acid (10^{-7} M and 10^{-4} M)	Gene-specific: DMRs of *SNRPN*, *PEG10* and *MEST* — MS-HRM	NA	* Folic acid decreased *SNRPN* methylation in JEG-3, HTR-8/SVneo and in cytotrophoblasts. At 10^{-7} M, methylation decreased respectively by 1.5 ($p<0.01$), 1.2 ($p<0.05$) and 1.2 ($p<0.05$) and at 10^{-4} by 1.8 ($p<0.001$), 2.2 ($p<0.001$) and 1.4 ($p<0.01$) fold. * Folic acid did not change *PEG10* or *MEST* methylation.	NA
Rahat (2022)* — India (32)	In vitro study	JEG-3 and HTR-8/SVneo cell lines	Incubation for 48 hours with folic acid (10^{-7} M and 10^{-4} M)	Global methylation: —ELISA based kit and LINE-1 methylation — MS-HRM Gene-specific: *RASSF1A*, *P16*, *RB1*, *PRKCDBP*, *APC*, *c-myc*, *c-jun*, *VEGF*, *EGFR*, *hTERT*, *MMP2*, *MMP-9*, *TIMP1*, *TIMP2* — MS-HRM	NA	* Folic acid incubation did not change LINE-1 methylation. * Global methylation decreased in a dose-dependent manner. • JEG-3 cells: - 3.7% and -4.9% at 10^{-7} M and 10^{-4} M. • HTR-8/SVneo cells: - 4.3% and -5.7% at 10^{-7} M and 10^{-4} M ($p<0.001$). * In JEG-3 cells, folic acid increased methylation of *APC* (1.14x at 10^{-7} M; 1.28x at 10^{-4} M) and *P16* (5x at 10^{-7} M) and decreased for *c-jun* (1.1x at 10^{-7} M). * In HTR-8/SVneo cells, methylation increased for *APC* (4.1x at 10^{-7} M) and decreased for *P16 (*2.4x at 10^{-7} M and 3.5x at 10^{-4} M), *c-myc* (1.8x at 10^{-7} M and 2.5x at 10^{-4} M) and *c-jun* (2.14 x at 10^{-7} M and 5.5x at 10^{-4} M).	NA

* Same study. Abbreviations: WGBS = Whole Genome Bisulfite Sequencing, LINE-1 = Long Interspersed Nuclear Element-1, ASD = autism spectrum disorder, SGA = small for gestational age, AGA = appropriate growth for gestational age, SQLNS = small quantity lipid-based micronutrient, CpG = Cytosine-phosphate-Guanine, DMR = differentially methylated region, SAM = S-adenosylmethionine, SAH = S-adenosylhomocysteine, PE = preeclampsia, FGR = fetal growth restriction, PTB = preterm birth, NA = not applicable, LC-MS/MS = Liquid Chromatography with tandem mass spectrometry, MS-HRM = Methylation-sensitive high-resolution melt analysis, MS = mass spectrometry, PCR = polymerase chain reaction

Table 2: Direct associations between one-carbon moieties and global methylation, genome-wide methylation or gene-specific methylation in included clinical studies with an ErasmusAGE quality score of at least 5 out of 10 points.

Outcome	Exposure	Methylation	Ref
Global methylation	Dietary folate	=	(33)
	Folate in placental villi	=	(32)
	Umbilical cord RBC folate	↑/=	(38)
	Vitamin B12 supplements	=	(39)
	Umbilical cord vitamin B12	=	(38)
	Choline supplements	↑	(41)
	Choline in maternal blood: first trimester/ third trimester/ term	↓ / ↓ / =	(42)
	Choline in cord blood	↓	(42)
	Umbilical cord ▪ SAM ▪ SAH ▪ SAM:SAH	 = = ↑/=	(38)
	Dietary patterns: ▪ ↑B-vitamins, choline, methionine ↓betaine ▪ ↑B6, folate, betaine ↓ B12 ▪ ↑Betaine ↓B2, B6, folate	 = = =	(44)
	Vitamin use in first month of pregnancy	=	(33)
		↓/ =	(46)
	Vitamin use before and during pregnancy Vitamin use before or during pregnancy	↑ / = = / =	(44)
Genome-wide	**Exposure**	**Genes linked to identified CpGs**	
	Umbilical cord RBC Folate	=	(38)
	Umbilical cord vitamin B12	=	
	Umbilical cord ▪ SAM ▪ SAH ▪ SAM:SAH	 *LOXL3 GNL1* *ZC3H12D* *RAD54L2 ETS2 ZNF836 ABAT*	
	Dietary patterns ▪ ↑B-vitamins, choline, methionine ↓betaine ▪ ↑B6, folate, betaine ↓ B12 ▪ ↑Betaine ↓B2, B6, folate	**Genes linked to identified DMRs** *NPDC1* *DLL1 FAR1* *AS3MT GSDMD SLC25A46 ZNF175*	(44)
	Dietary patterns	Individual CpGs =	
	Vitamin use in first month of pregnancy	Individual CpGs = *	(46)

Outcome	Exposure	Methylation	Ref
Gene-specific	**Exposure**	**Gene of interest**	
	Start folic acid preconceptional compared to start during pregnancy	*FAM198A* ↓(boys)/= (girls) *ANKRD20B* =	(34)
	Folic acid use in first trimester	*H19* promoter DMR↓ Two *IGF2* DMRs *IGF2/H19ICR* =	(35)
	Maternal serum folate	*MMP-9* ↑	(37)
		LEP =/↑	(36)
	Vitamin B12 supplements	*VEGF* =	(39)
	Vitamin B12 in maternal blood	*MMP-9* ↑	(37)
		MTR ↓	(40)
		Four *IGF2/H19* DMRs =	(35)
	Homocysteine in maternal blood	Four *IGF2/H19* DMRs =	(35)
	Choline supplements	*CRH NR3C1* ↑ *GNAS-AS1* ↓/= *IGF2 IL-10 LEP* =	(41)
	Choline in first trimester maternal blood	*AdipoQ PPARG1A NR3C1, HSD11β2 PPARA RXRA* ↓ *MEG3 H19 MEST LEP NDUFB6* =	(42)
	Choline in third trimester maternal blood	*AdipoQ PPARG1A NR3C1, HSD11β2 PPARA NDUFB6 RXRA* ↓ *MEG3 H19 MEST LEP* =	(42)
	Choline in term maternal blood	*PPARG1A NR3C1 HSD11β2, PPARA RXRA* ↓ *AdipoQ MEG3 H19 MEST LEP NDUFB6* =	(42)
	Choline in cord blood	*NR3C1 PPARA* ↓ *AdipoQ PPARG1A HSD11β2 RXRA MEG3 H19 MEST LEP NDUFB6* =	(42)
	Placenta SAM and SAM:SAH	*PIGF* ↑	(43)
	Small quantity lipid-based micronutrient use	*IGF1* =	(45)

* No associations with individual CpGs after FDR correction. Using a less stringent threshold of p<0.01, 9,216 (MARBLES) and 3,442 (EARLI) differentially methylated CpGs of which ±95% was hypomethylated in vitamin groups (average -4%) were identified. Identified CpGs were enriched in neuronal developmental pathways. DMR = differentially methylated region, CpG = Cytosine-phosphate-Guanine, RBC = Red Blood Cell, SAM = S-adenosylmethionine, SAH = S-adenosylhomocysteine, FDR = False Discovery Rate.

cells, where a 4.3% decrease (p<0.001) was found at 10^{-7} M folic acid, comparable to the physiological range of 400-600 µg/day, and a 5.7% decrease (p<0.001) was found at a much higher concentration of 10^{-4} M folic acid. Taking a gene-specific approach, promoter methylation of the tumor suppressor gene *APC* increased in both cell lines in response to folic acid, while for *P16* an increase was observed in JEG-3 cells while methylation decreased in HTR-

8/SVneo cells. Methylation of the promoter regions of oncogenes *c-myc* and *c-jun* decreased in both cell lines (32). No changes were found in the *MMP-9* promoter region. In a comparable in vitro study, methylation of the promoter regions of *SNRPN*, *PEG10* and *MEST* was studied (31). Folic acid suppletion decreased methylation of *SNRPN* in a dose-dependent manner in JEG-3, HTR-8/SVneo cell lines and in cytotrophoblasts. No changes were found for *PEG10* and *MEST* (31).

Interim conclusions – Folate/folic acid

Mixed results have been reported for the relationship between folate/folic acid and markers of global placental DNA methylation. Only one study took a genome-wide approach and identified no significant associations between methylation of single CpGs and cord blood folate. When taking a gene-specific approach, several – but not all – investigated sites associated with folate/folic acid (Table 2). This indicates site-specific relationships between DNA methylation and folate/folic acid.

Vitamin B12

Supplement use

In a randomized trial in Indian women with low vitamin B12 levels, LINE-1 methylation and *VEGF* promoter methylation did not differ between women who used vitamin B12 supplements from end first trimester till delivery as compared to women without vitamin B12 supplements (ErasmusAGE score 8) (39).

Blood

Maternal

No associations were found between vitamin B12 levels at 28 weeks of gestation and four investigated *IGF2/H19* DMRs (ErasmusAGE score 6) (35). Contrary, vitamin B12 levels in first half of pregnancy associated with lower methylation of 2 out of 13 investigated CpGs at the transcription start site of *LEP* (β=-0.8, p=0.02; β=-0.02, p=0.02) (36) and vitamin B12 associated with *MMP-9* promoter methylation (r=0.68, p <0.001) (both ErasmusAGE score 5) (37). A negative association was found between plasma vitamin B12 at time of delivery and methylation of 3 out of 7 investigated CpGs in the *MTR* promoter region (r=-0.208, p=0.049; r=-0.248, p=0.017; r=-0.298, p=0.004) (ErasmusAGE score 5) (40).

Fetal (umbilical cord)

Van Otterdijk *et al*. found no significant associations between vitamin B12 and methylation of CpG islands, 'shores', 'shelves', or 'open sea' regions or with methylation of individual CpGs when taking a genome-wide approach (ErasmusAGE score 5) (38).

Interim conclusions – Vitamin B12

Vitamin B12 did not associate with different markers of global placental DNA methylation or with methylation of individual CpGs taking a genome-wide approach. In a gene-specific approach, several investigated sites were associated with vitamin B12, underlining again the site-specific relationship between DNA methylation and vitamin B12 (Table 2).

Homocysteine

Blood

Maternal

No associations were found between homocysteine and four studied *IGF2/H19* DMRs (ErasmusAGE score 6) (35).

Choline

Supplement use

Jiang *et al*. randomized women in third trimester to either 480mg or 930mg choline per day for 12 weeks (ErasmusAGE score 6). They studied global DNA methylation and methylation of regions related to cortisol-regulating genes *CRH* and *NR3C1*, methylation of DMR0 of *IGF2* and methylation of *LEP*, *GNAS-AS1* and *IL10* promoter regions. Women in the 930mg group had higher global methylation compared to the 480mg group (4.4 ±0.2% VS 3.6 ±0.2%, p=0.02) and higher methylation of the *CRH* promoter region (p=0.05) and the 5= untranslated exon 1F (and flanking regions) of *NR3C1* (p=0.002). Of the individual CpGs, 4 out of 15 studied *NR3C1* CpGs showed higher methylation while none of the five studied individual CpGs for the *CRH* region reached significance. Although 1 out of 11 studied *GNAS-AS1* CpGs was lower methylated in the 930mg group (p=0.02), average *GNAS-AS1* methylation did not differ between groups. No differences were found in methylation of *IGF2*, *IL10* or *LEP* promoter regions (41).

Blood

Maternal

One study determined maternal plasma choline in first trimester, third trimester and at term, and investigated methylation levels of twelve genes involved in fetal growth, lipid and energy metabolism, or adipogenesis and DNA methylation of LINE-1 elements (ErasmusAGE score 6). A negative modest association was found between DNA methylation of *PPARG1A*, *NR3C1*, *HSD11ß2*, *PPARA* and *RXRA* and maternal choline at all three time points (range: r=-0.188 — r=-0.452). For *AdipoQ* a negative association with maternal choline levels was observed in first and third trimester (r=-0.307, p=0.001; r=-0.201, p=0.030) and for *NDUFB6* only in third trimester (r=-0.214, p=0.021). No associations were found with *MEG3*, *H19*, *MEST* and *LEP* methylation.

LINE-1 methylation was negatively associated with maternal choline in first and third trimester (r=-0.205, p=0.022; r=-0.207, p=0.026) (42).

Fetal (umbilical cord)

The same study showed a negative association between choline in cord blood and methylation of *NR3C1*, *PPARA* and LINE-1 (r=-0.20, p=0.033; r=-0.223, p=0.017; r=-0.248, p=0.008). No associations were found with methylation of *MEG3*, *H19*, *MEST*, *LEP*, *AdipoQ*, *NDUFB6*, *PPARG1A*, *HSD11β2* or *RXRA* (42).

In vitro studies

In an in vitro study using BeWo cells as a model for first trimester trophoblast, global DNA methylation was not altered by choline treatment (30).

Interim conclusions – Choline

Mixed results have been reported for choline and markers of global placental DNA methylation as well as for choline and *NR3C1* methylation. Several other relationships between DNA methylation of specific genes and choline intake or blood levels were found depending on studied site and moment of measuring choline, indicating site-specific relationships between DNA methylation and choline which may differ depending on timing of exposure (Table 2).

S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH)

Blood

Fetal (umbilical cord)

The SAM:SAH ratio was positively associated with methylation of 'open sea' and 'shore' regions only in mother-infant dyads homozygous for the *MTHFR* C677T genotype. There were no significant associations between SAM:SAH and CpG island or 'shelve' regions and the observed positive associations with SAM and negative associations with SAH did not reach significance for the different genomic regions. When investigating individual CpGs genome-wide, limited associations were found. The SAM:SAH ratio associated with individual CpGs located in *RAD54L2*, *ETS2*, *ZNF836* and *ABAT*, SAM associated with two loci located in *LOXL3* and *GNL1* gene bodies and SAH associated with methylation of one CpG in a *ZC3H12D* CpG island. Several other identified associations did not reach significance after correcting for multiple testing (ErasmusAGE score 5) (38).

Other measures

Heil *et al*. (ErasmusAGE score 5) studied SAM and SAH levels in placentas and methylation of LINE-1 and one CpG in the 5' flanking region of *PlGF*. SAM and SAM:SAH ratio were positively

associated with *PlGF* methylation (r=0.31, p=0.006; r=0.24, p=0.04, respectively). LINE-1 methylation associated with *PlGF* methylation (r=0.40, p<0.001) but no association between LINE-1 and SAM or SAH was studied (43).

Interim conclusions – SAM/SAH

A higher SAM:SAH ratio indicates higher availability of methyl group donors (Figure 1). Although only two studies investigated SAM and SAH, the SAM:SAH ratio in umbilical cord blood associated positively with markers of global methylation depending on genotype, and SAM, SAH, and SAM:SAH associated with methylation of a few individual CpGs in a genome-wide study. Both placental SAM and SAM:SAH ratio associated positively with *PlGF* methylation (Table 2). This suggests that SAM and SAH can impact placental DNA methylation both at a regional/global level as well as at gene-specific sites.

Combinations

Dietary patterns

Lecorguille *et al*. (ErasmusAGE score 7) assessed dietary intake in the year before pregnancy and distinguished three dietary patterns (44). After adjustment for vitamin use, the 'varied and balanced' diet which is rich in B-vitamins, choline and methionine, but low in betaine showed a positive association with a DMR related to *NPDC1* (p=0.02). A 'vegetarian tendency' diet which is rich in vitamin B6, folate and betaine but low in vitamin B12 showed a significant negative association with two DMRs related to *DLL1* ($p=5.3x10^{-7}$, $p=7.0x10^{-9}$) and *FAR1* ($p=3.7x10^{-9}$, $p=7.8x10^{-3}$). Lastly, 'bread and starchy food' diet which is rich in betaine but low in vitamin B2, B6 and folate associated negatively with DMRs located near *AS3MT* and *GSDMD*, and positively with DMRs related to *SLC25A46* and *ZNF175* (p=0.02; p=0.004; p=0.01; p=0.006, respectively). No associations were found with individual CpGs or global DNA methylation (44).

Supplement use

An intervention study (ErasmusAGE score 6) randomized women to daily use of small quantity lipid-based micronutrient (SQLNS) starting at least three months before conception until delivery versus no supplements. SQLNS contain essential fatty acids, proteins and multiple vitamins including folate, vitamin B6 and B12. Methylation of two CpGs in the *IGF1* promoter region were assessed. No differences were found between study groups (45).

Composition of prenatal vitamins varies but they usually contain folic acid, vitamin B6 and B12 as well as other micronutrients. Lecorguille *et al*. (ErasmusAGE score 7) found higher methylation of Alu repetitive elements in women taking vitamin supplements before and during

pregnancy compared to women not taking any vitamins (β=0.40, p=0.005) although no differences in LINE-1 methylation were found. Vitamin use only before or during pregnancy was not associated with Alu or LINE-1 methylation (44).

Two studies investigated placental DNA methylation and self-reported maternal vitamin intake in the first month of pregnancy. Dou *et al*. (ErasmusAGE score 8) studied DNA methylation in the MARBLES and in the EARLI cohort, both comprise couples at increased risk for offspring with autism spectrum disorder (ASD). Mean array methylation for MARBLES women taking vitamins was -0.60% (CI -1.08 — - 0.13) and the same magnitude of effect was seen in the EARLI cohort, although not significant -0.52% (CI -1.04 — 0.01). No individual CpGs associated with vitamin intake using the FDR-corrected significance threshold ($p<1.0^{-7}$). With a less stringent significance threshold of p<0.01, they found 9,216 and 3,442 differentially methylated CpGs in the MARBLES and EARLI cohort, respectively. About 95% of these showed lower methylation in the vitamin group in both cohorts (average -4%). The identified CpGs were enriched in neuronal developmental pathways (46). Schmidt *et al*. (ErasmusAGE score 6) found no associations between vitamin use and methylation of predefined partially- or highly methylated domains or methylation of enhancers, active promoters and bivalent promoters (33).

Interim conclusions – Combinations

Global methylation was not associated with dietary patterns reflecting differences in intake of one-carbon moieties and use of vitamin supplements showed both increased and decreased global methylation as well as no differences depending on used technique and/or timing of supplement use. A genome-wide study revealed a few DMRs associated with dietary patterns, but dietary patterns or vitamin supplement use did not associate with individual CpGs after FDR correction. *IGF1* promoter methylation was not altered in women taking SQLNS (Table 2). In general, dietary patterns and vitamin supplement use did not substantially impact placental DNA methylation in included studies.

Discussion

This review systematically summarizes associations between one-carbon metabolism and placental DNA methylation. Included studies suggest that one-carbon moieties can impact placental DNA methylation at multiple specific genomic regions rather than affecting global methylation. On the other hand, this review shows that there is a lot of heterogeneity among studies regarding investigated exposures, studied outcomes, sample size and study design. Additionally, differences in DNA methylation have been found between female and male offspring (34), depending on which part of the placenta was sampled (47), timing of exposure

(42, 44) and underlying genotype (38), which could also explain parts of observed differences between studies. Based on the use of ErasmusAGE quality score, we discussed studies with a quality score of at least 5 out of 10 points.

Global Methylation

A wide variety of techniques to study global methylation has been used by included studies (Table 1). Global DNA methylation was not altered by the use of vitamin B12 supplements, while higher intake of choline did increase global DNA methylation. One study found associations between one-carbon moieties and average DNA methylation to differ between genomic regions (i.e. CpG islands, 'shores', 'shelves' and 'open sea') (38). Overall, most studies found no associations between one-carbon moieties and measures of global DNA methylation (Table 2). Hence, higher availability of methyl donors provided by one-carbon metabolism does not just lead to increased global placental methylation.

Genome-Wide Approach

The number of included genome-wide studies is limited, and only three had a quality score of at least five. All three studies used the Illumina Infinium HumanMethylation450 (450K) BeadChip array, which covers >480,000 CpGs and one partly used the Infinium methylation EPIC array covering >850,000 CpGs. All three studies investigated different exposures (Table 2). CpGs associated with maternal vitamin use in the first month of pregnancy could only be identified when a less stringent FDR-corrected p-value was applied and 95% of these were hypomethylated in women who did take vitamins in the first month of pregnancy. Identified CpGs were enriched in neuronal developmental pathways (46). A few DMRs linked to different genes could be identified based on dietary patterns reflecting differences in intake of one-carbon moieties (44) and methylation of a few CpGs associated with umbilical cord levels of SAM, SAH and SAM:SAH, but not with RBC folate or vitamin B12 levels in cord blood (38).

Gene-Specific Approach

Included studies mostly investigated different loci, hampering comparison of results. Overall, most gene-specific studies did find relationships between one-carbon moieties and placental DNA methylation. On the other hand, most studies reported both significant and non-significant associations and there is a lack of uniformity in directionality of identified associations, indicating site-specific relationships (Table 2).

Genes involved in growth and metabolism

Genes involved in growth and metabolism have been of particular interest in included studies. The imprinted *IGF2/H19* cluster plays an important role in fetal and placental growth (48).

Methylation of *IGF2/H19* has been investigated in multiple studies, with most reporting no significant associations with investigated one-carbon moieties (35, 42). In intervention studies, higher intake of choline did also not alter *IGF2* methylation (41) and the use of SQLNS did not affect methylation of *IGF1*, another major growth factor (45). This is in contrast with other studies showing that maternal intake of methyl-group donors including folate/folic acid associated negatively with *IGF2* methylation in buccal epithelial cells in 6 months old children and positively with *IGF2* methylation in infant blood and cord blood suggesting a tissue-specific response (15, 49, 50).

LEP encodes the hormone Leptin which plays a central role in energy homeostasis including appetite regulation, and mutations in *LEP* can contribute to the development of obesity and diabetes type 2 (51). Different results for *LEP* methylation have been reported depending on studied one-carbon moiety: positive associations with serum folate, negative associations with serum vitamin B12 (36) and no associations with choline have been reported (42) (Both ErasmusAGE score 6).

Cortisol regulating genes could play a role in multiple diseases including cardiovascular complications. Jiang *et al*. investigated choline intake (480mg/daily versus 930mg/daily) and found increased methylation in the higher dose group of the *CRH* promotor and the 5' untranslated exon 1F of *NR3C1* (41). In contrast, *NR3C1* methylation associated negatively with choline levels in a study by Nakanishi *et al*. (ErasmusAge score 6). A possible explanation is that both studies targeted a different genomic region of *NR3C1* or there may be lack of comparability between blood levels of choline and use of choline supplements.

Nakanishi *et al*. investigated several other gene-specific sites related to growth and metabolism and choline in maternal blood and cord blood. Negative associations with choline levels were also found for *AdipoQ*, *PPARG1A*, *HSD11Ƅ2*, *PPARA*, *NDUFB6* and *RXA*, while no associations were found with methylation of *MEG3* and *MEST*, all involved in fetal growth. Differences have been found for different time points during pregnancy (42). In an in vitro study, *MEST* methylation was also not altered in response to folic acid (31).

MMP-9 codes for a matrix metalloproteinase induced during labor and MMP-9 is also increased in multiple cardiovascular diseases including hypertension and myocardial infarction (52). Serum vitamin B12 and folate associated positively with *MMP-9* promoter methylation and with lower MMP-9 RNA and protein levels (37). Contrary, folic acid treatment did not change *MMP-9* methylation in an in vitro model (32).

Genes related to placental development and function

One-carbon metabolism has also been linked to placental angiogenesis which is crucial for a well-functioning placenta. The placenta itself produces several angiogenic factors involved in endothelial growth and function, including vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), both of which are decreased in preeclampsia (PE) (53, 54). For example, increased maternal choline intake can suppress anti-angiogenic soluble fms-like tyrosine kinase 1 (sFLT-1) expression in human placentas and promotes angiogenesis in mice placentas (10). Vitamin B12 supplements did not alter *VEGF* methylation in a high-quality intervention study (39), while another study showed *PlGF* methylation associated with SAM and SAM:SAH in the placenta (43). Since a higher SAM:SAH ratio indicates higher availability of methyl group donors (Figure 1), a deranged one-carbon metabolism may be involved in the pathogenesis of placental-related complications partly through inducing epigenetic changes resulting in impaired placental functioning.

Genes directly related to one-carbon metabolism

Plasma vitamin B12 was negatively associated with *MTR* promoter methylation. This can have an impact on one-carbon metabolism, since *MTR* codes for methionine synthase which is crucial in the conversion of homocysteine to methionine (40) (Figure 1).

Genes selected based on prior genome-wide analysis

Lastly, Liu *et al*. selected gene-specific sites of interest based on DMRs identified through genome-wide analysis. Methylation of *FAM198A* associated negatively with preconceptional start of folic acid only in male offspring indicating a sex-specific impact on DNA methylation. No associations were found between timing of folic acid supplements and methylation of two *ANKRD20B* CpGs (34).

In summary, most studies reported both significant and non-significant associations depending on the studied genes and moieties, indicating a site-specific relationship between one-carbon moieties and DNA methylation at various genes in the placenta (Table 2).

Previous Reviews

Our review is largely in line with previously published human and animal reviews. James *et al*. focused predominantly on DNA methylation in cord blood and showed several associations between maternal one-carbon metabolism and methylation of specific genes. Overlapping genes with the current review include *IGF2/H19*, *LEP*, *NR3C1*, *RXRA*, *MEG3*, *MEST*, *IL10* and *GNAS-AS1* and several other associations were reported. For most genes, differences were found depending on studied tissue, moiety, specific loci and timing of exposure (12). In rodents,

maternal folic acid supplements (24) and choline intake or methyl group supplemented diets (14) have been shown to impact DNA methylation at several gene-specific sites, leading to both hypo- and hypermethylation depending on studied genomic region. Additionally, these studies found mixed outcomes for global methylation in multiple offspring tissues including brain, mucosa and liver. For homocysteine, associations with hypomethylation were more prominent in animal models as compared to human studies, where mixed results have been reported (25). Likewise, multiple studies using livestock showed that dietary restrictions or excesses, or a methyl supplemented diet can affect both gene-specific and global DNA methylation in the offspring, depending on studied tissue, offspring sex and timing of exposure (26, 55). A systematic review focusing on physical activity and dietary intake of carbohydrates, fats and proteins during pregnancy showed similar mixed associations with DNA methylation in placental tissues and cord blood (56). This could indicate a role for a variety of lifestyle behaviors affecting the epigenome, however, one-carbon metabolism is influenced by lifestyle behaviors which could also be (part of) the underlying biological mechanism for observed associations.

Strengths and Limitations

In the present article, we systematically review studies investigating relationships between different one-carbon moieties and DNA methylation focusing on the human placenta. We included a wide range of exposures and outcomes and were not limited to a specific timing in relation to delivery, to be able to give a comprehensive overview of current literature. Consequently, heterogeneity between studies limits comparability of results and a meta-analysis is therefore not possible. Besides, most included studies face several challenges which should be taken into account. First, dietary patterns and vitamin use are often associated with other (lifestyle) behaviors potentially affecting DNA methylation, including smoking and BMI. Most studies are observational and do not adequately adjust for potential confounders. Second, one-carbon moieties are usually not consumed independently of each other and of other nutrients in the diet, creating difficulties when studying the effect of a single moiety. Additionally, most studies were performed in healthy cohorts without major nutritional deficiencies. The effect of differences in one-carbon moieties could be limited within a normal range. Sample size of most included studies is relatively small and self-reported exposures like food questionnaires and vitamin intake could be less reliable. Differences in sampling methods and storage conditions of placental tissues could also lead to differences between studies. Lastly, the timing between exposure and measuring outcome may affect results. Placental DNA methylation is only studied postpartum in all included studies, and might not accurately reflect

methylation profiles at time of earlier exposures because of accumulating exposures during gestation also impacting DNA methylation profiles (57).

Clinical Implications and Future Research

Current studies investigating one-carbon metabolism and placental DNA methylation display low overlap in terms of studied exposures and genomic regions, and the number of high-quality intervention studies is limited. Therefore, high-quality intervention studies in combination with a genome-wide readout for DNA methylation and preferably randomized and blinded, are warranted to further investigate the impact of an optimized maternal one-carbon metabolism on DNA methylation profiles and health in the offspring. Ideally, studies will include follow-up after birth to assess the impacts on lifelong health.

Furthermore, DNA methylation is highly cell type specific. Investigating placental DNA methylation per cell type could therefore decrease heterogeneity both within and between studies. Moreover, placental tissue might not be an appropriate model for some research questions and other tissues or cell types could also be incorporated (58); however, tissues from internal organs are largely inaccessible and the placenta could provide insight in both placental function and fetal epigenetic programming. Generally, placental DNA methylation is only studied at one time point, i.e. after delivery. In the future, the presence of cfDNA originated from placental cell types derived from maternal blood might be an unique in vivo opportunity to noninvasively study placental DNA methylation profiles already during pregnancy (23). Moreover, cfDNA could potentially provide targets to improve future prenatal diagnosis and obstetrical care by identifying modifiable epigenetic derangements during pregnancy, contributing to improved health outcomes across the life course.

Conclusions

Most included studies indicate relationships between several measures of one-carbon moieties in the prenatal period and site-specific DNA methylation in the placenta rather than changes in global DNA methylation. Several gene-specific associations with DNA methylation were found, especially in genes involved in growth and metabolism. A deranged one-carbon metabolism leading to detrimental changes in the fetal epigenome in genes involved in for example the cardiovascular system may support the DOHaD paradigm. Changes in DNA methylation could not only lead to altered placental functions but could also serve as a proxy for fetal epigenetic programming, with both consequences for fetal development as well as future health outcomes.

On the other hand, conflicting outcomes were reported and there is a lack of uniformity between studies in geographically study populations, investigated loci, exposure measures, and study designs. Moreover, DNA methylation is only studied in postpartum placentas, though this may be overcome by investigating methylation of cfDNA in future studies. High-quality intervention studies tackling common limitations of included studies are needed to elucidate the relationship between one-carbon metabolism and specific differences in epigenetic programming in the offspring, ideally with longitudinal follow-up after birth. This could enable us to assess the corresponding impact on health outcomes across the life course and identify preventative or therapeutic measures contributing to improved health outcomes for future generations.

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Supplemental Material

Table S1: Used search strategies

Database	Searching strategies	Results (n)
Medline	(exp Vitamins/ OR exp Avitaminosis/ OR Homocysteine/ OR Life Style/ OR Diet/ OR "Diet, Food, and Nutrition"/ OR Nutritional Sciences/ OR Malnutrition/ OR Feeding Behavior/ OR Diet, Healthy/ OR exp Dietary Supplements / OR Antioxidants/ OR Metabolome/ OR Choline/ OR Maternal Nutritional Physiological Phenomena/ OR Oxidants/ OR Nutritional Status/ OR "Methylenetetrahydrofolate Reductase (NADPH2)"/ OR ((carbon ADJ3 metabol*) OR one-carbon OR 1-carbon OR vitamin* OR avitaminos* OR folic-acid* OR folate* OR homocystein* OR life-style* OR lifestyle* OR diet OR nutrition* OR malnutrition* OR nutrient* OR micronutrient* OR supplement* OR antioxidant* OR anti-oxidant* OR methylenetetrahydrofol* OR mthfr OR metabolom* OR cholin* OR ((parent* OR maternal* OR paternal* OR mother* OR father*) ADJ3 (intake* OR deficien* OR status)) OR (zinc ADJ3 (status* OR metabol* OR intake* OR deficien*)) OR (dietar* ADJ3 ratio*) OR cyanocobalamin* OR mecobalamin* OR methylcobalamin* OR cobalamin* OR cobamamid* OR hydroxocobalamin*).ab,ti.) AND (exp Placenta / OR exp Placenta Diseases / OR (placenta* OR Uteroplacenta*).ab,ti.) AND (DNA Methylation / OR Epigenomics / OR Epigenome / OR Histone Code / OR DNA Damage / OR Epigenesis, Genetic / OR (((DNA OR cfDNA OR cffDNA OR histone OR gene) ADJ6 (methylat* OR hypermethylat* OR hypomethylat* OR modificat* OR damage)) OR epigenetic* OR epigenom* OR methylome* OR (genetic* ADJ3 epigenes*)).ab,ti.) AND english.la.	699
Embase	('carbon metabolism'/de OR vitamin/exp OR homocysteine/de OR lifestyle/exp OR diet/de OR nutrition/de OR 'feeding behavior'/de OR 'healthy diet'/de OR 'unhealthy diet'/de OR supplementation/de OR 'vitamin supplementation'/de OR 'mineral supplementation'/de OR antioxidant/de OR metabolome/de OR choline/de OR 'maternal nutrition'/de OR 'nutritional deficiency'/exp OR 'oxidizing agent'/de OR 'diet supplementation'/de OR 'nutritional status'/de OR 'methylenetetrahydrofolate reductase (NADPH2)'/de OR 'zinc metabolism'/de OR 'zinc deficiency'/de OR 'zinc intake'/de OR ((carbon NEAR/3 metabol*) OR one-carbon OR 1-carbon OR vitamin* OR avitaminos* OR folic-acid* OR folate* OR homocystein* OR life-style* OR lifestyle* OR diet OR nutrition* OR malnutrition* OR nutrient* OR micronutrient* OR supplement* OR antioxidant* OR anti-oxidant* OR methylenetetrahydrofol* OR mthfr OR metabolom* OR cholin* OR ((parent* OR maternal* OR paternal* OR mother* OR father*) NEAR/3 (intake* OR deficien* OR status)) OR (zinc NEAR/3 (status* OR metabol* OR intake* OR deficien*)) OR (dietar* NEAR/3 ratio*) OR cyanocobalamin* OR mecobalamin* OR methylcobalamin* OR cobalamin* OR cobamamid* OR hydroxocobalamin*):Ab,ti) AND (placenta/exp OR 'placenta tissue'/de OR 'placenta function'/exp OR 'placenta disorder'/exp OR (placenta* OR Uteroplacenta*):Ab,ti) AND ('DNA methylation'/exp OR epigenetics/exp OR epigenome/exp OR 'histone modification'/exp OR 'DNA damage'/exp OR 'genetic epigenesis'/de OR (((DNA OR cfDNA OR cffDNA OR histone OR gene) NEAR/6 (methylat* OR hypermethylat* OR hypomethylat* OR modificat* OR damage)) OR epigenetic* OR epigenom* OR methylome* OR (genetic* NEAR/3 epigenes*)):Ab,ti) NOT ([conference abstract]/lim AND [2000-2019]/py) AND [english]/lim	1046
Web of Science	TS=((((carbon NEAR/2 metabol*) OR one-carbon OR 1-carbon OR vitamin* OR avitaminos* OR folic-acid* OR folate* OR homocystein* OR life-style* OR lifestyle* OR diet OR nutrition* OR malnutrition* OR nutrient* OR micronutrient* OR supplement* OR antioxidant* OR anti-oxidant* OR methylenetetrahydrofol* OR mthfr OR metabolom* OR cholin* OR ((parent* OR maternal* OR paternal* OR mother* OR father*) NEAR/2 (intake* OR deficien* OR status)) OR (zinc NEAR/2 (status* OR metabol* OR intake* OR deficien*)) OR (dietar* NEAR/2 ratio*) OR cyanocobalamin* OR mecobalamin* OR methylcobalamin* OR cobalamin* OR cobamamid* OR hydroxocobalamin*)) AND ((placenta* OR Uteroplacenta*)) AND ((((DNA OR cfDNA OR cffDNA OR histone OR gene) NEAR/5 (methylat* OR hypermethylat* OR hypomethylat* OR modificat* OR damage)) OR epigenetic* OR epigenom* OR methylome* OR (genetic* NEAR/2 epigenes*)))) AND DT=(article) AND LA=(english)	639
Cochrane CENTRAL Register of Controlled Trials	(((carbon NEAR/3 metabol*) OR one-carbon OR "1-carbon" OR vitamin* OR avitaminos* OR folic-acid* OR folate* OR homocystein* OR life-style* OR lifestyle* OR diet OR nutrition* OR malnutrition* OR nutrient* OR micronutrient* OR supplement* OR antioxidant* OR anti-oxidant* OR methylenetetrahydrofol* OR mthfr OR metabolom* OR cholin* OR ((parent* OR maternal* OR paternal* OR mother* OR father*) NEAR/3 (intake* OR deficien* OR status)) OR (zinc NEAR/3 (status* OR metabol* OR intake* OR deficien*)) OR (dietar* NEAR/3 ratio*) OR cyanocobalamin* OR mecobalamin* OR methylcobalamin* OR cobalamin* OR cobamamid* OR hydroxocobalamin*):Ab,ti) AND ((placenta* OR Uteroplacenta*):Ab,ti) AND ((((DNA OR cfDNA OR cffDNA OR histone OR gene) NEAR/6 (methylat* OR hypermethylat* OR hypomethylat* OR modificat* OR damage)) OR epigenetic* OR epigenom* OR methylome* OR (genetic* NEAR/3 epigenes*)):Ab,ti)	27
Google Scholar	"carbon metabolism"|"one-carbon"|vitamins|"folic-acid"|folate|homocystein|"life-style"|lifestyle|diet|nutrition|nutrients|supplements placenta "DNA|histone|gene methylation|hypermethylation|hypomethylation|modification|damage"|epigenetics|epigenomics	200

Table S2: ErasmusAGE quality score form for systematic reviews

Original: ErasmusAGE, 24 June 2013

This quality score can be used to assess the quality of studies included in systematic reviews and meta-analyses and is applicable to both interventional and observational studies. The score was designed based on previously published scoring systems (Carter et al, 2010 and the Quality Assessment Tool for Quantitative Studies). The quality score is composed of 5 items, and each item is allocated 0, 1 or 2 points. This allows a total score between 0 and 10 points, 10 representing the highest quality. The version presented below needs to be adapted for each review separately.

1. **Study design**
   0 for studies with cross-sectional data collection
   1 for studies with longitudinal data collection (both retrospective and prospective)
   2 for intervention studies

2. **Study size** (predefined)*
   0 small population for analysis (<50 patients)
   1 intermediate population for analysis (50-150 patients)
   2 large population for analysis (>150 patients)

3. **Exposure**
   Observational studies
   0 if the study used no appropriate exposure measurement method or if not reported
   1 if the study used moderate quality exposure measurement methods
   2 if the study used adequate exposure measurement methods

   Intervention studies
   0 if the intervention was not described or not blinded
   1 if the intervention was adequately single blinded.
   2 if the intervention was adequately double-blinded.

4. **Outcome**
   0 if the study used no appropriate outcome measurement method or if not reported
   1 if the study used moderate quality outcome measurement methods
   2 if the study used adequate outcome measurement methods

5. **Adjustments** †*
   0 if findings are not controlled for at least key confounders (fetal sex, gestational age)
   1 if findings are controlled for key confounders (fetal sex, gestational age)
   2 if findings are additionally controlled for additional covariates or when an intervention is adequately randomized


3

Chapter 3

First-trimester maternal folate and vitamin B12 concentrations and their associations with first-trimester placental growth: The Rotterdam Periconception Cohort

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Human Reproduction. 2025

Abstract

Study question: Are maternal folate and vitamin B12 concentrations associated with first trimester placental growth?

Summary answer: Maternal folate concentrations and commencement of folic acid supplements prior to conception, as compared to following conception, are positively associated with first trimester placental volume, whereas no associations were found for maternal vitamin B12 concentrations.

What is known already: Besides the protective effect of folic acid supplement use against neural tube defects and other adverse birth outcomes, the preconceptional commencement of folic acid supplements is positively associated with postpartum placental size, although conflicting outcomes have been reported. Studies in mice show an association with vitamin B12 deficiency and decreased placental weight postpartum.

Study design, size, duration: Between January 2010 and December 2020, 480 pregnancies (727 longitudinal ultrasound measurements) with known maternal folate and/or vitamin B12 blood concentrations in the first trimester and 875 pregnancies (1430 longitudinal ultrasound measurements) with known timing of folic acid supplement initiation were included in the Rotterdam Periconception Cohort, a prospective, hospital-based observational cohort.

Participants/materials, setting, methods: Red Blood Cell (RBC) folate and serum vitamin B12 concentrations were determined in first trimester maternal blood, and the timing of folic acid supplement use was collected using validated questionnaires. Placental volume (PV) was measured from serial three-dimensional (3D) ultrasounds performed at 7, 9 and 11 weeks of gestation. Linear mixed models were used to assess the associations between maternal folate and vitamin B12 concentrations with first trimester PVs. Analyses were adjusted for gestational age at ultrasound, maternal age, BMI, geographical background, education level, parity, smoking, mode of conception and the other B-vitamin. For validation, the association between the timing of folic acid supplement initiation (pre- or postconception) and PV was assessed.

Main results and the role of chance: The median RBC folate concentration was 1,395 nmol/l (IQR 1,169-1,588) and the median serum vitamin B12 concentration was 314 pmol/l (IQR 241-391). For RBC folate, the smallest PVs were found in women in the lowest quartile, with the largest difference as compared to women in the 4th quartile: ^{3}√PV (β = -0.141, 95% CI = -0.249 to -0.033, p=0.010), corresponding to a 1.79 cm^3 (-18.7%) and a 6.99 cm^3 (-9.9%) smaller PV at 7 and 11 weeks of gestation, respectively. Additionally, PV was significantly smaller in women who initiated folic acid supplements following rather than prior to conception: ^{3}√PV (β = -0.129, 95% CI = -0.207 to -0.051, p=0.001) corresponding to a 1.69 cm^3 (-16.9%) and a 6.62 cm^3 (-8.9%) smaller PV at 7 and 11 weeks of gestation, respectively. We found no significant association between maternal serum vitamin B12 concentrations and PV.

Limitations, reasons for caution: The observational design of this study does not exclude residual confounding, and our hospital-based study population, with mostly adequate RBC folate and serum vitamin B12 concentrations, could limit the generalizability of our results.

Wider implications of the findings: Our results emphasize the importance of the preconceptional commencement of folic acid supplements to achieve adequate maternal RBC folate concentrations, which could support optimal placental growth during the first trimester and also protect against neural tube defects and other adverse birth outcomes.

Introduction

Folate and vitamin B12 are critical nutrients involved in one-carbon metabolism. Through the provision of methyl donors, one-carbon metabolism plays an essential role in cellular processes such as DNA synthesis and DNA methylation (1). The periconceptional period is characterized by major epigenetic reprogramming, including the removal of most DNA methylation marks following conception, followed by a wave of remethylation in developing embryonic lineages (2-4). This influences both fetal and placental development, and necessitates an increased demand for methyl donors such as folate in pregnant women. Moreover, the use of periconceptional folic acid supplements reduces the prevalence of neural tube defects and is therefore recommended for women from preconception until at least the end of the first trimester (5).

A well-functioning placenta is essential for fetal growth and development, and placental dysfunction is involved in the pathogenesis of various obstetric complications (6). Although conflicting outcomes have been reported, several studies have found associations between low folate and vitamin B12 concentrations and adverse pregnancy outcomes such as preeclampsia and low birthweight (7-11). Folic acid has been shown to promote placental trophoblast invasion by affecting DNA methylation in placental tissues and cell lines (12, 13), and folate deficiency increases the apoptosis rate in human trophoblasts (14), while maternal vitamin B12 has been associated with the expression of angiogenic markers in the placenta (15). Additionally, folate and vitamin B12 are involved in the remethylation of homocysteine to methionine, and can subsequently decrease homocysteine concentrations (10, 16). Elevated homocysteine concentrations can impair endothelial function and have been associated with smaller first trimester placental growth in our cohort as well as in pregnancies with placental mediated complications (17, 18). Folate and vitamin B12 may thus also promote placental development by decreasing homocysteine concentrations.

A recent study showed a positive relationship between periconceptional folic acid use and measures of placental size postpartum but mixed results have been reported for placental weight (19, 20). Likewise, no association was found between maternal vitamin B12 concentrations and postpartum placental weight in humans (19), but vitamin B12 deficiency has been suggested to impair placental development (21) and decreased placental weight has been found in mice fed a vitamin B12 deficient diet (22). Previous studies have mainly investigated periconceptional folic acid use or first trimester maternal folate and vitamin B12 concentrations in relation to postpartum placental growth. However, currently placental growth can noninvasively be measured in the first trimester of pregnancy using three-

dimensional (3D) ultrasound. Studying periconceptional measures of folate/folic acid and vitamin B12 with first trimester placental growth is less biased by a timing difference in measuring the outcome and exposure. We hypothesize that lower maternal blood concentrations of both folate and vitamin B12 are negatively associated with early serial placental volume (PV) measurements as a feature of placental growth. Therefore, the aim of this study was to investigate, in the first trimester of pregnancy, the associations between maternal Red blood Cell (RBC) folate (an indicator of the long-term 2-4 months folate status) and serum vitamin B12 concentrations, and placental growth. For the purpose of validation, we also aimed to investigate the associations between first trimester maternal serum folate concentrations (an indicator of 1-3 days short-term folate status) and the timing of folic acid supplement initiation (pre- or postconception), and placental growth. The results may further indicate how to optimize placental development in the future.

Methods

Study population

All data were collected from the Rotterdam Periconceptional Cohort (Predict study). The Predict study is an ongoing prospective cohort study conducted at the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center, The Netherlands, since November 2010 (23, 24). Women aged ≥18 years with singleton pregnancies before 10 weeks of gestation were eligible for participation. Both parents signed a written informed consent form at enrollment.

Study parameters

Participant characteristics were obtained through validated self-reported questionnaires addressing topics including medical and obstetrical history, education and lifestyle behaviours, such as smoking, alcohol use and the use of folic acid. Dietary intake was obtained using a validated food frequency questionnaire (FFQ) in the first trimester, from which dietary folate intake was determined using the Dutch food composition table by Wageningen University (25). Weight and height were measured at study entry by a research nurse. For naturally conceived pregnancies in women with a regular cycle between 25 and 31 days, gestational age was dated based on their first day of their last menstrual period (LMP). In case of an irregular cycle or unknown LMP, pregnancy dating was based on the crown-rump length (CRL) measured at the 9th week ultrasound. If the gestational age calculated using the LMP differed by ≥6 days from that calculated based on the CRL, dating was based on the CRL. Pregnancies conceived after intrauterine insemination were dated based on the insemination date and were considered naturally conceived. The date of embryo transfer minus the number of days the embryo was

cultured, was used to determine the conception date used to date pregnancies following in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI).

Folate and vitamin B12

A non-fasting blood sample was taken at study inclusion during the first trimester. For pregnancies included between November 2010 and December 2016, maternal RBC folate and serum folate and vitamin B12 concentrations were determined. The maternal RBC folate concentration was calculated using an in house developed protocol in which the hemolysate folate was corrected for hematocrit and the serum folate concentration. Both hemolysate and serum folate as well as total vitamin B12 concentrations were determined using an electrochemiluminescent immuno assay (ECLIA) on a Cobas E801 analyser (Roche Diagnostics, Switzerland).

Placental volume measurement

The placental volume (PV) was measured at 7, 9 and 11 weeks of gestation using 3D ultrasonography to assess volumes of the whole pregnancy. These measurements were performed with standardized settings as described previously using a 6–12 MHz transvaginal probe compatible with the GE Voluson E8 Expert system (26). PV was measured using Virtual Organ Computer-aided AnaLysis (VOCAL) software as described previously (27). In short, the placental outline (Figure 1A) was repeatedly traced in a rotational step of 15° to calculate the total pregnancy volume. Similarly, the contours of the gestational sac (Figure 1B) were traced to establish the volume of the gestational sac, which was subsequently deducted from the total pregnancy volume to obtain the PV (Figure 1).

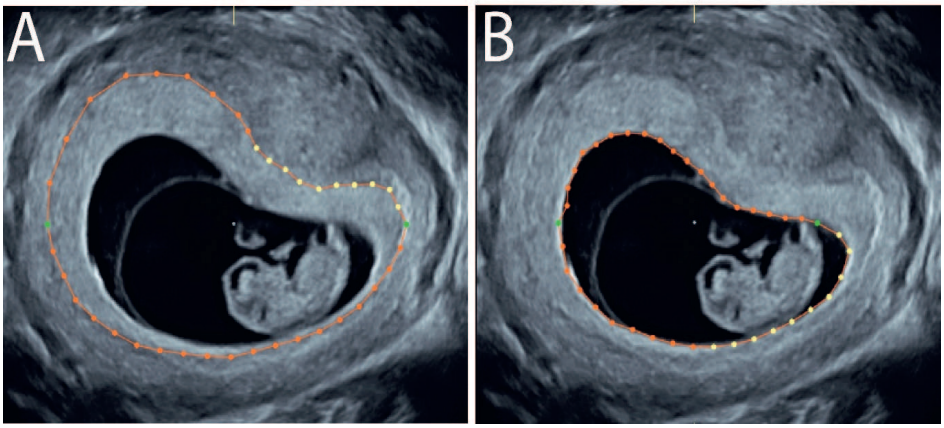


Figure 1: Measuring placental volume (PV) using 3D ultrasound volumes. The PV is calculated by the total pregnancy volume **(Figure A)** minus the volume of the gestational sac **(Figure B)**.

Statistical analysis

We excluded pregnancies in case of miscarriage, termination of pregnancy, intrauterine fetal death or study withdrawal. In cases where women participated in the Predict study across multiple pregnancies, only the first pregnancy was included in the analyses. Parental characteristics were described using medians and interquartile ranges for continuous variables, and percentages were used for categorical variables. Differences in baseline characteristics between women who initiated folic acid supplement use prior to conception and those who initiated folic acid supplement use following conception were assessed using the chi-square test for categorical variables, and the Mann-Whitney U test for continuous variables. Linear mixed models were used to assess the associations between maternal RBC folate and serum vitamin B12 concentrations and longitudinal measurements of PV. In addition to linear models, maternal folate and vitamin B12 concentrations were divided into quartiles and the lowest quartiles (Q1s) were used as a reference. Because of the skewed distribution of PV, a third root transformation was applied to the PV data.

For the linear mixed models investigating associations between maternal folate and vitamin B12 concentrations with PV, we first adjusted only for a cubic spline function of gestational age at the time of the ultrasound (model 1). In model 2, we additionally adjusted for fetal sex, parity, maternal age, maternal BMI, mode of conception, education level, geographical origin and periconceptional smoking status. For validation, we investigated the association between serum folate and PV. Additionally, we investigated the relationship between the timing of folic acid supplement initiation (pre- or postconception) and RBC folate concentrations using the Mann-Whitney U test, and we investigated the association between the timing of folic acid supplement initiation and PV using linear mixed models with the same covariates as used in model 1 and model 2. For the association between the timing of folic acid supplement initiation and PV, we performed a sensitivity analysis additionally adjusting for dietary folate intake obtained through the FFQ. Only women with a total daily energy intake above the Goldberg cut-off (28) were included, to exclude participants with unrealistic low intake of energy. Furthermore, we adjusted for RBC folate concentrations when analyzing the association with serum vitamin B12, and for serum vitamin B12 when studying the association with RBC or serum folate. P-values ≤0.05 were considered statistically significant and all analyses were performed using SPSS version 28.0.1.0 (142) (IBM SPSS Statistics, Armonk, NY) and R (Version 4.3.2).

Results

Study population

There were 1,003 pregnancies with available ultrasound data included in the Predict study between November 2010 and December 2020. We excluded 108 pregnancies prior to analysis because of: miscarriage or termination of pregnancy (n=27), study withdrawal (n=9), participation in multiple pregnancies (n=20), intrauterine fetal death (n=5), no available PV measurement (n=21), or no available RBC folate and serum vitamin B12 concentrations and unknown timing of folic acid supplement initiation (n=26); in case women participated in the Predict study during multiple pregnancies, only the first pregnancy was included (exclusion of n=20). Of the 895 remaining pregnancies, RBC folate concentrations were available for 429 pregnancies (642 PV measurements), serum vitamin B12 concentrations were available for 472 pregnancies (713 PV measurements), and the timing of folic acid supplement initiation was known for 875 pregnancies (1430 PV measurements) (Figure 2).

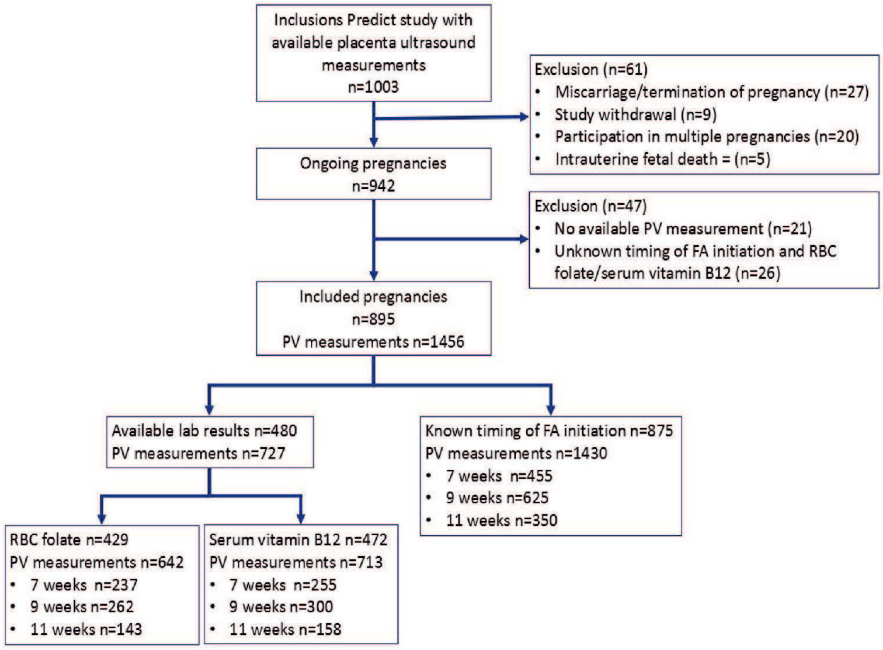


Figure 2: Flowchart of the included study population. RBC = red blood cell, PV = placental volume, FA = folic acid.

Periconceptional maternal characteristics

Baseline characteristics of included pregnancies with available RBC folate and/or serum vitamin B12 concentrations (n=480) are depicted in Table 1. Mean maternal age of this study population was 32.0 years (IQR 28.8 – 35.1) and mean periconceptional maternal BMI was 24.5 kg/m^2 (IQR 22.0 – 28.4). Our study population was predominantly of Dutch origin (79.6%) and highly

educated (54.4%). Around half (56.3%) were nulliparous and 63.5% of pregnancies were conceived spontaneously. During the periconceptional period, 16.9% of women smoked and 32.9% used alcohol. For 98.3% of the study population, it was known that they used folic acid supplements during pregnancy, and 80.6% of women initiated folic acid supplements prior to conception.

Table 1: Baseline characteristics of included pregnancies with available RBC folate and/or serum vitamin B12 concentrations (n=480). Data are presented as median [interquartile range (IQR)] or n (%).

	Study sample (n=480)
Age at conception	32.0 [28.8 – 35.1]
Geographical background	
Dutch	382 (79.6)
Western, other	26 (5.4)
Non-Western	58 (12.1)
Missing	*14 (2.9)*
Education level	
Low	36 (7.5)
Moderate	169 (35.2)
High	261 (54.4)
Missing	*14 (2.9)*
Nulliparous	270 (56.3)
Missing	*8 (1.7)*
Conception mode	
Spontaneous	305 (63.5)
IVF/ICSI	175 (36.5)
Body mass index (kg/m^2)	24.5 [22.0 – 28.4]
Missing	*3 (0.6)*
Periconceptional smoking	81 (16.9)
Missing	*11 (2.3)*
Periconceptional alcohol use	158 (32.9)
Missing	*11 (2.3)*
Folic acid supplement use	472 (98.3)
Missing	*8 (1.7)*
Of which preconceptional initiation of folic acid supplements	387 (80.6)
Missing	*20 (4.2)*

Baseline characteristics for women with known timing of folic acid supplement initiation (n=875) are depicted in Table 2 and are largely comparable. Women who initiated folic acid supplements prior to conception were older, more often of Dutch origin, more highly educated, more often nulliparous, conceived more often after IVF/ICSI treatment, and smoked less often, as compared to women who initiated folic acid supplements following conception (all $p<0.001$) (Table 2). Additionally, periconceptional maternal BMI was lower in women who initiated folic acid supplements prior to as compared to following conception (24.1 versus 25.5 kg/m^2, $p=0.002$). Dietary intake of folate assessed using the FFQ did not differ between groups.

Table 2: Baseline characteristics of included pregnancies based on timing of folic acid supplement initiation (n=875). Data are presented as median [interquartile range (IQR)] or n (%). *Significance at p ≤ 0.05 assessed by chi-square test or Mann-Whitney U test as appropriate. FA = folic acid. FFQ = Food Frequency Questionnaire.

	Total study population (n=875)	Initiated FA preconceptional (n=732)	Initiated FA postconceptional (n=143)	p-value
Age at conception	32.2 [29.1–35.5]	32.5 [29.3-35.7]	30.1 [27.4 – 34.8]	<0.001*
Geographical background				<0.001*
Dutch	715 (81.7)	614 (83.9)	101 (70.6)	
Western, other	47 (5.4)	38 (5.2)	9 (6.3)	
Non-Western	112 (12.8)	80 (10.9)	32 (22.4)	
Missing	*1*	*0*	*1*	
Education level				
Low	65 (7.4)	40 (5.5)	25 (17.5)	<0.001*
Moderate	306 (35.0)	247 (33.7)	59 (41.3)	
High	502 (57.4)	443 (60.5)	59 (41.3)	
Missing	*2*	*2*	*0*	
Nulliparous	503 (57.5)	452 (61.7)	51 (35.7)	<0.001*
Missing	*11*	*10*	*1*	
Conception mode				
Spontaneous	497 (56.8)	364 (49.7)	133 (93.0)	<0.001*
IVF/ICSI	378 (43.2)	368 (50.3)	10 (7.0)	
Body mass index	24.2 [22.1 – 28.0]	24.1 [22.0-27.6]	25.5 [22.2-30.9]	0.002*
Missing	*3*	*1*	*2*	
Periconceptional smoking	130 (14.9)	88 (12.0)	42 (29.4)	<0.001*
Missing	*1*	*1*	*0*	
Periconceptional alcohol use	270 (30.9)	219 (29.9)	51 (35.7)	0.177
Missing	*1*	*1*	*0*	
Dietary folate intake (µg/day) (FFQ)	*262 [211-324]*	*263 [215-324]*	*255 [198-322]*	0.306
Unknown or below Goldberg cut-off	*426*	*347*	*79*	

Maternal folate and vitamin B12 concentrations and placental volumes

The median RBC folate concentration was 1,395 nmol/l (IQR 1,169 – 1,588), and the median serum vitamin B12 concentration was 314 pmol/l (IQR 241 – 391) (Table 3). In our linear models, there were no significant associations between RBC folate or serum vitamin B12 concentrations and PV (Table 4). However, the quartile analyses show that for RBC folate, women in the lowest quartile had the smallest PVs (Table 5). Compared to women in the lowest quartile, PV was significantly larger in women with RBC folate concentrations in the second quartile or in the highest quartile in model 1 (Q2 $^3\sqrt{PV}$: β = 0.137 (95% CI 0.041 – 0.233), p=0.005); Q4 $^3\sqrt{PV}$: β = 0.126 (95% CI 0.029 – 0.223), p=0.011). Comparable results were found after adjustment for potential confounders in model 2 (Q2 $^3\sqrt{PV}$: β = 0.129 (95% CI 0.027 – 0.232), p=0.014); Q4 $^3\sqrt{PV}$: β = 0.141 (95% CI 0.033 – 0.249), p=0.010). For women with RBC folate concentrations in Q3 as compared to Q1, positive effect estimates were found for PV, although these were not statistically significant (model 1 $^3\sqrt{PV}$: β = 0.071 (95% CI -0.025 – 0.167), p=0.147); model 2 $^3\sqrt{PV}$: β = 0.088 (95% CI -0.019 – 0.195), p=0.107).

For maternal serum folate concentrations, an indicator of short-term folate status, we again found the smallest PVs in women with the lowest serum folate concentrations. However, no significant associations were found for women with serum folate concentrations in Q2 or Q4 as compared to Q1 (Table S1). However, compared to women with serum folate concentrations in the lowest quartile, PV was significantly higher in women with serum folate concentrations in the third quartile (model 1 $\sqrt[3]{PV}$: β = 0.104 (95% CI 0.010 – 0.198), p=0.030); model 2 $\sqrt[3]{PV}$: β = 0.132 (95% CI 0.027 – 0.237, p=0.014). No significant associations were found between maternal serum vitamin B12 concentrations and PV (Table 5).

Table 3: First trimester maternal red blood cell (RBC) folate and serum vitamin B12 concentrations in quartiles.

	RBC folate (nmol/l) (n=429)	Serum vitamin B12 (pmol/l) (n=472)
Q1	449 – 1169 (n=108)	109 – 241 (n=118)
Q2	1170 – 1395 (n=107)	242 – 313 (n=118)
Q3	1396 – 1588 (n=107)	314 – 389 (n=117)
Q4	1589 – 2919 (n=107)	390 – 897 (n=119)

Table 4: Effect estimates for associations between first trimester maternal Red Blood Cell (RBC) folate and serum vitamin B12 concentrations and first trimester placental volumes ($PV\ \sqrt[3]{cm^3}$). Model 1 is adjusted for gestational age at the ultrasound. Model 2 is additionally adjusted for fetal sex and maternal covariates age, geographic origin, education, periconceptional BMI, mode of conception, parity and the other B-vitamin.

	RBC folate				Serum vitamin B12			
	Model 1		Model 2		Model 1		Model 2	
	Bèta (95% CI) (10^{-3})	p-value	Bèta (95% CI) (10^{-3})	p-value	Bèta (95% CI) (10^{-3})	p-value	Bèta (95% CI) (10^{-3})	p-value
Continuous	0.0901 (-0.0639 – 0.1866)	0.067	0.0948 (-0.0106 – 0.2002)	0.078	0.0861 (-0.1769 – 0.3491)	0.520	0.1102 (-0.1783 – 0.3987)	0.453

Table 5: Effect estimates for associations between first trimester maternal Red Blood Cell (RBC) folate and serum vitamin B12 concentrations and first trimester placental volumes ($PV\ \sqrt[3]{cm^3}$). Quartiles 1 are taken as reference. Model 1 is adjusted for gestational age at the ultrasound. Model 2 is additionally adjusted for fetal sex and maternal covariates age, geographic origin, education, periconceptional BMI, mode of conception, parity and the other B-vitamin.

	RBC folate				Serum vitamin B12			
	Model 1		Model 2		Model 1		Model 2	
	Bèta (95% CI)	p-value	Bèta (95% CI)	p-value	Bèta (95% CI)	p-value	Bèta (95% CI)	p-value
Q1	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*	*Reference*
Q2	0.137 (0.041 – 0.233)	0.005*	0.129 (0.027 – 0.232)	0.014*	-0.034 (-0.125 – 0.057)	0.461	-0.053 (-0.154 – 0.049)	0.308
Q3	0.071 (-0.025 – 0.167)	0.147	0.0879 (-0.019 – 0.195)	0.107	0.050 (-0.042 – 0.142)	0.282	0.011 (-0.093 – 0.116)	0.831
Q4	0.126 (0.029 – 0.223)	0.011*	0.1411 (0.033 – 0.249)	0.010*	0.033 (-0.058 – 0.124)	0.482	0.056 (-0.047 – 0.158)	0.286

Timing of folic acid supplement use initiation

We assumed higher RBC folate concentrations in women who initiated the use of folic acid supplements prior to conception, compared to following conception. As expected, in our study population with known RBC folate concentrations, higher RBC folate concentrations were found in women who initiated folic acid supplement use prior to conception than in women who initiated folic acid supplement use following conception (1,458 vs 1,047 nmol/l, p<0.001) (Figure 3). Therefore, as a validation of the observed results for RBC folate concentrations, we investigated the association between the timing of folic acid supplement initiation (pre- or post-conception) and PV in a larger cohort (n=875).

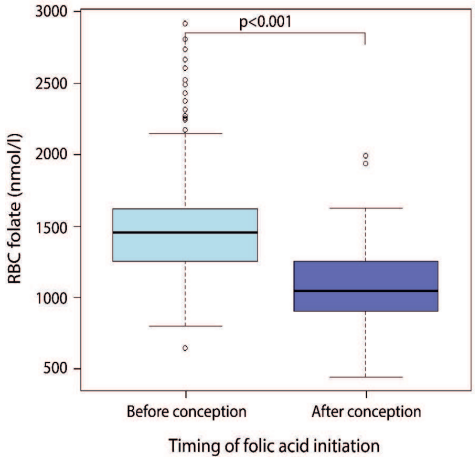


Figure 3: Red blood cell (RBC) folate (nmol/l) by timing of folic acid supplement initiation.

As for lower RBC folate concentrations, PV was significantly smaller in women who initiated the use of folic acid supplements following conception, as compared to those who initiated folic acid supplements prior to conception (model 1 $^3\sqrt{PV}$: β = -0.117 (95% CI -0.185 – -0.049), p=0.0007; model 2 $^3\sqrt{PV}$: β = -0.129 (95% CI -0.207 – -0.051), p=0.0012). After additionally adjusting for dietary folate intake in a subset of pregnancies with reported energy intake (FFQ) above the Goldberg cut-off (n= 449, PVs = 735), comparable results were found: $^3\sqrt{PV}$: β = -0.124 (95% CI -0.233 – -0.014), p=0.027.

Retransformation

Retransformation of the effect estimates to the original scale shows that in women with RBC folate concentrations in Q1 as compared to Q4, PV was 1.78 cm^3 (-18.7%) smaller at 7 weeks of gestation and 6.99 cm^3 (-9.9%) smaller at 11 weeks of gestation in the fully adjusted model (model 2). For women who initiated folic acid supplements following conception, as compared to preconception, PV was 1.69 cm^3 (-16.9%) and 6.62 cm^3 (-8.9%) smaller at 7 and 11 weeks of

gestation, respectively (model 2). Corresponding effect plots are shown in Figure 4. Additionally, effect plots for the whole study population including individual data points by maternal RBC folate quartile and by timing of initiation of folic acid supplement use are depicted in Supplemental Figure S1.

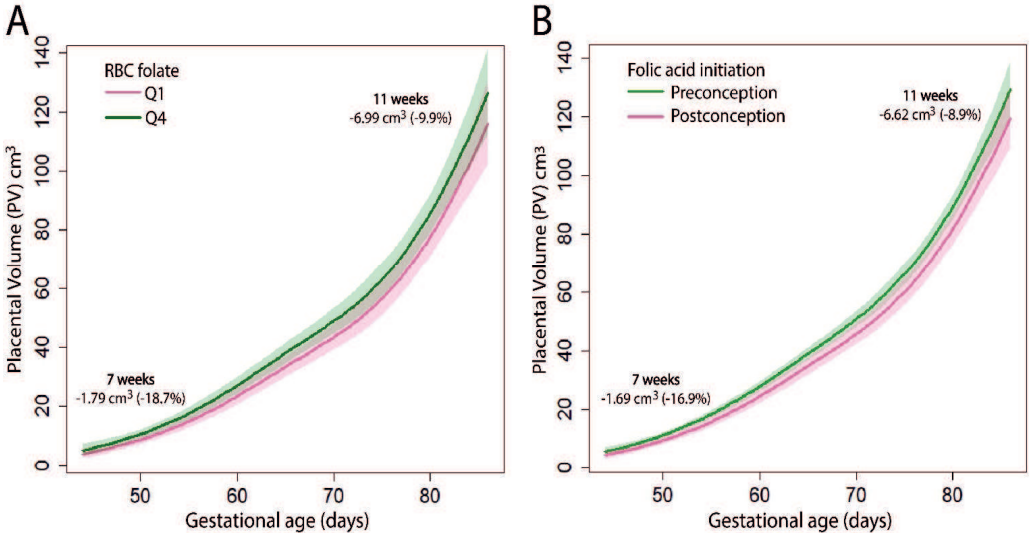


Figure 4: Effect plots showing the placental volume (PV) trajectories during the first trimester of pregnancy. (A) Red blood cell (RBC) folate Quartile 1 and Quartile 4; **(B)** Women who initiated the use of folic acid supplements prior to or following conception. Effect plots are based on model 2.

Discussion

This study is the first to investigate the associations between maternal folate and vitamin B12 concentrations and first trimester placental growth. We found smaller first trimester placental volumes in pregnancies with the lowest maternal RBC folate concentrations. PV was 18.7% and 9.9% smaller at 7 and 11 weeks of gestation, respectively, in women in the lowest quartile compared to women in the highest quartile. These effect sizes were comparable to the smaller PVs found in women who initiated folic acid supplements following conception compared to women who initiated folic acid supplement use prior to conception. No significant associations were found between serum vitamin B12 concentrations and PV, in our population with mostly adequate vitamin B12 concentrations. These results support the role of folate in placental growth during early pregnancy.

Our results are largely in line with other studies reporting 1-12% differences in placental weight or growth measured after birth, in association with maternal folate concentrations or the timing of folic acid supplement use (19, 29-31). Especially considering the relative diminishing

difference we observed over time, between 7 and 11 weeks of gestation, which could indicate an increase in the effect of other (maternal) factors on placental growth across gestation. In contrast, a systematic review reported no significant relationship between folic acid supplement use and placental weight; however, this might be due to the small sample size included in this systematic review and difficulties in measuring placental weight in an uniform manner (20). In mice, maternal dietary folate has been shown to increase placental weight (32, 33). Another study found inhibited signalling of mTORC1 and mTORC2, stimulators of placental amino acid transport, in folate deficient mice. This resulted in altered placental nutrient transport and lower fetal birth weight, but they reported no difference in placental weight (34).

The positive associations between maternal folate concentrations and early folic acid supplementation with first trimester placental growth may be explained by the key role of folate in one-carbon metabolism. In one-carbon metabolism, folate provides methyl donors required for DNA synthesis and epigenetic processes including DNA and histone methylation (1). For example, several studies have shown associations between measures of folate or folic acid and human placental DNA methylation of specific genes involved in growth and development (35). Also, in in vitro studies, folic acid has been shown to affect DNA methylation, leading to an increased invasive potential of trophoblasts (12), and the apoptosis rate of human trophoblasts was higher when cultured without folate possibly due to impaired DNA synthesis and repair required for cell replication (14). Moreover, pregnancy (especially the first trimester) is characterized by epigenetic reprogramming and rapid cell replication and proliferation leading to an increased demand for methyl groups. Therefore, this period is considered a particularly vulnerable time span for epigenetic disruptions that can impair placental and fetal growth and development, and even health outcomes later in life (3).

Another potential underlying biological mechanism explaining the positive association between folate/folic acid and first trimester placental growth could be the role of folate in the remethylation of homocysteine in the one-carbon metabolism (10, 16). Higher plasma concentrations of maternal homocysteine have in turn been associated with smaller first trimester placentas in our cohort (18) and with decreased placental weight postpartum by others (19). Moreover, hyperhomocysteinemia is associated with placental mediated complications such as preeclampsia (17). Elevated homocysteine concentrations are associated with oxidative stress, leading to impaired endothelial function which can negatively affect placentation and placental growth (17, 36). Folate may thus be protective against these processes by decreasing homocysteine concentrations and, as such, promote placental growth.

Serum folate concentrations are generally positively correlated with RBC folate concentrations (37) and are more often used in clinical settings, as they are easier to measure. However, RBC folate is a more reliable indicator of long-term folate status, while serum folate is more sensitive to recent folate intake (1-3 days), which likely has a smaller impact on overall placental growth. This may explain the less evident relationship found between serum folate and PV, with only significant larger PVs in women with Q3 as compared to Q1 serum folate concentrations.

We found no significant association between maternal serum vitamin B12 concentrations and placental growth. This is in line with another study in humans, which found no association between maternal vitamin B12 concentrations and postpartum placental weight (19). In contrast, smaller placentas have been found in vitamin B12 deficient mice (22). However, vitamin B12 concentrations were adequate for the majority of the participants in our study population. Therefore, our results suggest that vitamin B12 concentrations do not affect first trimester placental growth when vitamin B12 concentrations are sufficient. It would be of interest to investigate the relationship between vitamin B12 and placental growth in a population with a high prevalence of vitamin B12 deficiency. Notably, vitamin B12 deficiency could induce a functional folate deficiency, while folate concentrations could be normal or even high, which is referred to as the methyl-folate trap. This 'trap' is caused if the conversion from 5-methyl-tetrahydrofolate into tetrahydrofolate, in which vitamin B12 acts as cofactor, is hampered (38). In mice, oversupplementation with folic acid in vitamin B12 deficient mice, still resulted in smaller placental and fetal weights, which could be explained by a functional folate deficiency (22). This should be taken into account when comparing our results to populations with high rates of vitamin B12 deficiency, and supports the need for further research on the associations between low vitamin B12 concentrations and placental development.

Importantly, excessive folic acid intake may also pose potential risks. Several studies have observed neurodevelopmental changes in the offspring of mice oversupplemented with folic acid. In humans, conflicting outcomes have been reported, although most studies have found positive associations with neurodevelopment in the offspring (reviewed in (39)). In relation to placental weight, excessive supplementation of folic acid has been associated with a lower placental weight in rats (40) and this trend was also observed in mice (41). Generally, the doses used in these studies largely exceeded the recommended daily intake of folate and were not representative of our study population. A previous study estimated that RBC folate concentrations above 1300 nmol/L had little additional benefit for the prevention of neural tube defects (42). This supports the detrimental effects specifically of a folate deficiency, comparable to our results showing smaller placental volumes particularly in women with the

lowest folate concentrations, while caution is required to prevent oversupplementation in humans.

Optimal first trimester placental growth is not defined and a larger first trimester placenta does not necessarily indicate a better functioning placenta. Yet, first trimester placental growth is positively associated with embryonic and fetal growth (26). Whether a larger placenta results in a larger embryo or whether their growths are independent of each other during the first trimester (largely before the feto-maternal circulation is established after unplugging of the spiral arteries), still remains to be elucidated. However, smaller first trimester placental growth has been associated with several obstetric complications (43, 44), indicative of the positive effects of a larger first trimester placental volume.

The strengths of our study include early measurements of PV using 3D ultrasounds enabling us to study the associations between first trimester maternal folate and vitamin B12 concentrations directly with first trimester growth, which has not been undertaken previously. We validated our results for RBC folate concentrations with serum folate concentrations, and in a larger cohort by investigating the association between the timing of folic acid supplement initiation and PV. These results demonstrate the robustness of our results. Limitations of our study are that, despite correcting for several confounders, potential residual confounding cannot be excluded because of the observational nature of this study. Also, the study was performed in a hospital-based setting, explaining the relatively high number of pregnancies conceived after IVF/ICSI and the high rates of preconceptional folic acid use. Moreover, vitamin B12 concentrations were adequate for the vast majority of our study population. Therefore, it would be of interest to investigate the studied associations in a population-based cohort as well as in populations with a high prevalence of vitamin B12 and/or folate deficiencies.

Conclusions

We observed smaller first trimester placental volumes particularly in women with the lowest RBC folate concentrations. As a validation, we show comparable results with smaller placental volumes in women with the lowest serum folate concentrations, and in women who initiated folic acid supplements following as compared to prior to conception. In our study population with mostly adequate vitamin B12 serum concentrations, we found no associations between serum vitamin B12 concentrations and first trimester placental growth. Our study emphasizes the importance of the preconceptional initiation of folic acid supplements to acquire adequate maternal folate concentrations, which can support placental growth during the first trimester.

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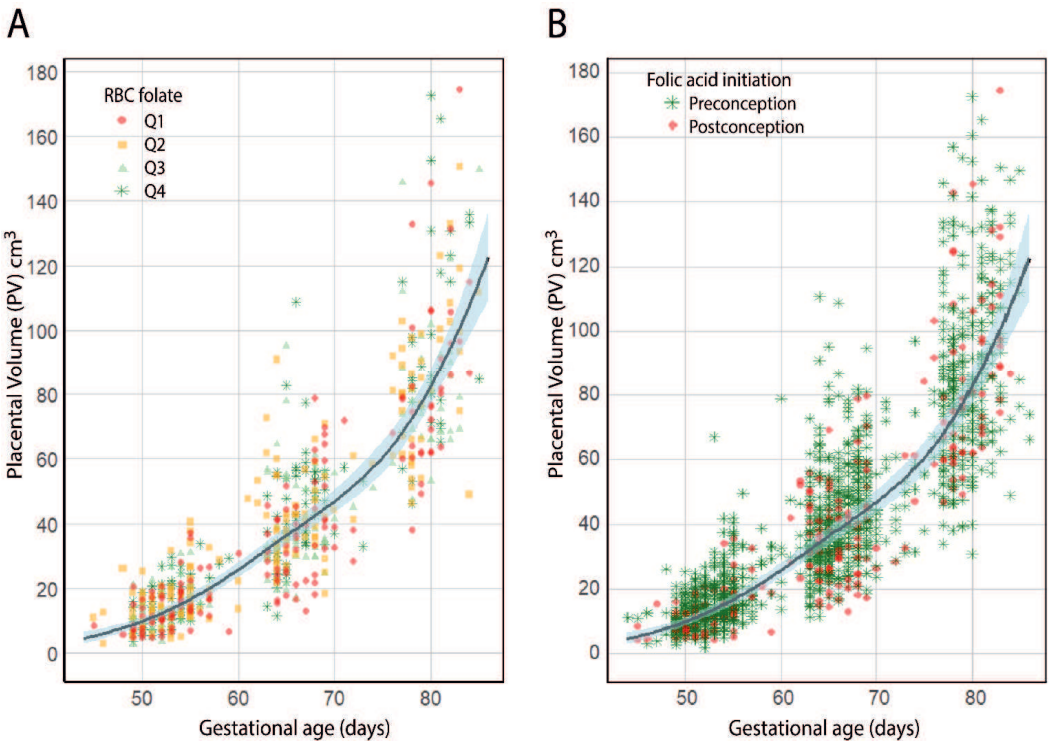
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Supplemental Material

Table S1: Effect estimates for associations between first trimester maternal serum folate concentrations and first trimester placental volumes ($PV\ \sqrt[3]{cm^3}$). Quartile 1 is taken as reference. Model 1 is adjusted for gestational age at the ultrasound. Model 2 is additionally adjusted for fetal sex and maternal covariates age, geographic origin, education, periconceptional BMI, mode of conception, parity and first trimester vitamin B12 concentration.

	Serum folate			
	Model 1		Model 2	
	Bèta (95% CI)	p-value	Bèta (95% CI)	p-value
Q1 (11.0 – 32.7 nmol/l) (n=116)	*Reference*	*Reference*	*Reference*	*Reference*
Q2 (32.8 – 39.2 nmol/l) (n=117)	0.069 (-0.023 – 0.162)	0.142	0.071 (-0.028 – 0.170)	0.161
Q3 (39.3 – 45.3 nmol/l) (n=113)	0.104 (0.010 – 0.198)	0.030*	0.132 (0.027 – 0.237)	0.014*
Q4 (45.4 – 141.6 nmol/l) (n=114)	0.088 (-0.006 – 0.182)	0.066	0.082 (-0.023 – 0.187)	0.127



Supplemental Figure S1: **Effect plots showing the placental volume (PV) trajectories during the first trimester of pregnancy for the total study population. (A)** Individual data points based on red blood cell (RBC) folate Quartiles; **(B)** Individual data points based on timing of folic acid supplement use initiation. Effect plots are based on model 2.


4

Chapter 4

Paternal age and first trimester placental size and growth: The Rotterdam Periconceptional Cohort

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Abstract

Introduction: Despite a noticeable trend of delayed fatherhood, less is known about the impact of paternal age on the paternally programmed placenta. We hypothesize that paternal aging affects seminal quality and as such induces ageing-related epigenetic alterations that influence placental growth. Our main aim is to investigate associations between paternal age and first trimester (vascular) placental growth trajectories.

Methods: Pregnant women were enrolled before 10 weeks of gestation in the Rotterdam Periconceptional Cohort (Predict study). Placental volumes (PV) and utero-placental vascular volumes (uPVV) were measured at 7, 9 and 11 weeks gestation. Associations between paternal age and PV and uPVV were investigated using linear mixed models and the maximum likelihood ratio test to test non-linear relationships. We adjusted for gestational age, fetal sex, parental smoking and maternal age, BMI, education and parity, and stratified for conception mode.

Results: From 808 pregnancies we obtained 1,313 PV and from 183 pregnancies 345 uPVV measurements. We show no associations between paternal age and PV (p=0.934) and uPVV (p=0.489) in our total population or in pregnancies conceived naturally (PV p=0.166; uPVV p=0.446) and after IVF/ICSI (PV p=0.909; uPVV p=0.749). For example, PV was 0.9% smaller (95% CI -5.7%—7.1%) in fathers aged 40 compared to 30 years old at 9 weeks gestation in the total study population.

Discussion: We are not demonstrating a significant impact of paternal age on first trimester placental growth in a tertiary care population. Given the trend of increasing paternal age, our study should be repeated in the general population.

Introduction

Appropriate placental growth and development are essential for a healthy pregnancy and both underweight and overweight placentas are associated with unfavorable pregnancy outcomes (1, 2). While developed countries have witnessed a noticeable trend of delayed parenthood (3), pregnancies of older women tend to have a higher placental weight and placental weight-to-birth weight ratio (4). On the other hand, advanced maternal age is also associated with increased risks of pregnancy complications, such as miscarriage, hypertensive disorders of pregnancy (HDP) and fetal growth restriction, outcomes that are associated with lower placental weights (5-10). Although paternal age is also increasing, limited and conflicting data are available on the associations between paternal age and placental growth or pregnancy outcomes. A small increase in placental weight and placental weight-to-birth weight ratio has been reported among older fathers (>50 years old) (11), while a lower placental weight and placental weight-to-birth weight ratio were found in mice with advanced paternal age (12). Additionally, some studies found no associations between paternal age and low birth weight (13, 14) or HDP (14, 15), while others reported so-called U-shaped relationships with increased risks for these adverse outcomes in pregnancies with both younger (<25 years) or older (45+ years) fathers (15-20).

Epigenetic modifications are suggested as a potential mechanism between the potential impact of paternal conditions and placental growth and function (21). Epigenetic mechanisms such as DNA methylation can alter gene expression and play a crucial role in various biological processes, including cell differentiation and genomic imprinting (22). Overall, paternally expressed imprinted genes are particularly expressed in the placenta and largely stimulate placental growth (23, 24). Since age-related DNA methylation differences are observed in sperm cells, aging-induced epigenetic alterations are hypothesized to result in abnormal gene expression and could consequently affect placental growth and pregnancy outcomes (25, 26).

Placental growth is typically only studied after delivery by measuring placental weight. However, innovative 3D ultrasound techniques using V-scope software enable the in vivo assessment of utero-placental (vascular) development during pregnancy already in the first trimester of pregnancy (27, 28). We hypothesize that smaller placental (vascular) growth occurs in pregnancies conceived by older fathers, because of potential aging-induced epigenetic alterations leading to dysregulation of paternally expressed genes which generally promote placental growth (23-25). This would be in line with the previous study conducted in mice which took place in a well-controlled setting (12). We further hypothesize that paternal age has the largest measurable effect very early in pregnancy because of accumulating effects of (maternal)

conditions on placental growth across pregnancy. Therefore, our aim is to investigate associations between paternal age and features of first trimester (vascular) placental growth. A second aim is to investigate associations between paternal age and placental-related complications including HDP and small for gestational age (SGA) offspring.

Methods

Study population

All data were collected in the Rotterdam Periconceptional Cohort (Predict study), an ongoing prospective tertiary hospital-based birth cohort conducted at the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center, The Netherlands (29, 30). Women of at least 18 years old with a singleton pregnancy before 10 weeks of gestation were eligible for participation. For this study, participants were recruited from November 2010 till December 2020. The VIRTUAL placenta study was embedded as subcohort between January 2017 to March 2018 and had the same eligibility criteria as the Predict study. In our analyses, we excluded pregnancies in case of miscarriage, intrauterine fetal death, known fetal congenital anomalies, twins, gamete donation, pregnancies after the transfer of a cryopreserved embryo >1 year old, unknown paternal age or in case of study withdrawal. Both parents signed a written informed consent form at enrollment.

Study parameters

Maternal and paternal characteristics were obtained through validated self-reported questionnaires. Weight and height were measured at study entry. Naturally conceived pregnancies in women with a regular cycle between 25 and 31 days were dated based on their first day of their last menstrual period (LMP). For women with irregular cycles or unknown LMP, the dating was based on the crown-rump length (CRL) measured at the 9th week ultrasound. If the gestational age based on LMP differed ≥6 days from the gestational age as calculated by the CRL, dating was based on the CRL. Pregnancies conceived through ovulation induction or intrauterine insemination (IUI) were considered naturally conceived. IUI pregnancies were dated based on insemination date. In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) pregnancies were dated based on conception date (29, 30). For IVF/ICSI pregnancies, paternal age was based on the date of sperm retrieval or ejaculation date where appropriate.

Ultrasound variables

Placental volume (PV) was measured in both the Predict study and the Virtual Placenta Study, while the utero-placental vascular volume (uPVV) was measured only in this subcohort (29).

Measurement of PV and uPVV have extensively been described previously and have good to excellent intra- and inter-observer agreement (27, 28). In short, PV and uPVV were measured using internal 3D ultrasound volumes of the whole pregnancy with standardized settings, where 3D power Doppler ultrasound was used to specifically visualize the uPVV. All measurements were performed in the 7th, 9th and 11th week of gestation by using a 6-12 MHz transvaginal probe compatible with the GE Voluson E8 Expert system.

Afterwards, PV was measured offline by using Virtual Organ Computer-aided AnaLysis (VOCAL). Using the VOCAL algorithm, twelve segments of the trophoblast were made using a rotational step of 15°. Total pregnancy volume and the volume of the gestational sac were manually measured for all twelve sections. PV was calculated as total pregnancy volume minus the volume of the gestational sac. The uPVV was measured offline using a Virtual Reality (VR) desktop system. This projects the ultrasound datasets as a hologram and allows for accurate removal of embryonic and myometrial blood vessels. The uPVV is the volume of the remaining blood vessels up to the utero-placental border. Quality of the images was scored and volumes rated as insufficient quality due to, for example, movement artefacts, acoustic shadowing or incompleteness, were excluded before analysis (27).

Pregnancy outcome variables

All pregnancy outcomes, i.e., birth weight, fetal sex, date of delivery, pregnancy induced hypertension (PIH) and preeclampsia (PE) were retrieved from medical records. PIH was defined as newly onset systolic blood pressure above 140 mmHg and/or a diastolic blood pressure above 90 mmHg (31). PE was defined as newly onset hypertension after 20 weeks of gestation and the presence of proteinuria (>300mg) in 24 hours urine (32). We defined small for gestational age (SGA) as birth weight percentile <p10 according to Hoftiezer birthweight charts (33).

Statistical analysis

We stratified the analysis according to mode of conception into pregnancies naturally conceived and after IVF/ICSI treatment, since assisted reproductive technologies have been reported to affect placental weight and parental age is associated with use of fertility treatment (34). Baseline characteristics between groups were compared using the Mann-Whitney U test for continuous variables and the chi-square test for categorical variables.

Linear mixed models were used to assess associations between paternal age and repeated measurements of PV and uPVV. Because of the skewed distributions of PV and uPVV, we used

a cubic root transformation to obtain linearity and normality of residuals and random effects. The likelihood ratio test was used to compare between models without paternal age and paternal age plus paternal age^2 to account for potential non-linear or U-shaped relationships. An interaction term containing paternal age and gestational age at the moment of the ultrasound was included since we hypothesized that the effect of paternal age on placental growth trajectories differs over gestation, with the largest effect very early in pregnancy because of accumulating effects of other (maternal) conditions and exposures on placental growth over gestation.

In model 1 we adjusted for gestational age at the moment of the ultrasound and model 2 was additionally adjusted for parity, fetal sex, periconceptional parental smoking, and maternal age, BMI, and education based on previous literature and insight of correlations between the covariates. Since first trimester placental growth is associated with adverse pregnancy outcomes, a sensitivity analysis was performed excluding pregnancies complicated by PE, PIH and SGA (9, 10, 28).

The associations between paternal age and HDP and SGA were assessed using logistic regression analyses. We made a crude model (model 1) and a multivariable model adjusted for above mentioned covariates and mode of conception (model 2). When investigating SGA, we did not adjust for fetal sex since Hoftiezer birth weight charts are sex-specific. P-values ≤0.05 were considered statistically significant and all analyses were performed using SPSS package 28.0 (IBM SPSS Statistics, Armonk, NY) and R (Version 4.2.1).

Results

Baseline

We included 2,051 first trimester pregnancies in the Predict study between November 2010 and December 2020. We excluded 526 pregnancies prior to analysis because of miscarriage or termination of pregnancy (TOP) (n=175), unknown paternal age (n=95), pregnancy after the transfer of a cryopreserved embryo >1 year old (n=35), twin pregnancies (n=66), fetal congenital malformations (n=54), gamete donation (n=46), study withdrawal (drop-outs) (n=39) and intrauterine fetal deaths (IUFD) (n=16). Of the 1,525 remaining pregnancies, 825 had at least one PV or uPVV measurement available with a total of 1,313 PV and 345 uPVV measurements. For our secondary outcomes, medical reports were available for 1,449 of 1,525 included pregnancies (Figure 1).

Baseline characteristics of couples with available ultrasound data and available medical reports are depicted in Table 1 and S1, respectively. Of couples with available ultrasounds, 475 pregnancies were naturally conceived and 350 after IVF/ICSI. Compared to naturally conceived pregnancies, the median age at conception was slightly higher for pregnancies conceived after IVF/ICSI for both men (34.9 vs 33.3 years, p<0.001) and women (33.0 vs 31.2 years, p<0.001), fathers had a slightly higher BMI (26.7 kg/m^2 vs 25.7 kg/m^2, p = 0.003) and women were more often nulliparous (73.4% vs 45.3%, p<0.001), started with folic acid supplements more often before conception (94.0% vs 72.0%, p<0.001) and were less likely to consume alcohol in the periconceptional period (22.0% vs 37.3%, p<0.001).

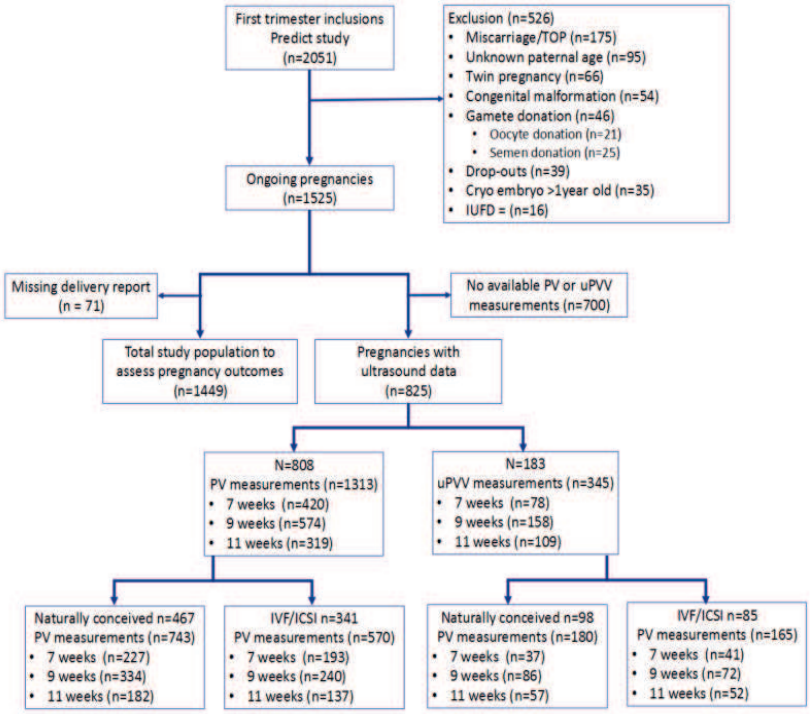


Figure 1: Flowchart of total study population. TOP = termination of pregnancy, IUFD = intra-uterine fetal death, PV = placental volume, uPVV = utero-placental vascular volume

In the total study population, we found no significant associations between paternal age and PV in model 1 (p=0.742) and model 2 (p=0.934). After stratification for mode of conception, we also found no significant association for naturally conceived pregnancies (model 1 p=0.179; model 2 p=0.166) and for pregnancies conceived after IVF/ICSI (model 1 p=0.101; model 2 p=0.909). To visualize the relationship between paternal age and PV, effect plots are shown for model 2 for the total study population and stratified for mode of conception at a gestational age of 7 weeks, 9 weeks and 11 weeks (Figure 2A-C). In the total study population and in IVF/ICSI

Table 1. Baseline characteristics of included couples with available ultrasound data stratified for mode of conception. Data are presented as median [interquartile range (IQR)] or n (%). *Significance at p ≤ 0.05 assessed by chi-square test or Mann-Whitney U test as appropriate.

	Total study population	Naturally conceived pregnancies	IVF/ICSI pregnancies	p-value
Men	n = 825	n = 475	n = 350	
Age (years)	33.9 [30.9–38.3]	33.3 [30.2–37.6]	34.9 [31.4–39.0]	<0.001*
Geographic origin				0.317
Dutch	613 (74.3)	357 (75.2)	256 (73.1)	
Western	24 (2.9)	17 (3.6)	7 (2.0)	
Non-Western	88 (10.7)	56 (11.8)	32 (9.1)	
Missing	*100 (12.1)*	*45 (9.5)*	*55 (15.7)*	
Educational level				0.304
Low	95 (11.5)	55 (11.6)	40 (11.4)	
Intermediate	282 (34.2)	159 (33.5)	123 (35.1)	
High	340 (41.2)	212 (44.6)	128 (36.6)	
Missing	*108 (13.1)*	*49 (10.3)*	*59 (16.9)*	
BMI at study entry (kg/m^2)	26.0 [23.7–28.6]	25.7 [23.5–28.1]	26.7 [24.0–29.3]	0.003*
Missing	*106*	*56*	*50*	
Periconceptional alcohol use	534 (64.7)	320 (67.4)	214 (61.1)	0.609
Missing	*99 (12.0)*	*44 (9.3)*	*55 (15.7)*	
Periconceptional smoking	222 (26.9)	139 (29.2)	83 (23.7)	0.237
Missing	*99 (12.0)*	*44 (9.3)*	*55 (15.7)*	
Women	n = 825	n = 475	n = 350	
Age (years)	32.1 [29.0–35.4]	31.2 [28.7–34.7]	33.0 [29.8–36.1]	<0.001*
Nulliparous	472 (57.2)	215 (45.3)	257 (73.4)	<0.001*
Missing	*6 (0.7)*	*6 (1.3)*	-	
Geographic origin				0.716
Dutch	653 (79.2)	383 (80.6)	270 (77.1)	
Western	44 (5.3)	24 (5.1)	20 (5.7)	
Non-Western	98 (11.9)	54 (11.4)	44 (12.6)	
Missing	*30 (3.6)*	*14 (2.9)*	*16 (4.6)*	
Educational level				0.663
Low	54 (6.5)	33 (6.9)	21 (6.0)	
Intermediate	273 (33.1)	153 (32.2)	120 (34.3)	
High	468 (56.7)	276 (58.0)	192 (54.9)	
Missing	*30 (3.6)*	*13 (2.7)*	*17 (4.9)*	
BMI at study entry (kg/m^2)	24.5 [22.1–28.2]	24.6 [22.2–28.5]	24.3 [22.0–28.0]	0.235
Missing	*13*	8	*5*	
Folic acid supplement use	809 (98.1)	465 (97.9)	344 (98.3)	NA
Missing	*16 (1.9)*	*10 (2.1)*	*6 (1.7)*	
Started preconceptionally	671 (81.3)	342 (72.0)	329 (94.0)	<0.001*
Missing	*39 (4.7)*	*23 (4.8)*	*16 (4.6)*	
Periconceptional alcohol use	54 (30.8)	177 (37.3)	77 (22.0)	<0.001*
Missing	*20 (2.4)*	*12 (2.5)*	*8 (2.3)*	
Periconceptional smoking	116 (14.0)	68 (14.3)	48 (13.7)	0.795
Missing	*20 (2.4)*	*12 (2.5)*	*8 (2.3)*	

pregnancies, no significant associations are observed between paternal age and PV (Figure 2A, 2C). In naturally conceived pregnancies, a non-significant (p=0.166) association is observed with a tendency to larger PVs in both younger and older fathers (Figure 2B). The number of fathers with more extreme ages, especially <25 years or >45 years old, is limited in all study groups (Figure 2A-C). To illustrate the magnitude of effects, the back-transformed PV of for example 40 years old fathers compared to 30 years old fathers is 0.9% smaller (95% CI -5.7%—7.1%) at a gestational age of 9 weeks in the total study population. The effect plots for model 1 are shown in Figure S1.

In the subpopulation with available uPVV measurements, no significant association was found between paternal age and uPVV in the total study population for both models (model 1: p=0.809; model 2: p=0.489) or stratified for naturally conceived pregnancies (model 1: p=0.722; model 2: p=0.446) and IVF/ICSI pregnancies (model 1: p=0.887; model 2: p=0.749). Comparable results were found in the sensitivity analyses for the relation between paternal age and PV or uPVV after excluding pregnancies complicated by HDP or pregnancies with SGA offspring (data not shown).

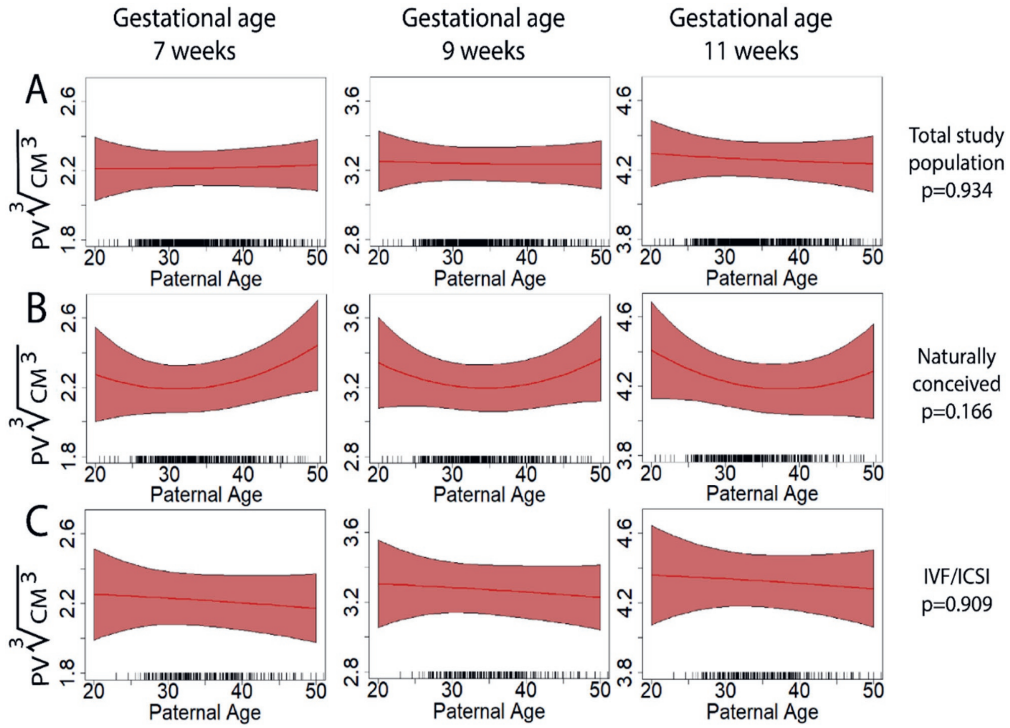


Figure 2: **Effect plots showing the relationship between paternal age and placental volume (PV) $\sqrt[3]{CM^3}$ at 7 weeks, 9 weeks and 11 weeks of gestation including 95% confidence intervals according to model 2**. Rug plot at x-axis depicts the distribution of paternal age in our study population. Model adjusted for gestational age at the ultrasound, fetal sex,

periconceptional parental smoking and maternal age, BMI, education and parity. P-values are outcomes of likelihood ratio test investigating the joined effect of paternal age, paternal age^2 and an interaction term including gestational age at the ultrasound and paternal age. **A)** The total study population; **B)** Naturally conceived pregnancies; **C)** Pregnancies conceived via IVF/ICSI.

Secondary outcomes

Table 2 shows the pregnancy outcomes in the study population stratified by paternal age categories. The number of fathers aged <25 years or >45 years old was again limited (Table 2). Hypertensive disorders complicated 9.6% of pregnancies (n=139). The median gestational age at birth was 39 weeks and 1 day [IQR 38+0–40+1] and the median birth weight was 3340 grams [IQR 2965gr–3680gr]. Based on Hoftiezer birth weight percentiles, 12.6% of newborns (n=182) were born SGA and 5.3% (n=77) had a birth weight <p3.

Due to a lack of power, we pooled PIH and PE in the analyses and did not stratify for mode of conception, but included mode of conception as covariate in the adjusted model. Paternal age >50 years of age was significantly association with less HDP in model 1 (p=0.048) (Figure S2A), but this association attenuated after adjustments in model 2 (p=0.109) (Figure 3A). Paternal age <28 years of age was significantly associated with an increase of SGA in model 1 (p=0.028) (Figure S2B), but this association attenuated in model 2 (p=0.092) (Figure 3B).

Table 2: Pregnancy outcomes in the total study population with available delivery reports and stratified by paternal age categories. Data are presented as median [interquartile range (IQR)] or n (%). HDP = hypertensive disorder of pregnancy, PIH = pregnancy induced hypertension, PE = preeclampsia, SGA = small for gestational age (birth weight <p10), birth weight percentiles are according to Hoftiezer birth weight charts.

Pregnancy outcome	**Total (n = 1449)**	**<25 years (n = 47)**	**25-35 years (n = 775)**	**35-45 years (n = 544)**	**>45 years (n = 83)**
HDP	139 (9.6)	4 (8.5)	80 (10.3)	51 (9.4)	4 (4.8)
PIH	81 (5.6)	1 (2.1)	51 (6.6)	26 (4.8)	3 (3.6)
PE	58 (4.0)	3 (6.4)	29 (3.7)	25 (4.6)	1 (1.2)
Gestational age at delivery	39+1 [38+0–40+1]	38+2 [37+4–39+4]	39+1 [38+1–40+1]	39+1 [37+6–40+1]	39+2 [38+3–40+4]
Missing	*5*	-	*3*	*1*	*1*
Birth weight (gram)	3340 [2965 – 3680]	3090 [2530–3600]	3340 [2960–3680]	3330 [2938–3690]	3420 [3088–3773]
Missing	*12*	-	*7*	*3*	*2*
SGA	182 (12.6)	12 (25.5)	98 (12.6)	65 (11.9)	7 (8.4)
Birth weight <p3	77 (5.3)	5 (10.6)	40 (5.2)	28 (5.1)	4 (4.8)
Missing	*20*	*1*	*10*	*7*	*2*

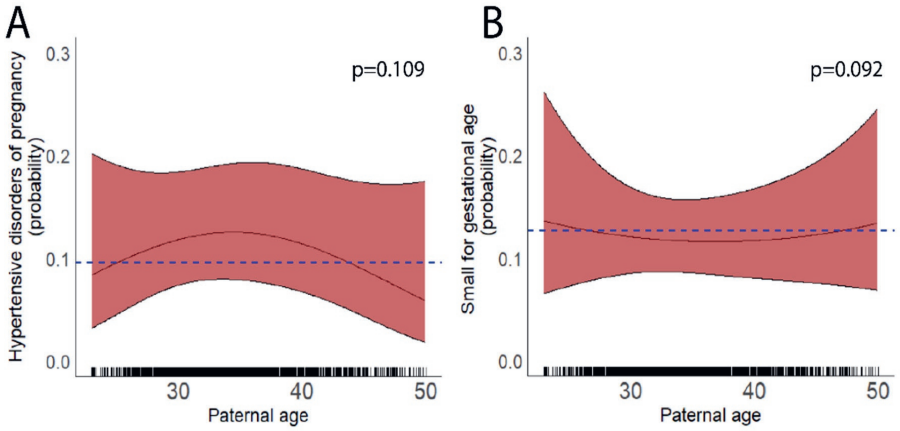


Figure 3: Effect plots showing the relationship between paternal age and investigated pregnancy outcomes including 95% confidence intervals according to our adjusted model (model 2). Dotted blue line represents population average. Model input parameters: maternal age=32.1 years, maternal BMI=24.6, Parental smoking=no, fetal sex=boy (only for HDP), education level=intermediate, parity=nulliparous, mode of conception=naturally. P-values are outcomes of likelihood ratio test investigating the joined effect of paternal age and paternal age^2. Rug plot at x-axis depicts the distribution of paternal age in our study population. **A)** Hypertensive disorders of pregnancy (HDP) (p=0.109), **B)** Small for gestational age (SGA) offspring (p=0.092).

Discussion

This is the first study investigating the association between paternal age and PV and uPVV in the first trimester of pregnancy. We hypothesized that older fathers would show smaller first trimester placental growth trajectories, because of potential aging-induced epigenetic alterations leading to dysregulation of paternally-expressed genes. However, no significant associations were shown in our study population between paternal age and first trimester PV or uPVV. Secondary, we found no significant associations between paternal age and the occurrence of HDP or SGA after adjusting for potential confounders.

Previous studies investigating paternal age and placental growth are limited and inconsistent and have only considered placental weight postpartum. Our study is in line with another study that found no association between paternal age and placental weight (35). However, another study including almost 600,000 pregnancies found a higher placental weight in fathers aged >50 years as compared to fathers aged 20-24 years, but the absolute difference was small, especially after adjustment for potential confounders (range 6.8-14 grams; 1-2% of placental weight) (11). On the contrary, a 25% reduced placental weight was found in mice with advanced paternal age (11-15 months old) compared to mice with younger fathers (4-5 months old), but a comparable controlled setting is not possible in humans (12).

A proposed mechanism between paternal characteristics and placental growth is the sperm epigenome. DNA methylation patterns of imprinted genes are passed on to the offspring and paternally-expressed imprinted genes which are particularly expressed in the placenta largely stimulate placental growth (23, 24, 36). Different preconceptional paternal exposures like nutrition have been found to impact imprinted genes in rodent placentas (21, 37). This indicates that paternal characteristics can affect epigenetic programming of the placenta and consequently placental growth and function. However, although significant changes in DNA methylation have been observed in sperm from older men, these alterations were generally found in non-imprinted regions and could therefore largely be removed during demethylation processes after fertilization, mitigating its potential effect on placental growth and explaining our negative results (25, 26, 38).

We hypothesized that paternal age has the largest effect early in pregnancy, because of accumulating effects of other factors influencing PV and uPVV development over gestation, but this was not observed in our study. Other factors, such as oocyte DNA and the maternal environment including nutrition can substantially impact placental growth already in the first trimester and especially in a tertiary hospital population, underlying parental conditions are suggested to have a more prominent effect on placental growth compared to paternal age (39, 40). Additionally, paternal age is strongly correlated with maternal age which has been shown to enhance placental growth (4). Consequently, the effect of paternal age on PV/uPVV may be masked by other (maternal) factors already in early pregnancy.

(Early) placental growth is clinically important since aberrant growth is associated with adverse pregnancy outcomes, including fetal growth restriction and HDP [9, 10]. Excluding pregnancies with HDP or SGA offspring did not change our outcomes in the sensitivity analysis. Additionally, we did not find an association between paternal age and the occurrence of SGA or HDP after adjustment for potential confounders, which is in line with other studies (13-15). However, others reported U-shaped relationships where pregnancies with younger and/or older fathers showed an increased risk for hypertensive disorders (16-18, 20) or low birth weight (15, 19, 20, 41) and the largest effect was generally found for fathers aged >45 years. Since most of these studies comprised over a million couples, we would need a larger study population to detect such small differences. Additionally, differences between studies in statistical analysis including used age categories, the definition of low birth weight and handling of potential confounders could impact the results and explain parts of observed differences between studies.

Strengths and limitations

To our knowledge, this is the first study investigating the association between paternal age and PV and uPVV in the first trimester. One of the main strengths of this study is the use of standardized 3D ultrasound combined with VR to longitudinally assess early placental growth measurements in more than 800 pregnancies. These techniques are proven to be valid and well repeatable (27). Other strengths of this study are the prospective design and our statistical approach. Although our model outcomes are more difficult to interpret as compared to linear regression models, our comprehensive models would allow for the identification of a non-linear relationship between paternal age and our studied outcomes, without categorizing paternal age which would inevitably lead to loss of information. Additionally, our models allow for the identification of a gestational age-dependent effect from paternal age on PV and uPVV. However, this study also has limitations. The effect estimates show that our study was not powered to find differences as small as found in a previous study conducted in humans (11) or to investigate the associations between paternal age and the risk of placental-related complications. Especially the number of fathers in our study population with more extreme ages is limited. For pregnancies with available ultrasound data, only 2.3% (n=19) of fathers were <25 years old and 6.2% (n=51) were 45+ years old. For pregnancies with available delivery reports to assess our secondary outcomes, 5.7% (n=47) and 10.1% (n=83) of fathers were aged <25 years old or 45+ years old, respectively. Although the paternal age distribution in our study is comparable to that in the general population, this hampers the robustness of our results for younger and older fathers. Second, multiple factors including maternal adiposity, uterine position and embryonic movements can impact the quality of the ultrasound. Consequently, using our technical approach, 25-30% of PV and uPVV measurements are of insufficient quality and therefore not included in our analyses, limiting the number of (longitudinal) measurements (28). Additionally, as for every observational study, residual confounding cannot be excluded. Our study population was enrolled at a tertiary hospital limiting generalizability of our results to the general population.

Conclusions

The clear trend of delayed fatherhood in many countries emphasizes the importance of investigating the potential impact of advanced paternal age on the course and outcome of pregnancy. In this study, we did not find an effect of paternal age on first trimester placental growth, utero-placental vascularization volume and placental-related pregnancy complications. Although accumulating evidence associates paternal conditions such as age to health and development of the offspring, underlying mechanisms might not mainly work through changes in placental (vascular) growth but rather through functional, histopathological changes, while

changes in early placental growth might be smaller than what we could detect in our study. However, we provide reassuring evidence that paternal age, particularly between 25 and 45 years old, does not substantially impact placental growth in the first trimester in a tertiary care population. Future studies should validate our findings in the general population in larger cohorts, ideally including more fathers with a younger and more advanced age.

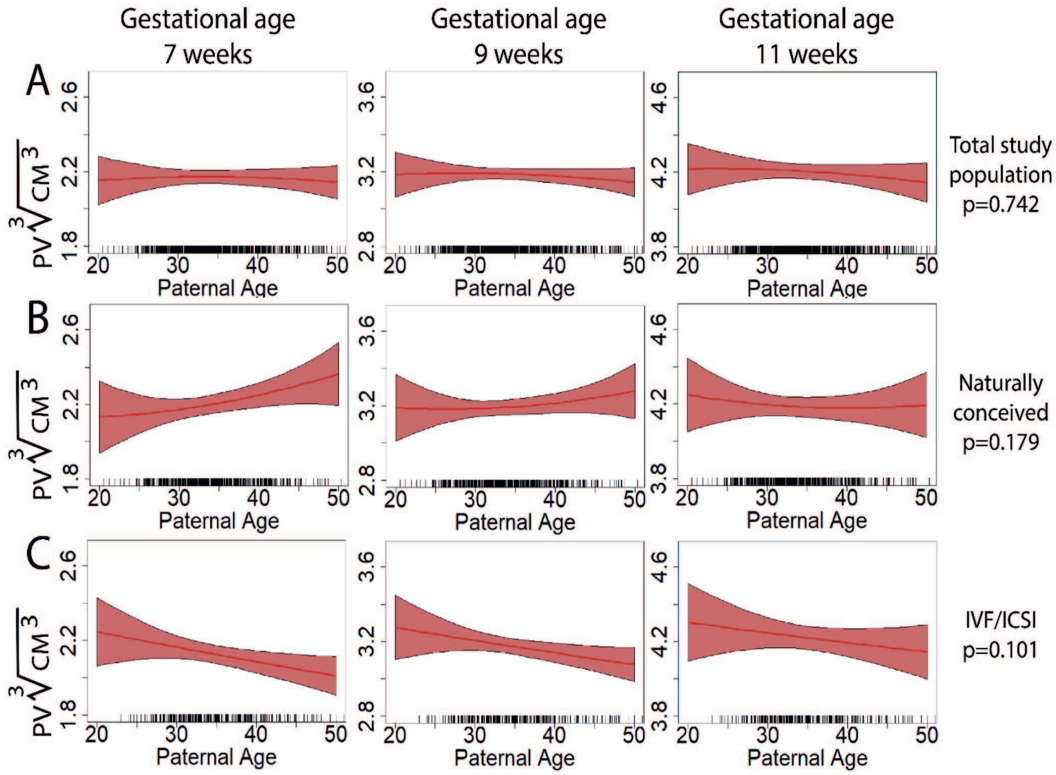
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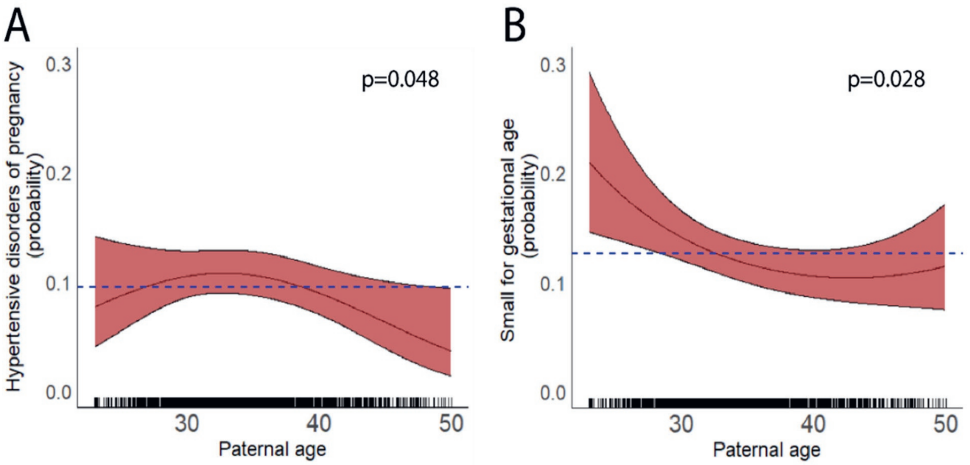
Supplemental Material

Table S1: Baseline characteristics of included couples with available delivery reports stratified for mode of conception. Data are presented as median [interquartile range (IQR)] or n (%). *Significance at p ≤ 0.05 assessed by chi-square test or Man- Whitney U test as appropriate.

	Total study population	Naturally conceived	IVF/ICSI pregnancies	p-value
Men	n = 1449	n = 968	n = 481	
Age (years)	34.1 [30.9–38.2]	33.7 [30.5–37.8]	35.0 [31.7–39.0]	<0.001*
Geographic origin				0.050*
Dutch	1018 (70.3)	660 (68.2)	358 (74.4)	
Western	37 (2.6)	29 (3.0)	8 (1.7)	
Non-Western	159 (11.0)	115 (11.9)	44 (9.1)	
Missing	*235 (16.2)*	*164 (16.9)*	*71 (14.8)*	
Educational level				0.087
Low	166 (11.5)	112 (11.6)	54 (11.2)	
Intermediate	452 (31.2)	281 (29.0)	171 (35.6)	
High	586 (40.4)	402 (41.5)	184 (38.3)	
Missing	*245 (16.9)*	*173 (17.9)*	*72 (15.0)*	
BMI at study entry (kg/m^2)	25.9 [23.7–28.3]	25.7 [23.6–28.1]	26.5 [24.0–29.2]	0.002*
Missing	*179*	*112*	*67*	
Periconceptional alcohol use	907 (62.6)	611 (63.1)	296 (61.5)	0.171
Missing	*233 (16.1)*	*162 (16.7)*	*71 (14.8)*	
Periconceptional smoking	376 (25.9)	268 (27.7)	108 (22.5)	0.014*
Missing	*233 (16.1)*	*162 (16.7)*	*71 (14.8)*	
Women	n =1449	n = 968	n = 481	
Age (years)	32.1 [29.0–35.4]	31.4 [28.7–34.8]	33.0 [30.0–36.2]	<0.001*
Nulliparous	739 (51.0)	394 (40.7)	345 (71.7)	<0.001*
Missing	*10 (0.7)*	*7 (0.7)*	*3 (0.6)*	
Geographic origin				0.548
Dutch	1084 (74.6)	713 (73.7)	371 (77.1)	
Western	69 (4.8)	41 (4.2)	28 (5.8)	
Non-Western	175 (12.1)	113 (11.7)	62 (12.9)	
Missing	*121 (8.4)*	*101 (10.4)*	*20 (4.2)*	
Educational level				0.400
Low	94 (6.5)	66 (6.8)	28 (5.8)	
Intermediate	456 (31.5)	289 (29.9)	167 (34.7)	
High	775 (53.5)	510 (52.7)	265 (55.1)	
Missing	*124 (8.6)*	*103 (10.6)*	*21 (4.4)*	
BMI at study entry (kg/m^2)	24.6 [22.1–28.3]	24.6 [22.2–28.7]	24.4 [21.7–27.7]	0.016*
Missing	*22*	15	*7*	
Folic acid supplement use	1374 (94.8)	900 (93.9)	474 (98.5)	0.468
Missing	*74 (5.1)*	*67 (6.9)*	*7 (1.5)*	
Started preconceptionally	1067 (73.6)	618 (63.8)	449 (93.3)	<0.001*
Missing	*147 (10.1)*	*127 (13.1)*	*20 (4.2)*	
Periconceptional alcohol use	413 (28.5)	314 (32.4)	99 (20.6)	<0.001*
Missing	*86 (5.9)*	*76 (7.9)*	*10 (2.1)*	
Periconceptional smoking	196 (13.5)	139 (14.4)	57 (11.9)	0.083
Missing	*85 (5.9)*	*75 (7.7)*	*10 (2.1)*	



Supplemental Figure S1: Effect plots showing the relationship between paternal age and placental volume (PV) $\sqrt[3]{CM^3}$ at 7 weeks, 9 weeks and 11 weeks of gestation including 95% confidence intervals according to model 1. Rug plot at x-axis depicts the distribution of paternal age in our study population. Model adjusted for gestational age at the moment of the ultrasound. P-values are outcomes of likelihood ratio test investigating the joined effect of paternal age, paternal age^2 and an interaction term including gestational age at the ultrasound and paternal age. **A)** The total study population; **B)** Naturally conceived pregnancies; **C)** Pregnancies conceived via IVF/ICSI.



Supplemental Figure S2: Effect plots showing the relationship between paternal age and investigated pregnancy outcomes including 95% confidence intervals according to model 1. Dotted blue line represents population average. P-values are outcomes of likelihood ratio test investigating the joined effect of paternal age and paternal age^2. Rug plot at x-axis depicts the distribution of paternal age in our study population. **A)** Hypertensive disorders of pregnancy (HDP) (p=0.048), **B)** Small for gestational age (SGA) offspring (p=0.028).

11

Part II

Using MeD-seq to assess DNA methylation in placental tissues and cell-free DNA from maternal plasma in health and disease


5

Chapter 5

Genome-wide methylation profiling of maternal cell-free DNA using Methylated DNA Sequencing (MeD-seq) indicates a placental and immune cell signature

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Abstract

Background: Placental-originated cell-free DNA (cfDNA) provides unique opportunities to study (epi)genetic placental programming remotely, but studies investigating the cfDNA methylome are scarce and usually technologically challenging. Methylated DNA sequencing (MeD-seq) is well-compatible with low cfDNA concentrations and has a high genome-wide coverage. We therefore aim to investigate the feasibility of genome-wide methylation profiling of first trimester maternal cfDNA using MeD-seq, by identifying placental-specific methylation marks in cfDNA.

Methods: We collected cfDNA from non-pregnant controls (female n=6, male n=12) and pregnant women (n=10), first trimester placentas (n=10), and paired preconceptional and first trimester buffy coats (total n=20). Differentially methylated regions (DMRs) were identified between pregnant and non-pregnant women. We investigated placental-specific markers in maternal cfDNA, including *RASSF1* promoter and Y-chromosomal methylation, and studied overlap with placental and buffy coat DNA methylation.

Results: We identified 436 DMRs between cfDNA from pregnant and non-pregnant women which were validated using male cfDNA. *RASSF1* promoter methylation was higher in maternal cfDNA (fold change 2.87, unpaired t-test $p<0.0001$). Differential methylation of Y-chromosomal sequences could determine fetal sex. DMRs in maternal cfDNA showed large overlap with DNA methylation of these regions in placentas and buffy coats. Sixteen DMRs in maternal cfDNA were specifically found only in placentas. These novel potential placental-specific DMRs were more prominent than *RASSF1* promoter methylation.

Conclusions: MeD-seq can detect (novel) genome-wide placental DNA methylation marks and determine fetal sex in maternal cfDNA. Our results indicate a placental and immune-cell contribution to the pregnancy-specific cfDNA methylation signature. This study supports future research into maternal cfDNA methylation.

Background

A well-functioning placenta is essential for healthy fetal development, but the placenta is largely inaccessible during gestation. The direct study of placental tissue necessitates invasive procedures (1), or postpartum placental examination. Therefore, less invasive techniques to study placental development throughout gestation are warranted.

Turnover and shedding of placental cells result in release of placental-originated cell-free DNA (cfDNA) in the maternal blood stream. Maternal plasma-derived cfDNA is widely used to perform noninvasive prenatal testing (NIPT), enabling screening for chromosomal abnormalities (2). However, delving into the epigenetic landscape of maternal cfDNA could allow for applications beyond current practice (3). DNA methylation is an important epigenetic mechanism playing a crucial role in placental growth, development and function, by regulating gene expression (4-6). Differences in placental DNA methylation have been associated with gestational age and adverse obstetrical outcomes including preeclampsia (7-9). Maternal plasma-derived cfDNA could facilitate the noninvasive study of placental DNA methylation in health and disease already from the first trimester onwards.

A major challenge in using maternal cfDNA as a proxy for placental DNA is the relatively low contribution of placental-derived cfDNA to total maternal cfDNA. The largest contributor to maternal cfDNA is the maternal hematopoietic system, with a placental-derived fraction of roughly 10% at the end of the first trimester (10-12). However, since DNA methylation is highly cell type specific, cfDNA methylation profiles can aid in determining their tissue of origin (4, 13).

Previous studies identifying placental-specific DNA methylation markers mostly focused on methylation differences on chromosomes 13, 18 and 21, which have been shown to be potentially useful in screening for chromosomal abnormalities (14-23), and/or focused on methylation differences between placental tissues and maternal whole blood (24-26). Nonetheless, our understanding of the genome-wide impact of pregnancy directly on the cfDNA methylome, and how this reflects changes in maternal and placental tissues, is limited. The scarce studies on cfDNA are mostly performed with whole genome bisulfite sequencing (27-29), which is costly and technologically challenging due to degradation of already low DNA levels in cfDNA samples, or achieved a limited genome-wide coverage (30). At the same time, recent studies support the use of the cfDNA methylome as early predictive biomarker for preeclampsia development (31-33). This emphasizes the need to further explore the epigenetic landscape of cfDNA already during the early stages of pregnancy, ideally using less costly methods while ensuring a high genome-wide coverage.

We therefore used Methylated DNA sequencing (MeD-seq) to investigate its feasibility to study the maternal cfDNA methylome. MeD-seq is compatible with cfDNA and covers >50% of genome-wide cytosine-guanine dinucleotides (CpGs), detecting DNA methylation at >99% of all CpG islands and promoters (34, 35). We aim to investigate the impact of pregnancy on the first trimester maternal cfDNA methylome, and explore the placental and hematopoietic origins of identified differences. We further aim to identify placental-specific methylation marks in cfDNA. As proof-of-principle, we show the possibility to determine fetal sex in maternal cfDNA based on Y-chromosomal methylation marks.

Methods

Study design

We included pregnant women who participated in the Rotterdam Periconception Cohort (Predict study) at the Erasmus Medical Centre, Rotterdam. The Predict study is an ongoing prospective cohort study where women with a singleton pregnancy are recruited before 10 weeks of gestation and longitudinally followed throughout pregnancy (36, 37). Blood samples for cfDNA isolation were obtained around 11 weeks of gestation between November 2022 and March 2023.

Blood samples for cfDNA isolation from non-pregnant controls were previously obtained for research purposes from anonymous, healthy blood donors (HBDs) enrolled via the Dutch National blood bank (Sanquin). Additionally, we prospectively collected first trimester (around 9-12 weeks of gestation) placental tissues after elective surgical abortions at a Dutch abortion clinic between July and August 2023. All pregnancies had to be without known congenital abnormalities. Lastly, maternal paired buffy coats were collected from women who participated within the Predict study both before pregnancy (preconception) as in the first trimester of a subsequent pregnancy.

Both the Predict study (MEC-2004-227) and the research protocol to collect placental tissues (MEC-2022-0788) were approved by the Medical Ethics Committee of the Erasmus Medical Centre. All participants provided written informed consent.

Processing of samples

Blood samples for cfDNA isolation were collected in CellSave preservative tubes. Plasma was subsequently isolated by two centrifugation steps (10 minutes at 1,711g — 2,000g followed by 10 minutes 12,000g — 16,000g) (38). Blood samples for DNA isolation of buffy coats were collected in EDTA tubes. Buffy coats were isolated after centrifugation of the blood sample (10

minutes at 2,000g). Plasma and buffy coats were stored at −80 °C prior to (cf)DNA isolation. Placental tissues were stored in buffered 4% formaldehyde solution.

(cf)DNA isolation

cfDNA was isolated from 4 ml of plasma using the QIAamp Circulating Nucleic Acid Kit. DNA extraction and isolation from buffy coats were performed using the TECAN freedom EVO robot combined with the Promega ReliaPrep™ Large Volume HT gDNA Isolation System. Placental samples were formalin-fixed and full thickness biopsies were taken followed by embedding in formalin-fixed paraffin-embedded (FFPE) blocks. Four to six slides of 4-6 µm were cut. One slide per sample was stained using hematoxylin and eosin (H&E) and placental parenchyma was evaluated by a perinatal pathologist (LEM). DNA was extracted and isolated from subsequent slides with normal first trimester placental parenchyma using the QIAamp DSP DNA FFPE Tissue kit according to the manufacturer's protocol.

MeD-seq assay

MeD-seq assays were essentially performed as previously described (34, 35, 39). First, genomic DNA and plasma-derived cfDNA were digested with the methylation-dependent restriction enzyme LpnPI (New England Biolabs, Ipswich, MA) generating 32 bp DNA fragments containing the methylated CpG in the middle. The TruPLEX DNAseq 96D kit (Rubicon Genomics, Takara Bio Europe, Saint-Germain-en-Laye, France) was used to prep samples for sequencing and samples were purified on a Pippin HT system with 3% agarose gel cassettes (Sage Science, Beverly, MA). Samples were sequenced on the Illumina NextSeq2000 platform. Samples were first sequenced until ±2 M reads, and continued to a total of ±20 M reads if at least 20% of reads passed the LpnPI filter (see data processing and analysis).

Data processing and analysis

Dual indexed samples were demultiplexed using bcl2fastq software (Illumina). Differentially methylated regions (DMRs) were detected using a previously established bioinformatics pipeline (34, 35, 39). Custom python scripts were used to process acquired DNA methylation profiles. Raw fastq files were subjected to Illumina adaptor trimming and reads were filtered based on LpnPI restriction site occurrence between 13–17 bp from either the 5' or 3' end of the read to filter out DNA fragments that were methylated. Reads that passed the filter were mapped to hg38 using bowtie2 and BAM files were generated using SAMtools version 0.1.19. Genome-wide individual LpnPI site scores were used to generate read count scores for: transcription start sites (TSS, 1 kb before and 1 kb after), CpG-islands and gene bodies (1 kb after TSS till TES). Gene and CpG-island annotations were downloaded from ENSEMBL (Homo_sapiens_hg38.GRCh38.79.gtf, www.ensembl.org).

Additionally, a sliding window technique was used to detect DMRs and the chi-squared test on read counts was used for statistical testing. A Bonferroni corrected p-value ≤0.05 was considered statistically significant. Z-score transformation of the read count data was applied for unsupervised hierarchical clustering analyses.

DMRs were identified between cfDNA from pregnant and non-pregnant women. cfDNA from male HBDs were used as validation. Y-chromosomal methylated DNA sequences were identified between cfDNA from male and female HBDs and subsequently applied to maternal cfDNA to determine fetal sex. Methylation of the *RASSF1* promoter, known to be hypermethylated specifically in placental tissues (40), was compared between cfDNA from pregnant and non-pregnant women using an unpaired t-test standardized for number of reads per million (rpm). For DMRs identified in cfDNA, overlap with first trimester placental tissues and buffy coats was studied to explore potential tissues of origin of identified DMRs. To determine the presence or absence of cfDNA methylation signatures associated specifically with pregnant or with non-pregnant women, receiver operating characteristic (ROC) curves were calculated for each sample for each individual DMR. ROC curves of reference sets of pregnant women compared to non-pregnant women were used to calculate the optimal threshold (using the "scikit-learn" package Python) for each individual DMR. Samples above the threshold scored '1', samples under the threshold scored '0', leading to a binary score for each DMR. To investigate overlap of identified DMRs in cfDNA with placental tissues and with buffy coats, a cut-off value of ≥80% of samples scoring '1' or '0' was used for hypermethylated and hypomethylated DMRs, respectively.

Results

An overview of used samples and their technical performance is provided in Table S1.

DMRs between pregnant and non-pregnant cfDNA

To identify differences in cfDNA methylation related to pregnancy, we compared cfDNA from pregnant (n=10) and non-pregnant women (HBDs, n=6) (Figure 1A). For pregnant women, baseline characteristics are depicted in Table 1, and cfDNA was collected between 10+5 – 11+5 weeks of gestation (Figure 1B). Using MeD-seq (Figure 1C), we identified 436 autosomal DMRs with a fold change (FC) ≥2, of which 338 (77.5%) were hypermethylated in pregnant women (Table S2). Unsupervised hierarchical clustering using identified DMRs shows clear clusters between pregnant and non-pregnant women (Figure 1D). As a validation, we show large overlap of male cfDNA (n=12) with non-pregnant women showing robustness of our results (Figure S1).

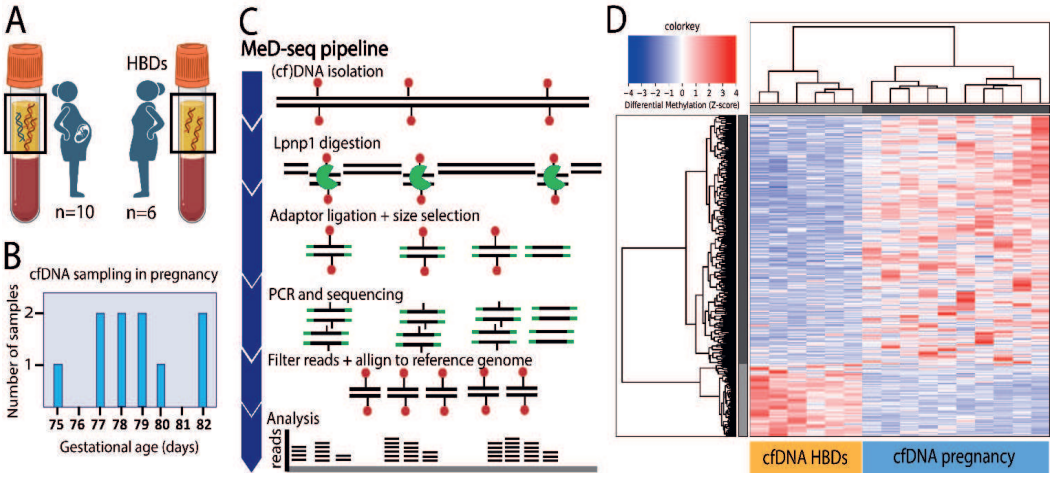


Figure 1: Identification of DMRs between cfDNA from pregnant (first trimester) and non-pregnant women (HBDs). A) Study population consists of pregnant women at the end of the first trimester and non-pregnant women. cfDNA in plasma from pregnant women partly originates from the placenta. **B)** Gestational age at time of cfDNA sampling in pregnancy. **C)** Schematic overview of the MeD-seq pipeline. (cf)DNA is digested by LpnPI which recognizes methylated CpGs and cuts the DNA 16 bp up- and downstream leading to 32 bp fragments. This is followed by adaptor ligation, size selection of cfDNA fragments (32 bp), amplification and sequencing using the Illumina NextSeq2000 platform. Reads are filtered based on a central methylated CpG and aligned to a reference genome (hg38) prior to analysis. **D)** Heatmap showing an unsupervised hierarchical clustering using autosomal DMRs with a Fold Change ≥2 between cfDNA from pregnant women in first trimester and non-pregnant women (HBDs) showing clear separation between the two groups. Z-scores on normalized MeD-seq data are used to visualize the data, red represents hypermethylation, blue represents hypomethylation.

Table 1: Baseline characteristics of pregnant women with cfDNA samples (n=10). Categorical variables are described using absolute numbers and percentages (%). Continuous variables are described using medians and interquartile ranges (IQR). IVF/ICSI = in vitro fertilization/intracytoplasmic sperm injection.

Maternal age (years)	32.4 (30.7 – 34.3)
BMI at study entry	25.1 (22.8 – 26.8)
Country of birth	
Dutch	8 (80%)
Non-Dutch	2 (20%)
Nulliparous	6 (60%)
Smoking during pregnancy	1 (10%)
Mode of conception	
Spontaneous	4 (40%)
IVF/ICSI	6 (60%)
Folic acid supplement use	10 (100%)
Started preconception	9 (90%)
Male fetus	5 (50%)

Placental origin of methylation profiles in cfDNA from pregnant women

To explore which DMRs in maternal cfDNA could be of placental-origin, we compared DMRs identified in cfDNA with MeD-seq data from first trimester placental tissues (n=10) (Figure 2A). Placental tissues were collected between 9+2 – 12+2 weeks of gestation (Figure 2B, Table S1).

Unsupervised hierarchical clustering analysis shows that first trimester placental tissues cluster between cfDNA from pregnant and non-pregnant women (Figure 2C). Based on our cumulative methylation score, the majority of DMRs hypermethylated in cfDNA from pregnant women were in general also detected in placental tissues, while DMRs hypermethylated in cfDNA from non-pregnant women were not found in placental tissues (Figure 2D, Table S3). More strictly, of 338 DMRs hypermethylated and 98 DMRs hypomethylated in cfDNA from pregnant women, 143 (42.3%) and 88 (89.8%) were respectively consistently also hypermethylated or hypomethylated in ≥80% of placental tissues (Table S3). DMRs that are hyper- or hypomethylated in both maternal cfDNA and placental tissues, as compared to cfDNA from non-pregnant women, could indicate a placental-origin of these DMRs in maternal cfDNA.

A well-studied placental-specific DNA methylation mark is hypermethylation of the *RASSF1* promoter (41). As expected, the *RASSF1* promoter displayed a higher methylation level in first trimester placental tissues as compared to cfDNA from pregnant or non-pregnant women (Figure 2E-F, Table S4). Moreover, the *RASSF1* promoter displayed a higher methylation level in cfDNA from pregnant as compared to non-pregnant women (FC 2.87, unpaired t-test p<0.0001) (Figure 2E-F, Table S4), although this did not remain statistically significant in our genome-wide analyses after Bonferroni correction.

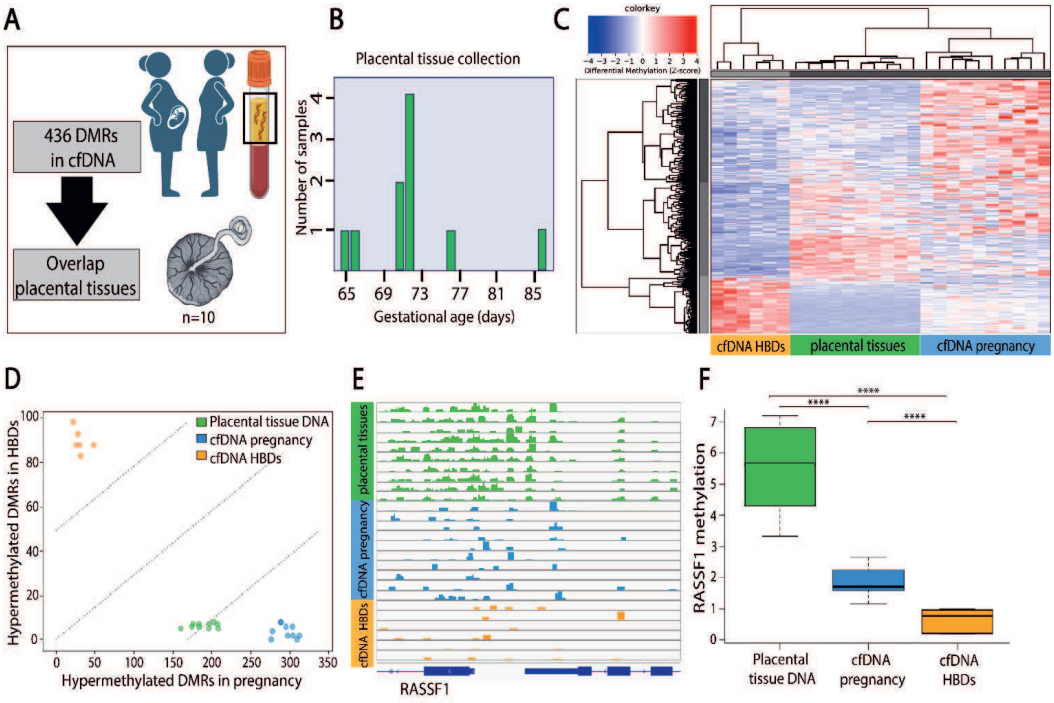


Figure 2 (previous page): Placental-origin of methylation profiles in cfDNA from pregnant women. A) DMRs were identified in cfDNA between non-pregnant women (HBDs) and pregnant women in the first trimester. For identified DMRs, overlap with first trimester placental tissue biopsies is studied. **B)** Gestational age at time of placental tissue collection. **C)** Heatmap visualizing unsupervised hierarchical clustering of first trimester placental tissue biopsies between cfDNA from pregnant women and non-pregnant women (HBDs). Selected autosomal DMRs were identified between pregnant and non-pregnant women. Red represents hypermethylation, blue represents hypomethylation. **D)** After generating a cumulative methylation score, most DMRs hypermethylated in cfDNA from pregnant women are also hypermethylated in first trimester placental tissue biopsies (x-axis). DMRs specifically hypermethylated in cfDNA from non-pregnant women (HBDs) are not hypermethylated in first trimester placental tissue biopsies (y-axis). Each dot represents one sample. **E, F)** Gene-tracks and boxplots show hypermethylation of the *RASSF1* promoter in placental tissue biopsies, and higher methylation of the *RASSF1* promoter in cfDNA from pregnant women compared to cfDNA from non-pregnant women (HBDs) (t-test $p<0.0001$). *In our genome-wide analysis, the difference in *RASSF1* promoter methylation between cfDNA from pregnant and non-pregnant women did not remain statistically significant after Bonferroni correction.

Fetal sex determination based on DNA methylation

To assess the feasibility of determining fetal sex using cfDNA methylation profiles, we first identified 147 Y-chromosomal regions containing DNA methylation with a FC ≥2 in cfDNA from male (n=6) compared to female HBDs (n=6) (Figure 3A, Table S5). Using identified Y-chromosomal regions, two clear clusters were identified in maternal cfDNA (Figure 3B). Women bearing boys (n=5) showed hypermethylation at numerous Y-chromosomal regions as compared to women pregnant with girls (n=5) (Figure 3B-C). Epigenetic marks can thus be used to determine fetal sex in maternal cfDNA. The minimal detection of Y-chromosomal reads in women pregnant with girls (Figure 3B-C), can be explained by mapping errors caused by large regions displaying high levels of homology between parts of the X- and Y-chromosome.

Overlap identified DMRs with buffy coats and novel placental-specific markers

Not all DMRs identified in maternal cfDNA were detected in placental tissues (Figure 2C-D, Table S3). This could indicate pregnancy-induced changes in maternal cfDNA from other tissues. During pregnancy, physiological changes result in an increase in white blood cells while simultaneously a change in composition of maternal immune cells occurs (42). Since most cfDNA originates from maternal hematopoietic cells (10), we hypothesized that pregnancy-induced physiological immunological changes might explain part of the observed methylation differences between cfDNA from pregnant and non-pregnant women.

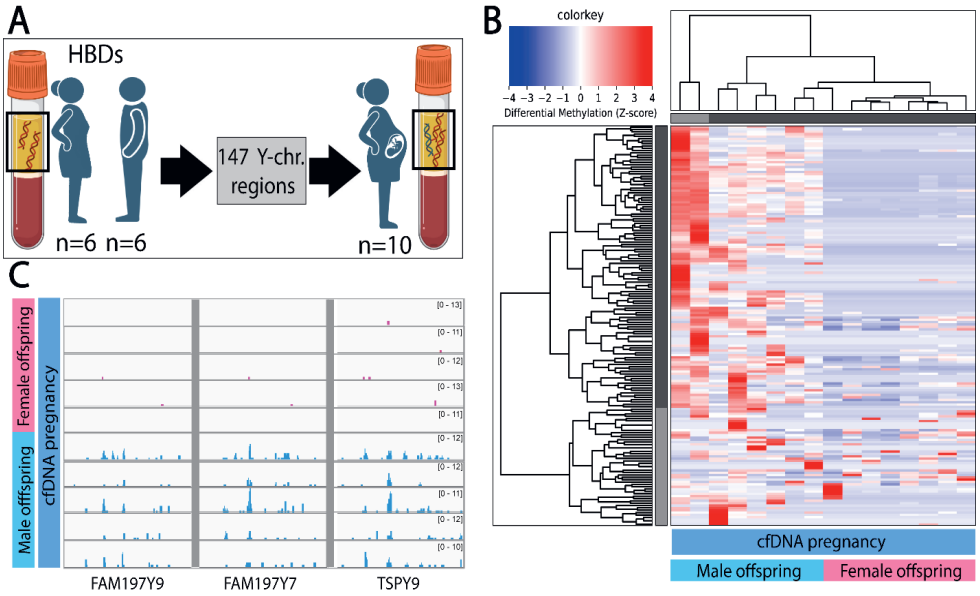


Figure 3: Fetal sex determination in cfDNA from pregnant women. A) Y-chromosomal regions containing DNA methylation were identified between male and female healthy blood donors (HBDs). Identified Y-chromosomal regions were studied in cfDNA from pregnant women. **B)** Heatmap visualizing unsupervised hierarchical clustering of cfDNA from pregnant women based on identified Y-chromosomal regions shows clear clusters of women bearing male offspring (blue bar, left) and female offspring (pink bar, right). Red represents hypermethylation, blue represents hypomethylation. **C)** Example gene-tracks of Y-chromosomal regions in cfDNA from pregnant women showing methylation only in women bearing male offspring.

To first identify DNA methylation changes in buffy coats related to pregnancy, we compared DNA methylation profiles between paired buffy coats collected preconception (n=10) and in the first trimester of a subsequent pregnancy (n=10) (Figure 4A). Baseline characteristics of included women are depicted in Table S6. In contrast to cfDNA, we identified no DMRs between preconception and first trimester buffy coats.

To further explore possible origins of DMRs identified in cfDNA, next to overlap with placental tissues, we studied overlap of identified DMRs with pooled buffy coats (n=20) (Figure 4B). Surprisingly, the majority of DMRs identified in maternal cfDNA were also present in buffy coats (Figure 4C-D). Based on our cumulative methylation score, 226 (66.9%) of DMRs hypermethylated and 85 (86.7%) of DMRs hypomethylated in maternal cfDNA were respectively also hyper- or hypomethylated in ≥80% of buffy coat samples (Table S3). While 231 (53.0%) of DMRs identified in maternal cfDNA were present in ≥80% of placental tissue biopsies, 177 of these were also present in ≥80% of buffy coat samples (Figure 4D, Table S3). These DMRs are therefore not placental-specific, but could also be of hematopoietic origin. The large overlap of DMRs in maternal cfDNA with DNA methylation of buffy coats might reflect an overall increased immune response during pregnancy.

DMRs in maternal cfDNA that overlap with DNA methylation in placental tissues but not with buffy coats, are potential placental-specific DMRs. We identified 16 DMRs that were hypermethylated in ≥80% of both maternal cfDNA and placental tissue samples, while hypomethylated in both ≥80% of HBD cfDNA and buffy coat samples (Table S3). These DMRs are therefore likely to represent placental-specific markers in cfDNA. To illustrate, Figure 4E shows gene tracks for three DMRs with the highest FC between cfDNA from pregnant and non-pregnant women. The three top-ranked DMRs correspond to *TMEM240* (FC 10.2), *DHRS3* (FC 7.6) and a CpG island 40 kb upstream of *PCMTD2* (FC 6.5).

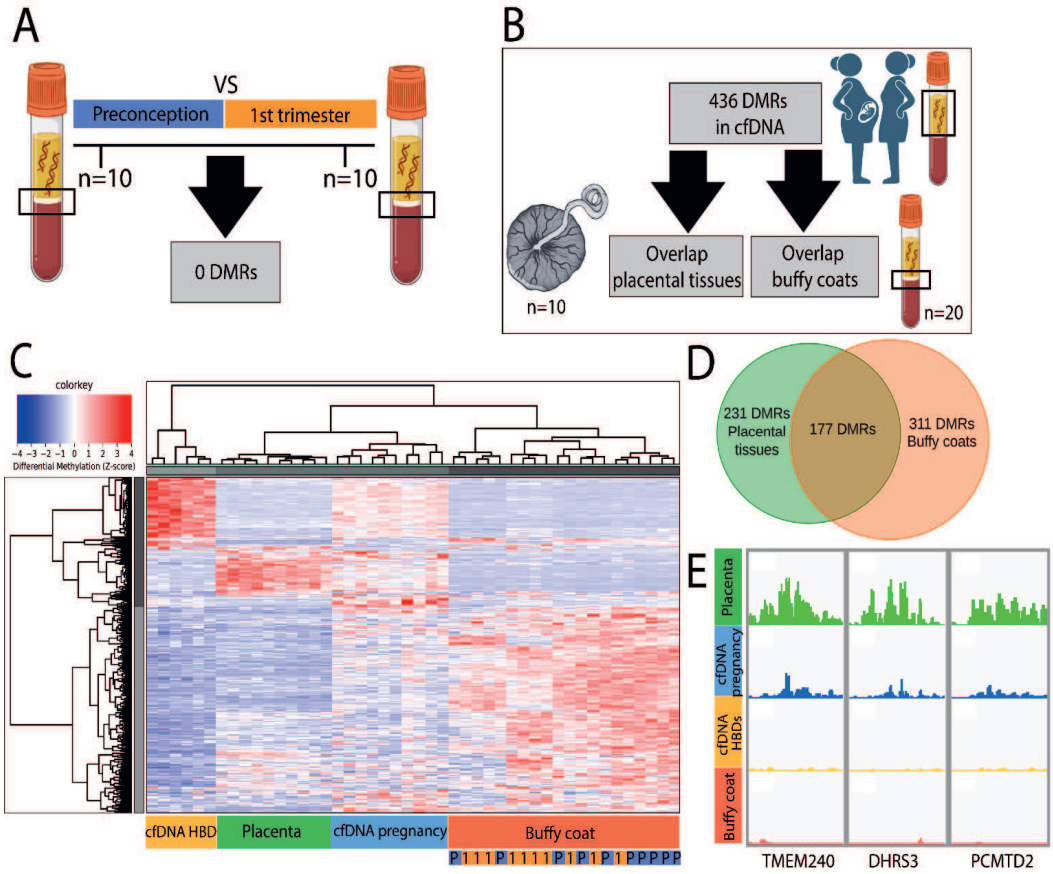


Figure 4: Origins of DMRs identified in cfDNA: overlap with placental tissues and buffy coats and identification of novel placental-markers in cfDNA. A) No DMRs were found between paired buffy coats collected preconception and in the first trimester of pregnancy. **B)** Overlap with DNA methylation in placental tissue biopsies and pooled buffy coats was studied for DMRs identified in cfDNA. **C)** Heatmap visualizing unsupervised hierarchical clustering for DMRs found between cfDNA from pregnant women and non-pregnant women (HBDs), shows larger overlap of buffy coats with cfDNA from pregnant women as with cfDNA from non-pregnant women. Selected autosomal DMRs were identified with a Fold Change ≥2. Red represents hypermethylation, blue represents hypomethylation. Buffy coats collected during the first trimester of pregnancy (P, blue) cluster between buffy coats collected preconceptionally (1, orange). **D)** Venn diagram visualizing overlap

of DMRs identified in cfDNA from pregnant women compared to non-pregnant women with first trimester placental tissue biopsies and buffy coats. Used cutoff is DMRs present in ≥80% of placental tissue biopsies or buffy coats based on our cumulative methylation score (Table S3). **E)** Example gene-tracks of potential placental-specific DMRs in maternal cfDNA. Gene-tracks for DMRs with highest fold change between cfDNA from pregnant and non-pregnant women. DMRs are hypermethylated in cfDNA from pregnant women and placental tissue biopsies as compared to cfDNA from non-pregnant women (HBDs). DMRs corresponding to *TMEM240*, *DHRS3* and a CpG island upstream of *PCMTD2* show no hypermethylation in buffy coats indicative of a placental-origin.

Discussion

We applied MeD-seq to investigate the genome-wide impact of first trimester pregnancy on the maternal plasma-derived cfDNA methylome. We identified numerous DMRs between cfDNA from pregnant and non-pregnant women. Next, we confirmed identification of placental-specific methylation marks in maternal cfDNA by showing hypermethylation of the *RASSF1* promoter – a known placental-specific marker – and the possibility to determine fetal sex based on Y-chromosomal DNA methylation. Moreover, we identified novel potential placental-specific methylation marks in maternal cfDNA, such as hypermethylated DMRs in *TMEM240*, *DHRS3* and a CpG island upstream of *PCMTD2*, that were more robust as compared to *RASSF1* promoter hypermethylation.

Although the majority of DMRs identified in maternal cfDNA overlapped with placental DNA methylation, most DMRs also overlapped with DNA methylation of buffy coats. Our results suggest a more prominent immune cell type(s) related contribution to DMRs in cfDNA from pregnant as compared to non-pregnant women, and thus both a placental and immune cell contribution to the pregnancy-specific cfDNA methylation signature. This is in line with other studies that recently found that the increased cfDNA levels in patients with several types of cancers is predominantly caused by increased levels of leukocyte-derived cfDNA instead of tumor-derived cfDNA (43,44), as is also found for patients with acute pancreatitis or sepsis. Our findings therefore suggest that the cfDNA methylation profile reflects an overall increased immune response during pregnancy (10,45). Although the presence of placental-originated cfDNA fragments influences the maternal cfDNA methylome, it is important to acknowledge the large contribution of other maternal tissues to total maternal cfDNA when interpreting methylation changes related to pregnancy.

DMRs in maternal cfDNA that were not shared with either placental tissues or buffy coats, could be attributable to physiological pregnancy-related changes in other (high turnover) maternal tissues that contribute to cfDNA, such as liver- or endothelium (10). Another explanation could be a different contribution of hematopoietic and placental cell types to buffy coats or tissue biopsies as compared to their contribution to cfDNA. For example, in placental tissue biopsies,

DNA methylation reflects the average methylation of all cells in the selected sample, while for cfDNA the contribution of different placental cell types could be influenced by apoptosis rate and anatomical position in relation to the maternal blood stream.

Although fetal sex can already be determined in maternal cfDNA based on the presence of Y-chromosomal genetic material (2), we were able to establish the fetal sex by combining numerous methylated Y-chromosomal regions in maternal cfDNA. In the future, we aim to investigate the use of DNA methylation data from all chromosomes for the detection of aneuploidies as previously shown by others (15,16,21-23). The potential added value of combining genetic and epigenetic information in NIPT warrants further study.

MeD-seq can be used to discover genome-wide DMRs directly in cfDNA, instead of using differences between whole blood and placental tissues. This may more reflect the turnover of specific cell types and can lead to additional markers relevant for cfDNA applications in pregnancy. For example, the previously mentioned hypermethylation of DMRs in *TMEM240*, *DHRS3* and a CpG island upstream of *PCMTD2* were more robust in cfDNA of pregnant compared to non-pregnant women, as compared to *RASSF1* hypermethylation. Application of the UCSC database shows enrichment for regulatory regions (ReMap database) for two of our DMRs (*DHRS3* and *PCMTD2*), possibly related to a functional role for DNA methylation in gene regulation of nearby genes. The three top-ranked DMRs show enrichment for binding of numerous proteins, mainly including BRD4 which is involved in regulating cell differentiation, and ESR1 and CTCF, which play a role in transcription regulation. Additionally, comparable to hypermethylation of *RASSF1*, hypermethylation of *TMEM240* and *DHRS3* have been described in several types of cancers and these genes have been proposed as tumor suppressor genes (46-48). Similarities in DNA methylation between the placenta and cancers are well known, and are suggested to be involved in their mutual properties such as proliferation, cell invasion and immune modulation (49).

A previous study identified differentially methylated CpGs between cfDNA from pregnant and non-pregnant women (30). They found 8,277 differently methylated CpGs, of which 1,704 CpGs, corresponding to 501 genes, were hypermethylated in cfDNA from pregnant compared to non-pregnant women, as well as in chorionic villus samples compared to maternal leukocytes. Although we focused on DMRs instead of individual CpGs, 11 of these genes overlapped with genes corresponding to our identified DMRs, of which for six the previously identified CpGs were captured by our DMRs: *DCAF10*, *DENND2D*, *OSR2*, *RNF126P1*, *TMEM17* and *TMEM233*

(Figure S2). These DMRs are therefore also likely robust placental-specific DNA methylation markers in maternal cfDNA.

In contrast to this previous study, most DMRs identified in our study were hypermethylated instead of hypomethylated in cfDNA from pregnant as compared to non-pregnant women. This bias towards hypermethylated regions when using MeD-seq could be explained by technological considerations combined with a 'diluted' placental signal within the total pool of maternal cfDNA. For example, for regions highly methylated in blood cells and thus in cfDNA, but hypomethylated in placental tissues, the relative decrease in methylation of maternal cfDNA caused by hypomethylated placental-originated cfDNA fragments will often be limited and remain undetected.

The relatively low contribution of placental-derived cfDNA remains a major challenge when using cfDNA as a proxy for placental DNA (11,12). In future studies, fragment size of cfDNA may be used to enrich for placental-originated cfDNA, since placental-originated cfDNA fragments are shorter than maternal cfDNA fragments (50). Combining DNA methylation analyses with other cfDNA features, referred to as "fragmentomics", could improve tissue of origin identification and analyses (50,51). Importantly, the contribution of different placental cell types (e.g. syncytiotrophoblast, cytotrophoblast, extravillous trophoblast) to cfDNA is currently unclear and probably also changes during gestation, driven by developmental changes in the placenta composition itself, as is observed for cell-free RNA (52). Therefore, future studies should strive to establish DNA methylation profiles for specific placental cell type as reference.

Recent studies have shown promising results for the use of the cfDNA methylome as predictor of obstetric complications, such as preeclampsia (32,33). More research is warranted to establish robust, easy to use biomarkers for obstetric health based on the cfDNA methylome, and to investigate whether these DNA methylation biomarkers could for example be incorporated into early prenatal genetic screening (53). Of note, cfDNA methylation differences for biomarker development do not have to originate from placental cfDNA, but could thus also originate from maternal tissues such as hematopoietic cells.

This study is one of the first to study genome-wide methylation of cfDNA in pregnant women. As compared to other techniques, MeD-seq could be a less costly and easier to perform method for low concentration cfDNA methylation profiling while ensuring a high genome-wide coverage. However, limitations of this pilot study are that we did not correct for potential confounders such as age, ethnicity and smoking which can impact DNA methylation (54-56).

For HBDs and placental tissues, we had no access to detailed participant's characteristics. Furthermore, MeD-seq has a bias towards hypermethylated regions and the highly fragmented nature of cfDNA may influence the coverage of the genome. Lastly, differences in placental cell type contributions in tissue biopsies and cfDNA could have distorted our results. Future studies should therefore aim to establish reference methylation profiles for different placental cell types.

Conclusion

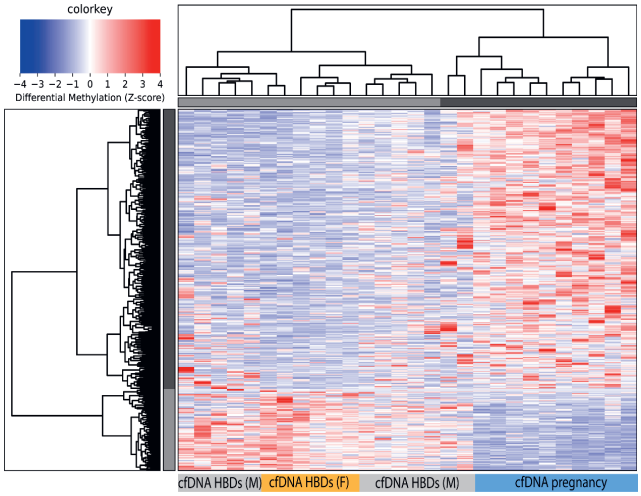
Research into the cfDNA methylome could allow for cfDNA applications beyond current practice; however, exploring the epigenetic landscape of cfDNA during pregnancy is still in its infancy. This pilot study revealed feasibility of genome-wide methylation profiling of maternal cfDNA using the MeD-seq technology. As a proof-of-concept, we showed that genome-wide placental DNA methylation marks can be identified in first trimester maternal cfDNA and fetal sex can be determined based on Y-chromosomal DNA methylation. Moreover, our results indicate not only a placental-origin but also a significant immune cell contribution to the pregnancy-specific cfDNA methylation signature. The added value of epigenome-wide analyses of cfDNA using MeD-seq in the current practice of prenatal genetic testing and in relation to obstetric complications and gestational age warrants further study.

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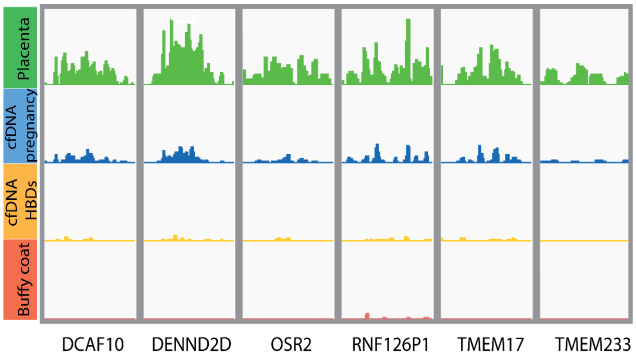
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Supplemental Material



Supplemental Figure S1: Heatmap visualizing overlap between cfDNA from male (M) and female (F) non-pregnant healthy blood donors (HBDs) compared to cfDNA from pregnant women, based on DMRs identified between pregnant women (P) and non-pregnant female HBDs. Red represents hypermethylation, blue represents hypomethylation.



Supplemental Figure S2: Gene-tracks for DMRs between cfDNA from pregnant women and non-pregnant female healthy blood donors (HBDs) that overlap with CpGs previously identified by Chu *et al.* (30)

Supplemental Tables: Can be accessed via the website of the scientific journal in which this manuscript has been published**.**


6

Chapter 6

Methylome profiling of cell-free DNA during the early life course in (un)complicated pregnancies using MeD-seq: protocol for a cohort study embedded in the prospective Rotterdam Periconception Cohort

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Abstract

Introduction: Placental DNA methylation differences have been associated with timing in gestation and pregnancy complications. Maternal cell-free DNA (cfDNA) partly originates from the placenta and could enable the minimally invasive study of placental DNA methylation dynamics. We will for the first time longitudinally investigate cfDNA methylation during pregnancy by using Methylated DNA Sequencing (MeD-seq), which is compatible with low cfDNA levels and has an extensive genome-wide coverage. We aim to investigate DNA methylation in placental tissues and cfDNA during different trimesters in uncomplicated pregnancies, and in pregnancies with placental-related complications, including preeclampsia and fetal growth restriction. Identified gestational age- and disease-specific differentially methylated regions (DMRs) could lead to numerous applications including biomarker development.

Methods and analysis: Our study design involves three sub-studies. Sub-study 1 is a single-centre prospective, observational subcohort embedded within the Rotterdam Periconception Cohort (Predict study). We will longitudinally collect maternal plasma in each trimester and during delivery, and sample postpartum placentas (n=300). In sub-study 2, we will prospectively collect first and second trimester placental tissues (n=10 per trimester). In sub-study 3 we will retrospectively collect plasma after noninvasive prenatal testing (NIPT) in an independent validation case-control cohort (n=30-60). A methylation-dependent restriction enzyme (LpnPI) will be used to generate DNA fragments followed by sequencing on the Illumina NextSeq2000 platform. DMRs will be identified in placental tissues and cell types, and in cfDNA related to gestational age or placental-related complications. (Paired) placental methylation profiles will be correlated to DMRs in cfDNA to aid tissue of origin analysis. We will establish a methylation score to predict associated diseases.

Discussion: This study will provide insights in placental DNA methylation dynamics in health and disease, and could lead to clinical relevant biomarkers.

Introduction

DNA methylation is an important epigenetic mechanism involved in the regulation of gene expression and plays a vital role in placental development and function (1-3). The genome-wide distribution of DNA methylation in placental tissue is known to change with increasing gestational age (4-6) and we and others have shown changes in placental DNA methylation in postpartum placentas associated with major obstetric complications, including preeclampsia, intrauterine growth restriction and preterm birth (7-17). Therefore, the study of placental DNA methylation potentially provides more insight in normal placental development and in underlying pathophysiology of placental-related complications, and can be used to develop both gestational age-specific as well as disease-specific biomarkers.

Previous DNA methylation studies mainly focussed on postpartum placental tissues. Placental methylation profiles of early- and mid-gestation placentas are largely understudied due to the impossibility to noninvasively investigate placental tissue during (uncomplicated) pregnancy. However, a promising alternative to study placental DNA methylation is by use of blood plasma derived cell-free DNA (cfDNA) originating partly from turnover and spill of placental tissue into the maternal circulation. This is also referred to as cell-free fetal DNA, since placental and fetal tissues largely share the same DNA sequence. cfDNA is already widely used for noninvasive prenatal testing (NIPT) for chromosomal aneuploidies as part of prenatal screening (18).

Research into the epigenetic features of (fetal) cfDNA is suggested to allow for cfDNA applications beyond current practice (19, 20). Thus far, studies investigating genome-wide methylation of cfDNA during pregnancy are scarce, but promising results have been reported recently indicating the feasibility of predicting the risk for preeclampsia by cfDNA methylome profiling at an earlier pregnancy stage (21-23). Early identification of pregnancies at high risk of developing preeclampsia is of the utmost importance to enable timely targeted preventative interventions such as the initation of low-dose aspirin and subsequently decrease maternal and fetal morbidity and mortality (24-26).

A previous developed ‘placental epigenetic clock’ showed the possibility to estimate gestational age using DNA methylation differences in placental tissues (27). Although studies using cfDNA are limited, differences in methylation levels of cfDNA fragments between trimesters are reported (28). This indicates the feasibility of identifying gestational age-specific methylation markers in cfDNA. This could increase our understanding of placental epigenetic programming during development, and could be used to assess gestational age using maternal blood. This could be relevant in circumstances where the widely used first trimester ultrasound is not

always available or when pregnancies are discovered in second or third trimester, when assessing gestational age using ultrasound is less reliable (29).

Specificity of cfDNA as a placental-specific marker is hampered by a background of cfDNA originating from maternal tissues. The placental-derived fraction in maternal cfDNA is about 10% at the end of the first trimester and increases with gestational age (30-32). Since DNA methylation patterns are cell type specific (1), identification of methylation patterns in cfDNA specific for placental cell types will increase specificity of cfDNA as placental-specific marker.

Most techniques used to study DNA methylation are limited to a subset of cytosine-guanine (CpG) dinucleotides and rely on bisulfite conversion which substantially degrades DNA (33-35). This challenges DNA methylation analyses of cfDNA which is generally only present in low amounts. We will therefore use the Methylated DNA sequencing (MeD-seq) technique. By omitting bisulfite conversion, MeD-seq is well compatible with low amounts of (cf)DNA as shown in previous studies performed using MeD-seq (36-39). MeD-seq makes use of a methylation-dependent restriction enzyme (LpnPI) which activity is restricted by short fragment size. In contrast to other methylation dependent enzymes, which overdigest CpG dense regions of the genome, LpnPI does not have this problem and is able to generate DNA fragments from methylated CpG islands that are still large enough (around 32 bp) to be isolated, sequenced and properly mapped. This limits the required sequencing depth while allowing for genome-wide methylation profiling of >50% of all potentially methylated CpGs, detecting >99% of all CpG islands and promoters, a more extensive coverage as compared to most other techniques (40, 41).

In summary, research gaps exist regarding dynamics of the placental DNA methylome over gestation and in the context of placental-related complications. Placental-originated cfDNA has the potential to facilitate minimally invasive placental methylome profiling during pregnancy and recent studies indicate the feasibility of its use in early preeclampsia prediction (22, 23). Because of the extensive genome-wide coverage of the MeD-seq technology, we expect to identify additional differentially methylated regions (DMRs) related to normal placental development and placental-related complications that can improve the current use of cfDNA.

Aims and objectives

We hypothesize that our study can identify several novel trimester-specific and disease-specific DMRs in placental tissues and (fetal) cfDNA. This could lead to numerous (pre)clinical applications including improved biomarker development for the prediction of obstetric

complications. To this end, we aim to investigate genome-wide DNA methylation in both placental tissues and (fetal) cfDNA during different trimesters of gestation and in the context of placental-related complications in three different sub-studies, using the MeD-seq technology.

The study objectives include:

1. Identification of DNA methylation profiles in placental tissues and specific placental cell types in uncomplicated pregnancies in each trimester (**sub-study 1 and 2**);
    a. Identify DMRs between trimesters and between different placental cell types
    b. Use identified DNA methylation profiles as reference in tissue of origin analyses of DMRs in cfDNA
2. Identification of methylation profiles in cfDNA in uncomplicated pregnancies in each trimester and at delivery (**sub-study 1**);
    a. Identify DMRs between trimesters and at birth in cfDNA
    b. Investigate overlap of DMRs with DNA methylation marks in (paired) placental tissues
3. Identification of methylation profiles in cfDNA in pregnancies with obstetric complications including preeclampsia and fetal growth restriction in each trimester and at delivery (**sub-study 1 and 3**);
    a. Identify disease-specific DMRs in each trimester and at birth in cfDNA from complicated pregnancies compared to uncomplicated pregnancies
    b. Investigate overlap of DMRs with DNA methylation marks in (paired) placental tissues
    c. Establish a methylation score able to predict disease-risk of associated complications based on top-ranked DMRs
    d. Validate first trimester findings in an independent case-control cohort (**sub-study 3**)

Subsequently, underlying genetic pathways related to identified DMRs involved in normal placental development or disease development will be investigated to increase our understanding of epigenetic regulation during placental development.

Methods and analysis

Study design

Our study design involves three simultaneously conducted sub-studies (Figure 1). Sub-study 1 is a single-centre, prospective, observational subcohort which is embedded in the Rotterdam

Periconceptional Cohort (Predict study) (42, 43) in which we longitudinally collect cfDNA in each trimester during pregnancy and during delivery, and we sample placental tissues postpartum. Inclusions for this subcohort started November 2022 and the total study duration is expected to take 30 months. Sub-study 2 is a cross-sectional study in which we prospectively collect placental tissues of uncomplicated pregnancies of the first and second trimester at an elective abortion clinic from July 2023 onwards. Lastly, sub-study 3 is a retrospectively selected case-control study in which we will collect residual plasma samples previously collected for NIPT to validate our first trimester cfDNA findings in relation to obstetric complications in an independent cohort. The Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) checklist for this protocol can be found in Supporting Information File S1.

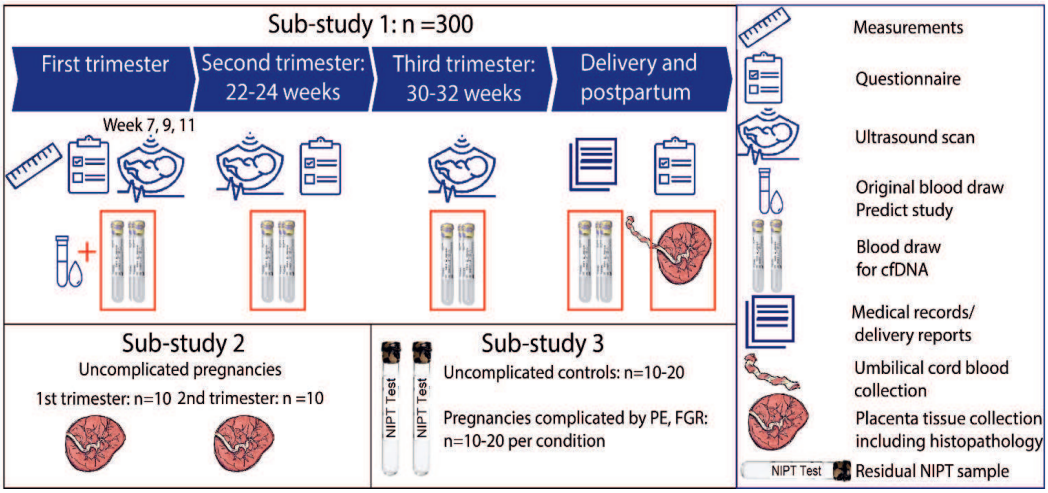


Figure 1: Overview of collected tissue materials and study procedures in the different sub-studies. Procedures in the current subcohort (sub-study 1) in addition to regular procedures in the Predict study during pregnancy are outlined in red. PE = preeclampsia, FGR = fetal growth restriction, cfDNA = cell-free DNA, NIPT = noninvasive prenatal testing

Ethics

Approval of all different parts of this protocol was obtained by the Medical Ethics Committee from the Erasmus MC, Rotterdam, The Netherlands. The Predict study was prospectively registered previously (NL4115 Dutch Trial Register). Approval for sub-study 1 which is embedded in the Predict study, was received in October 2022 (MEC-2004-0227, amendment A-0003). Approval for sub-study 2 version 1.1 and for amendment A-0001 was obtained in April 2023 (MEC-2023-0028). For sub-study 3 version 2.0 and amendment A-0001, approval was obtained in December 2023 (MEC-2022-0788). A copy of the approved protocols can be found in Supporting Information File S2. Any study amendments will first be submitted for approval by the Medical Ethics Committee.

All samples collected within sub-study 1 and 3 are pseudo-anonymously coded with a unique study number. All samples within sub-study 2 are anonymously collected. The monitoring of the Predict study is followed for sub-study 1, which includes an independent monitor of the data monitoring committee of the Erasmus MC (42, 43). The final trial dataset is accessible for the research team of the Predict study.

Study population and recruitment

In sub-study 1, the same in- and exclusion criteria are followed as for the Predict study (42, 43). All participants will be included at the outpatient clinic of the Erasmus MC, Rotterdam. Women of at least 18 years old with a singleton pregnancy who are <10+0 weeks pregnant are eligible for participation if they have sufficient understanding of the Dutch language and are willing to give written informed consent. Women pregnant after oocyte donation will be excluded. Written informed consent is asked by a research nurse at the Erasmus MC.

In sub-study 2, participants are included at a Dutch abortion clinic. Women of at least 18 years old with a singleton pregnancy with no known birth defects and a gestational age between 9+0–12+0 or 16+0–17+6 are eligible for participation. To ensure anonymity of participants, written informed consent is asked by a local nurse or medical doctor not involved in this study. Written informed consent is asked after choosing for elective, surgical abortion for social indications. Potential participants are excluded if they are infected with HIV, Hepatitis B or other blood borne infectious diseases.

Lastly, in sub-study 3, women will retrospectively be selected if they gave written informed consent to use residual NIPT material for research purposes. Between April 2017 and April 2023, all pregnant women in the Netherlands were offered NIPT on a trial basis (TRIDENT-2 study) (44, 45). Women who previously participated in the Predict study and/or delivered at the Erasmus MC and who developed preeclampsia or fetal growth restriction will be matched with women who had uncomplicated pregnancies.

Preeclampsia will be defined according to the ISSHP guidelines (46). Preeclampsia will be diagnosed if there is new-onset hypertension (≥140/90 mmHg) after 20 weeks of gestation, combined with at least one of the following new-onset complications: proteinuria; maternal end-organ dysfunction including thrombocytopenia, elevated liver enzymes, acute kidney injury, pulmonary edema or neurological symptoms (such as eclampsia, severe headaches, visual scotomata, stroke); or uteroplacental dysfunction (such as fetal growth restriction, abnormal umbilical artery Doppler wave form analysis, placental abruption). In women with

chronic hypertension, superimposed preeclampsia will be diagnosed after development of new proteinuria, one of the above mentioned maternal organ dysfunctions or signs of uteroplacental dysfunction. We will further distinguish early-onset (<34 weeks of gestation) and late-onset (≥34 weeks of gestation) preeclampsia (46).

Study procedures: sub-study 1

For women participating in sub-study 1, there will be multiple study visits including different measurements and procedures. The design and logistics of the Predict study have extensively been described earlier (42, 43). Figure 1 gives an overview of visits and procedures as part of the Predict study and adaptations to the protocol for women in our subcohort.

In short, sub-study 1 consist of the following measurements and procedures:

Anthropometrics

Height, weight, body circumferences and blood pressure will be measured at study entry by a research nurse.

Questionnaires

In the first trimester, a food frequency questionnaire and a general questionnaire focusing on lifestyle and general (health) information including geographical background will be filled out. At 24 weeks of gestation and after delivery, general questionnaires will be filled out focusing on lifestyle and pregnancy outcomes.

Ultrasound scans

At 7, 9 and 11 weeks of gestation, 3D ultrasounds scans are performed using a GE Voluson E10 Expert system with a 6–12 megahertz transvaginal probe and 4D View software (General Electric Medical Systems, Zipf, Austria) focusing on embryonic and placental (vascular) development. At 22–24 weeks and 30–32 weeks of gestation, fetal growth will be assessed by fetal biometry and ultrasound Dopplers of the umbilical and uterine arteries and the middle cerebral artery will be performed.

Blood samples

Blood samples will be collected in each trimester at the same day as the ultrasound. In addition to the standard Predict protocol, two CellSave preservative tubes (10mL each) are used to collect blood for subsequent cfDNA analyses at each time point. In the first trimester, blood is collected at 11 weeks of gestation which is comparable to timing of the national protocol for the NIPT (44). If the delivery takes place at the Erasmus MC, blood will be collected after

admission at the day of delivery before childbirth. Plasma will be isolated by two centrifugation steps (10 minutes at 1,711 g — 2,000 g followed by 10 minutes 12,000 g — 16,000 g) within 96 hours after blood collection and stored at −80 °C before cfDNA isolation takes place.

Postpartum tissues

After delivery, umbilical cord blood will be collected in one EDTA tube and a separator tube (both 10ml) and placentas will be formalin fixed. Histopathological examination of the placenta will be performed and formalin-fixed paraffin-embedded (FFPE) blocks containing placental tissues are stored.

Medical records

Medical records including delivery reports will be used to obtain detailed information regarding pregnancy outcomes, such as date of birth, birthweight, fetal sex and the occurrence of obstetric complications such as preeclampsia.

Study procedures: sub-study 2

In sub-study 2, participation of women will be anonymized and the only data collected is the gestational age at the time of elective abortion and the placental tissues. Participants will not undergo additional study procedures, since only leftover tissues will be collected. After the surgical procedure, placental tissues will be stored in formaldehyde solution 4%, buffered.

Study procedures: sub-study 3

In sub-study 3 there will be no additional study procedures for participants, since only residual NIPT samples will be used.

Laser capture microdissection

DNA methylation patterns are cell type specific (1). To be able to correlate DNA methylation profiles in placental tissues and cfDNA to specific placental cell types, we aim to obtain reference DNA methylation profiles for different placental cell types. This will increase specificity of cfDNA as placental-specific marker. An experienced technician and expert perinatal pathologist will perform laser capture microdissection (LCM) to separate different placental cell types, including extravillous trophoblasts and the double trophoblast layer consisting of syncytiotrophoblast (SCT) and cytotrophoblast (CTB) in first and second trimester, and the single trophoblast layer and knob-shaped trophoblasts (syncytial knots) in third trimester (Figure 2) (47). A previous study showed compatibility of MeD-seq with samples isolated after LCM (48). Cell types will be separated from three samples per trimester from

uncomplicated pregnancies. First and second trimester samples are collected in sub-study 2 and third trimester samples are collected in sub-study 1 (Figure 1).

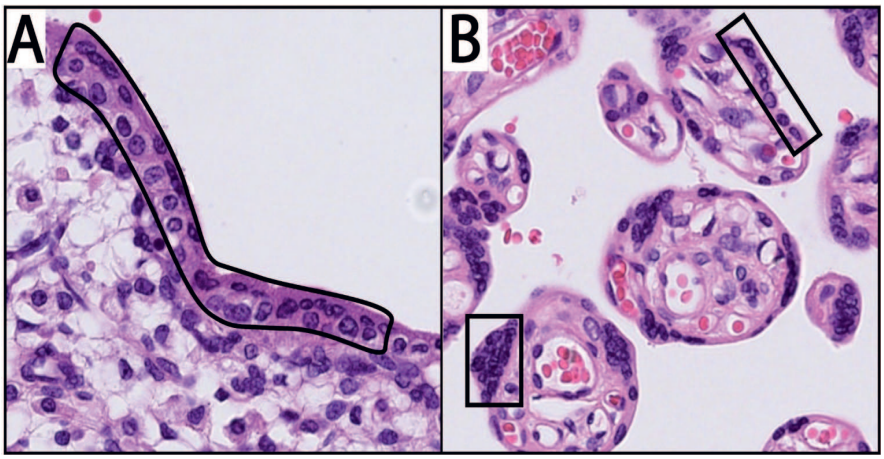


Figure 2: **Part of placental cell types that will be separated by laser capture microdissection, Hematoxylin and Eosin (H&E) Stain, 40x magnification. A)** First trimester placenta: outlined is the double layer of trophoblasts consisting of syncytiotrophoblast (outer layer) and cytotrophoblast (inner layer). **B)** Third trimester placenta: outlined are knob-shaped trophoblast (syncytial knots) (under, left) and the single trophoblast layer (up, middle).

(cf)DNA isolation

Both bulk DNA isolation and cell type specific DNA isolation of LCM samples will be performed using the QIAamp DSP DNA FFPE Tissue kit according to the manufacturer's protocol. All cfDNA will be isolated from 4 ml of plasma using the QIAamp Circulating Nucleic Acid Kit according to the manufacturer's protocol.

Sample size

Based on our previous experiments to generate reference profiles using MeD-seq, the number of reliable DMRs stabilizes when having a sample size of ±8-9 samples, as for example shown in (48). Based on preliminary data, we also observed this stabilization in number of identified DMRs between cfDNA collected in the first trimester in sub-study 1 compared to cfDNA from non-pregnant controls (Supplemental Figure S1). This intended sample size is also supported by two recent cfDNA studies which identified robust methylation differences between women with preeclampsia and uncomplicated control pregnancies (n=5-6 per group) (21, 23). Therefore we aim to include 10 samples per time point for both cfDNA and direct placental tissues, and for both complicated and uncomplicated pregnancies.

In the Predict study, around 4% of pregnancies are complicated by preeclampsia and around 5% of children in this cohort is born with a birthweight <3th percentile. To include enough

women who will develop above mentioned complications, we aim to prospectively include at least 300 pregnant women in sub-study 1.

In sub-study 2, we will collect 10 placentas in first and 10 placentas in second trimester and compare these with 10 third trimester placentas collected postpartum from uncomplicated pregnancies in sub-study 1 (total n=30).

In sub-study 3, we will retrospectively collect samples from: 1. women who developed preeclampsia, distinguishing women with early-onset as well as late-onset preeclampsia, 2. women with fetal growth restriction and 3. uncomplicated control pregnancies (n=10-20 per group).

Data analysis

MeD-seq assays will be performed as previously described (40, 41). In short, genomic DNA and plasma-derived cfDNA are digested with the methylation-dependent restriction enzyme LpnPI (New England Biolabs, Ipswich, MA) generating 32 bp DNA fragments containing the methylated CpG in the middle, followed by sequencing on the Illumina NextSeq2000 platform. Custom Python scripts will be used to process the acquired DNA methylation profiles for the different samples using a previously established pipeline (40, 41). Raw data files (fastq) will be filtered to be able to distinguish methylation data from background data and will be mapped to the human genome (hg38) using bowtie. We will generate regional (promoter, gene body and CpG Island regions) methylation scores. For the genome-wide analysis, a sliding window technique will be used to detect DMRs and the chi-squared test will be used for statistical testing with a Bonferroni correction. A Bonferroni adjusted p-value ≤0.05 will be considered significant. After DMRs are determined, Z-score transformation of the read count data will be applied for unsupervised hierarchical clustering analyses.

Our primary outcomes are DMRs in placental tissues and specific placental cell types between trimesters, DMRs in cfDNA in uncomplicated pregnancies between trimesters and at delivery, and DMRs in cfDNA in pregnancies with obstetric complications including fetal growth restriction and preeclampsia in each trimester and at delivery. (Paired) cfDNA and placental DMRs will be correlated to investigate the placental-origin of identified DMRs in cfDNA.

The intended timeline of data collection and data analysis is depicted in Figure 3. We start our data analysis with the characterisation of DNA methylation profiles in bulk placental DNA and in different placental cell types in each trimester to create a reference placental methylation

map. Used placental tissues are collected in sub-study 1 and sub-study 2 from uncomplicated pregnancies. DMRs will be identified between placental cell types and between trimesters. Next, DMRs will be identified between cfDNA collected from uncomplicated pregnancies at the four different time points in sub-study 1. Direct comparison between placental tissues and paired cfDNA collected at delivery will allow for accurate identification of the placental contribution to cfDNA and overlap with the established reference placental DNA methylation map will be investigated.

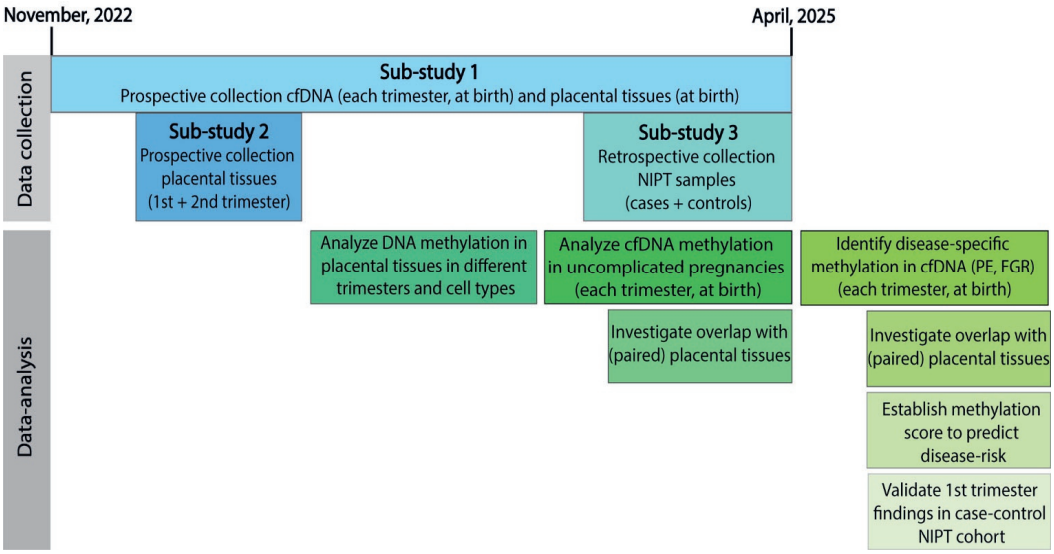


Figure 3: Timeline of data collection in the different sub-studies and data-analysis.

Lastly, DMRs will be identified in cfDNA between (retrospectively collected) uncomplicated pregnancies and pregnancies complicated by major obstetric complications, including preeclampsia or fetal growth restriction. Sensitivity analysis will be performed for women with early- or late-onset preeclampsia where possible. In sub-study 1, DMRs will be investigated for each trimester and cases and controls will be matched based on maternal age, maternal BMI, parity, mode of conception, fetal sex, ethnicity, presence of gestational diabetes and smoking status. Overlap of identified DMRs with placental DNA methylation will be studied as described previously. Identified differences in methylation profiles in cfDNA could – at least partly – originate from methylation differences in the placenta (22). Moreover, total cfDNA including maternal-originated cfDNA could also make an important contribution to biomarker development, as demonstrated by others for preeclampsia prediction (21). Depending on the identification of disease-specific DMRs, we will develop a disease-prediction tool based on top-ranked DMRs for each trimester. DMRs will be used to generate receiver operating

characteristic (ROC) curves based on the reference samples, this will allow us to score individual samples on a specific threshold per DMR. Samples having methylation scores above the threshold will score a 1, while samples below the threshold score a 0, leading to a cumulative methylation score per individual sample. In sub-study 3, we aim to validate the first trimester disease-associated DMRs identified in sub-study 1 in an independent cohort using residual NIPT samples. Cases and controls will additionally be matched for gestational age at NIPT sampling.

Subsequently, pathway analyses of genes containing identified DMRs will be performed using http://geneontology.org.

Dissemination

Findings from this study will be disseminated by publication of peer-reviewed manuscripts and presentations at scientific conferences. All study data leading to publications will be available upon reasonable request. Raw sequencing data (fastq files) will be deposited on the Sequence Read Archive (SRA) database. Participants of the Predict study will be informed about the progress and results of this study through a yearly newsletter.

Research implications

The placenta is a crucial but largely inaccessible organ during development, and plays an important role in the pathophysiology of several obstetric complications. Unravelling the epigenetic programming of the placenta already during pregnancy in health and disease could be of great interest to increase our understanding of how epigenetic mechanisms influence placental and fetal health and development.

We are the first study longitudinally investigating cfDNA methylation dynamics during gestation using MeD-seq, a technology with an extensive genome-wide coverage. Identified DNA methylation changes can provide more insight in normal placental development. Estimating gestational age using the cfDNA methylome with a clinically relevant precision would require the identification of methylation profiles specific for a small gestational age window (e.g. 1-2 weeks), which would also require more sampling moments throughout gestation. However, this study could serve as a first proof of concept by the identification of trimester-specific methylation profiles in cfDNA. Additionally, placental DNA methylation differences have been associated – either as cause, consequence or both – with several obstetric complications with a major burden for fetal and maternal health and implications for health outcomes across the life course. For example, several studies investigating postpartum placentas from pregnancies complicated by preeclampsia have shown DNA methylation differences in genomic regions

relevant to the pathophysiology of preeclampsia, such as angiogenesis and cell proliferation (7). Identified disease-specific DMRs in our study could allow for further unravelling of underlying (epigenetic) mechanisms contributing to the development of associated diseases at different time points in pregnancy. Moreover, identified DMRs may be used as biomarker for (early) prediction of disease. Early detection of high-risk pregnancies is key to improve health outcomes for both mother and child, by enabling timely initiation of adequate treatment and prevention (24-26).

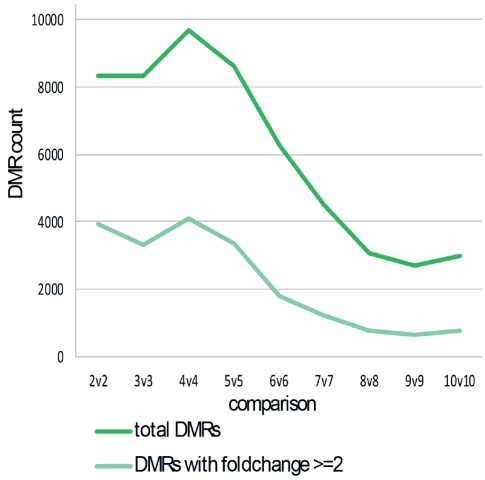
Although genome-wide analysis of the cfDNA methylome during pregnancy is largely understudied, cfDNA holds great promise to study epigenetic (placental) programming during pregnancy and to develop clinically relevant biomarkers for obstetrical care. Our study could therefore form a basis for future studies into cfDNA applications with the ultimate aim to improve prenatal and future health for mother and child.

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Supplemental Material



Supplemental Figure S1: Number of identified DMRs between cfDNA from pregnant women in the first trimester, as compared to cfDNA from non-pregnant controls with increasing sample size (preliminary data). The number of identified DMRs stabilizes after including 8 pregnant and 8 non-pregnant cfDNA samples (8v8).



Chapter 7

Profiling (placental) DNA methylation in cell-free DNA across gestation: The Rotterdam Periconception Cohort

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 # Equal contribution

Molecular Human reproduction. 2025

Abstract

Placental DNA methylation varies across gestation and is associated with obstetrical complications. Cell-free DNA (cfDNA) from maternal plasma could provide a noninvasive approach to study placental DNA methylation in ongoing pregnancies. However, research on maternal cfDNA methylation is limited and technologically challenging. Therefore, we aimed to investigate DNA methylation in maternal cfDNA and placental tissues across gestation using the innovative Methylated DNA Sequencing (MeD-seq) technology. Secondly, we explored the origins of methylation differences in maternal cfDNA across gestation, and aimed to identify gestational age-associated placental DNA methylation markers directly in cfDNA. We longitudinally collected maternal cfDNA in all three trimesters and at birth (n=10), alongside placental tissues from first trimester, second trimester and term pregnancies (all n=10), and used previously collected maternal blood buffy coat samples (n=20). Different placental cell types, including syncytiotrophoblasts/cytotrophoblasts (SCTs/CTBs) (n=10), extravillous trophoblasts (EVTs) (n=7) and syncytial knotting (SK) (n=3), and maternal cell types including spiral arteries (n=3) and endometrial epithelium (n=3), were isolated using laser capture microdissection. Differentially methylated regions (DMRs) identified in cfDNA from pregnant compared to non-pregnant women (n=6) ranged from 798 to 2163 in first and third trimester, respectively. Gradual DNA methylation changes were observed across gestation in cfDNA, placental tissues and trophoblasts. We showed an increase in DMRs in cfDNA, that overlap with DNA methylation in placental tissues and especially trophoblasts, and in DNA methylation of placenta-specific markers across gestation, reflecting an increased placental-originated cfDNA fraction. Among 110 DMRs between first trimester and term placental tissues, those related to *NXPH4*, *EPS8L2*, *AMOTL1* and *IRX2* had the strongest association with gestational age in cfDNA, for which comparable associations were found in SCTs/CTBs. These DMRs were all hypomethylated in maternal buffy coat samples. This study indicates the feasibility of identifying gestational age-dependent placental DNA methylation marks in maternal cfDNA, and can serve as reference for future studies.

Introduction

DNA methylation is an epigenetic mechanism with a vital role in placental development and function (1-3). Differences in placental DNA methylation profiles have been found in relation to gestational age (4-7) and have been associated with several obstetrical complications such as preeclampsia (8, 9). Hence, studying placental DNA methylation dynamics across pregnancy can increase our understanding of placental development in both health and disease and may lead to the development of clinically relevant biomarkers for associated complications.

Studies investigating placental DNA methylation at early- and mid-gestation are limited, since acquiring placental tissue during pregnancy is historically limited by both logistic and ethical barriers. Moreover, the need to obtain physical placental tissue limits the clinical applicability of studying placental DNA methylation during pregnancy. An alternative approach to investigate placental DNA methylation could be the analysis of cell-free DNA (cfDNA) in maternal plasma. Although most cfDNA fragments in maternal blood originate from maternal tissues, placental-originated cfDNA fragments can also be detected in maternal blood during pregnancy. Importantly, the epigenetic features of the placenta are preserved in placental-originated cfDNA fragments (10, 11). Until now, cfDNA derived from maternal plasma has been already widely used for noninvasive prenatal testing (NIPT), an antenatal blood test primarily used to screen for fetal chromosomal abnormalities (12).

Studies investigating the maternal cfDNA methylome are limited and generally technologically challenging, due to degradation of DNA isolated from already limited amounts of cfDNA as side-effect of the necessary bisulfite treatment (13). In our previous study, we therefore used Methylated DNA Sequencing (MeD-seq). By using a methylation-dependent restriction enzyme, MeD-seq is well-compatible with low amounts of cfDNA while providing full genome-wide coverage (14-16). MeD-seq has extensively been validated and compared to other techniques, including whole-genome bisulfite sequencing (WGBS), MeDIP and the 450K Infinium array (14, 17). We previously showed numerous methylation differences between cfDNA from non-pregnant women and pregnant women in the first trimester using MeD-seq (18). Other studies have reported differences in cfDNA methylation levels between trimesters (19), or estimated the placental-derived cfDNA fraction in different trimesters based on genome-wide cfDNA methylation profiles (20, 21). In addition, a recent study showed the feasibility of predicting preeclampsia using the first trimester maternal cfDNA methylome (22). However, genome-wide methylation dynamics in cfDNA in relation to the duration of gestation, and the possibility of detecting gestational age-associated placental methylation markers in cfDNA, have not been investigated before.

We hypothesized that both placental and maternal cfDNA methylation are altered with the duration of gestation and that placental DNA methylation differences related to gestational age can be detected in maternal cfDNA. Therefore, our first aim was to investigate DNA methylation in cfDNA from maternal blood and in placental tissues in association with gestational age using MeD-seq. Secondly, we aimed to explore the origins of methylation differences in maternal cfDNA across gestation, and to identify gestational age-associated placental DNA methylation markers directly in cfDNA. This study increases our understanding of the understudied cfDNA methylome across gestation and the role of epigenetic programming during normal (placental) development. Moreover, our data can serve as a reference for future studies including pregnancies with obstetrical complications such as preeclampsia.

Methods

Ethics statement

All participants provided written informed consent and our study was approved by the Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam (Predict study: MEC-2004-227, collection of first/second trimester placentas: MEC-2023-0028).

Study design

We prospectively collected cfDNA from maternal plasma in all three trimesters and at birth and collected postpartum placental tissues from women who participated in the Rotterdam Periconceptional Cohort (Predict study) (n=10) at the Erasmus Medical Centre, Rotterdam (23-25). Women who were at least 18 years old with a singleton pregnancy and who were <10+0 weeks pregnant were eligible for participation. Blood samples were collected in each trimester, around 11, 22–25, and 30–33 weeks of gestation, and during delivery (n=10). For the current study, blood samples were specifically selected from women who had an uncomplicated pregnancy (e.g. term pregnancy, birthweight percentile >10 and <90, no obstetrical complications such as hypertensive disorders of pregnancy and no congenital malformations).

In addition to data collected within the Predict study, we prospectively collected placental tissues after elective abortions of singleton pregnancies without congenital abnormalities in a Dutch abortion clinic. These samples were collected from first and second trimester pregnancies at approximately 9-12 weeks (n=10) and 16-18 weeks (n=10) of gestation. We further used our reference sets of cfDNA obtained from non-pregnant women (n=6) and maternal buffy coat samples (leukocytes) (n=20) as described previously (18, 26).

Sample processing

Blood samples were collected in CellSave preservative tubes (Menarini Silicon Biosystems Inc, Huntingdon Valley, PA). Plasma was isolated by two centrifugation steps (10 minutes at 1,711 g — 2,000 g followed by 10 minutes at 12,000 g — 16,000 g) within 96 hours and stored at −80 °C prior to cfDNA isolation (27). Placental tissues were collected after surgical abortion (first and second trimester) and after term birth (Predict study) and stored in buffered 4% formaldehyde solution. Full-thickness biopsies were taken followed by embedding in formalin-fixed paraffin-embedded (FFPE) blocks. Histopathological examination of the placentas was performed by a perinatal pathologist (LM).

Laser capture microdissection (LCM)

To validate our bulk placental analyses and to evaluate the (placental) cell type of origin in the maternal cfDNA methylation signatures, we isolated specific placental cell types using LCM. PALM membrane slides 1.0 PEN were UV treated (254 nm) and coated with poly-L-lysine (0.1%) according to the manufacturer's protocol before use, to improve tissue adhesion to the membrane. Sections of 16 µm were cut from the FFPE blocks, mounted on the pretreated membrane slides and incubated at 60 °C for two hours. Subsequently, the sections were dewaxed, dehydrated and stained with hematoxylin. Regions of interest were annotated by a perinatal pathologist (LM) and included syncytiotrophoblasts and cytotrophoblasts (SCTs/CTBs) in first and second trimester and term placental tissues, extravillous trophoblasts (EVTs) in first and second trimester placental tissues and syncytial knotting (SK) from term placental tissues. Furthermore, we isolated spiral arteries and endometrial epithelium as maternal tissues from first and second trimester placental tissues, to explore a possible contribution of these cell types to the maternal cfDNA signature in pregnancy. The annotated regions were cut using the Leica Laser Microdissection system and we dissected 5-20 mm^2 per sample including minimal 1000 cells to obtain between 10-50 ng DNA per 8.66 µl of sample. DNA concentrations were measured using NanoDropTM.

(cf)DNA isolation

cfDNA was isolated from 4 ml of plasma using the QIAamp Circulating Nucleic Acid Kit according to the manufacturer's protocol. For isolation of bulk placental DNA, four to six slides of 4 – 6 µm were cut from the FFPE blocks. One slide per sample was stained using hematoxylin and eosin (H&E), and DNA from normal placental parenchyma was isolated from subsequent slides. DNA isolation from both bulk placenta and LCM samples was performed using the QIAamp DSP DNA FFPE Tissue Kit according to the manufacturer's protocol.

MeD-seq assay and data analysis

MeD-seq assays were performed as described previously (14-16, 18, 25). (cf)DNA was digested with the methylation-dependent restriction enzyme LpnPI (New England Biolabs, Ipswich, MA, USA) generating 32 bp DNA fragments containing the methylated CpG in the middle. LpnPI is unable to digest its own fragments preventing loss of fragments coming from CpG dense regions (14). The TruPLEX DNAseq 96D kit (Rubicon Genomics, Takara Bio Europe, Saint-Germain-en-Laye, France) was used to prep samples for sequencing and samples were purified on a Pippin HT system with 3% agarose gel cassettes (Sage Science, Beverly, MA, USA). Samples were sequenced on the Illumina NextSeq2000 platform. Samples having <20 million reads or <20% CpG reads were considered failed. Dual indexed samples were demultiplexed using bcl2fastq software (Illumina).

Differentially methylated regions (DMRs) were identified using a previously established bioinformatics pipeline. Custom python scripts were used to process the acquired DNA methylation profiles. Raw fastq files were subjected to Illumina adaptor trimming. We filtered reads based on LpnPI restriction site occurrence between 13–17 bp from either the 5′ or 3′ end of the read to filter out DNA fragments that were methylated, and mapped reads that passed the filter to hg38 using bowtie2. BAM files were generated using SAMtools version 0.1.19 for visualization in IGV. To detect DMRs, a full genome-wide sliding window was used to detect sequentially differentially methylated LpnPI sites between two groups of the derivation cohort, and genome-wide read counts were normalized (rpm, reads per million) for coverage and compared using the chi-squared test. Neighbouring significantly called LpnPI sites were binned and reported. Overlap of genome-wide detected DMRs was reported for TSS (1 kb before and 1 kb after), CpG island or gene body regions (1kb after TSS till TES) using the annotations of the UCSC database downloaded from ENSEMBL (Homo_sapiens_hg38.GRCh38.79.gtf, http://www.ensembl.org). A Bonferroni-corrected p-value ≤0.05 was considered statistically significant. Z-score transformation of the read count data was applied for unsupervised hierarchical clustering analyses.

To identify cfDNA methylation associated with duration of gestation, DMRs were identified between maternal cfDNA in each trimester and at delivery and cfDNA from non-pregnant women, as well as between trimesters and delivery in maternal cfDNA. Additionally, DMRs were identified between first trimester, second trimester and term placental tissues and SCTs/CTBs, and between different trophoblast populations with a comparable gestational age.

To explore the tissue or cell type of origin of identified DMRs, we developed a binary methylation score for each DMR. Receiver operating characteristic (ROC) curves of reference sets were used to calculate the optimal DNA methylation threshold (using the "scikit-learn" package in Python) that best distinguished the two reference sets for each individual DMR (Figure 1A). We compared this threshold to DNA methylation of that specific region in other tissues. Samples with a DNA methylation level above the threshold for the specific DMR scored '1', and samples with a DNA methylation level below the threshold for the specific DMR scored '0', leading to a binary score for each DMR (Figure 1B). A cumulative methylation score was subsequently generated for each sample of interest based on the binary scores for all identified DMRs. For example, for identified DMRs that were hypermethylated in first trimester cfDNA as compared to cfDNA of non-pregnant women, we counted, in each individual placenta and buffy coat sample, how many of these DMRs had a DNA methylation level of this specific region above the established threshold. Likewise, for all identified DMRs that were hypomethylated in first trimester cfDNA as compared to cfDNA of non-pregnant women, we counted, in each individual placenta and buffy coat sample, how many of these DMRs had a DNA methylation level of this specific region below the established threshold. Together, they formed the proportion of DMRs in first trimester maternal cfDNA that, based on the binary score, overlaps with DNA methylation in placenta and buffy coats, which is another major source of cfDNA (10) (Figure 1B).

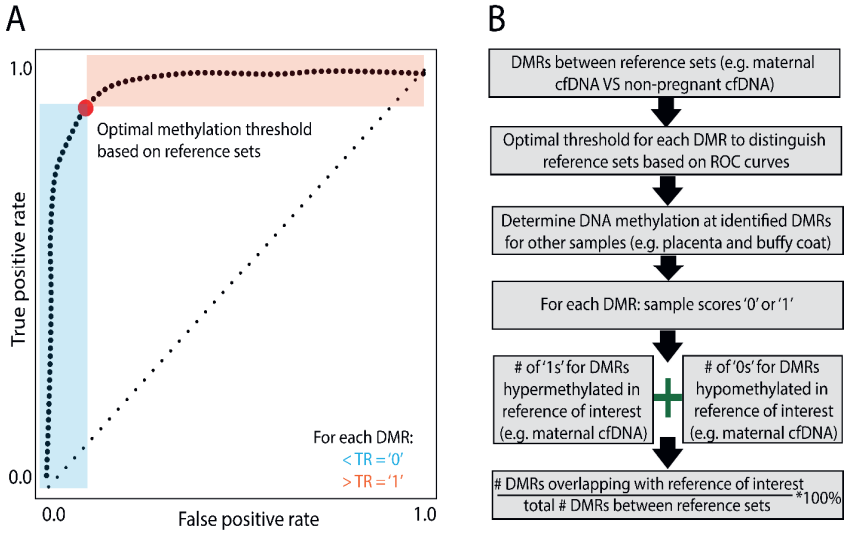


Figure 1: Obtaining a cumulative methylation score based on a binary score for each differentially methylated region (DMR) of interest. **A)** Receiver operating characteristic (ROC) curves were generated for each individual DMRs identified between reference sets of interest to determine the optimal methylation threshold (TR) that best distinguished both groups (e.g. maternal cell-free (cf)DNA compared to cfDNA from non-pregnant women). This threshold is defined based on the highest combination of the sensitivity (y-axis) and specificity (x-axis is 1-specificity). **B)** Workflow: first, DMRs between reference sets were identified (e.g. between maternal cfDNA and cfDNA from non-pregnant women). The calculated optimal

threshold (Figure 1A) was applied to all other samples of interest (e.g. placenta tissue and cell types, and buffy coat samples) leading to a binary methylation score for each DMR for these samples of interest. Samples with methylation levels below the threshold for the specific DMR scored '0', and samples with methylation levels above the threshold for the specific DMR scored '1'. The cumulative methylation score for a specific sample (e.g. placenta or buffy coat) consists of the total number of DMRs above the set threshold ('hypermethylated') that were also hypermethylated in the reference of interest (e.g. maternal cfDNA), together with the total number of DMRs for which the methylation level is below the set threshold ('hypomethylated') that were also hypomethylated in the reference of interest. The total number of DMRs that overlap with the reference of interest, divided by the total number of DMRs between the reference sets is the proportion of overlapping DMRs.

Next, methylation of the *RASSF1* promoter, a placental-specific DNA methylation marker (28), and methylation of our previously identified top-ranked placental-specific DMRs were compared between collection periods of cfDNA using an unpaired t-test standardized for the number of reads per million (rpm). These top-ranked DMRs were previously identified in first trimester cfDNA and are related to *TMEM240*, *DHRS3* and a CpG island upstream of *PCMTD2* (18).

Lastly, we determined the presence of specific placental DNA methylation markers associated with the duration of gestation in cfDNA. We calculated the Pearson correlation score for each DMR identified between first trimester and term placental tissues and SCTs/CTBs with gestational age in all cfDNA samples. A Pearson correlation score ≥0.7 was used to define a positive association. A cumulative methylation score was calculated for cfDNA samples based on methylation levels of placental DMRs positively associated with gestational age in cfDNA. High DNA methylation levels for identified placental DMRs in hematopoietic cells can mask the detection of these placental DMRs in cfDNA, since maternal blood cells account for the majority of cfDNA fragments (10). Therefore, we additionally selected DMRs between first trimester and term placental tissues that were hypomethylated in buffy coat samples (≥80% of samples scored '0' based on the binary score) to perform targeted analyses in maternal cfDNA.

Population statistics are presented as absolute numbers and percentages (%) for categorical variables and as medians and interquartile ranges (IQRs) for continuous variables.

Results

Technical performance

An overview of the samples and their technical performance is provided in Supplementary Table S1. For cfDNA collected in the first and second trimester or at delivery, 9 out of 10 samples per collection period passed quality control. DNA methylation profiles were established for all

third trimester cfDNA samples, all bulk placental tissues and all specific placental cell types isolated using LCM.

Methylation differences in maternal cfDNA during gestation

The baseline characteristics of pregnant women included in this study are depicted in Table 1. Plasma samples for cfDNA isolation were collected at 10+4–11+6 weeks (n=9), 22+0–25+6 weeks (n=9), and 28+6–32+6 weeks (n=10) of gestation for the different trimesters. Sampling at delivery occurred between 37+4 and 41+0 weeks of gestation (n=9) (Figure 2A, Supplementary Table S1).

Table 1. Baseline characteristics of pregnant women with cfDNA samples (n=10). Categorical variables are described as absolute numbers and percentages (%). Continuous variables are described as medians and interquartile ranges (IQRs). IVF/ICSI = in vitro fertilization/intracytoplasmic sperm injection.

Age at conception (years)	32.0 (30.5 – 35.4)
BMI at study entry	25.3 (23.8 – 25.8)
Country of birth	
The Netherlands	8 (80%)
Europe, other	2 (20%)
Nulliparous	5 (50%)
Periconceptional smoking	1 (10%)
Mode of conception	
Spontaneous	5 (50%)
IVF/ICSI	5 (50%)
Folic acid supplement use	10 (100%)
Started before conception	9 (90%)
Education level	
Intermediate	6 (60%)
High	4 (40%)
Male fetus	4 (40%)
Gestational age at birth	39+2 (39+1 – 40+3)
Birth weight (grams)	3655 (3458 – 3825)
Birth weight (percentile)	66 (54-79)

In comparison to cfDNA from non-pregnant women, the number of identified DMRs in maternal cfDNA increased over the three trimesters, from 798 in the first trimester, 1,494 in the second trimester to 2,163 DMRs in the third trimester, whereas 1,600 DMRs were identified in cfDNA collected at delivery (Figure 2B, Supplementary Table S2). The majority of the identified DMRs were hypermethylated in cfDNA collected during pregnancy as compared to cfDNA from non-pregnant women, with only 11 (1.4%), 80 (5.4%), 89 (4.1%) and 100 (6.3%) DMRs hypermethylated in cfDNA from non-pregnant women in the three trimesters or at delivery, respectively (Figure 2B, Supplementary Table S2). The numbers of DMRs identified between the trimesters and delivery were considerably lower, ranging from no DMRs between the first and

second trimester, to 235 DMRs between the first trimester and delivery (Figure 2C, Supplementary Table S3). Additionally, only one DMR was found between the second and third trimester, six DMRs were found between the third trimester and delivery, 13 DMRs were identified between the first and third trimester and 144 DMRs were found between the second trimester and delivery (Figure 2C, Supplementary Table S3).

To study the course of identified DMRs during gestation, Figure 2D shows an unsupervised hierarchical clustering analysis for DMRs identified in cfDNA between the first trimester and delivery. This shows a gradual change in DNA methylation for identified DMRs related to the duration of gestation, with an overall clustering of samples collected during the same time periods and in consecutive trimesters. Comparable results for these DMRs were obtained using a cumulative methylation score (Methods, Figure 1) when distinguishing overlap with DMRs specifically hypermethylated in cfDNA collected in the first trimester or at delivery (Figure 2E, Supplementary Table S4), as well as for DMRs identified in cfDNA between non-pregnant women and cfDNA collected at delivery (Supplementary Figure S1).

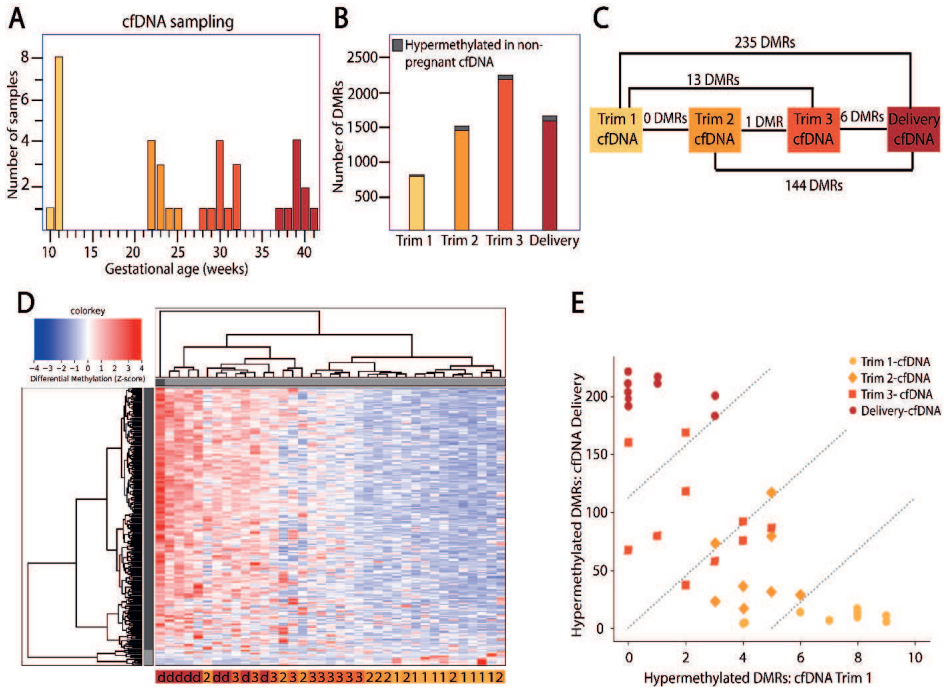


Figure 2: DNA methylation in maternal cell-free DNA (cfDNA) related to the duration of gestation. A) Gestational age at the time of cfDNA collection. **B)** Number of identified differentially methylated regions (DMRs) between cfDNA collected at different periods during pregnancy and cfDNA from non-pregnant women. The majority of DMRs were hypermethylated in maternal cfDNA, with a small proportion of DMRs hypermethylated in cfDNA from non-pregnant women (gray). **C)** Number of identified DMRs between cfDNA collected in different trimesters and at delivery. **D)** Heatmap visualizing unsupervised

hierarchical clustering of maternal cfDNA samples shows general clustering of samples collected during the same and consecutive time points. Selected autosomal DMRs were identified between cfDNA collected in the first trimester and cfDNA collected at delivery (d) with a fold change ≥2. The three trimesters are depicted as 1, 2 and 3. **E)** Cumulative methylation score based on DMRs between first trimester (Trim) and delivery cfDNA for all maternal cfDNA samples. For each sample, the cumulative number of '1s' for DMRs hypermethylated in first trimester cfDNA are on the x-axis, and the cumulative number of '1s' for DMRs hypermethylated in cfDNA collected at delivery are on the y-axis (Methods).

DNA methylation differences in placental tissues during gestation

Next, we investigated differences in DNA methylation between placental tissues collected at different periods during gestation. Placental tissues were collected between 9+2–12+2 and 16+0–17+6 weeks of gestation in the first and second trimester, respectively, and after term deliveries between 37+4–40+6 weeks of gestation (all n=10) (Figure 3A, Supplementary Table S1). We identified 110 autosomal DMRs between first trimester and term placentas, four DMRs between first and second trimester placentas, and 41 DMRs between second trimester and term placentas (Figure 3B, Supplementary Table S5). Unsupervised hierarchical clustering analysis for DMRs identified between first trimester and term placental tissues showed that placental tissues from second trimester cluster between first trimester and term placental tissues (Figure 3C), indicating a gradual, progressive change in placental DNA methylation during gestation.

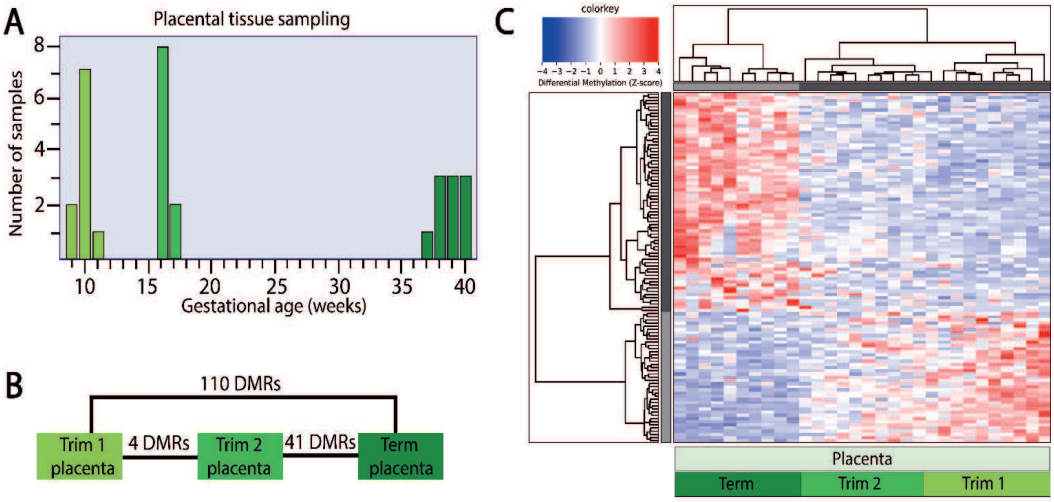


Figure 3: DNA methylation in placental tissues in relation to the duration of gestation. A) Gestational age at the time of placental tissue collection. **B)** Number of identified DMRs between placental tissues collected in the first and second trimester (Trim) and after term delivery. **C)** Heatmap visualizing unsupervised hierarchical clustering of second trimester placental tissues between first trimester and term placental tissues. Selected autosomal differentially methylated regions (DMRs) were identified between first trimester and term placental tissues with a fold change ≥2.

DNA methylation differences in relation to duration of gestation and between placental cell types

We further zoomed in on different placental cell types to explore the cell type of origin of DNA methylation differences and monitor potential changes of contribution of these cell types in bulk placental tissues and maternal cfDNA. Using LCM, we isolated syncytiotrophoblasts and cytotrophoblasts (SCTs/CTBs) from first (n=4) and second (n=3) trimester, and from term (n=3) placentas, extravillous trophoblasts (EVTs) from first (n=4) and second (n=3) trimester placentas, and syncytial knotting (SK) (n=3) from term placentas. Additionally, maternal-originated endometrial epithelium and spiral arteries from first (both n=2) and second (both n=1) trimester placentas were isolated. Using a cumulative methylation score based on binary scores for all DMRs between first trimester and term bulk placental tissues, we found that the methylation profiles of different trophoblast populations (SCTs/CTBs, EVTs and SK) are most comparable to the methylation profiles in bulk placental tissues collected at a comparable gestational age (Figure 4A). For first and second trimester endometrial epithelium and spiral arteries, a relationship between identified placental DMRs and gestational age was not observed (Supplementary Figure S2, Supplementary Table S6), so these cell types cannot explain the gestational age-associated differences observed in bulk placental DNA. Additionally, we identified 3,122 DMRs between first trimester and term SCTs/CTBs, 1,320 DMRs between first and second trimester SCTs/CTBs and 1,911 DMRs between second trimester and term SCTs/CTBs (Supplementary Table S7). Using DMRs between first trimester and term SCTs/CTBs, second trimester SCTs/CTBs cluster in between in an unsupervised hierarchical clustering analysis (Figure 4B). Both showed that DNA methylation changes related to gestational age are also observed in trophoblasts, indicating a gradual, progressive change in DNA methylation of trophoblast cells during placental development.

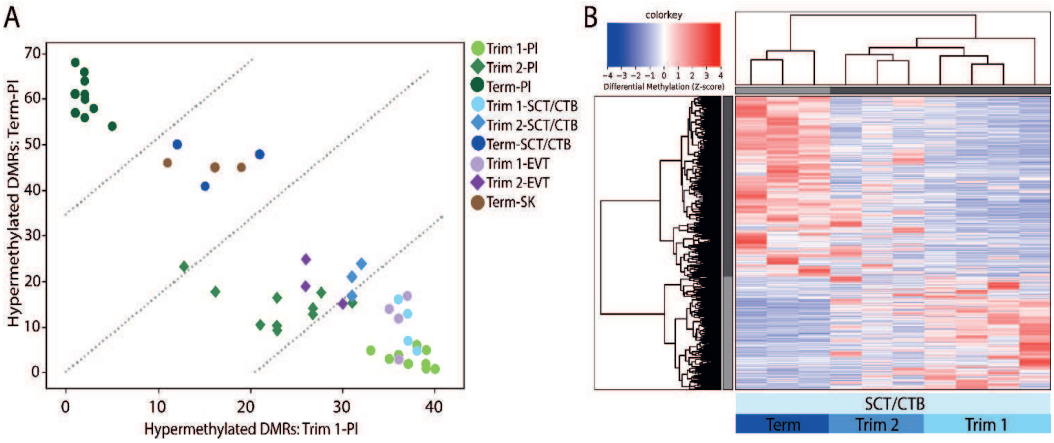


Figure 4 (previous page): DNA methylation associated with the duration of gestation in trophoblasts. A) Cumulative methylation scores for placental cell types isolated after laser capture microdissection (LCM), based on methylation of differentially methylated regions (DMRs) identified between bulk term placenta (Term-Pl) and first trimester placenta (Trim 1-Pl). For each LCM sample, the cumulative number of '1s' for DMRs hypermethylated in first trimester placental tissues are on the x-axis, and the cumulative number of '1s' for DMRs hypermethylated in term placental tissues are on the y-axis. The different trophoblast populations show the greatest overlap with placental tissues collected at a comparable gestational age. **B)** Heatmap visualizing unsupervised hierarchical clustering of second trimester SCTs/CTBs between first trimester and term SCTs/CTBs. Selected autosomal DMRs were identified between first trimester and term SCTs/CTBs with a fold change ≥2. Pl = (bulk) placenta, SCT/CTB = syncytiotrophoblasts and cytotrophoblasts), SK = syncytial knotting, EVT= extravillous trophoblasts.

Despite the large overlap between different trophoblast populations from the same trimester for DMRs identified in bulk placental tissues related to the duration of gestation (Figure 4A), we identified numerous differences in DNA methylation between different trophoblast populations within the same trimester. We identified 601 and 1,610 DMRs between SCTs/CTBs and EVTs in the first and second trimester, respectively, and 558 DMRs between term SCTs/CTBs and SK (Supplementary Figure S3, Supplementary Table S8). These cell type-specific DNA methylation profiles may be used to evaluate the contribution of these specific cell types to the methylation signature in maternal cfDNA.

Origins of DMRs in maternal cfDNA

Besides the presence of placental-originated cfDNA in maternal cfDNA, physiological pregnancy-induced changes in other tissues contributing to maternal cfDNA could be important drivers of observed DMRs in maternal cfDNA. To explore the (placental-)origin of DMRs identified in maternal cfDNA, we therefore studied the overlap with DNA methylation in bulk placental tissues, placental cell types and maternal blood buffy coats, another major source of cfDNA (10), for DMRs identified between pregnant and non-pregnant women (Figure 5A). Using our cumulative methylation scores based on a binary score for each identified DMR (Methods, Figure 1A-B), the mean proportion of identified DMRs in maternal cfDNA compared to cfDNA from non-pregnant women that overlapped with DNA methylation in our placental tissue samples increased from 41.8% (SD 4.97) in the first trimester, to 59.9% (SD 7.73) at delivery. Comparable results were found for placental tissues collected at different periods during gestation (Figure 5B). Conversely, the mean proportion of DMRs that overlapped with DNA methylation of buffy coats was 72.4% (SD 5.68) in first trimester cfDNA, and this decreased with advancing gestational age, especially after the third trimester, to 25.4% (SD 2.24) in cfDNA at delivery (Figure 5B, Supplementary Table S9A-D).

Zooming in on different placental cell types, a comparable course in overlapping DMRs as for the bulk placental samples was found for the different types of trophoblasts collected in each

trimester (Figure 5C). The mean proportion of identified DMRs in maternal cfDNA that overlapped with DNA methylation of trophoblasts was higher as for bulk placenta, and increased from 52.4% (SD 3.8) in first trimester to 76.2% (SD 3.9) at delivery (Supplementary Table S9A-D, Figure 5C). Conversely, there was considerably less overlap with DNA methylation of spiral arteries and the endometrial epithelium throughout gestation (Supplementary Figure S4, Supplementary Table S9A-D). Based on DMRs identified between different trophoblast populations, we did not specifically distinguish DNA methylation markers from a specific trophoblast population over another trophoblast population (EVTs, SCT/CTBs or SK) in maternal cfDNA (Supplementary Figure S5).

As a measure of the placental-originated fraction in total cfDNA, we investigated the methylation of the *RASSF1* promoter, a placental-specific marker, during gestation (28) (Supplementary Table S10). As compared to cfDNA from non-pregnant women, *RASSF1* promoter methylation was higher in cfDNA collected in the different trimesters and at birth. In first and second trimester cfDNA, the mean *RASSF1* promoter methylation increased with a fold change (FC) of 3.07 (p=0.0008) and 3.01 (p=0.034), respectively (Figure 5D). No difference in *RASSF1* methylation was found between first and second trimester cfDNA (mean FC= 0.98, p=0.93). As compared to cfDNA from non-pregnant women, the mean *RASSF1* promoter methylation increased with a FC of 4.20 (p<0.0001) in third trimester cfDNA, and a FC of 7.06 (p<0.0001) in cfDNA collected at delivery. Between first and third trimester cfDNA, *RASSF1* promoter methylation increased with a FC of 1.37 (p=0.054). As compared to cfDNA collected in the three different trimesters, the mean *RASSF1* promoter methylation was higher in cfDNA at delivery (FC=2.30, p=0.0002; FC=2.35, p=0.0001; FC 1.68, p=0.0031, respectively) (Figure 5D). For our previously identified, additional, top-ranked placental-specific DMRs related to *TMEM240*, *DHRS3* and a CpG island upstream of *PCMTD2* (18), a comparable gestational age-related increase in DNA methylation was found (Figure 5D, Supplementary Figure S6, Supplementary Table S10). The cumulative methylation of these four markers shows a clear, consequent increase with advancing gestational age (Figure 5D).

The increase in placental-derived cfDNA was most apparent at delivery, particularly considering the increased total cfDNA concentration in plasma at delivery. While the total cfDNA concentration did not differ between non-pregnant women and the three trimesters, the total cfDNA concentration per ml of plasma was about 3-4 times higher at delivery as compared to the other sampling periods (p-values between 0.001 – 0.019) (Figure 5E).

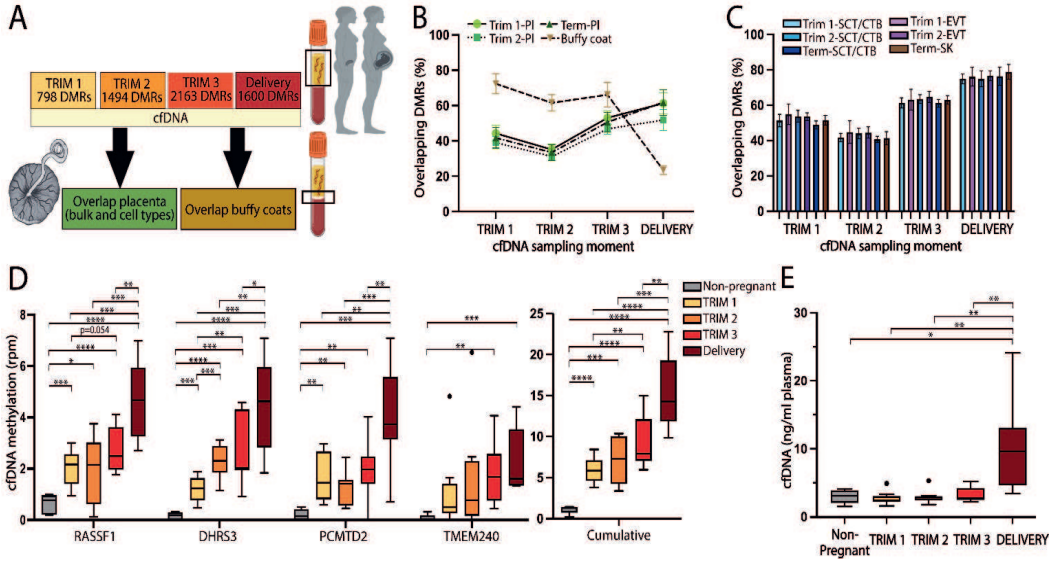


Figure 5: (Placental-)origin of DMRs identified in maternal cell-free (cf)DNA as compared to cfDNA from non-pregnant women. A) Overlap of differentially methylated regions (DMRs) identified in maternal cfDNA across gestation as compared to cfDNA from non-pregnant women was studied in placental bulk tissues, specific placental cell types and buffy coats. **B, C)** The proportion of DMRs identified in maternal cfDNA in the different trimesters (Trim) and at delivery as compared to cfDNA from non-pregnant women, that overlap with DNA methylation in placental tissues and buffy coats **(B)**, and with different trophoblast populations **(C)** based on our cumulative methylation scores. The means and SDs are depicted. **D)** Box plot showing methylation of the *RASSF1* promoter, methylation of three previously identified placental-specific methylation markers (DMRs related to *DHRS3*, *PCMTD2* and *TMEM240*), and methylation of these four markers combined in cfDNA across gestation. Y-axis depicts the normalized number of reads per million reads (rpm), see also Supplementary Table S10. **E)** Box plot showing the cfDNA concentration per milliliter of plasma in samples from non-pregnant women and from pregnant women for each trimester and at delivery. No differences were found in the cfDNA concentration between cfDNA from non-pregnant women and cfDNA collected in the three trimesters during pregnancy. * p≤0.05, ** p≤0.01, *** p≤0.001, **** p≤0.0001, all based on t-test. PI = (bulk) placenta, SCT/CTB = syncytiotrophoblast/cytotrophoblast, EVT= extravillous trophoblast, SK = syncytial knotting.

Presence of trimester-specific placental DMRs in maternal cfDNA

Besides the increase in methylation of universal placental-specific markers during gestation, we further investigated the presence in maternal cfDNA of DNA methylation profiles that were not found in all placental tissues, but that were related to gestational age in placental tissue. Most gestational age-specific DMRs identified in placental tissues were not specifically present in cfDNA collected at a comparable gestational age. Based on a cumulative methylation score for all DMRs identified between first trimester and term placental tissues, maternal cfDNA collected at different periods in pregnancy show largely comparable overlap (Supplementary Figure S7, Supplementary Table S11). However, a change in placental DNA methylation could be masked in maternal cfDNA if methylation of these regions is high in hematopoietic cells, which account for the majority of cfDNA fragments. We therefore selected DMRs identified

between first trimester and term placental tissues with low DNA methylation levels in buffy coats. Of 110 DMRs, 32 DMRs were selected which were hypomethylated in ≥80% of buffy coat samples based on the binary scores. Of those, 11 were hypermethylated in first trimester placental tissues, and 21 were hypermethylated in term placental tissues (Supplementary Table S12). We observed a clear increase in DNA methylation in cfDNA across gestation of DMRs specifically hypermethylated in term placental tissues, while a decrease in DNA methylation of DMRs hypermethylated in first trimester was less clear (Figure 6A).

When all individual DMRs were investigated, six DMRs identified between first trimester and term placental tissues showed high correlations with gestational age in cfDNA (Pearson correlation ≥0.7) Supplementary (Table S13). These DMRs were related to *NXPH4*, *EPS8L2*, *AMOTL1*, *IRX2*, *ERICH2-DT*/*SP5* and *LBX2* (Figure 6B) and all had low DNA methylation levels in buffy coat samples. Moreover, Figure 6C shows a cumulative methylation score based on these six DMRs for all cfDNA samples. After investigating individual DMRs identified between first trimester and term SCT/CTBs, five DMRs were associated with gestational age in cfDNA (Pearson correlation ≥0.7) (Supplementary Table S14). Four of these DMRs overlapped with the six DMRs identified in bulk placental tissues, and were related to *NXPH4*, *EPS8L2*, *AMOTL1* and *IRX2* (Figure 6D).

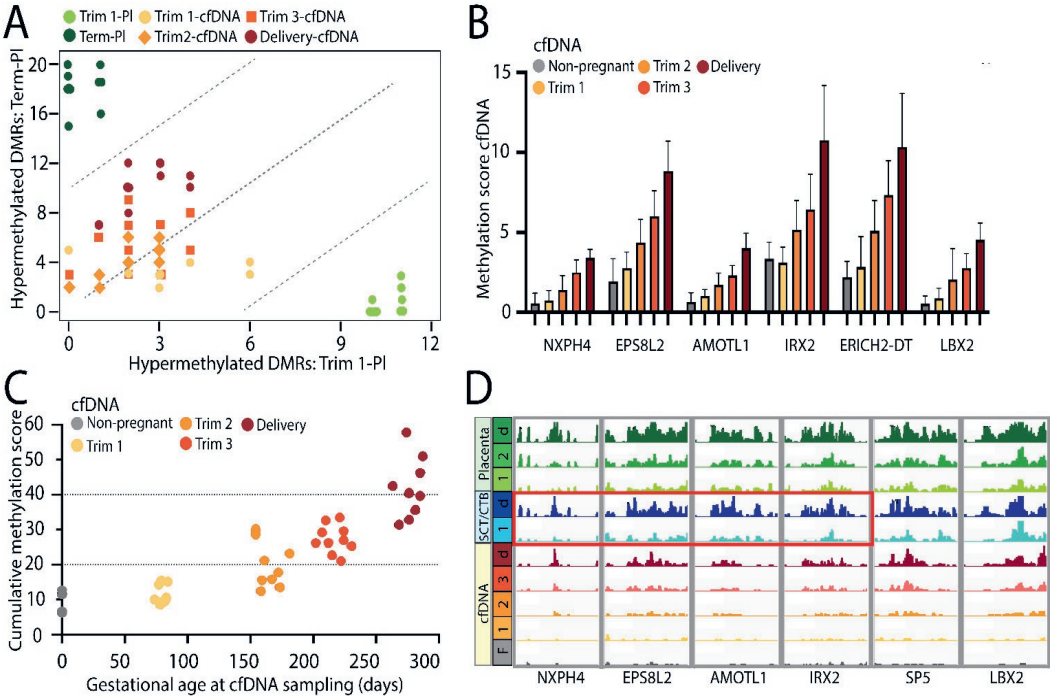


Figure 6 (previous page): Differentially methylated regions (DMRs) associated with gestational age in placental tissues and in maternal cell-free DNA (cfDNA). A) The overlap of DMRs identified between first trimester and term placental tissues was studied in maternal cfDNA collected in the different trimesters and at delivery using a cumulative methylation score. Only DMRs with low DNA methylation levels in buffy coat samples were selected (n=32). The y-axis represents overlap with DMRs hypermethylated in term placental tissues, and the x-axis represents overlap with DMRs hypermethylated in first trimester placental tissues. Each dot represents one sample. **B)** Mean methylation score in cfDNA for six DMRs between first trimester and term placental tissues, that are positively associated with gestational age in cfDNA (Pearson correlation score ≥0.7). (DMR five is associated with *ERICH2-DT* and *SP5*). The error bars depict SDs. **C)** Cumulative methylation score for each cfDNA sample, based on the six identified placental DMRs associated with gestational age in cfDNA. **D)** Average gene-tracks for the six identified placental DMRs associated with gestational age in cfDNA. The first four DMRs were also found between the first trimester and term SCTs/CTBs (outlined in red). (DMR five is associated with *ERICH2-DT* and *SP5*). SCT/CTB = syncytiotrophoblasts/cytotrophoblasts, F = non-pregnant women, 1 = first trimester, 2 = second trimester, 3 = third trimester, d = delivery.

This indicates that, besides the detection of universal placental-specific markers, also placental methylation marks related to the duration of gestation, particularly those originating from SCT/CTBs, could be identified in maternal cfDNA. A prerequisite for the detection of these placental DNA methylation markers in maternal cfDNA is hypomethylation for these DMRs in hematopoietic cells.

Discussion

We used MeD-seq to longitudinally investigate genome-wide maternal cfDNA methylation across pregnancy, including each trimester and at birth. To understand and identify which cfDNA methylation markers originate from placental tissue, we investigated DNA methylation in placental tissues and specific placental cell types in each trimester.

We showed a gradual change in DNA methylation across gestation in maternal cfDNA and in placental tissues and trophoblasts. With advancing gestational age, we observed an increase in DMRs identified in maternal cfDNA that overlapped with DNA methylation of placental tissues, particularly in trophoblast populations, and an increase in DNA methylation of known placental-specific markers. These findings suggest that the identified DMRs in maternal cfDNA during gestation are largely attributable to the known increasing placental-derived cfDNA fraction especially after 20 weeks of gestation (20, 21, 29), and underline the trophoblast-origin of the placental signature in maternal cfDNA. We did not specifically distinguish DNA methylation markers from a specific trophoblast population over another trophoblast population (Supplementary Figure S5), which could indicate that the different trophoblast populations all contribute to the methylation signature in maternal cfDNA.

Additionally, we found a large overlap of DMRs identified in maternal cfDNA in all three trimesters with DNA methylation in buffy coats, suggesting an increased immune response during pregnancy (18). This also underlines that there are different tissues of origins driving the DMRs that are identified in maternal cfDNA, and this should be taken into account when interpreting cfDNA methylation differences related to pregnancy. Since an increase in placental-originated cfDNA fragments will lead to a relatively decreased contribution of cfDNA fragments from maternal blood cells to total maternal cfDNA, this can explain the decrease in overlap with DMRs found in buffy coats during gestation.

The clear increase during gestation in methylation of our previously identified placental-specific DMRs underlines their placental-origin. A combined methylation score for these different placental-specific markers in addition to *RASSF1* promoter methylation could potentially be used in the determination of the placental-fraction in total maternal cfDNA, for which multiple methods exist (30).

Lastly, we focused on 32 DMRs identified between first trimester and term placental tissues that were hypomethylated in buffy coat samples. Methylation markers specifically present in term placental tissues showed increasing methylation levels in cfDNA across gestation, while a decrease in first trimester placental methylation markers was less clear. Six DMRs hypermethylated in term as compared to first trimester placental tissues showed the strongest correlation with gestational age in maternal cfDNA, four of which were also found in SCTs/CTBs. These DMRs were related to *NXPH4*, *EPS8L2*, *AMOTL1* and *IRX2*. The preselection of markers identified directly in placental tissues supports the feasibility of identifying specific gestational age-related placental DNA methylation markers in maternal cfDNA. However, the difference in detection between first trimester and term placental methylation markers is likely due to the simultaneously increasing placental-derived cfDNA fraction, enhancing the detectability of placental DNA methylation markers with advancing gestational age.

The large differences in the number of identified DMRs between cfDNA from pregnant versus non-pregnant women, compared with the number of DMRs identified between trimesters suggest that most DNA methylation changes in cfDNA during pregnancy are minor compared to the DNA methylation changes invoked by the pregnancy itself, which are already present at the end of the first trimester. Moreover, to prevent the identification of false positive results, the MeD-seq pipeline uses the Bonferroni correction and a minimal fold change of two for identified DMRs. This rather stringent approach combined with a relatively small difference in

the placental-originated fraction between subsequent sampling periods could explain the lack of acquiring this threshold for most DMRs.

A 'diluted' placental signature in total cfDNA and/or specific methylation profiles of cfDNA fragments originating from maternal tissues can also impact the detectability of placental methylation in total cfDNA. For example, for regions highly methylated in maternal blood cells and thus in cfDNA, the change in average methylation of total cfDNA caused by changes in placental DNA methylation will often be below two and remain undetected. Focusing on regions with low methylation levels in leukocytes as compared to placental tissues, as done for the gestational age-specific placental markers and as done by (22), or an enrichment of placental-originated cfDNA based on the shorter fragment size of placental-originated cfDNA as compared to maternal-originated cfDNA (11), could partly overcome this technical issue in future studies.

DMRs identified in placental tissues and cell types in relation to the duration of gestation are, as expected, related to genes with important roles in various cellular functions and developmental processes, such as the regulation of gene transcription and differentiation. Differentially methylated CpGs related to *NXPH4* and *LBX2* have previously been described in a study that developed a placental epigenetic clock (7), whereas aberrant methylation and/or expression of *NXPH4*, *EPS8L2*, *AMOTL1*, *IRX2* and *LBX2* have also been described in multiple types of cancers (31-35). Similarities between the placenta and cancers in terms of DNA methylation have frequently been described and are suggested to play a role in mutual properties, including their invasive character, cell proliferation and immune modulation (36).

Apart from gestational age differences in DNA methylation driven by placental development and differentiation over time as also shown by others (5, 6), the varying composition of different cell types likely plays a significant role in driving the observed differences. For example, the abundance of SCTs is higher than CTBs in term placenta as compared to first trimester placenta (37). However, DNA methylation changes caused by differences in cell composition would likely also lead to methylation differences in cfDNA and could therefore be of great value in identifying placental-originated methylation differences in cfDNA. This is supported by our data suggesting that different trophoblast types (SCTs/CTBs, EVTs, SK) have unique DNA methylation patterns and all contribute to the methylation signature in maternal cfDNA.

Strengths of the current study include that we longitudinally studied genome-wide maternal cfDNA methylation dynamics across gestation, which has been largely understudied thus far.

DNA methylation profiles in placental tissues and cell types across gestation were correlated with DNA methylation in maternal cfDNA. Moreover, the high genome-wide coverage of MeD-seq leads to the identification of novel methylation markers, and our stringent approach minimizes false positive results. On the other hand, our rather strict approach could also lead to fewer identified regions and more false negative results. Furthermore, owing to the substantial efforts required for the isolation of placental cell types, our sample size for specific placental cell types was relatively small. We also did not account for potential confounders such as smoking, maternal age and ethnicity, since we had no access to detailed information about the participants' characteristics for first and second trimester placental tissues or non-pregnant women. However, the longitudinal sampling of maternal cfDNA minimizes bias in our cfDNA analyses and we used data from specific placental cell types to validate our results from bulk placental tissues. Lastly, the fragmented nature of cfDNA could impact the coverage of the genome.

Although the genome-wide cfDNA methylome during pregnancy is largely understudied, cfDNA holds great promise for studying epigenetic (placental) programming during pregnancy and to develop clinically relevant biomarkers to further improve obstetrical care. We show the possibility to detect robust placental methylation markers in maternal cfDNA in relation to gestational age. Moreover, promising results have been reported recently indicating the feasibility of predicting the risk for preeclampsia by genome-wide cfDNA methylome profiling at an early pregnancy stage (22, 38). Also, the placental-derived fraction has been described to differ in pregnancies with preeclampsia (39, 40). Both differences in the placental cfDNA fraction and actual DNA methylation differences in placental cells (or perhaps also in maternal tissues) (41), could jointly lead to improved biomarker development in future studies. Since cfDNA is already widely used for noninvasive prenatal testing (NIPT), in the future, cfDNA methylation profiling could possibly be combined with the current NIPT (42), moving forward to what we would call an Epigenome-wide noninvasive prenatal test (Epi-NIPT) (18).

Conclusion

This study increases our understanding of the maternal cfDNA methylome across gestation. We showed that most DNA methylation differences in maternal cfDNA across gestation are attributable to an increased placental fraction. Additionally, we identified specific gestational age-dependent placental DNA methylation marks in maternal cfDNA, paving the way for further research aimed at detecting placental methylation markers in maternal cfDNA. The study of cfDNA methylome profiling in relation to obstetrical complications warrants further study, for which this study could serve as a reference.

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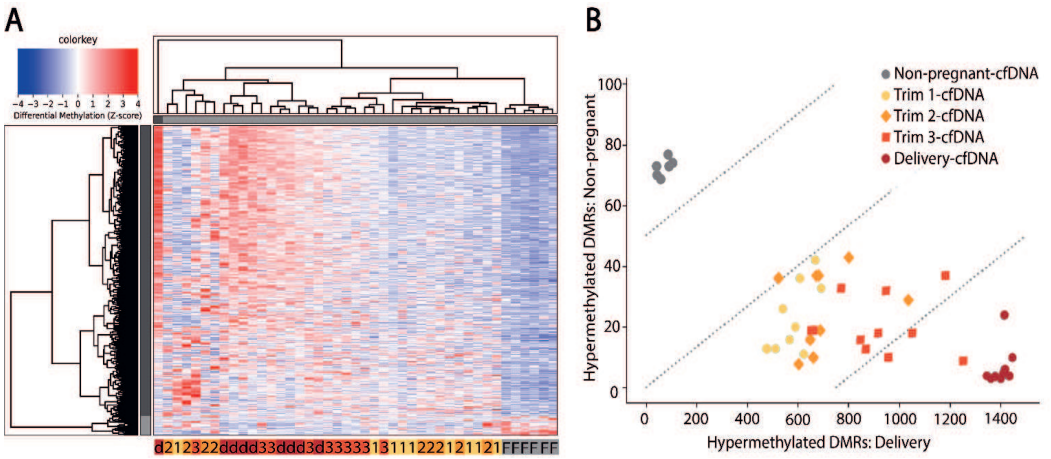
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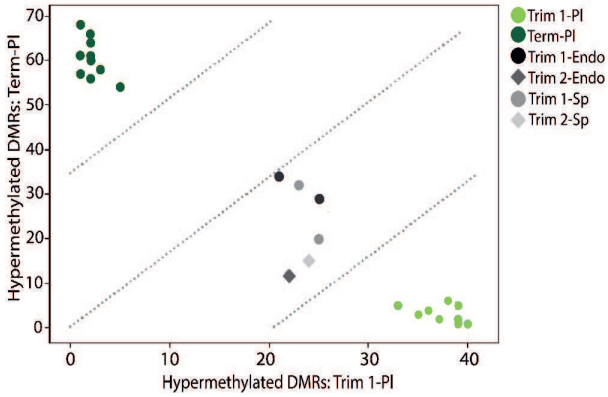
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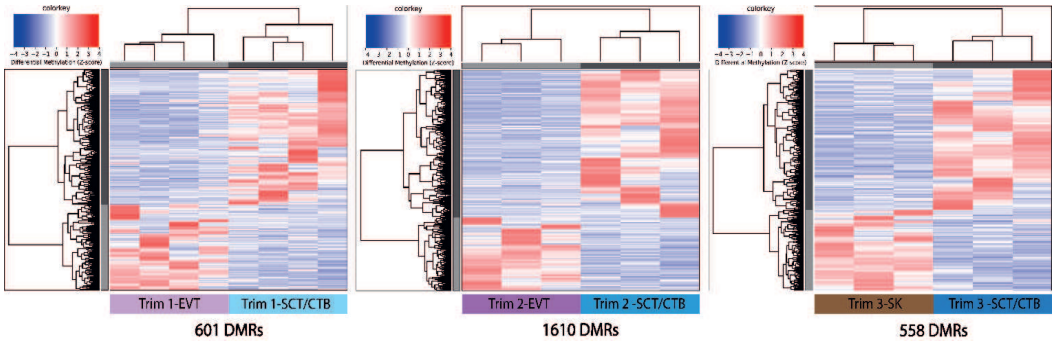
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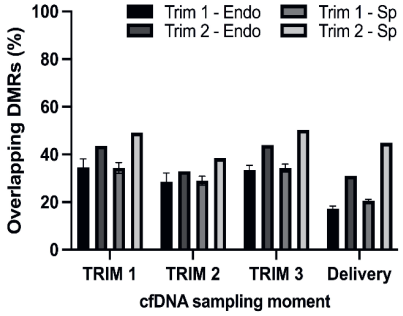
Supplemental Figure S1: DNA methylation in cfDNA related to the duration of gestation. A) Heatmap visualizing unsupervised hierarchical clustering of all cfDNA samples shows generally clustering of samples collected during the same and consecutive time points. Selected autosomal DMRs were identified between cfDNA collected in non-pregnant women (F) and cfDNA collected from pregnant women at delivery (d) with a fold change ≥2. The three trimesters are depicted as 1, 2 and 3. **B)** Cumulative methylation score based on DMRs between cfDNA from non-pregnant women and cfDNA collected at delivery for all cfDNA samples. For each sample, the cumulative number of '1s' for DMRs hypermethylated in cfDNA at delivery are on the x-axis, and the cumulative number of '1s' for DMRs hypermethylated in cfDNA from non-pregnant women are on the y-axis (Methods).



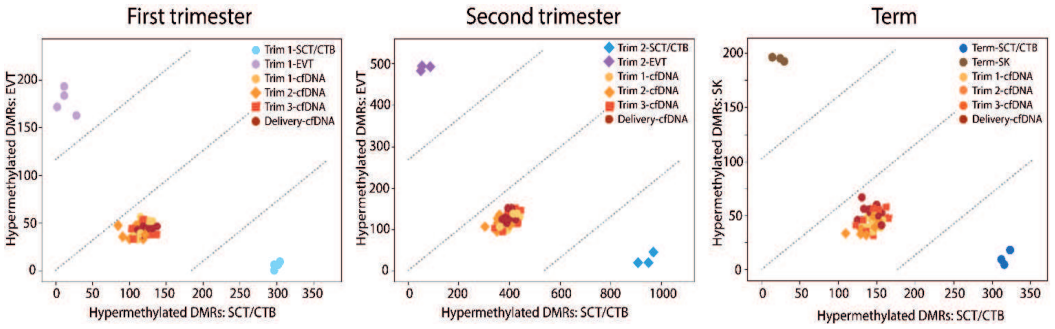
Supplemental Figure S2: DNA methylation associated with the duration of gestation in endometrial cells and spiral arteries. Cumulative methylation scores for endometrial cells and spiral arteries isolated after laser capture microdissection (LCM), based on methylation of DMRs identified between bulk term placenta (Term-PI) and first trimester placenta (Trim 1-PI). For each LCM sample, the cumulative number of '1s' for DMRs hypermethylated in first trimester placental tissues are on the x-axis, and the cumulative number of '1s' for DMRs hypermethylated in term placental tissues are on the y-axis (Methods). No relationship was found with gestational age in spiral arteries or endometrial cells for identified DMRs in placental tissues. Endo = endometrial cells, Sp = spiral arteries.



Supplemental Figure S3: DMRs identified between trophoblast populations collected at a comparable gestational age. Heatmaps visualizing unsupervised hierarchical clustering for autosomal DMRs with a fold change ≥2 between **1)** first trimester extravillous trophoblasts (EVTs) and syncytiotrophoblasts/cytotrophoblasts (SCTs/CTBs) (n=601) (left); **2)** second trimester EVTs and SCTs/CTBs (n=1,610) (middle); and **3)** term Syncytial knotting (SK) and SCTs/CTBs (n=558) (right).

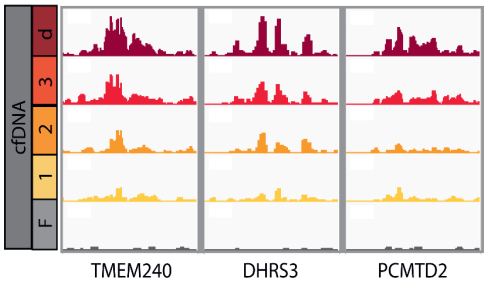


Supplemental Figure S4: Overlap of DNA methylation of endometrial cells and spiral arteries with DMRs identified in maternal cfDNA. The proportion of DMRs identified in maternal cfDNA in the different trimester and at delivery as compared to cfDNA from non-pregnant women, that overlap with DNA methylation in endometrial cells and spiral arteries based on our cumulative methylation score (Methods). The means and SDs are depicted. Overlap is considerably lower as compared to overlap with trophoblast cells (Figure 5C) and no increase is observed related to gestational age. Endo = endometrial cells, Sp = spiral arteries.

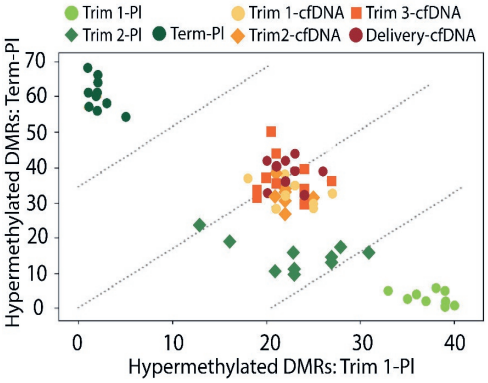


Supplemental Figure S5: DNA methylation in maternal cfDNA associated with specific trophoblast populations. Cumulative methylation scores for maternal cfDNA samples based on methylation of DMRs identified between different trophoblast populations with a comparable gestational age. We calculated a binary score for each identified DMR related to trophoblast population (Methods). In maternal cfDNA, we do not specifically distinguish DNA methylation markers from

first (left) or second (middle) trimester extravillous trophoblasts (EVTs) as compared to first (left) or second (middle) trimester syncytiotrophoblasts/cytotrophoblasts (SCTs/CTBs), or from term (right) Syncytial knotting (SK) as compared to term SCTs/CTBs. Each dot represents one sample.



Supplemental Figure S6: Gene-tracks for three top-ranked placental-specific DMRs that were previously identified in cfDNA. F = non-pregnant women, 1 = first trimester, 2 = second trimester, 3 = third trimester, d = delivery.



Supplemental Figure S7: DNA methylation in maternal cfDNA for all (n=110) DMRs identified between first trimester and term placental tissues. Overlap of DMRs identified between first trimester and term placental tissues was studied in maternal cfDNA collected in the different trimesters and at delivery. After generating a cumulative methylation score based on all identified placental DMRs, cfDNA samples from all trimesters show largely comparable overlap with DMRs hypermethylated in first trimester (x-axis) and term (y-axis) placental tissues. Each dot represents one sample.

Supplemental Tables S1-S14: Can be accessed upon request or through the publisher's website. The MeD-seq datasets generated and/or analysed during the current study are available in the National Center for Biotechnology Information (SRA database) with accession numbers PRJNA1108949 and PRJNA1147893 (see Supplementary Table S1).



Chapter 8

Cell-free DNA methylation profiling in early-onset preeclampsia using Methylated DNA sequencing

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Under review

Abstract

Introduction: Distinct DNA methylation profiles have been identified in maternal plasma cell-free DNA (cfDNA) in early-onset preeclampsia (EOPE). However, findings across studies often lack concordance and the biological mechanisms driving methylation differences are largely unknown. We aimed to validate previously reported EOPE-associated regions, identify novel differentially methylated regions (DMRs), and investigate changes in tissue-specific cfDNA contributions.

Methods: First trimester cfDNA (n=35) and cfDNA after diagnosis (n=21) were collected from EOPE pregnancies and matched controls. Placental tissues were collected from EOPE (n=5) and control (n=7) pregnancies. MeD-seq assays were performed and methylation levels at previously identified EOPE-associated regions, placental contribution to cfDNA, cfDNA concentration, and identified EOPE-specific placental markers were compared between cases and controls. Additionally, liver-, kidney-, and endothelium-specific hypermethylated regions were identified using publicly available datasets and compared in cfDNA.

Results: Only 2.7-8.4% of previously identified regions showed significant differential methylation in our cohort. We identified one novel DMR in the first trimester and 249 novel DMRs in our after diagnosis cohort. Total cfDNA concentration was 20-fold higher after EOPE diagnosis compared to controls ($p<0.001$). We identified 606 DMRs in EOPE placentas, which unexpectedly were almost not present in cfDNA after EOPE diagnosis. Also, the placental-, liver- and kidney-specific contributions were relatively decreased, while endothelium-specific methylation was increased.

Discussion: Lack of concordance across studies remains a significant limitation, potentially explained by methodological heterogeneity. Differential tissue contributions to cfDNA likely account for DMRs identified after EOPE diagnosis. Further research is required to clarify the (tissue) origins of identified cfDNA DMRs in more detail.

Introduction

Preeclampsia (PE) affects 4-5% of pregnancies and remains a major contributor to maternal and neonatal morbidity and mortality. It is a heterogeneous syndrome defined by hypertension accompanied by at least one additional complication such as proteinuria, maternal end-organ dysfunction or uteroplacental dysfunction (1).

DNA methylation is an epigenetic mechanism affecting gene expression by binding methyl groups to the DNA sequence (2). DNA methylation is tissue-specific (3) and methylation differences have been found in relation to various pathological conditions, including altered placental methylation in PE (4, 5). Cell-free DNA (cfDNA) in maternal plasma partially originates from the placenta. Recent studies have identified distinct cfDNA methylation profiles associated with PE as early as the first trimester (6-9), particularly in early-onset preeclampsia (EOPE), a severe form diagnosed before 34 weeks of gestation (1). However, findings across different studies often lack concordance, and the biological mechanisms or sources driving these methylation differences are largely unknown.

We aimed to investigate cfDNA methylation profiles associated with EOPE in the first trimester and after clinical EOPE diagnosis using Methylated DNA sequencing (MeD-seq), which, unlike most techniques, provides full genome-wide coverage (10, 11). We assessed the presence of methylation differences at previously identified regions (6, 7), and aimed to identify novel regions in EOPE potentially not covered previously. Finally, we investigated changes in tissue-specific contributions to cfDNA in EOPE as potential cause of cfDNA methylation differences and to gain insight into the underlying disease pathophysiology.

Methods

Study design

We set up two case-control studies. First, we selected first trimester residual NIPT samples collected presymptomatically between January 2021-May 2023 from women who subsequently developed EOPE (n=15) and gestational age-matched controls (n=20). These women participated in the Rotterdam Periconception Cohort (Predict study) (12, 13) and/or gave birth at the Erasmus Medical Centre in Rotterdam, The Netherlands. Second, we collected cfDNA and postpartum placental tissues between April 2023-November 2024 from women admitted to the Erasmus Medical Centre after clinical EOPE diagnosis (n=10) and gestational age-matched controls from the Predict study (n=11) (14) (Figure 1). (EO)PE was defined according to the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (15).

Written informed consent for re-use of NIPT material was provided in the TRIDENT-2 study (16) and both parents provided written informed consent within the Predict study. The Medical Ethics Committee of the Erasmus Medical Centre approved this study (re-use NIPT material: MEC-2022-0788, Predict study: MEC-2004-227: Addendum 3).

To identify tissue-specific methylation markers, we used publicly available MeD-seq data from maternal buffy coats (n=10), liver (n=7), kidney (renal tubular epithelial cells) (n=3) and endothelium (spiral arteries) (n=3) (17-20).

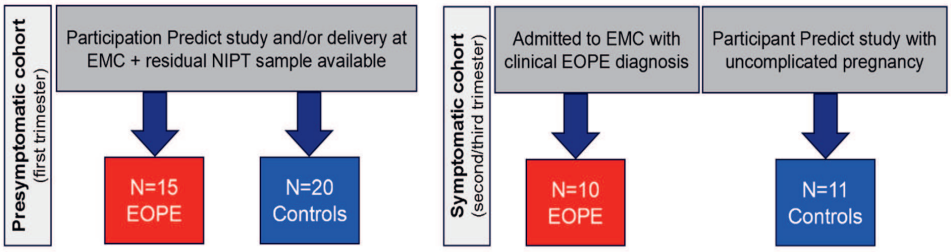


Figure 1: Samples included in cfDNA analyses. Selected controls were required to have a term delivery (≥37 weeks of gestation), no congenital malformations and no symptoms of preeclampsia. EMC = Erasmus Medical Centre, NIPT = noninvasive prenatal testing, EOPE = early-onset preeclampsia.

Sample collection and processing

cfDNA samples were collected in BCT CE tubes (Streck) (NIPT) and CellSave preservative tubes (Predict study). Plasma was isolated within respectively 5 or 4 days, and stored at −80 °C. cfDNA was isolated using the QIAamp Circulating Nucleic Acid Kit and concentrations were measured using the Quant-it HS kit. Placental full-thickness biopsies were embedded in formalin-fixed paraffin-embedded (FFPE) blocks and DNA was subsequently isolated using the QIAamp DSP DNA FFPE Tissue Kit as previously described (17, 20).

MeD-seq assays

MeD-seq assays were performed as described previously (10, 11, 14). In short, (cf)DNA was digested with the methylation-dependent restriction enzyme LpnPI (New England Biolabs, Ipswich, MA, USA) generating 32 bp DNA fragments containing the methylated CpG in the middle. The TruPLEX DNAseq 96D kit (Rubicon Genomics, Takara Bio Europe, Saint-Germain-en-Laye, France) was used to prep samples for sequencing and samples were purified on a Pippin HT system with 3% agarose gel cassettes (Sage Science, Beverly, MA, USA). Samples were sequenced on the Illumina NextSeq2000 platform.

Data analysis

Descriptive statistics were used to report baseline characteristics using absolute numbers and percentages (%) for categorical variables, and medians and interquartile ranges (IQR) for continuous variables. Differences between cases and controls were assessed by chi-squared or Mann-Whitney U test, respectively. P-values ≤0.05 were considered statistically significant. The Mann-Whitney U test was used to compare total cfDNA concentrations and the placental-derived signal in cfDNA based on a cumulative methylation score of placental-specific hypermethylated markers, including the *RASSF1* promoter and previously identified placental-specific DMRs standardized for number of reads per million (rpm) (17, 20). For NIPT samples, the placental-derived fraction was determined based on Veriseq and Wisecondor as part of regular prenatal testing (21, 22). After clinical EOPE diagnosis, the correlation between cfDNA concentration and placental-derived cfDNA signal was investigated using the Spearman's rank correlation coefficient.

Validation of previous findings

We compared the normalized number of reads for previously identified EOPE-associated regions (6, 7) in cfDNA between cases and controls using the Mann-Whitney U test. For reported regions, all LpnPI sites were added together and for identified probes, we included all LpnPI sites in a 100 bp region around the central CpG. Additionally, for each region we calculated the methylation threshold that best distinguished cases and controls in our cohorts using receiver operating characteristic (ROC) curves (using the "scikit-learn" package in Python). Samples with methylation above the threshold for the specific region scored '1', and samples with methylation below the threshold scored '0', leading to a binary score for each region. The outcomes of these binary scores were used for unsupervised hierarchical clustering analyses.

Novel DMR detection

Differentially methylated regions (DMRs) based on MeD-seq data were identified using a previously established bioinformatics pipeline (10, 11, 14). Custom Python scripts were used to process acquired DNA methylation profiles. Raw fastq files were subjected to Illumina adaptor trimming. We filtered reads based on LpnPI restriction site occurrence between 13–17 bp from either the 5' or 3' end of the read to filter out methylated DNA fragments and mapped reads that passed the filter to hg38 using bowtie2. A full genome-wide sliding window was used to detect sequentially differentially methylated LpnPI sites between cases and controls. Genome-wide read counts were normalized (rpm) for coverage and compared using the chi-square test. Neighboring significantly called LpnPI sites were binned and reported using the UCSC database annotations (Homo_sapiens_hg38.GRCh38.79.gtf, www.ensembl.org). A Bonferroni corrected

p-value ≤0.05 was considered statistically significant and DMRs with a fold change of ≥2 were selected. Z-score transformation of the read count data was applied for unsupervised hierarchical clustering analyses.

Potential origins of DMRs

We compared methylation levels in cfDNA for DMRs hypermethylated in EOPE compared to control placental tissues, while hypomethylated in maternal buffy coats (≥90% of samples below ROC-established threshold). Hereby, placental-derived DNA methylation of these regions in cfDNA would not be masked by high methylation levels of maternal hematopoietic cells (20). Additionally, tissue-specific hypermethylated regions were identified for liver, kidney and endothelium, as MeD-seq captures methylated DNA fragments and these tissues are known contributors to cfDNA and/or are involved in PE (1, 23). The cumulative normalized number of reads for six selected top regions per tissue were compared between cases and controls to explore differences in the contributions of these tissues to cfDNA.

Results

Patient characteristics

Table S1 provides an overview of included samples and their technical performance, and Table 1 shows our patient characteristics for both case-control cohorts.

Previously identified regions

Presymptomatic

De Borre *et al*. (6) investigated 28,898 regions with low DNA methylation in blood and normalized for the placental (fetal)-derived cfDNA fraction. They identified six differentially methylated regions after False Discovery Rate (FDR) correction in first trimester cfDNA, but showed predictive values of additional top-ranked regions. Of their top 200 regions, 191 have at least one LpnPI recognition site and are thus covered using MeD-seq. We found methylation differences for 9/191 regions (4.7%) in our presymptomatic cohort, of which 2/6 (33.3%) corresponded to their FDR-corrected regions (Table S2). Despite the low number of significant regions, clustering analysis using thresholds (Methods) for these 191 regions allowed for good distinction between cases and control. This shows that different methylation levels could at least be detected at part of investigated regions (Figure 2A). Baetens *et al*. (7) identified 42 DMRs with an absolute methylation difference of >10% using reduced representation bisulfite sequencing (cf-RRBS). Only 1/37 (2.7%) with LpnPI recognition sites showed differential methylation in our first trimester cohort (Table S3), and corresponding clustering analysis did

not adequately distinguish our cases and controls (Figure 2B). There was no overlap in identified regions or corresponding genes between these top 200 regions identified by De Borre *et al*. (6) and these 42 DMRs identified by Baetens *et al*. (7).

	Presymptomatic cohort			Symptomatic cohort		
	EOPE (n=15)	**Control (n=20)**	**p-value**	**EOPE (n=10)**	**Control (n=11)**	**p-value**
Maternal age	30.3 (27.7 – 33.9)	32.5 (29.2 – 35.0)	0.214	30.6 (28.9 – 32.5)	31.1 (29.6 – 35.8)	0.481
Periconceptional BMI	26.5 (23.2 – 28.7)	23.8 (22.9 – 28.7)	0.657	25.8 (19.8 – 30.8)	25.4 (23.2 – 26.1)	0.888
Periconceptional smoking	3 (20.0)	1 (5.0)	0.167	1 (10.0)	1 (9.1)	0.943
Nulliparous	12 (80.0)	13 (65.0)	0.331	8 (80.0)	5 (45.5)	0.104
Geographical origin			0.347			0.468
Dutch	12 (80.0)	18 (90.0)		7 (70)	9 (81.8)	
European, other	1 (6.7)	-		1 (10)	2 (18.2)	
Hispanic	-	-		1 (10)	-	
Asian	-	1 (5.0)		1 (10)	-	
Western, other	1 (6.7)	-		-	-	
Surinam	-	1 (5.0)		-	-	
Other/mixed	1 (6.7)	-		-	-	
Conception mode			<0.001*			0.141
Spontaneous	15 (100)	8 (40.0)		8 (80)	6 (45.5)	
IVF/ICSI	-	10 (50.0)		1 (10)	5 (45.5)	
OI	-	2 (10.0)		1 (10)	0 (0)	
Gestational diabetes	0 (0)	0 (0)	NA	0 (0)	0 (0)	NA
Preexisting hypertension	2 (13.3)	0 (0)	0.093	1 (10)	0 (0)	0.283
Fetal sex			0.118			0.835
Boy	10 (66.7)	8 (40.0)		5 (50)	5 (54.5)	
Girl	5 (33.3)	12 (60.0)		5 (50)	6 (45.5)	
Gestational age at EOPE diagnosis	28+2 (25+4 – 30+6) *Missing n=2*	-		29+4 (28+2 – 30+1)	-	
Gestational age at birth	29+6 (27+6 – 33+1)	39+3 (38+4 – 40+2)	<0.001*	31+2 (31+0 – 33+1)	39+2 (39+1 – 40+5)	<0.001*
Birth weight (grams)	1040 (775 – 1270)	3455 (3225 – 3551)	<0.001*	1175 (1098 – 1467)	3520 (3435 – 3830)	<0.001*
Gestational age at blood withdrawal	12+2 (11+3 – 12+3)	12+3 (11+2 – 12+6)	0.831	30+2 (29+4 – 30+5)	30+2 (29+4 – 31+4)	0.572

Table 1: Baseline characteristics from early-onset preeclampsia (EOPE) and control pregnancies for our presymptomatic (left) and symptomatic (right) cohort. Data are presented as median (interquartile range) or n (%). *Significance at p≤ 0.05 assessed by chi-square test or Mann-Whitney U test as appropriate. IVF = in vitro fertilization, ICSI = intracytoplasmic sperm injection, OI = ovulation induction.

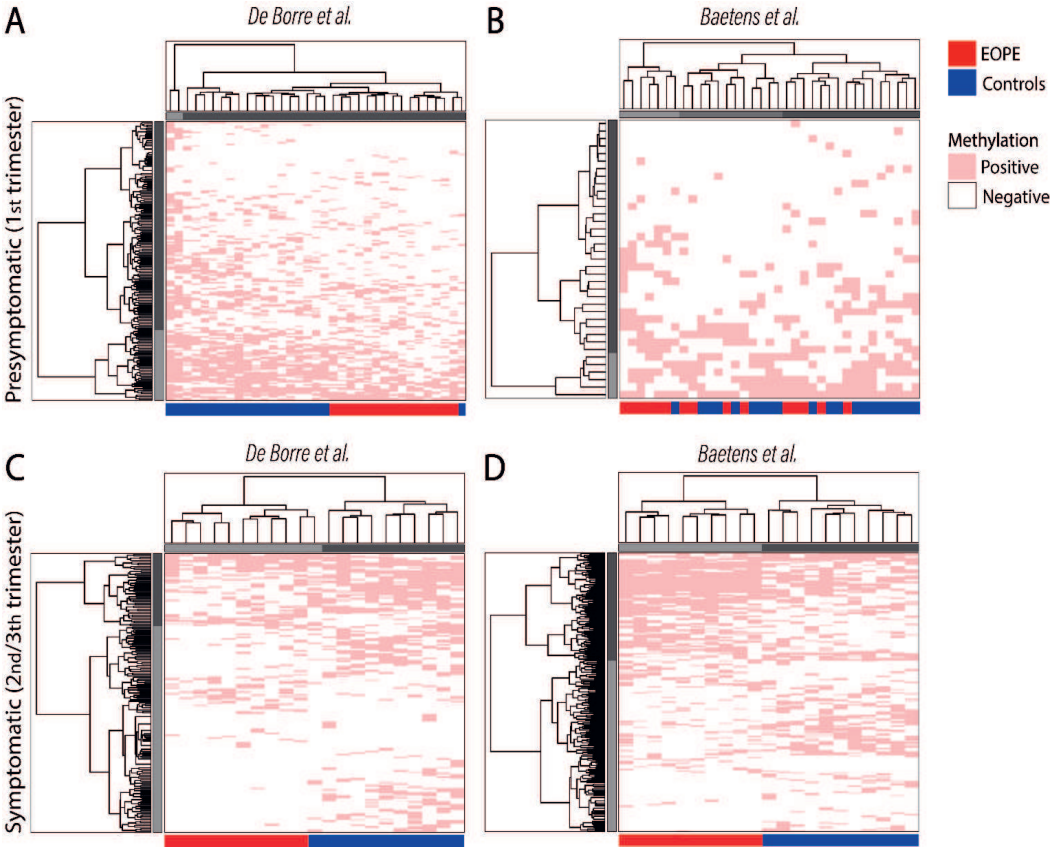


Figure 2: **Heatmaps based on previously identified regions differentially methylated in cfDNA between pregnancies complicated by early-onset preeclampsia (EOPE) and controls.** For each previously identified region with LpnPI recognition sites, the optimal threshold that best distinguished cases and controls in our cohort was determined and used to score the samples. **A)** Clustering based on the top 191 regions identified by De Borre *et al.* in the first trimester (of which originally only six passed False Discovery Rate correction) for our presymptomatic cohort. **B)** Clustering based on 37 DMRs identified by Baetens *et al*. in the first trimester for our presymptomatic cohort. **C)** Clustering based on 190 regions identified by De Borre *et al.* after EOPE diagnosis for our symptomatic cohort. **D)** Clustering based on the top 250 DMRs identified by Baetens *et al*. after EOPE diagnosis for our symptomatic cohort.

Symptomatic

After EOPE diagnosis, De Borre *et al*. (6) identified 231 FDR-corrected regions. In our symptomatic cohort, we found cfDNA methylation differences in 16/190 of corresponding regions with LpnPI recognition sites (8.4%) (Table S4). Baetens *et al*. (7) identified 21,499 DMRs with an absolute methylation difference of >10%. Of their top 250 DMRs with LpnPI recognition sites (of 21,499 DMRs), we found significant methylation differences in 17 (6.8%) DMRs in our symptomatic cohort (Table S5). Clustering analyses using these 190 (6) and top 250 (7) regions performed well in our cohort (Figure 2C-D). There was no overlap between the 231 regions (6) and top 250 DMRs (7), but both identified a DMR located in the gene body of *ANK1* (Supplemental Figure S1).

Novel findings: Presymptomatic cohort

We next identified methylation differences using our pipeline. Figure 3A shows gestational age at cfDNA sampling for our presymptomatic cohort. We identified only one DMR in first trimester maternal cfDNA, which was located in the gene body of *R3HDM2* and had a mean fold change of 55.5 (chr12: 57,400,347-57,402,220, hg38. This region showed consistently higher methylation in 14 out of 15 EOPE cfDNA samples, than in all controls (Figure 3B). This DMR did not overlap with regions or genes previously reported by De Borre *et al*. (top 200 regions) (6) or by Baetens *et al*. (42 DMRs) (7). No significant differences were found in cfDNA concentrations (p=0.711, Figure 3C), or placental-specific methylation markers (methods) (p=0.250, Figure 3D), nor in placental-derived fractions based on Veriseq or Wisecondor (p=0.062 and p=0.294, respectively) (Supplemental Figure S2).

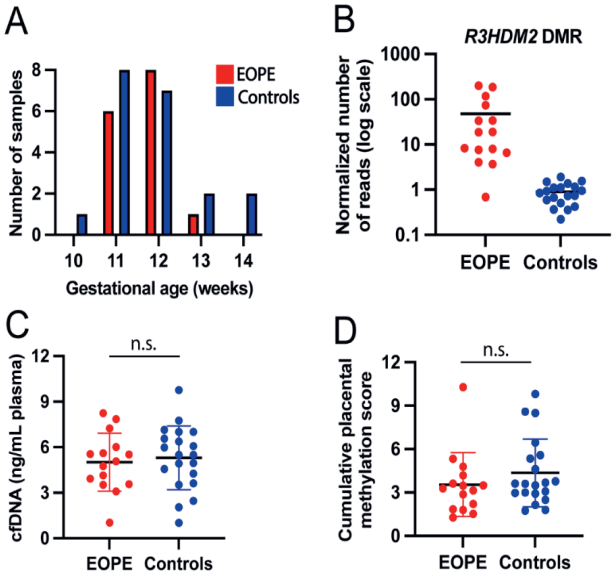


Figure 3: **Findings of our presymptomatic cohort. A)** Gestational age at cfDNA collection. **B)** The normalized number of reads for each maternal cfDNA sample for the one DMR identified between cases and controls in first trimester cfDNA using MeD-seq (chr12: 57,400,347-57,402,220, HG38). Y-axis is log scaled. Bars depict mean. The DMR is located in the gene-body of *R3HDM2*. **C)** Concentration of cfDNA isolated per milliliter plasma between cases and controls, bars depict mean with SD. **D)** Cumulative methylation score based on four placental-specific methylation markers (*RASSF1* promoter, DMRs related to *TMEM240, DHRS3* and a CpG island upstream of *PCMTD2*, see methods), as a measure of the placental-derived signal in total cfDNA. Bars depict mean with SD. EOPE = early-onset preeclampsia. N.s. = non-significant (Mann-Whitney U test).

Novel findings: Symptomatic cohort

Figure 4A shows gestational age at sampling for our symptomatic cohort. We identified 249 DMRs with a fold change of ≥2, of which 237 (95.2%) were hypermethylated in controls (Table S6). Unsupervised hierarchical clustering analysis based on these DMRs separated cases and controls (Figure 4B). Multiple general developmental pathways were identified using gene-set

enrichment analysis of associated genes, but no tissue-specific pathways directly related to PE pathogenesis were identified (Table S7).

Importantly, mean plasma cfDNA concentration was ~20-fold higher after clinical EOPE diagnosis compared to in controls ($p<0.001$), with high variability in EOPE (range 18.8 – 158.1 ng/ml plasma) (Figure 4C). Notably, two EOPE cases with accompanying elevated liver enzymes yielded the highest cfDNA concentrations. Mean methylation of placental-specific markers was 36.0% lower in EOPE than control pregnancies (Figure 4D, $p=0.018$), indicative of a lower placental-derived cfDNA fraction and thus a relatively larger increase of maternal-originated fragments.

There was a negative, although non-significant, trend between cfDNA concentration and placental-specific hypermethylation in EOPE cfDNA (R^2=-0.552, $p=0.098$) (Figure 4E), suggesting a potential overrepresentation of maternal-originated cfDNA especially when total cfDNA concentration is high. Lastly, based on DMRs identified after EOPE diagnosis, we were not able to adequately distinguish cases and controls in first trimester samples (Figure 4F). Also, the DMR identified in first trimester cfDNA was not found after diagnosis. This could indicate that the source(s) driving cfDNA methylation differences after EOPE diagnosis are not yet present in the first trimester.

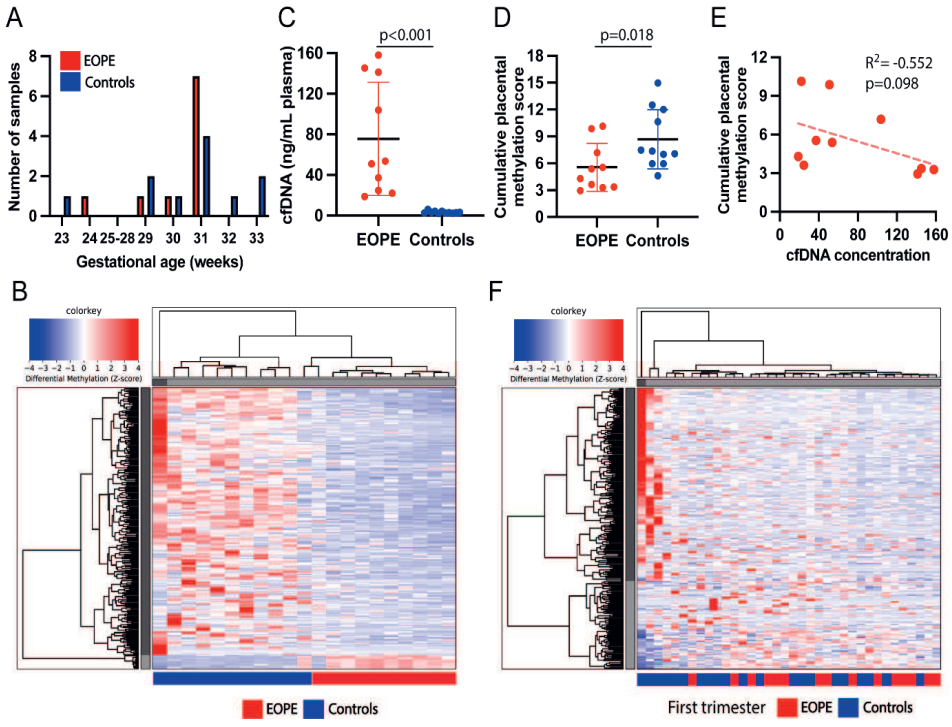


Figure 4 (previous page): Findings of our symptomatic cohort. **A)** Gestational age at cfDNA collection. **B)** Heatmap showing the unsupervised hierarchical clustering analysis based on DMRs identified between EOPE pregnancies and matched controls after diagnosis. **C)** Concentration of cfDNA isolated per milliliter plasma between cases and controls, bars depict mean with SD. P-value based on Mann-Whitney U test. **D)** Cumulative methylation score based on four placental-specific methylation markers (*RASSF1* promoter, DMRs related to *TMEM240, DHRS3* and a CpG island upstream of *PCMTD2*, see methods), as a measure of the placental-derived signal in total cfDNA. Bars depict mean with SD. P-value based on Mann-Whitney U test. **E)** The correlation between the cumulative methylation score (as in Figure D) and the cfDNA concentration (ng/ml plasma) for cfDNA from EOPE pregnancies. Correlation assessed using Spearman's rank correlation coefficient. **F)** Heatmap showing the unsupervised hierarchical clustering analysis for cfDNA samples obtained in the first trimester, based on DMRs identified in maternal cfDNA after EOPE diagnoses. EOPE = early-onset preeclampsia.

No exact overlap was found between our DMRs and regions previously identified by De Borre *et al*. or the top 250 regions by Baetens *et al*. (6, 7) Our 249 DMRs corresponded to 226 genes, of which four were found by De Borre *et al*. (6) (*TBCD*, *CTIF*, *MTUS1* and *PLXND1*) or by Baetens *et al*. (7) (top 250 DMRs) (*TPRM*, *PER2*, *CACNA2D3* and *RUNX1*) (Supplemental Figure S1).

Potential origins of DMRs after EOPE diagnosis

Placental methylation changes

We initially hypothesized that differences in placental DNA methylation could explain part of identified cfDNA DMRs. We identified 606 DMRs between EOPE (n=5) and control (n=7) placentas from our symptomatic cohort (Table S1, Table S8). Due to preterm births in EOPE, gestational age at placenta collection and time between cfDNA and placental tissue collection is inevitably different between groups (Table S1). Only 2/249 cfDNA DMRs in our symptomatic cohort overlapped with the 606 placental DMRs (chr14:104,429,589-104,431,042 and chr21:45,223,673-45,225,203 (hg38), Table S6 and S8) and both were hypermethylated in controls as compared to EOPE pregnancies. Of genes corresponding to our 249 cfDNA DMRs, 11/260 overlapped (Figure 5A). Unsupervised hierarchical clustering analysis using placental DMRs (n=606) could not distinguish cases from controls in cfDNA (Figure 5B). Specifically, 57 DMRs were hypermethylated in EOPE placentas while hypomethylated in buffy coats. Placental-derived DNA methylation of these regions in cfDNA would therefore not be masked by DNA methylation of hematopoietic cells (20). However, methylation of these regions was not increased in EOPE cfDNA (Figure 5C, Table S9). DNA methylation differences in EOPE placentas were thus not an important driver of methylation differences found in cfDNA.

1-versus-11 comparisons

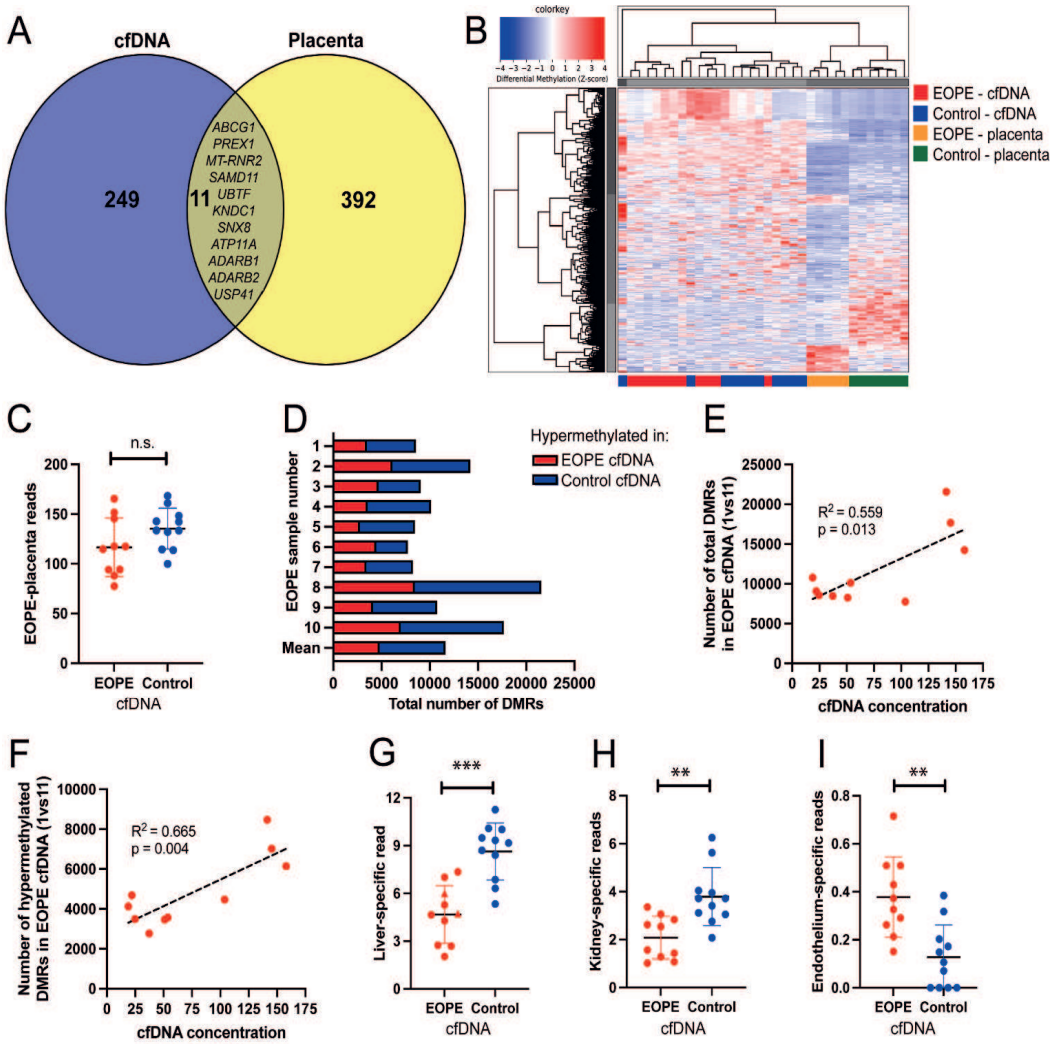
Only 12 (4.8%) DMRs in cfDNA were hypermethylated in EOPE pregnancies after diagnosis. We hypothesized that this may be caused by disease-heterogeneity. Specifically, our pipeline is designed to identify DMRs consistently differently methylated between at least 70% of cases and controls. However, influx of cfDNA fragments from both placental and maternal tissues

appear to vary considerably after EOPE diagnosis (Figure 4C-E). By comparing each individual cfDNA sample after EOPE diagnosis (n=10) against the set of control samples (n=11), we identified on average 11,652 DMRs per sample, of which 42.1% were hypermethylated in EOPE (Figure 5D). This indeed indicates that the hypermethylation in EOPE cfDNA is heterogenous and not consistent enough across samples to be identified as DMRs using our pipeline. Furthermore, we observed a positive correlation between the total number of DMRs and number of hypermethylated DMRs with cfDNA concentration in EOPE samples (Figure 5E-F, R^2=0.559, p=0.013 and R^2=0.685, p=0.004, respectively).

Tissue-specific markers in cfDNA

Next, we compared cfDNA methylation levels in our symptomatic cohort for regions specifically hypermethylated in liver (n=6), kidney (n=6) or endothelium (n=6) (Supplemental Figure S3). The relative contributions of liver- and kidney markers to cfDNA were approximately halved in EOPE, with mean fold changes of 0.542 (p=0.0003) and 0.548 (p=0.0011), respectively (Figure 5G-H), suggesting dilution by cfDNA fragments from other tissues. In 2/10 EOPE patients with accompanying liver enzyme disturbances, liver-specific hypermethylation in cfDNA was around average (Figure 5G), indicating cfDNA methylation may not directly correspond with liver disturbances. In contrast, endothelium-specific hypermethylated regions were nearly three-fold higher in cfDNA after EOPE diagnosis compared to in controls (mean fold change 2.962, p=0.0027) (Figure 5I).

Figure 5 (next page): Exploring potential origins of differentially methylated regions (DMRs) identified in cfDNA after diagnosis of early-onset preeclampsia (EOPE). A-C: EOPE-related differences in placental DNA methylation related to cfDNA. A) Venn-diagram showing overlap in corresponding genes for DMRs identified in cfDNA (left) and in placental tissues (right) between EOPE and control pregnancies. Overlapping genes were *ABCG1, PREX1, MT-RNR2, SAMD11, UBTF, KNDC1, SNX8, ATP11A, ADARB1, ADARB2* and *USP4*. **B)** Heatmap showing unsupervised clustering analysis of cfDNA samples collected after EOPE diagnosis and matched controls, and placental tissues from EOPE and control pregnancies. DMRs (n=606) were identified in placental tissues between cases and controls. **C)** The normalized total number of reads in cfDNA from EOPE patients and controls, for DMRs hypermethylated in EOPE placentas as compared to control placentas, while hypomethylated in buffy coat samples (n=57). Bars depict mean with SD. Statistical significance tested using Mann-Whitney U test. **D-F: Heterogeneity in EOPE cfDNA assessed using individual sample comparisons. D)** Total number of identified DMRs and number of hypermethylated DMRs for each EOPE cfDNA sample after diagnosis (n=10), compared to the total group of control samples (n=11). **E-F)** Correlations between the total number of DMRs **(E)** and the total number of hypermethylated DMRs **(F)** in each individual EOPE cfDNA sample as compared to the total group of controls (n=11), and the total cfDNA concentration in each EOPE cfDNA sample. Correlations are assessed using the Spearman's rank correlation coefficient. **G-I: Maternal tissue-specific contributions to cfDNA.** The normalized total number of reads for six tissue-specific DMRs in cfDNA between EOPE cases (after diagnosis) and controls. Bars depict mean with SD. P-values are based on Mann-Whitney U test. **G)** Liver-specific reads, p=0.0003. Triangles correspond to two samples obtained from women with liver enzyme disturbances. **H)** Kidney-specific reads, p=0.0011. **I)** Endothelium-specific reads, p=0.0027. N.s. = non-significant (Mann-Whitney U test). ** p<0.01, *** p<0.001.



Discussion

We investigated cfDNA methylation differences between EOPE and uncomplicated pregnancies using MeD-seq. While only 2.7-8.4% of previously reported regions (6, 7) showed differential methylation in our cohort, these regions could generally be used to distinguish cases from controls. We identified one (novel) DMR in the first trimester, likely due to the stringency of MeD-seq, and identified 249 novel DMRs after clinical EOPE diagnosis. Regions identified after diagnosis had poor predictive values in first trimester cfDNA, indicating that the source(s) driving cfDNA methylation differences after EOPE diagnosis are not yet present in first trimester cfDNA. Gene-set enrichment analysis of symptomatic DMRs identified multiple developmental pathways. EOPE-associated placental DMRs (n=606) did not appear to drive cfDNA DMRs. Total

cfDNA was ~20x higher after EOPE diagnosis, with relatively decreased placental-, liver- and kidney contributions and increased endothelial contribution.

Total cfDNA is a mixture of cfDNA fragments originating from different tissues all with unique methylation profiles (23). Maternal cfDNA methylation will therefore be highly affected by the relative contributions of its different tissues. In contrast to De Borre *et al*. (6) and similar to Baetens *et al*. (7), we did not correct for the placental-derived cfDNA fraction. Different cell type contributions from both placental and maternal tissues are likely important drivers of methylation differences and can provide pathophysiological insights. The combination with heterogeneity in used techniques and genomic coverage may explain the low overlap between studies.

The large inter-individual variation in cfDNA concentration after EOPE diagnosis presumably reflects disease-heterogeneity. Others have found 10-fold increases with up to 30-fold higher concentrations in an EOPE-subgroup (24-26), or 2-5 fold increases which mostly comprised of late-onset PE and/or showed positive associations with disease severity (27-29). However, De Borre *et al*. (6) also found a 2-fold increase.

The lower placental-derived cfDNA fraction after EOPE diagnosis and negative trend with cfDNA concentrations have also been reported previously (24). While total placental-originated cfDNA is increased in PE (26, 29-33), the relative contribution is reduced especially in EOPE (24, 32), indicating dilution by maternal-originated cfDNA fragments. In contrast to others (34, 35), we did not find a lower first trimester placental-derived cfDNA fraction in EOPE, possibly attributable to our small sample size.

Consistent with previous studies (6, 7), regions identified after EOPE diagnosis demonstrated poor predictive value in first trimester cfDNA. Increased total cfDNA specifically of maternal-originated fragments in EOPE likely reflects disease severity with increased cellular turnover and tissue injury not yet present in the first trimester. This could explain why EOPE-related placental DMRs did not drive cfDNA DMRs. The positive correlation between cfDNA concentration and number of DMRs in individual EOPE samples suggests that cases with higher cellular turnover and/or greater tissue damage have more pronounced methylation profiles. Although in our symptomatic cohort only 12 cfDNA DMRs (4.8%) were hypermethylated in EOPE, on average 42.1% of DMRs were hypermethylated in individual EOPE samples. Heterogeneity among EOPE samples may have prevented the identification of additional

hypermethylated DMRs in the total population. A consistent dilution of the 'normal' pregnancy signal may explain consistently hypermethylated regions in controls.

We expected increased liver- and kidney-specific hypermethylation after EOPE diagnosis due to corresponding organ damage. However, their relative cfDNA contributions were decreased. This indicates a larger increase from other tissues, one of which may be endothelium as previously found earlier in gestation (36) and implicated in PE-pathogenesis. Others found rank changes in cfDNA contributions in PE mainly involving different immune cells (37). In PE, decreases in regulatory T-cells and monocytes and increases in effector T-cells have been documented (38, 39). Changes in hematopoietic cells, the major contributors to cfDNA (23,37), may also be a potential source of cfDNA methylation differences, requiring further study.

Strengths of this study include efforts to validate previous findings. Additionally, the full-genome wide, non-targeted approach of MeD-seq leads to novel DMRs and allows for the detection of methylation differences that could originate from different tissues. Limitations include the limited sample size and the stringency of MeD-seq data analysis to prevent false-positive results. Consequently, previously identified regions were not detected using our pipeline, whereas we did show methylation differences for some of these regions (Figure 1). Additionally, we investigated tissue-specific cfDNA contributions for a limited number of tissues (placenta, liver, kidney and endothelium); however, these are expected to be substantial contributors besides hematopoietic cells.

More research is warranted to determine the relationship between cell type proportion changes and identified methylation differences, preferably in a cell type-specific manner including different immune cells. A more extensive atlas containing MeD-seq data of additional cell types would be useful to perform future deconvolution analyses, explaining origins of cfDNA methylation differences in EOPE in more detail. Although cfDNA methylation profiles have shown promising results for PE-prediction (6-9), understanding their origins could provide insight into underlying disease mechanisms, potentially improving specificity and use of these biomarkers. Moreover, given the heterogeneity of PE, it would be interesting to investigate cfDNA methylation patterns, tissues of origins and cfDNA concentration in relation to disease phenotype.

Since cfDNA originates mostly from dying, apoptotic cells, cfDNA reflects cellular turnover and tissue damage. Therefore, cfDNA methylation might be useful to monitor disease severity, e.g. the extent to which different organ systems are affected, and guide clinical decision-making

after (EO)PE diagnosis which deserves further study. Longitudinal follow-up is of interest to investigate the timing of onset of observed differences. Finally, integrating additional features of cfDNA fragments referred to as fragmentomics (40-42), such as end motif signatures (43) and nucleosome accessibility (36), which have recently demonstrated potential for EOPE prediction, may significantly increase the information gained from cfDNA and enhance biomarker development.

Conclusions

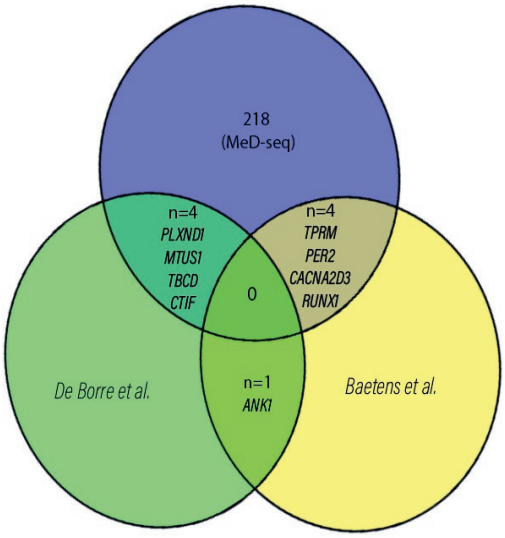
The limited concordance on EOPE cfDNA methylation differences across studies remains challenging, likely due to different applied techniques. We identified novel DMRs in cfDNA using MeD-seq, particularly after EOPE diagnosis. In addition to a large increase in total cfDNA concentration, changes in the relative contributions of different tissues to cfDNA were observed, which may drive identified methylation differences. Unexpectedly, placental DNA methylation differences were not a major contributor to cfDNA DMRs. More research is needed to determine the cellular origins of identified cfDNA DMRs in more detail and to investigate differences based on disease phenotype. This could enhance EOPE biomarker development.

References

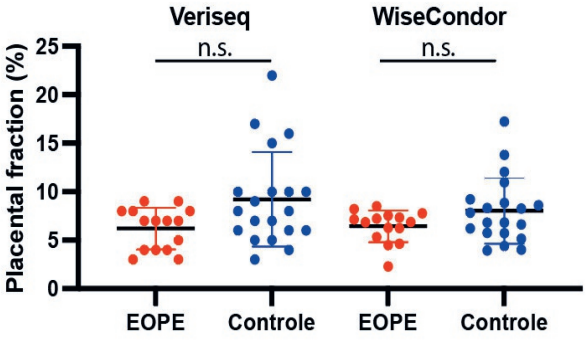
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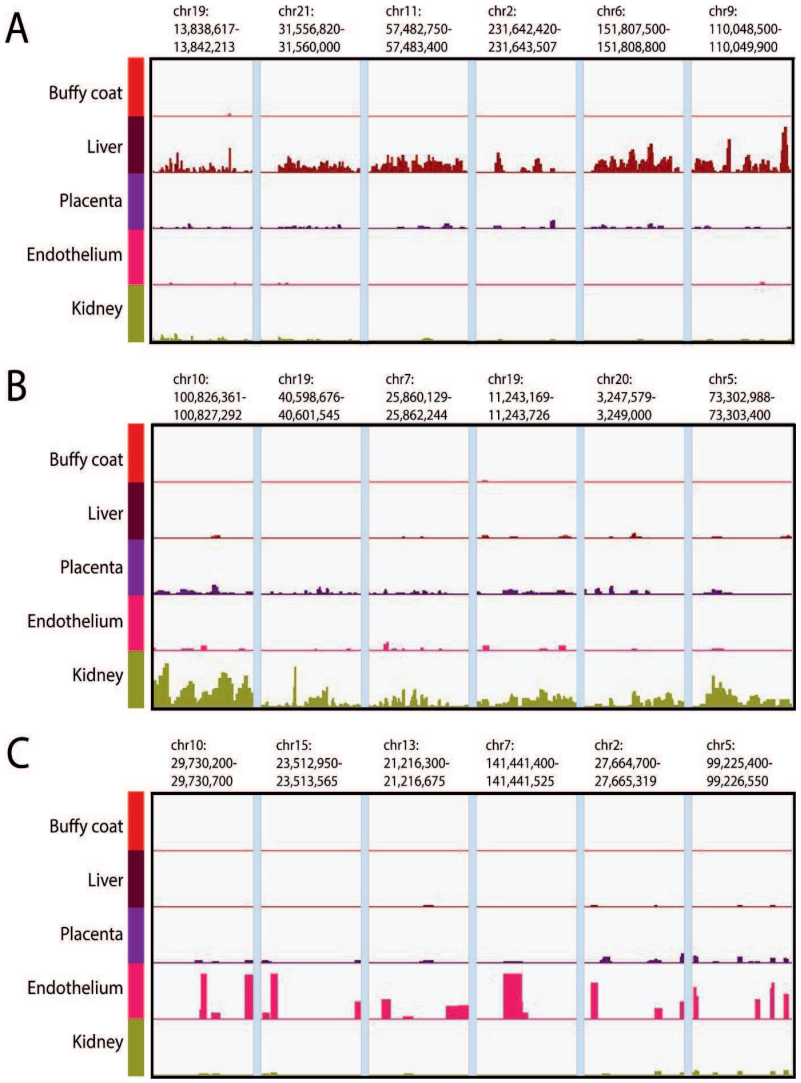
Supplemental Material



Supplemental Figure S1: **Venn-diagram showing overlap in genes corresponding to identified differentially methylated regions (DMRs) in our symptomatic cohort, by De Borre *et al.* and by Baetens *et al.* (top 250).** For *MTUS1* and *PLXND1*, we identified DMRs in the promotor regions, while De Borre *et al.* identified a differently methylated region in the gene bodies of these genes.



Supplemental Figure S2: Placental-derived cfDNA fraction in total maternal cfDNA between cfDNA from early-onset preeclampsia (EOPE) and control pregnancies in our presymptomatic cohort, as determined during NIPT using Veriseq (left, p=0.062) or WiseCondor (right, p=0.294). Based on the Mann-Whitney U test. Bars depict mean with SD. NIPT = noninvasive prenatal testing. N.s. = not significant.



Supplemental Figure 3: Gene-tracks of selected tissue-specific markers (n=6) for **A)** Liver, **B)** Kidney, and **C)** Endothelium.

Supplemental Tables: Can be accessed upon request.

Data availability: The MeD-seq datasets newly generated and analysed during the current study are available in the National Center for Biotechnology Information (SRA database) with accession number PRJNA1249230. Previously generated MeD-seq datasets which were used are available with accession numbers PRJNA1108949, PRJNA1147893, PRJNA1195705 and PRJNA600136.

III

Part III

Discussion and appendices


9



Chapter 9

General discussion

General discussion

The overall aim of this thesis is to enhance our understanding of placental development by use of different innovative approaches, and simultaneously gain insights into the dynamics of the largely understudied cfDNA methylome during pregnancy. The results of this thesis provide new evidence on associations between periconceptional parental exposures and early placental growth (Part I), and showed the (pre)clinical applicability of the novel MeD-seq technology to study the genome-wide cfDNA methylome in maternal plasma throughout pregnancy and provides evidence of associations in case of adverse pregnancy outcomes (Part II). Our results can guide future research into the prediction and prevention of adverse pregnancy outcomes and clinical management of identified high-risk pregnancies, and ultimately improve maternal and fetal health.

Parental preconception care

Many pregnancy complications originate early in gestation, emphasizing the periconceptional period as a critical window for preparing for a healthy pregnancy (1). Unhealthy lifestyle behaviours, such as smoking, alcohol consumption and inadequate nutrition, are common among the general population, including those planning pregnancy (2). Deficiencies of essential micronutrients and substance use around conception can lead to generalized excessive oxidative stress and disrupt epigenetic processes, a biological explanation of the potential lifelong and transgenerational health consequences for offspring. This underlines the importance of promoting and guiding healthy lifestyle choices among future parents (3, 4).

Despite widespread recommendations for folic acid supplement use among women planning to become pregnant (5), preconceptional use of folic acid remains suboptimal. In the Netherlands, only about 60% of women initiate folic acid supplement use before conception (6), with even considerably lower rates reported in many other parts of the world, especially in low- and middle income countries (LMIC) (6). Beyond its well-established role in preventing neural tube defects and other neural crest related malformations (5), our findings suggest that adequate maternal folate concentrations may support early placental growth. Other studies have shown that folate deficiency increases trophoblasts apoptosis (7), and have shown that folic acid enhances trophoblasts invasion through methylation changes (8, 9). Additionally, folic acid use has been associated with a reduced risk of preeclampsia (10) and low birthweight (11). While we found no association between vitamin B12 concentrations and placental volume in a population with adequate vitamin B12 concentrations, a vitamin B12 deficiency could negatively impact placental development and pregnancy outcomes (12). To promote awareness about the importance of adequate preparation for a healthy pregnancy – including

sufficient intake of essential micronutrients – education is warranted at a public level prior to reproduction (e.g. in primary and secondary schools). Additionally, education and provision of essential vitamins should be prioritized in LMICs, where concurrent deficiencies in multiple micronutrients are widespread and the potential for health gains is most significant.

We found no association between paternal age and first trimester placental growth; however, other aspects of paternal aging warrant consideration. Advanced paternal age has been associated with an increased risk of miscarriage, low birth weight, increased genetic abnormalities in offspring and declining fertility (13). Additionally, although paternal age around conception is not a factor which can be addressed, paternal lifestyle factors could also influence pregnancy outcomes through changes in the epigenetic setting of sperm cells (14, 15). These biological considerations, alongside social implications, underscore the importance of also addressing paternal factors in preconception care.

Maternal cfDNA methylation: lessons learned

The discovery of placental cfDNA fragments in maternal plasma by Lo *et al*. in 1997 (16) revolutionized the field of prenatal medicine. However, we are only at the early stages of exploring the potentials of applications related to establishment of the cfDNA epigenome in maternal plasma (17). The studies in this thesis aim to advance our understanding of the maternal cfDNA methylome and represents the first steps in using MeD-seq to detect and characterize (patho)physiological cfDNA methylation changes throughout gestation.

Besides placental-originated cfDNA fragments, most cfDNA fragments in maternal plasma originate from different maternal tissues. Notably, in Chapter 5 we report that pregnancy-induced changes in cfDNA methylation are far more complex than only the addition of placental-derived cfDNA fragments, indicating additional changes in maternal tissues that contribute to cfDNA, including turnover of immune cells. The insights gained into cfDNA dynamics in general have relevance beyond prenatal medicine, contributing to fields like oncology and transplantation. Analysis of cfDNA fragments is an example of so-called “liquid biopsies”, which provide information about the tumor or organ transplant through blood, and are a rapidly evolving research area (18). Recent oncology studies also showed that observed increases in cfDNA fragments do not primarily originate from tumor cells, but mainly originate from hematopoietic cells (19, 20). In addition, we found a large increase in total cfDNA in the maternal plasma in early-onset preeclampsia, with a relative increase in cfDNA from maternal tissues and a relative decrease in placental-originated cfDNA fragments. These studies highlight that cfDNA represents real-time information from a variety of tissues, besides the main tissue

of interest (e.g. placenta, organ transplant or tumor). All these tissues can subsequently be influenced by systemic processes including pregnancy or pathologic conditions. Moreover, recent findings indicate that the contribution of cfDNA fragments from a particular tissue is not solely determined by tissue-specific cell turnover; additional factors such as local cfDNA clearance by macrophages and the level of access to the bloodstream possibly affect the concentration and presence of tissue-specific cfDNA (21).

We demonstrated the feasibility of tracing specific placental DNA methylation marks related to gestational age in cfDNA (Chapter 7). Our results show that cfDNA analysis can not only identify the tissue of origin, but is also able to detect methylation differences within a specific tissue of interest. To ensure that the methylation signals of these markers in cfDNA are not overwhelmed by high methylation levels and relative overrepresentation of blood cells (22), they preferably should exhibit low methylation levels in hematopoietic cells. Overcoming these detection challenges, our findings pave the way for future applications of cfDNA methylome analysis in studying epigenetic differences within tissues of origin.

Methodological considerations

The data presented in this thesis were primarily collected as part of the Rotterdam Periconceptional Cohort (Predict study), an ongoing cohort study conducted at the Erasmus Medical Centre in Rotterdam (23, 24). As with all observational studies, our results are subject to potential biases. Selection bias (overall tertiary population of patients) and a limited sample size in certain studies may affect the external validity of our findings, e.g. the degree to which our results are representative for populations outside our study population. In general, study participants were predominantly of Dutch-origin, had higher prevalences of parental morbidities, were more highly educated, and exhibited healthier lifestyle behaviors compared to the general population. For example, maternal vitamin B12 levels were mostly adequate, and preconceptional use of folic acid was higher in our study population than the Dutch average. To promote the generalizability of our results, validation in other cohorts is recommended, particularly including participants from non-hospital settings, with higher prevalences of vitamin deficiencies, and with more diverse geographical backgrounds.

Another consideration is the significance of the first trimester placental volume. Optimal first trimester placental growth remains undefined, and a larger first trimester placenta does not necessarily indicate better functioning. However, because the placenta is largely inaccessible during pregnancy, it is often considered a “black box”. Ultrasound can be used to noninvasively visualize placental characteristics in early pregnancy, offering a “piece of the puzzle”. Notably,

first trimester placentas with smaller volumes have been observed in pregnancies later complicated by adverse outcomes such as preeclampsia (25-28), and early placental growth has positively been associated with fetal growth (29). These findings support the clinical significance of the first trimester placental volume and its dynamics.

Several methods can be used to study (cf)DNA methylation. This thesis focused on investigating the applicability of MeD-seq for studying the maternal cfDNA methylome, given its compatibility with low (cf)DNA concentrations and its ability to provide full genome-wide coverage (30). However, several methodological considerations should be addressed. MeD-seq relies on read mapping, which complicates the correction for methylation in repeat regions (e.g. copy number variations). To minimize this "noise", a sufficient sample size is required and a fold change of ≥2 is applied to identified DMRs to reduce the risk of false-positive findings. Additionally, MeD-seq captures only methylated DNA sequences. The absence of a methylation signal in a specific genomic region could reflect a true lack of methylation, but it may also be due to a poor representation of that region in the cfDNA sample. A combination of methylation markers will therefore often be preferred over a single marker in case of biomarker development using cfDNA. Furthermore, the full-genome wide coverage provided by MeD-seq possesses challenges in terms of correcting for multiple testing to avoid false-positive results. A more targeted approach could reduce the stringency of this correction, increasing the likelihood of identifying relevant regions that might otherwise be overlooked. In general, the stringency of MeD-seq leads to less identified regions as compared to other techniques (Chapter 8). At the same time, this stringency promotes only identifying robust, substantial methylation differences, and part of DMRs identified by MeD-seq may not be captured by other methods with a smaller genome-wide coverage (Chapter 8). The extend of (expected) (cf)DNA methylation differences and their intended use, could determine whether MeD-seq, other techniques, or a combination of approaches would be the most suitable study approach.

Lastly, we mainly focused on overlap of DMRs identified in cfDNA with DNA methylation profiles in other cell types or tissues to explore the (potential) tissues of origins underlying these DMRs. However, this is challenged by similarities in DNA methylation patterns in different cell types or tissues. It would be helpful to expand our current reference sets of genome-wide DNA methylation profiles assessed using MeD-seq, to include all tissues – or preferably cell types – that can contribute to cfDNA. Next, for each tissue or cell type, regions with unique methylation profiles would need to be identified (such as hypermethylation of the *RASSF1* promoter for placental tissue) to be able to determine the different origins of the cfDNA methylation signals

more specifically (deconvolution), and to be able to subsequently take cell type proportions into account in further analyses.

Clinical implications and considerations

This thesis emphasizes the importance of preconceptional care, particularly the timely initiation of folic acid supplement use to support early placental growth (part I). Part II builds up to investigating the potential of studying cfDNA methylation using MeD-seq as biomarker for predicting adverse pregnancy outcomes such as preeclampsia.

There is an urgent need to early identify pregnancies that are at high risk of adverse pregnancy outcome, such as preeclampsia. In case of preeclampsia, this will enable timely initiation of targeted preventative measures such as aspirin, which should be initiated before 16 weeks of gestation – well before symptoms arise (31, 32). Overall, currently first trimester risk assessment for preeclampsia relies primarily on maternal demographic characteristics and medical history, but this approach has poor predictive accuracy (33). An algorithm developed by the Fetal Medicine Foundation (FMF), which incorporates maternal characteristics, blood pressure, placental growth factor (PlGF), pregnancy associated plasma protein A (PAPP-A) and the Uterine artery pulsatility index (UtA-PI), performs better as a first trimester screening test. However, its use is limited by the need for trained sonographers (33). To improve preeclampsia prediction, various promising strategies are being explored. In addition to maternal cfDNA methylation differences (34, 35), approaches analyzing cell-free RNA (36), circulating miRNAs (37) and the vaginal microbiome (38) show potential. Moreover, 3D placental (vascular) volume imaging markers, as also investigated in this thesis, may serve as predictive biomarkers, given that smaller first trimester placental (vascular) volumes have been observed in women who later develop preeclampsia (25-28). As there is yet no holy grail in early preeclampsia prediction, maternal cfDNA methylation may contribute to future risk assessment while potentially shedding light on its underlying pathophysiology.

In addition to early prediction of preeclampsia, there is a critical need for tools to guide clinical decision making after preeclampsia diagnosis. Currently, women diagnosed with preeclampsia are usually admitted to the hospital for monitoring of maternal and fetal health until after delivery. While this approach enables prompt intervention when necessary, it also places a significant burden on healthcare resources, personnel and costs. Furthermore, preeclampsia is a heterogenous complex disease, and some patients may not require extended inpatient care. However, it remains impossible to predict when preeclampsia becomes so severe that short-term delivery is necessary. Therefore, a reliable prognostic test to assess disease severity, to

allow for optimal timing of fetal preparation by antenatal corticosteroids and to predict the need for short-term intervention, is warranted. Such a tool should distinguish between patients who require intensive inpatient care from those who could be safely managed in an outpatient setting or through methods like telemonitoring (39), and/or should be able to predict when delivery will be indicated to guide the use of antenatal corticosteroids. Antenatal corticosteroids are ideally administrated 2-7 days before delivery in case of birth before 34 weeks of gestation (40). Currently, the sFlt-1/PlGF ratio is already being evaluated as part of The PreRisk calculator, to determine its ability to identify high-risk preeclampsia cases that require hospitalization (41). Maternal cfDNA concentration and its methylation profiles may serve as a novel prognostic biomarker, reflecting not only placental turnover but also (generalized) maternal tissue damage. This is suggested by our data showing large increases in cfDNA concentrations with a large inter-individual variety after EOPE diagnosis, a relative increase in maternal-originated cfDNA fragments, and a positive correlation between the number of identified DMRs and the cfDNA concentration in individual EOPE cfDNA samples (Chapter 8). Additionally, previous studies have shown a relation between cfDNA concentration (hence, correlation with amount of tissue damage) and disease severity (42). Future research is needed to develop such a prognostic tool using cfDNA, which could enhance personalized care in preeclampsia management, minimize unnecessary hospitalizations and reduce healthcare costs, while ensuring maternal and fetal safety.

Given the complexity of preeclampsia which involves multiple biological pathways (43), it is likely that a combination of biomarkers and maternal characteristics will provide the most accurate predictions. As highlighted above, the ease of use and availability of a clinical test will also be crucial for its successful integration into different healthcare systems. Since noninvasive prenatal testing (NIPT) is already widely available, cfDNA methylome-based biomarkers could potentially be incorporated into this test as an accompanying tool for early disease prediction (44). Finally, the affordability and cost-effectiveness of any new test should be considered to prevent increasing health inequalities and ensure efficient use of medical resources.

Directions for future research

Artificial intelligence and imaging

The use of artificial intelligence (AI) to automate ultrasound measurements of the placenta is emerging (45). Validation and standardization of automatic measurements of placental imaging markers could make these measurements less time consuming and more widely available, with the potential of integration into clinical healthcare. In the future, this could enable the large-scale assessment of various placental imaging markers such as placental (vascular) volume. This

is critical for validating our findings related to first trimester placental volume and to investigate the clinical significance of measuring dynamics in early placental growth.

External validation

Ideally, populations with higher prevalences of deficiencies in micronutrients, such as vitamin B12 deficiency, would be included in future studies to explore the potential effects of these vitamins on (early) placental growth. This could include populations within the Netherlands with a more vulnerable health status including pregnant women with a lower social-economic status. Additionally, research could be conducted in LMIC with high prevalences of deficiencies in essential nutrients, especially in countries with predominantly vegetarian diets. Expanding to more ethnically diverse populations is also required to investigate the generalizability of our results in populations with other genetic backgrounds, particularly for our epigenetic analyses.

Future research using cfDNA and DNA methylation

The genome-wide study of the maternal cfDNA methylome is still in its early stages, but it is expected to continue and expand, offering a wide range of potential directions for future research.

Fragmentomics

One interesting direction for future research is the integration of additional characteristics of cfDNA fragments, referred to as fragmentomics, with DNA methylation profiles. In addition to differences in fragment size between maternal and placental cfDNA fragments, other characteristics such as end motif profiles (DNA sequences found at fragment ends), preferred ends (genomic location of fragmentation), nucleosomal patterns (protecting specific genomic regions from cleavage), and single-stranded jagged ends (overhanging single-stranded regions) have been associated with the tissue of origin of cfDNA fragments (Figure 1) (46-48). Notably, end motif signatures (49) and nucleosome accessibility (50) of cfDNA fragments in plasma samples collected for NIPT have recently been shown to have predictive value for early-onset preeclampsia. Ongoing advancements in sequencing technologies and bioinformatics – also driven by research in other fields such as oncology and transplantation – will enhance the study of cfDNA characteristics and increase the amount of information gained from these co-called liquid biopsies. As research on cfDNA continues to evolve, combining these approaches will improve the identification of tissues of origins of cfDNA, and could promote the technical separation of placental and maternal cfDNA fragments. This would also allow for the study of DNA methylation in (enriched) placental cfDNA fragments in relation to health and disease, as well as its associations with environmental or lifestyle factors, including one-carbon moieties.

Targeted approach

A more targeted approach could strengthen the signal of interest in future cfDNA studies as compared to full genome-wide analyses. For cfDNA research, this could involve preselecting regions that exhibit low DNA methylation in blood cells but are highly methylated in the cell type or tissue of interest. This strategy ensures that the methylation signal is not masked by high methylation levels in cfDNA fragments from background DNA methylation present in hematopoietic cells (Chapter 7).

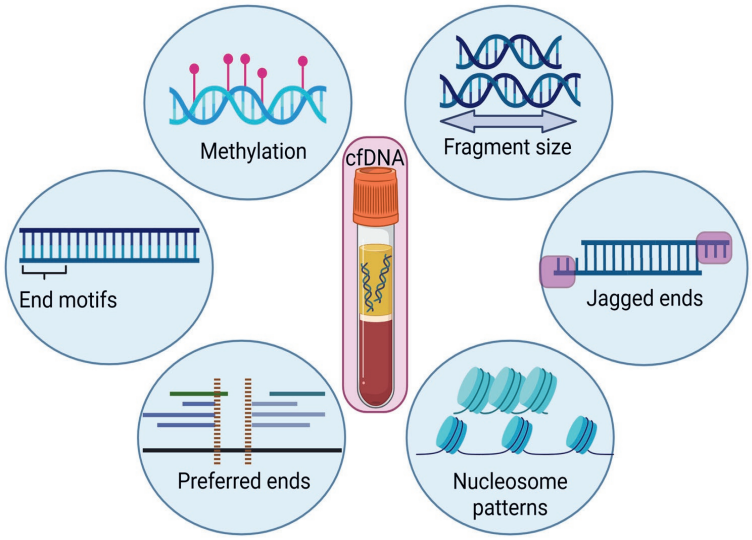


Figure 1: Schematic overview of biological characteristics of cell-free DNA fragments. All reflect diverse, nonrandom fragmentation processes and depend on the tissue of origin. Other characteristics besides DNA methylation profiles, also referred to as fragmentomics, include: overall fragment size distribution of cfDNA fragments; end motifs: characteristic sequences of nucleotides at the end of a cfDNA fragment; jagged ends: the presence and lengths of overhanging, single-stranded ends of a cfDNA fragment caused by incomplete or asymmetric nuclease activity during apoptosis and cfDNA degradation (for example more frequently found in placental than in maternal cfDNA fragments); preferred ends: genomic locations with preferred cleavage sites; nucleosome patterns: nucleosomes protect the DNA from cleavage and the genome coverage of these protected regions is expected to be higher in cfDNA, than that of linker DNA regions that are preferentially cut. In addition, fragment size is affected by nucleosome structures and positioning. Figure is created with BioRender.

Origins and consequences of DNA methylation differences

Another important area of research is to explore the biological significance of identified DNA methylation differences. The biological meaning of identified differences is often unclear, especially in genome-wide studies. For example, it remains unknown whether DNA methylation profiles in placental tissues associated with preeclampsia play a causative role in the development of preeclampsia, or are actually a response to the pathogenic preeclamptic environment (51). Moreover, DNA methylation profiles vary between cell types (22). Therefore, when studying heterogenous tissues that consist of different cell types, such as the placenta and cfDNA, DNA methylation differences may reflect differences in cell composition instead of

actual changes in DNA methylation within a cell type or tissue. This is illustrated by a previous study that showed differences in cell type proportions were the primary driver of gene expression changes previously linked to preeclampsia (52). Likewise, our studies showed that the contribution of cfDNA fragments originating from placental tissues and other maternal organ systems in maternal plasma is influenced by gestational age as well as by pathophysiological processes such as preeclampsia. Depending on the research question, it may be crucial to distinguish between cell-specific methylation differences and those driven by variations in cell-type composition. To specifically study DNA methylation differences within a tissue of interest, studying DNA methylation dynamics in a cell type-specific manner or correcting for differences in cell type contributions is required.

To be able to distinguish the contribution of all relevant cell types to cfDNA with MeD-seq, an extensive reference database is required containing DNA methylation profiles from additional tissues and preferably cell types (e.g. different immune cells), as well as gestational age-specific methylation profiles of placental cell types and maternal immune cells. These reference profiles would preferably all be established using MeD-seq, since the interchangeability between techniques is limited.

Other types of (fragmentomics) data could also be integrated to enhance these analyses. On the other hand, DNA methylation differences driven by variations in cell type compositions could also be of utmost importance for the development of disease-specific biomarkers.

Follow-up experiments are needed to investigate the consequence of tissue-specific DNA methylation differences on gene expression and cellular function, and thus the biological relevance. This may provide insights into the role of these methylation variations in (patho)physiological placental development, and potentially lead to novel therapeutic strategies for placental-originated diseases. Understanding the biological cause and/or consequence of DNA methylation differences on cell function may also improve predictive capabilities of biomarkers.

Clinical research into cfDNA biomarkers in pregnancy

In addition to potential future (technical) directions described above, future research into maternal cfDNA methylation and fragmentomics could expand to study their predictive values in other obstetrical complications, including late-onset preeclampsia, fetal growth restriction and preterm labor. Topics we also plan to investigate in our prospective cohort study with longitudinal sampling. As other disease-predicting biomarkers (e.g., ultrasound markers, cell-free RNA, miRNAs, maternal characteristics, vaginal microbiome) are being studied, the added

value of combining different strategies, as well as their ease to be used in different clinical settings, ideally early in pregnancy, deserves further investigation.

While our initial focus was mostly on placental-originated cfDNA fragments in total cfDNA, this thesis highlights the potential value of maternal-originated cfDNA fragments in understanding maternal adaptations during pregnancy and their associations with obstetric complications. Although not useful for first trimester predictions, we observed a substantial increase in total cfDNA after diagnosis of early-onset preeclampsia, with a relative increase in cfDNA from maternal origin such as endothelium. Similarly, others found a relationship between higher cfDNA concentrations, particularly from maternal tissues, and increased disease severity in preeclampsia (42). This suggests that cfDNA concentrations and/or methylation profiles could potentially serve as predictive biomarkers for classifying disease severity, by reflecting overall (maternal) tissue damage. This may improve clinical decision making in case of preeclampsia – which remains a leading cause of neonatal and maternal morbidity and mortality worldwide (43) – on for example the best timing of pregnancy termination. A combination of cfDNA concentration and characteristics could potentially also be an interesting target in other (obstetrical) systemic diseases, such as acute fatty liver disease or sepsis.

Collaborations between research groups could lead to the development of feasible, combined algorithms with optimal predictive capabilities. Importantly, the effectiveness of targeted preventative measures such as aspirin prophylaxis, and the effects of preeclampsia management on health care use and perinatal health outcomes guided by these biomarkers, needs to be established in pregnancies identified as high- or low-risk using these novel strategies. Ultimately, the usefulness of (early) prediction of complications is determined by the possibilities to act upon identified risks.

Conclusions

This thesis advances our understanding of factors influencing early placental growth and the dynamics of the maternal cfDNA methylome by using 3D ultrasound and MeD-seq, two noninvasive approaches. It emphasizes the importance of the preconceptional initiation of folic acid supplement use to support early placental growth. Additionally, we demonstrate the current opportunities and limitations of using MeD-seq to study the maternal cfDNA methylome, emphasizing that maternal plasma cfDNA carries real-time information from both placenta and maternal tissues. The analysis of the total maternal cfDNA methylome offers significant potential for identifying biomarkers that could enhance the prediction of adverse pregnancy outcomes, ultimately contributing to better maternal and fetal health.

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Chapter 10

Summary (English)

Samenvatting (Dutch)

Summary (English)

Chapter 1 outlines the background and aims of this thesis. A well-functioning placenta is essential for a healthy pregnancy, and placental dysfunction is involved in complications like preeclampsia. However, studying the (patho)physiological development of the placenta is challenged by its inaccessibility during pregnancy.

Placental development is a complex process that can be influenced by a range of parental and environmental factors. DNA methylation, a key epigenetic mechanism involved in cell differentiation and genomic imprinting, plays a crucial role in placental growth and development. Placental DNA methylation differences have been associated with gestational age and obstetric complications. One-carbon metabolism (1CM), which includes B-vitamins like folate and vitamin B12, has a crucial role in these developmental processes, as it provides the required methyl groups for DNA synthesis and methylation.

This thesis employs two noninvasive methods to study (early) placental development and placental DNA methylation. The first approach involves first trimester 3D ultrasound of the placental (vascular) volume. The second investigates genome-wide DNA methylation in placental tissues and cell-free DNA (cfDNA) using the Methylated DNA Sequencing (MeD-seq) technology. Besides a majority of cfDNA fragments from maternal tissues, placental-originated fragments can be found within maternal plasma. As compared to most other techniques, MeD-seq is better compatible with low cfDNA concentrations and has a more extensive genome-wide coverage.

The overall aim of this thesis is to enhance our understanding of (patho)physiological placental development and dynamics in maternal cfDNA methylation. This could lead to the identification of novel targets supporting placental growth and the development of biomarkers for the (early) prediction and prevention of placental-related complications such as preeclampsia, and ultimately improve maternal and fetal health outcomes.

Part I of this thesis investigates one-carbon moieties and parental characteristics in relation to placental DNA methylation and early placental growth.

In **Chapter 2**, the results of a systematic review on one-carbon moieties and placental DNA methylation in humans are described. In general, one-carbon moieties were not associated with global placental DNA methylation, and genome-wide approaches identified limited DNA

methylation differences associated with one-carbon moieties. In contrast, most studies with a gene-specific approach, did indicate site-specific associations between different one-carbon moieties and genomic regions, specifically in genes involved in growth and metabolism. This suggests that disrupted 1CM may induce lasting changes in the fetal epigenome, with potential implications for fetal development as well as for future health outcomes.

In **Chapter 3** it is shown that women with low folate concentrations (Q1) have smaller first trimester placental volumes. These results were validated in a larger cohort, by showing comparable smaller placental volumes in women who initiated folic acid supplement use following as compared to prior to conception. These findings emphasize the importance of preconceptional folic acid supplementation to acquire adequate maternal folate concentrations, supporting first trimester placental growth in addition to preventing neural tube defects. No differences in placental volume were reported in relation to vitamin B12 concentrations, possibly due to the adequate vitamin B12 concentrations in our study population (**Chapter 3**), nor between paternal age and early placental (vascular) growth (**Chapter 4**).

Part II of this thesis focusses on the use of MeD-seq to study the genome-wide maternal cfDNA methylome.

First, the results of a pilot study are described in **Chapter 5**. The possibility to detect placental DNA methylation markers in first trimester maternal cfDNA using MeD-seq is shown by the detection of Y-chromosomal methylation markers in pregnancies with male offspring, and by hypermethylation of the *RASSF1* promoter, a universal placental-specific marker. In addition, novel placental DNA methylation markers in maternal cfDNA are identified. Surprisingly, besides a placental-originated signature, the results also indicate an immune cell contribution to the pregnancy-induced changes in the maternal cfDNA methylome. This reveals that pregnancy-induced changes in the maternal cfDNA methylome go beyond the additional presence of placental-originated cfDNA fragments. This underlines the importance of considering the contribution of other tissues to total cfDNA for everyone studying the cfDNA methylome.

Subsequently, **Chapter 6** describes a study protocol for an observational subcohort, embedded in the Rotterdam Periconception Cohort, to study cfDNA methylation dynamics over time in uncomplicated pregnancies and in pregnancies complicated by preeclampsia and fetal growth restriction. The study aims to enroll a total of 300 pregnancies with longitudinal cfDNA collected

in all three trimesters and at birth. The results for a subgroup of 10 uncomplicated pregnancies are described in **Chapter 7**, and show that most changes in maternal cfDNA methylation across gestation are attributable to the increasing placental-derived cfDNA fraction. Additionally, it shows that several gestational age-specific placental DNA methylation markers were also associated with gestational age in maternal cfDNA. This suggests that not only the tissue of origin of cfDNA fragments can be identified, but that methylation differences within a specific tissue of interest can also be detected in cfDNA. This paves the way for future applications of cfDNA methylome analysis to study epigenetic differences within tissues of origin.

The first results for maternal cfDNA methylation analyses in pregnancies complicated by early-onset preeclampsia are described in **Chapter 8**. Although we only identified one differentially methylated region (DMR) in first trimester cfDNA, we identified 249 novel DMRs after EOPE diagnosis and partly validated findings from previous studies. After EOPE diagnosis, we show a large increase in total cfDNA, with a relative higher increase in cfDNA fragments from maternal (including endothelium) than placental origin. Combined, the studies in part II of this thesis increase our understanding of the largely understudied maternal cfDNA methylome and guide future research.

Finally, **Chapter 9** summarizes the main findings and discusses methodological and clinical considerations as well as directions for future research.

Summary (Dutch)

Hoofdstuk 1 schetst de achtergrond en de doelen van dit proefschrift. Een goed functionerende placenta is essentieel voor een gezonde zwangerschap en disfunctie van de placenta is betrokken bij complicaties zoals zwangerschapsvergiftiging. Het bestuderen van de (patho)fysiologische ontwikkeling van de placenta is echter bemoeilijkt doordat de placenta niet op een niet-invasieve manier bereikbaar is tijdens de zwangerschap.

De ontwikkeling van de placenta is een complex proces dat beïnvloed kan worden door een reeks ouderlijke en omgevingsfactoren. DNA methylatie, een belangrijk epigenetisch mechanisme dat o.a. betrokken is bij cel differentiatie en genomische imprinting, speelt een cruciale rol in de groei en ontwikkeling van de placenta. Verschillen in DNA methyleringsprofielen in de placenta zijn in verband gebracht met zwangerschapsduur en obstetrische complicaties. Het 1-koolstofmetabolisme, welke B-vitaminen zoals foliumzuur en vitamine B12 bevat, speelt een cruciale rol in deze ontwikkelingsprocessen, omdat het de benodigde methylgroepen levert voor DNA synthese en methylatie.

In dit proefschrift worden twee niet-invasieve methoden gebruikt om de (vroege) ontwikkeling van de placenta en DNA methylatie van de placenta te bestuderen. De eerste benadering maakt gebruik van 3D-echografie van het placenta (vasculair) volume in het eerste trimester. De tweede methode onderzoekt genoomwijd DNA methylatie in placentaweefsels en in celvrij DNA (cfDNA) met behulp van de Methylated DNA Sequencing (MeD-seq) technologie. Naast een meerderheid van cfDNA fragmenten afkomstig van maternale weefsels, kunnen cfDNA fragmenten afkomstig van de placenta worden gevonden in maternaal plasma. In vergelijking met de meeste andere technieken is MeD-seq goed bruikbaar bij lage cfDNA concentraties en heeft het een grotere genoomwijde dekking.

Het algemene doel van dit proefschrift is om kennis van de (patho)fysiologische placenta ontwikkeling en dynamiek in maternale cfDNA methylatie te vergroten. Dit zou kunnen leiden tot de identificatie van nieuwe aanknopingspunten die de groei van de placenta ondersteunen, tot de ontwikkeling van biomarkers voor de (vroege) voorspelling en preventie van placenta-gerelateerde complicaties zoals zwangerschapsvergiftiging, en uiteindelijk tot de verbetering van de gezondheidsuitkomsten van moeder en kind.

Deel I van dit proefschrift onderzoekt metabolieten van het 1-koolstofmetabolisme en eigenschappen van de ouders in relatie tot placentaire DNA methylatie en vroege placentaire groei.

In **Hoofdstuk 2** worden de resultaten van een systematisch review naar 1-koolstofmetabolisme en placentaire DNA methylatie bij mensen beschreven. In het algemeen waren metabolieten van het 1-koolstofmetabolisme niet geassocieerd met de mate van globale placentaire DNA methylatie, en met behulp van genoomwijde benaderingen werden slechts enkele DNA methyleringsverschillen geassocieerd met metabolieten van het 1-koolstofmetabolisme. De meeste onderzoeken met een gen-specifieke benadering wezen daarentegen wel op associaties tussen verschillende 1-koolstofmetabolieten en DNA methylatie van genomische regio's, specifiek in genen die betrokken zijn bij groei en metabolisme. Dit suggereert dat een verstoord 1-koolstofmetabolisme blijvende veranderingen in het foetale epigenoom kan veroorzaken, met mogelijke implicaties voor zowel de foetale ontwikkeling als voor toekomstige gezondheidsuitkomsten.

Hoofdstuk 3 laat zien dat vrouwen met lage foliumzuurconcentraties (Q1) kleinere placentavolumes hebben in het eerste trimester. Deze resultaten werden gevalideerd in een groter cohort, waarbij tevens kleinere placentavolumes werden gevonden in vrouwen die begonnen met foliumzuursuppletie na de conceptie in vergelijking met vóór de conceptie. Deze bevindingen benadrukken het belang van preconceptioneel foliumzuur gebruik om adequate maternale foliumzuurconcentraties te verkrijgen, om de groei van de placenta in het eerste trimester te ondersteunen naast de gekende beschermende effecten van foliumzuur in het voorkómen van neurale buisdefecten. Er werden geen verschillen in placentavolume gerapporteerd in relatie tot vitamine B12 concentraties, mogelijk als gevolg van de adequate vitamine B12 concentraties in onze studiepopulatie (**Hoofdstuk 3**), noch tussen de paternale leeftijd en de vroege placentaire (vasculaire) groei (**Hoofdstuk 4**).

Deel II van dit proefschrift richt zich op het gebruik van MeD-seq om het genoomwijde maternale cfDNA methyloom te bestuderen.

Eerst worden de resultaten van een pilotstudie beschreven in **Hoofdstuk 5**. De mogelijkheid om met behulp van MeD-seq placentaire DNA methyleringsmarkers te detecteren in maternaal cfDNA in het eerste trimester wordt aangetoond door de detectie van Y-chromosomale methyleringsmarkers in zwangerschappen met een mannelijke foetus en door de detectie van hypermethylatie van de *RASSF1* promotor, een universele placenta-specifieke marker. Daarnaast werden nieuwe placentaire DNA methyleringsmarkers in maternaal cfDNA geïdentificeerd. Verrassend genoeg wijzen de resultaten, naast een door de placenta veroorzaakte signatuur, ook op een bijdrage van immuun cellen aan de zwangerschap-geïnduceerde veranderingen in het maternale cfDNA methyloom. Dit onthult dat

zwangerschap-geïnduceerde veranderingen in het maternale cfDNA methyloom verder gaan dan de extra aanwezigheid van placentaire cfDNA fragmenten. Dit onderstreept het belang van het in ogenschouw nemen van de bijdrage van andere weefsels aan het totale cfDNA voor iedereen die cfDNA methylatie bestudeert.

Vervolgens wordt in **Hoofdstuk 6** een studieprotocol beschreven voor een observationeel subcohort, ingebed in het Rotterdam Periconception Cohort, om de veranderingen van cfDNA methylatie in de tijd te bestuderen in ongecompliceerde zwangerschappen en in zwangerschappen gecompliceerd door zwangerschapsvergiftiging of foetale groeirestrictie. Het doel van de studie is om 300 zwangerschappen te includeren en hierbij longitudinaal cfDNA te verzamelen in elk trimesters en bij de bevalling. De resultaten voor een subgroep van 10 ongecompliceerde zwangerschappen worden beschreven in **Hoofdstuk 7** en laten zien dat de meeste veranderingen in maternaal cfDNA methylatie tijdens de zwangerschap zijn toe te schrijven aan de toenemende cfDNA fractie die afkomstig is van de placenta. Daarnaast laat het zien dat verschillende zwangerschapsduur-specifieke placenta DNA methylatie markers ook geassocieerd waren met zwangerschapsduur in maternaal cfDNA. Dit suggereert dat niet alleen het weefsel van oorsprong van cfDNA fragmenten kan worden geïdentificeerd, maar dat methylatie verschillen binnen een specifiek weefsel van interesse ook kunnen worden gedetecteerd in cfDNA. Dit maakt de weg vrij voor toekomstige toepassingen van cfDNA methyloom analyses om epigenetische verschillen binnen weefsels van herkomst te bestuderen.

De eerste resultaten voor maternale cfDNA methyleringsprofielen in zwangerschappen gecompliceerd door vroege zwangerschapsvergiftiging worden beschreven in **Hoofdstuk 8**. Ondanks dat we maar één regio vonden die anders gemethyleerd was in eerste trimester cfDNA, hebben we 249 nieuwe regio's gevonden na diagnose en hebben we een deel van de gevonden regio's uit eerdere studies kunnen valideren. Na diagnose van vroege zwangerschapsvergiftiging laten we zien dat er een grote toename is van het totale cfDNA, met relatief een grotere toename afkomstig van maternale weefsels (zoals endotheel) dan van de placenta. Samen vergroten de studies in deel II van dit proefschrift ons begrip van het grotendeels onderbelichte maternale cfDNA methyloom en geven ze richting aan toekomstig onderzoek.

Tot slot, **Hoofdstuk 9** vat de belangrijkste bevindingen samen en bespreekt methodologische en klinische overwegingen en richtingen voor toekomstig onderzoek.



Chapter II

Appendices

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List of publications

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List of abbreviations

1CM	One-carbon metabolism
3D	Three-dimensional
ABAT	4-aminobutyrate aminotransferase
AdipoQ	Adiponectin, C1Q and collagen domain containing
AGA	Appropriate growth for gestational age
AI	Artificial Intelligence
AMOTL1	Angiomotin Like 1
ANKRD20B	Ankyrin repeat domain 20 family member A8, pseudogene
APC	APC regulator of WNT signaling pathway
AS3MT	Arsenite methyltransferase
ASD	Autism spectrum disorder
BMI	Body Mass Index
bp	base pair
BRD4	Bromodomain Containing 4
cfDNA	Cell-free DNA
CI	Confidence interval
CpG	Cytosine-phosphate-Guanine
CRH	Corticotropin releasing hormone
CRL	Crown-rump length
CTB	Cytotrophoblast
CTCF	CCCTC-Binding Factor
DCAF10	DDB1 and CUL4 Associated Factor 10
DENND2D	DENN Domain Containing 2D
DLL1	Delta like canonical Notch ligand 1
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DOHaD	Developmental origins of health and disease
DNMTs	DNA Methyltransferases
DHRS3	Dehydrogenase/Reductase 3
EDTA	Ethylenediaminetetraacetic acid
EPS8L2	EPS8 Signaling Adaptor L2
Epi-NIPT	Epigenome-wide noninvasive prenatal test
EOPE	Early-onset preeclampsia
ETS2	ETS proto-oncogene 2, transcription factor
ESR1	Estrogen receptor 1
EVT	Extravillous trophoblasts
FA	Folic acid
FAM198A	Family with sequence similarity 198 member A
FAR1	Fatty acyl-CoA reductase 1
FC	Fold change
FDR	False Discovery Rate
FFPE	Formalin-fixed paraffin-embedded
FFQ	Food Frequency Questionnaires
FMF	Fetal Medicine Foundation
GA	Gestational age
GNAS-AS1	GNAS antisense RNA 1
GNL1	G protein nucleolar 1 (putative)
GSDMD	Gasdermin D
H&E	Hematoxylin and eosin
H19	H19 imprinted maternally expressed transcript
HBD	Healthy blood donor
HDP	Hypertensive disorders of pregnancy
HSD11β2	Hydroxysteroid 11-beta dehydrogenase 2
ICSI	Intracytoplasmic sperm injection
ICR	Imprinted control region
IGF1	Insulin like growth factor 1
IGF2	Insulin like growth factor 2
IL10	Interleukin 10
ISSHP	Internation Society for the Study of Hypertension in Pregnancy
IVF	In vitro fertilization
IUFD	intrauterine fetal death
IUI	Intrauterine insemination
IQR	Inter-quartile range
IRX2	Iroquois Homeobox 2
kb	kilobase
LBX2	Ladybird homeobox 2
LCM	Laser capture microdissection
LEP	Leptin
LINE-1	Long interspersed nuclear elements 1
LMIC	Low- and middle income countries
LMP	Last menstrual period
LOPE	Late-onset preeclampsia
LOXL3	Lysyl oxidase like 3
MEG3	Maternally expressed 3
MeD-seq	Methylated DNA sequencing
MEST	Mesoderm specific transcript
MMP9	Matrix metallopeptidase 9
MTHFR	Methylenetetrahydrofolate reductase
MTR	5-methyltetrahydrofolate-homocysteine methyltransferase
n	Number
NA	Not applicable
NDUFB6	NADH:ubiquinone oxidoreductase subunit B6
NIPT	Noninvasive prenatal testing
nmol	nanomol
NPDC1	Neural proliferation, differentiation and control 1
NR3C1	Nuclear receptor subfamily 3 group C member 1
NXPH4	Neurexophilin
OSR2	Odd-Skipped Related Transcription Factor 2
P16	or CDKN2Aink4a: cyclin-dependent kinase inhibitor 2A
PAPP-A	Pregnancy associated plasma protein A
PCMTD2	Protein-L-Isoaspartate (D-aspartate) O-Methyltransferase Domain Containing 2
PE	Preeclampsia
PEG10	Paternally expressed 10

PlGF	Placental growth factor
PIH	Pregnancy induced hypertension
PPARA	Peroxisome proliferator activated receptor alpha
PPARG1A	Peroxisome proliferator activated receptor gamma
PV	Placental volume
Q	Quartile
RASSF1	Ras association domain family member 1
RAD54L2	RAD54 like 2
RBC	Red blood cell
Ref	Reference
RNF126P1	Ring Finger Protein 126 pseudogene 1
ROC	Receiver operating characteristic
rpm	Reads per million
RXRA	Retinoid X receptor alpha
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SCT	Syncytiotrophoblast
SD	Standard deviation
SGA	Small for gestational age
SK	Syncytial knotting
SLC25A46	Solute carrier family 25 member 46
SNRPN	Small nuclear ribonucleoprotein polypeptide N
SP5	Sp5 Transcription Factor
SQLNS	Small quantity lipid-based micronutrient
SRA	Sequence Read Archive
TES	Transcription End Site
TMEM17	Transmembrane Protein 17
TMEM233	Transmembrane Protein 233
TMEM240	Transmembrane Protein 240
TOP	Termination of pregnancy
TSS	Transcription start site
uPVV	Utero-placental vascular volume
UtA-PI	Uterine artery pulsatility index
VEGF	Vascular endothelial growth factor
VOCAL	Virtual Organ Computer-aided AnaLysis
VR	Virtual Reality
ZC3H12D	Zinc finger CCCH-type containing 12D
ZNF175	Zinc finger protein 175
ZNF836	Zinc finger protein 836

List of publications

This thesis

The one-carbon metabolism as an underlying pathway for placental DNA methylation - a systematic review
van Vliet MM, Schoenmakers S, Gribnau J, Steegers-Theunissen RPM.
Epigenetics. 2024 Dec;19(1):2318516

First-trimester maternal folate and vitamin B12 concentrations and their associations with first-trimester placental growth: The Rotterdam Periconception Cohort
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Human Reproduction, 2025 May 15:deaf095

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Placenta. 2024 May;150:1-7

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Eur J Clin Invest. 2024 Nov 26:e14363

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Profiling (placental) DNA methylation in cell-free DNA across gestation: The Rotterdam Periconception Cohort
Van Vliet MM, Boers RG, Boers JB, Schäffers OJM, van der Meeren LE, Gribnau J[#], Schoenmakers S[#], Steegers-Theunissen RPM[#]. *# Equal contribution*
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Under review

Other

Epidemiological impact and cost-effectiveness of providing long-acting pre-exposure prophylaxis to injectable contraceptive users for HIV prevention in South Africa: a modelling study.
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Erythrocyte-bound apolipoprotein B in atherosclerosis and mortality.
de Vries MA, van Santen SS, Klop B, van der Meulen N, van Vliet M, van de Geijn GM, van der Zwan-van Beek EM, Birnie E, Liem AH, de Herder WW, Castro Cabezas M.
Eur J Clin Invest. 2017 Apr;47(4):289-296

PhD portfolio

PhD student	Marjolein M. van Vliet
Departments	Obstetrics and Gynecology – Periconception Epidemiology Developmental Biology
PhD period	April 2022 – April 2025
Promotors	Prof. dr. Régine P.M. Steegers-Theunissen Prof. dr. Joost Gribnau
Co-promotors	Dr. Sam Schoenmakers Dr. Ruben G. Boers

Training	**Year**	**ECTS**
Courses		
PhD introduction course (Graduate School)	2022	0.20
Scientific Integrity (Erasmus MC)	2022	0.30
Basic course on regulations and organization for clinical investigators (BROK) (NFU)	2022	1.50
Biostatistics I (NIHES)	2022	4.50
SNPs and Human diseases (NIHES)	2022	1.4
Basic human genetics (NIHES)	2022	0.6
Advances in clinical epidemiology (NIHES)	2023	0.7
Practice of epidemiological analysis (NIHES)	2023	0.7
Principles of genetic epidemiology (NIHES)	2023	0.7
UCSC Genome browser (NIHES)	2024	0.3
Introduction to analysis of population proteomics and metabolomics (NIHES)	2024	0.7
Introduction to the analysis of population epigenomics and transcriptomics (NIHES)	2024	0.7
The Public Health Approach: from plague to COVID-19 (NIHES)	2024	0.7
Primary and Secondary prevention research (NIHES)	2024	0.7
Seminars, workshops and masterclasses		
'Design and pitch a good poster' workshop (Erasmus MC)	2023	0.15
Attended conferences and research meetings		
Weekly research meeting Department of Obstetrics and Gynecology (Erasmus MC)	2022-2025	3.00
Weekly research meeting Department of Developmental Biology (Erasmus MC)	2022-2025	3.00

Annual PhD day 2022 (Erasmus MC)	2022-2023	0.20
Wladimiroff award meeting (Erasmus MC)	2023-2025	0.45
2nd conference on Pandemic Preparedness (Erasmus MC)	2024	0.30

Presentations at (inter)national conferences

Sophia Research Day 2023 (Rotterdam, the Netherlands): oral presentation	2023	1.00
Wladimiroff award meeting 2023 (Erasmus MC): oral presentation	2023	1.00
Sophia Research Day 2024 (Rotterdam, the Netherlands): oral presentation	2024	1.00
71th Annual scientific meeting of the SRI 2024 (Vancouver, Canada): three poster presentations	2024	1.00
Clinical & Health Sciences Day 2023 (Rotterdam, the Netherlands): poster presentation, elevator pitch	2023	1.00
Sophia Research Day 2025 (Rotterdam, the Netherlands): poster presentation	2025	1.00

Teaching

Supervising Britt Haug (medical student Erasmus MC): Master thesis	2023	2.00
Supervising medical students of the Minor 'The Mystery of Creation' (Erasmus MC)	2021	1.00

Other

Intervision for PhD students	2022-2024	1.0
Abstract Committee PhD Day 2024	2024	1.0
Board member Arts-Assistentenvereniging	2022-2024	NA

About the author



Maaike Marjolein van Vliet was born on the 9th of May 1995 in Zwijndrecht, the Netherlands. She grew up in Middelharnis and graduated from secondary school (VWO) at the RGO Middelharnis in 2013. In 2013, she started medical school at the Erasmus University Medical Center in Rotterdam, the Netherlands.

After obtaining her Bachelor's degree in 2016, she started the Research Master Infection & Immunity at the Erasmus University Medical Center Rotterdam, the Netherlands which she finished in 2018. In 2017, she started the master Health Economics, Policy & Law at the Erasmus University which she completed in 2019. She wrote her master theses about modeling the cost-effectiveness of long-acting pre-exposure prophylaxis combined with injectable contraceptives for HIV prevention in South Africa, and about trends in the unmet need for contraceptives in Zimbabwe. As of 2018, she started with her master degree in medicine and she graduated as a medical doctor in 2021.

During her studies, she worked as a teamlead in a supermarket for several years, in a student team of the department of Radiology and at the GGD Rotterdam Rijnmond during the COVID-19 pandemic. After graduation, she worked for a year as resident not in training at the department of Obstetrics and Gynecology in the Maasstad Hospital, Rotterdam, The Netherlands. In 2022, she started her PhD research in the Erasmus University Medical Centre in Rotterdam, at the department of Obstetrics and Gynecology and the department of Developmental Biology. She was supervised by her promotors Prof. dr. Régine P.M. Steegers-Theunissen and Prof. dr. Joost Gribnau and co-promotors Dr. Sam Schoenmakers and Dr. Ruben G. Boers. During her PhD research, she was a board member of the Arts-Assistentenvereniging of the Erasmus MC for two years. Since September 2025, she started working as a medical doctor in Sexual Health at the GGD Haaglanden. Here, she aims to combine clinical work with research and policy making, continuing to contribute to (improve) reproductive health.

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