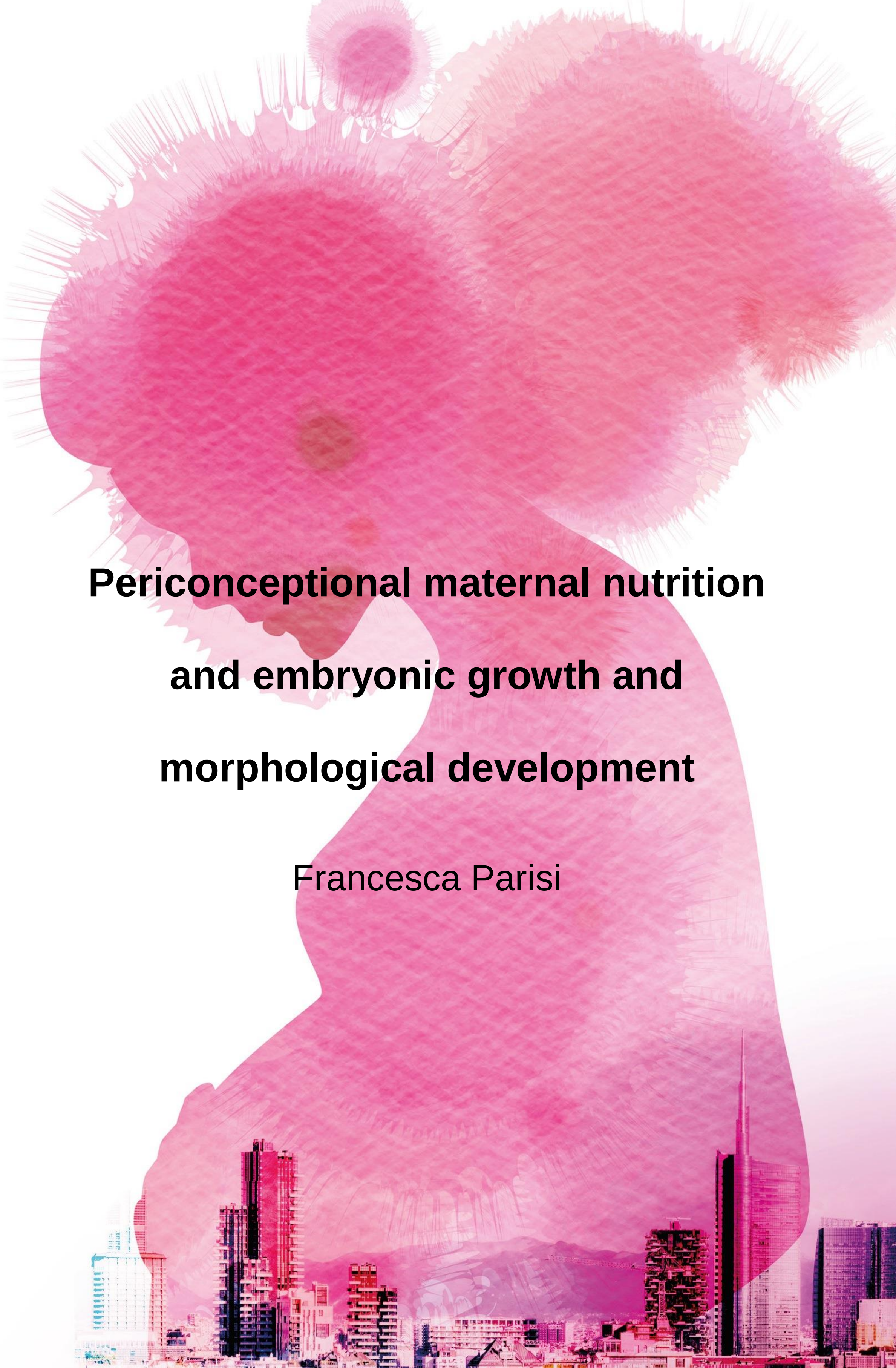

Periconceptional maternal nutrition and embryonic growth and morphological development

Francesca Parisi

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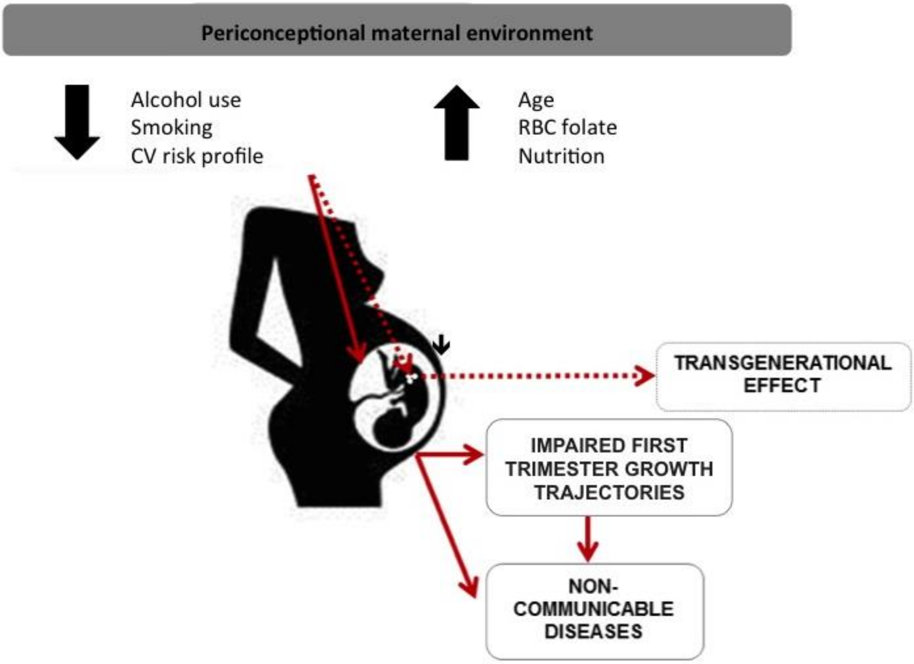
Chapter 1
Introduction

Over the past decades extensive research identified close associations between intrauterine fetal development, programming of postnatal phenotypes and the risk of non-communicable diseases in adult life (Developmental Origins of Health and Disease (DOHaD) hypothesis) (Hales et al., 2001; Gluckman et al., 2010). More recently, the research focus moved to the periconceptional period (time window: 14 weeks before up to and including 10 weeks after conception), covering the vulnerable early processes of gametogenesis, embryogenesis and placentation. During the periconceptional period, meticulous alignment of numerous molecular biological processes are involved, such as epigenetics, transcriptomics, and proteomics, which makes this a critical time window for exposures potentially resulting in a large shift in postnatal phenotype and homeostasis. Therefore, it is not surprising that significant associations have been detected between maternal periconceptional exposures, pregnancy outcome and chronic diseases in adult life (Steegers-Theunissen et al., 2013; Hart et al., 2013; Fleming et al., 2015). This emphasizes the importance of the implementation of periconceptional care in order to prevent and ameliorate pregnancy and future health outcomes in the offspring.

Maternal nutrition during pregnancy has been widely investigated in association with human fetal growth, birth weight and pregnancy outcome. Moving to the periconceptional period, animal studies demonstrated that both gamete maturation and preimplantation embryonic development are strongly influenced by parental nutrition, showing impaired reproductive competence, fertility, fetal and long-term health in case of periconceptional malnutrition (Watkins et al., 2008; Sinclair et al., 2013). The same model showed significant associations between adverse nutritional exposures during pregnancy, including restricted iodine, folate and protein intake, and impaired intrauterine cerebellar development, reporting altered growth of Purkinje cells, modified cerebellar methylation profiles and increased cerebellar lipoperoxidation in the offspring (Bonatto et al., 2006; Ranade et al., 2012; Yu et al., 2017). Furthermore, human studies showed improved embryo morphology scores after *in vitro* fertilization (IVF) in association with increased maternal fish and long chain polyunsaturated fatty acids intake (Hammiche et al., 2011). Finally, a curve linear association was shown between periconceptional maternal folate status and embryonic growth trajectories, whereas increased late first trimester crown-rump length (CRL) measurements were detected in mothers with strong adherence to an energy-rich dietary pattern (Bouwland-Both et al., 2013; van Uitert et al., 2014). The biological basis to support the plasticity of cells, tissues and organs seems to be mostly provided by the occurrence of epigenetic changes (e.g. DNA methylation) during early stages of development. In this perspective, epigenetics has gained interest as eventual interface between maternal dynamic environment, nutrition and fetal genome.

Maternal one-carbon (I-C) metabolism plays a crucial role in DNA methylation patterns in the offspring, possibly leading to permanent modifications of post-natal gene expression and phenotype and to modified outcomes of adult health and disease (Niculescu & Zeisel, 2002, Waterland et al., 2004; Steegers-Theunissen et al., 2013; Bouwland-Both et al., 2013; Bouwland-Both et al., 2015). Maternal I-C metabolism is influenced by common polymorphisms, including the methylene tetrahydrofolate-reductase (MTHFR) C677T polymorphism, as well as by environmental factors, like dietary folate intake, folic acid supplement use, lifestyle (i.e. smoking, coffee and alcohol consumption), and medication use (Steegers-Theunissen et al. 2013). Both genetic and environmental derangements in maternal I-C metabolism finally lead to alterations in intrauterine DNA methylation patterns, gene expression and transcriptomes, which could represent one of the causal links between maternal environment, reproductive failures and long-term health outcomes (Gluckman et al., 2005; Steegers-Theunissen et al., 2013). From this perspective, identifying the relevance of the periconceptional period and modifying parental exposures will present a unique opportunity for lifelong health effects in future generations (Uauy et al., 2011; Steegers et al., 2016) (Figure 1).

Figure 1. Periconceptional maternal characteristics and exposures and the impact on short-term embryonic, long-term and transgenerational health.



Periconceptional maternal characteristics and exposures, such as nutrition and lifestyle, have been associated with first trimester embryonic growth, with long-term implications for health and non-communicable diseases. In particular, maternal alcohol use, smoking habit and cardiovascular (CV) risk profile have been negatively associated with crown-rump length (CRL) trajectories (black arrows), whereas maternal age, adherence to an energy rich dietary pattern and an optimal red blood cell (RBC) folate status have been associated with increased embryonic growth. Early epigenetic programming of fetal gametes may additionally lead to a transgenerational effect of periconceptional maternal exposures.

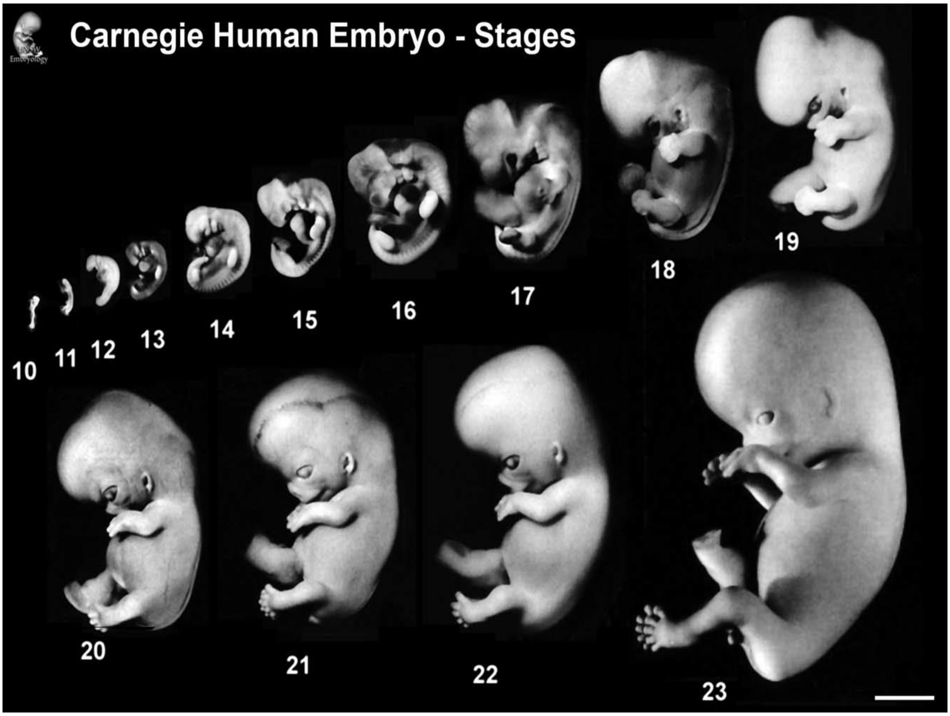
Another critical achievement of the last decade is that first trimester embryonic growth differs among women and pregnancies (van Uitert et al., 2013). So far, in routine clinical settings, the finding of a first trimester CRL measurement different from the expected according to the gestational age, commonly leads to the re-dating of that embryo based on the CRL measurement, instead of taking the last menstrual period (LMP) into consideration (Robinson, 1973). This assumes that embryonic growth must be the same in every woman and pregnancy and that only imprecise ovulation and implantation dates may eventually mislead the dating procedures. Nevertheless, as previously described, several maternal characteristics and exposures have been recently associated with longitudinal embryonic CRL measurements among very strictly dated pregnancies, reversing the idea of homogeneous first trimester embryonic growth (van Uitert et al., 2013; van Uitert et al., 2014; Wijnands et al., 2016). Furthermore, first trimester embryonic growth has been significantly associated with subsequent fetal growth parameters and pregnancy outcomes (Mook-Kanamori et al., 2010; van Uitert et al., 2013). This means that targeting first trimester embryonic growth can also prevent later risks during pregnancy. Moreover, the question about CRL accuracy in pregnancy dating should be targeted in clinical research and care.

The improvement in high-resolution three-dimensional ultrasound (3D US) techniques has tremendously increased our possibility to investigate the embryonic period, providing highly accurate length and volume measurements. This technology further gives a new critical role to the embryonic morphological assessment in order to improve pregnancy outcome through early screening and diagnosis (Karim et al., 2016). Reference curves of small embryonic structures, including the cerebellum, are now available as early as the first trimester of pregnancy (Koning et al., 2015). Moreover, the development of immersive virtual reality (VR) systems in combination with the V-scope volume rendering application provides an accurate visualization of embryonic structures through full depth perception and intuitive interaction with volumes (Koning et al., 2009; Rousian et al., 2011) (Figure 2).

Figure 2. Barco I-Space hologram of the embryo for embryonic volume measurement.

In particular, this system allows reliable, reproducible and automatic embryonic and fetal size measurements, provides reference charts for first trimester CRL and embryonic volume (EV) and enables *in vivo* embryonic staging according to the century old Carnegie classification system (O'Rahilly et al., 1987; O'Rahilly, 2010; Verwoerd-Dikkeboom et al., 2008; Rousian et al., 2013; Rousian et al., 2018). The Carnegie classification divides the embryonic period (58 post-conceptional days, 10^{+2} weeks of gestation) into 23 stages, strictly defined by external and internal morphological landmarks of development (Figure 3). Despite embryonic morphological development is a well-defined process, constantly occurring through all stages of development for every embryo and pregnancy, different times and velocities can occur, making comparisons worthwhile.

Figure 3. Carnegie stages of human embryonic development from stages 10 to 23.



Embryos are shown in a left lateral view and scaled for size comparison (scale bar 5 mm). Courtesy of M. Hill ("John Wiley and Sons" license number: 4384830666056).

Hypothesis

The maternal environment strongly impacts fetal development and pregnancy outcome, further modeling the future health of the offspring. I hypothesize that periconceptional maternal dietary patterns and I-C metabolism affect early stages of development, leading to remarkable changes in embryonic and cerebellar growth and to significant shifts in first trimester morphological development, with further effects on fetal outcome.

Aims of the thesis

In this thesis we aim to study human embryonic growth and morphological development in association with periconceptional maternal dietary patterns and I-C metabolism. The main research questions of this thesis are as follows:

- Are periconceptional maternal dietary patterns and blood biomarkers of I-C metabolism associated with first trimester embryonic growth?
- Are periconceptional maternal dietary patterns associated with prenatal cerebellar growth?
- Are periconceptional maternal dietary patterns and blood biomarkers of I-C metabolism associated with embryonic morphological development according to the Carnegie stages?
- Is embryonic morphological development according to the Carnegie stages associated with subsequent fetal growth as the main feature of pregnancy outcome?

Methods

The studies described in this thesis were performed in the Rotterdam Periconception Cohort (Predict study), a prospective periconceptional tertiary hospital-based birth cohort study conducted at the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Centre in Rotterdam, The Netherlands.
This ongoing cohort study started in 2009 aiming to investigate periconceptional determinants of first trimester and pregnancy outcome and the biological mechanisms associated with offspring health during the life course (Steegers-Theunissen et al., 2016). The protocol was approved by the local medical ethics committee and all women signed a written informed consent form before participation (METC Erasmus MC 2004-277).
All women of at least 18 years of age with an ongoing non-malformed early pregnancy (<8 weeks of gestation) were eligible for participation. Only spontaneous and intrauterine insemination (IUI) pregnancies with a

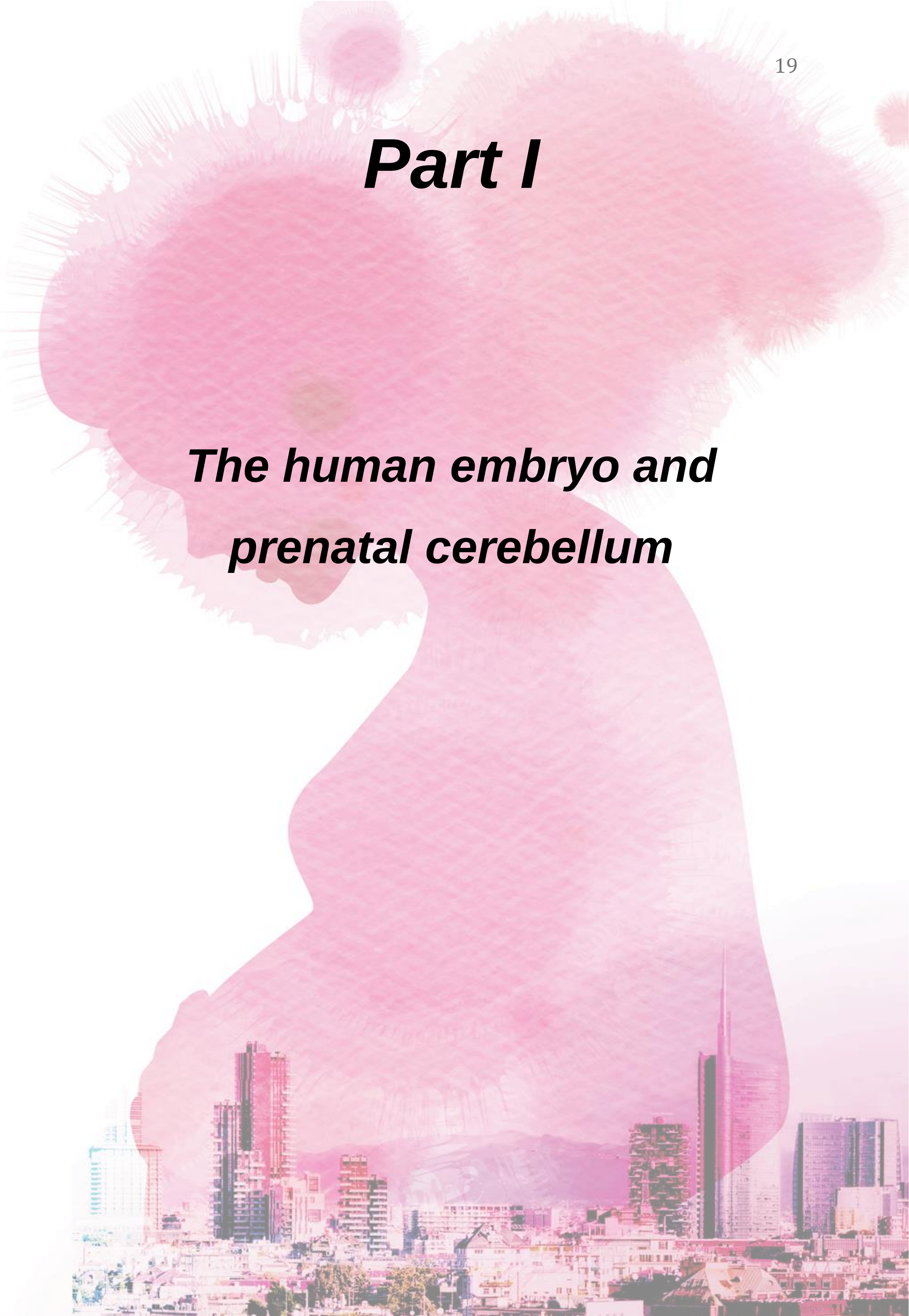
known first day of last menstrual period (LMP), a self-reported regular cycle and a CRL measurement observed <7 days different from the expected value according to the Robinson curve were included in the analysis (strictly dated spontaneous pregnancy subgroup). Pregnancies achieved after *in vitro* fertilization (IVF), intracytoplasmatic sperm injection (ICSI), or cryopreserved embryo transfer using homologous oocytes were also eligible (IVF/ICSI pregnancy subgroup). As subfertility and IVF/ICSI technique may possibly influence embryonic responses to maternal exposures through independent effects of culture media or epigenetic reprogramming, adjustment for conception mode and subgroup analysis were performed (Dumoulin et al., 2010; Nelissen et al., 2013; Wale et al., 2016; Hoeijmakers et al., 2016).
The dating procedure calculates gestational age from LMP for spontaneous pregnancies (with adjustment for cycle duration if <25 or >31 days), from LMP or insemination date plus 14 days for IUI pregnancies, from the day of oocyte retrieval plus 14 days for IVF/ICSI pregnancies and from embryo transfer day plus 17 or 18 days in pregnancies derived from transfer of cryopreserved embryos. In this way, the resulting study population included pregnancies with strict and reliable dating by definition

Outline

In Part I, I focus on human embryonic and prenatal cerebellar growth trajectories. *In vivo* longitudinal CRL and EV measurements were performed in a VR system in order to provide detailed first trimester embryonic growth trajectories. The second chapter investigates associations between periconceptional maternal dietary patterns and embryonic growth trajectories, whereas the third chapter investigates early first trimester maternal blood biomarkers of I-C metabolism in association with longitudinal CRL and EV measurements. Chapter 4 addresses the associations between periconceptional maternal dietary patterns and prenatal cerebellar growth trajectories assessed by repeated ultrasound scans from the first trimester of pregnancy onwards.

Part II evaluates the associations between periconceptional maternal dietary patterns (chapter 5), I-C biomarkers (chapter 6) and embryonic morphological development according to serial annotations of the Carnegie stages in a VR system. Finally, chapter 7 investigates the associations between the Carnegie stages of embryonic morphological development and subsequent fetal growth depicted by mid-pregnancy estimated fetal weight (EFW) and birth weight.

Part I



The human embryo and prenatal cerebellum

Chapter 2

Periconceptional maternal ‘high fish and olive oil, low meat’ dietary pattern is associated with increased embryonic growth: The Rotterdam Periconceptional Cohort (Predict Study)

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Ultrasound Obstet Gynecol. 2017;50(6):709-716

Abstract

Objective To investigate associations between periconceptional maternal dietary patterns and first trimester embryonic growth.

Methods 228 women with singleton ongoing pregnancies were enrolled in a prospective periconceptional cohort study, comprising of 135 strictly dated spontaneous pregnancies and 93 pregnancies achieved after *in vitro* fertilization or intracytoplasmatic sperm injection (IVF/ICSI pregnancies). All women underwent longitudinal transvaginal three-dimensional ultrasound (3D-US) scans from 6^{+0} up to 13^{+0} weeks of gestation. Crown-rump length (CRL) and embryonic volume (EV) measurements were performed using a virtual reality system. Periconceptional maternal dietary intakes were collected via food frequency questionnaires (FFQ). Principal component analysis was performed to identify dietary patterns. Associations between dietary patterns and CRL and EV trajectories were investigated using linear mixed models adjusted for potential confounders.

Results A median of five (range 1-7) 3D-US scans per pregnancy were performed. 991 out of 1162 datasets (85.3%) were of sufficient quality to perform CRL measurements and 899 for EV measurements (77.4%). A 'high fish and olive oil, low meat' dietary pattern comprising of high intakes of fish and olive oil, and very low intake of meat was identified. In strictly dated spontaneous pregnancies, a strong adherence to this dietary pattern was associated with a 1.9 mm (95% CI: 0.1, 3.63) increased CRL at 7 weeks (+14.6%) and 3.4 mm (95% CI: 0.2, 7.81) at 11 weeks (+6.9%), whereas EV increased by 0.06 cm^3 (95% CI: 0.01, 0.13) at 7 weeks (+20.4%) and 1.43 cm^3 (95% CI: 0.99, 1.87) at 11 weeks (+14.4%) respectively. No significant associations were observed in the total study population and IVF/ICSI pregnancies.

Conclusions Periconceptional maternal adherence to a 'high fish and olive oil, low meat' dietary pattern is positively associated with embryonic growth in strictly dated spontaneous pregnancies.

Introduction

Maternal nutrition is the main determinant of fetal nutrition known to influence pregnancy outcome as well as future health of the offspring (Barker, 2007; Gluckman et al., 2008). Nevertheless data are scarce about the influence of periconceptional maternal nutrition on embryonic growth (Robinson, 1973; Kramer, 1987; Gresham et al., 2014; Grieger et al., 2014; Timmermans et al., 2009). This is related to the fact that in clinical practice the embryonic period is often missed and to the widespread assumption that embryonic growth is the same in every pregnancy and woman (M'hamdi et al., 2016). However, in the last decade new insights reveal that first trimester embryonic growth differs and is significantly associated with periconceptional maternal characteristics, nutrition and lifestyle, including age, ethnicity, smoking and alcohol consumption (Steegers-Theunissen et al., 2015; van Uitert et al., 2013; Bottomley et al., 2009; Mook-Kanamori et al., 2010). In addition, a curvilinear association was shown between periconceptional maternal folate status and embryonic growth, while strong adherence to an energy-rich dietary pattern significantly increased late first trimester crown-rump length (CRL) measurements in the Generation R study (van Uitert et al., 2014; Bouwland-Both et al., 2013). These data emphasize the need for the development of customized embryonic growth curves. Since most reproductive failures and adverse pregnancy outcomes originate in the periconceptional period (time window: 14 weeks before up to 10 weeks after conception), customized growth curves may serve as early predictors of adverse pregnancy outcome in the future (Macklon et al., 2002; Steegers-Theunissen et al., 2013).

Over the last decades, safe, highly precise and reliable measurements of early embryonic structures have been performed using transvaginal three-dimensional ultrasound (3D-US) with high frequency probes and offline visualization in a virtual reality (VR) system (Verwoerd-Dikkeboom et al., 2008). Furthermore, the introduction of the Barco I-Space VR system and the V-Scope software allows automatic and precise embryonic volume (EV) measurements with high intra-observer and inter-observer agreement, thereby providing EV reference charts (Rousian et al., 2013). Nowadays principal component analysis (PCA) is a standard statistical analysis which derives dietary patterns from food frequency questionnaires (FFQs) by data-driven dimension reduction

techniques, further validated with biomarker concentrations and associated with complex diseases (Hu et al., 1999).
The aim of this study is to investigate associations between periconceptional maternal dietary patterns and first trimester embryonic growth, using longitudinal CRL and EV measurements as outcome.

Subjects and methods

This study was embedded in the ongoing Rotterdam Periconceptional Cohort (Predict Study), a prospective hospital-based birth cohort study, conducted at the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands (Steegers-Theunissen et al., 2016). The protocol was approved by the Medical Ethical and Institutional Review Board at the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands, and all participants signed a written informed consent (METC Erasmus MC 2004-277).
Pregnant women of at least 18 years of age were eligible for participation and were recruited before 8^{+0} weeks of gestation between November 2010 and July 2014 (Figure 1). From a total of 400 pregnancies, we excluded: 48 pregnancies complicated by twinning, miscarriage, ectopic implantation, congenital anomalies and intrauterine fetal death; 5 pregnancies conceived after oocyte donation; 26 pregnancies with missing (n=11) or unreliable (n=15) nutritional data. The remaining 321 patients included 93 IVF/ICSI pregnancies derived from *in vitro* fertilization (IVF), intracytoplasmatic sperm injection (ICSI) and cryo-embryo transfer. Among the 228 spontaneously and intrauterine insemination (IUI) conceived pregnancies, we selected women with strict pregnancy dating defined by a known first day of the last menstrual period (LMP), regular cycle and CRL observed < 7 days different from expected according to the Robinson curve (strictly dated spontaneous pregnancies, n=135) (Robinson, 1973). The gestational age was calculated from LMP for strictly dated spontaneous pregnancies (with adjustment for cycle duration if <25 or >31 days), from LMP or insemination date plus 14 days for IUI pregnancies, from the day of oocyte retrieval plus 14 days for the IVF/ICSI pregnancies and from

embryo transfer day plus 17 or 18 days in pregnancies derived from transfer of cryopreserved embryos. In this way, the total study population included pregnancies with reliable and strict dating by definition. Since an effect of conception mode on embryonic growth and responses to nutritional exposures cannot be excluded, we performed the analysis in the total study population and after stratification in the two subgroups of strictly dated spontaneous and IVF/ICSI pregnancies.

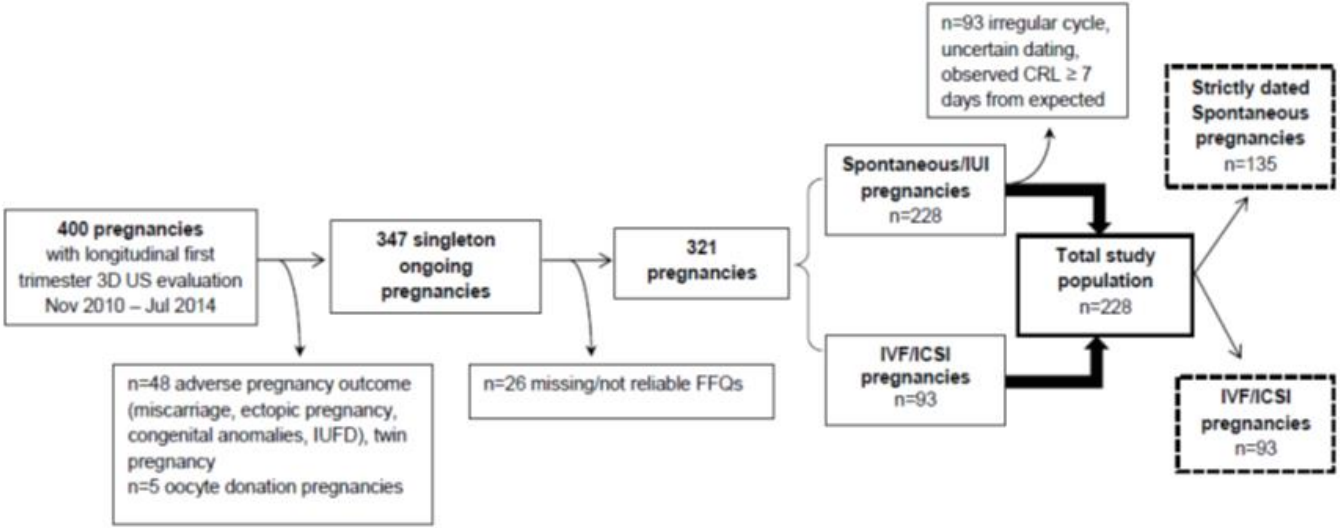
All women received longitudinal transvaginal 3D-US scans from 6^{+0} weeks up to 13^{+0} weeks of gestation with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D View software (General Electrics Medical Systems, Zipf, Austria). Since the pilot study showed an accurate modeling of embryonic growth curves with three scans per patients, 3D-US scans were generally performed every 7 days between 2010 and December 2012, and reduced to 3 scans per patient (at 7, 9, 11 weeks of gestation) after January 2013 (van Uitert et al., 2013). The obtained 3D-US datasets were transformed to Cartesian (rectangular) volumes and transferred to the BARCO I-Space (Barco N.V., Kortrijk, Belgium) at the Department of Bioinformatics, Erasmus MC, University Medical Centre, Rotterdam, in order to perform offline CRL and EV measurements using the V-Scope software. A length-measuring tool was used to perform length measurements in three dimensions (CRL). A semi-automated volume measuring application based on gray-scale differences was used to perform EV measurements. CRL and EV measurements and reliability have been extensively described elsewhere and excellent inter- and intra-observer agreement has been previously reported (Rousian et al., 2013; Rousian et al., 2010). CRL measurements were performed three times by a trained researcher and the average was used in the analysis. EV measurements were performed once in the same image selected for the CRL measurement.

At enrollment all participants filled out a general questionnaire providing details on age, ethnicity, educational level, obstetric and medical history and periconceptional lifestyle (smoking, alcohol use, folic acid or multivitamin supplements use). Anthropometric measurements were obtained by trained counselors (height, weight).

The validated semi-quantitative food frequency questionnaire (FFQ), developed by the division of Human Nutrition, Wageningen University, the Netherlands, and validated for women of reproductive age was used at enrollment to estimate habitual food intake over the previous four weeks (Siebelink et al., 2011; Verkleij-Hagoort et al., 2007). The FFQ consists of 196 food items structured according to meal patterns, including questions on consumption frequency, portion size, and preparation method. Energy and nutritional intakes were determined using the Dutch food composition table (Netherlands Nutrition Centre, NEVO-tabel 2011). The FFQs were checked in a standardized manner for completeness and consistency. First trimester fasting venous blood samples were collected at enrollment for serum folate and vitamin B12 and plasma total homocysteine (tHcy) assessment. The laboratory procedures have been extensively described elsewhere (van Uitert et al., 2014).

Data on birth outcomes were obtained from medical records (date of birth, gender, birth weight, congenital anomalies). Gestational age at birth was calculated from the dating procedure used in the first trimester as described above.

Figure 1. Flow chart of the study population.



IUFD: intrauterine fetal death, FFQ: food frequency questionnaire, IUI: intrauterine insemination; IVF: *in vitro* fertilization, ICSI: intracytoplasmatic sperm injection; CRL: crown-rump length.

Statistical analysis

Maternal characteristics were compared between included and excluded pregnancies using Chi-square or exact tests for ordinal variables and Mann-Whitney U test for continuous variables.

PCA was applied to identify dietary patterns in women with reliable FFQs as extensively described by Hoffmann and performed in several studies (Bouwland-Both et al., 2013; Vujkovic et al., 2007; Hoffmann et al., 2004). We reduced 196 food items from the FFQs to 11 predefined food groups based on origin and similar nutrient content (cereals, olive oil, solid fat, fish, fruit, grain, vegetables, meat, snacks, sugars, alcohol). Since maternal alcohol consumption has been associated with embryonic growth in our previous study, we excluded alcohol from dietary pattern extraction and consider its use as a confounder for further adjustment (van Uitert et al., 2013). Practically, PCA is a standard multivariate statistical technique that aggregates specific food groups on the basis of the degree to which food items are reciprocally correlated. Only dietary patterns (principal components) with eigenvalues ≥1.1 were extracted, in order to reduce bias of multiple testing and to identify the most common dietary patterns in the study population. When PCA is performed, a factor loading is automatically calculated for each food group, showing the extent to which each food group is correlated with the specific dietary pattern. We used three factor loadings with the highest absolute value to label the dietary patterns. Finally, all women automatically receive a factor score for every dietary pattern representing their adherence to that specific dietary pattern. Kruskal-Wallis test was used to compare the adherence to each dietary pattern between included and excluded women. Since the FFQ was validated for the assessment of folate and vitamin B12 intake, maternal biomarkers of one-carbon metabolism, including folate, vitamin B12, and tHcy, were compared between women with strong adherence (positive factor scores) *versus* weak adherence (negative factor scores) to each dietary pattern using Mann-Whitney *U*-tests (Verkleij-Hagoort et al., 2007). Lastly, the food intake level (FIL) was calculated as the ratio of energy intake divided by basal metabolic rate (BMR) and compared with a physical activity level (PAL) of a sedentary lifestyle in order to evaluate underreporting of food intake (new Oxford equations stratified by age) (Ramirez-Zea, 2005).

Linear mixed models, which allow the modelling of longitudinal measurements accounting for the dependent observations within the same pregnancy, were firstly estimated to evaluate associations between conception mode and embryonic growth in the total study population. Square root transformation of CRL data and third root transformation of EV data were performed to obtain a normal distribution of observations as required by linear mixed models and resulted in linearity with gestational age and a constant variance independent from gestational age. Linear mixed models were secondly estimated to evaluate associations between dietary patterns, food groups and embryonic growth in both total study population and strictly dated spontaneous and IVF/ICSI pregnancy subgroups. We performed a crude model using gestational age as predictor and with adjustment for energy intake. In the fully adjusted model, we additionally entered all potential confounders (parity, alcohol use, smoking, folic acid/multivitamin supplement use, maternal age, BMI and comorbidity, fetal gender). Maternal chronic comorbidities considered for adjustment were cardiovascular, autoimmune, metabolic and endocrine diseases.
P-values ≤0.05 were considered significant. All analyses were performed using SPSS Statistics for Windows, Version 21.0 (IBM Corp. Armonk, NY) and R version 3.2.1 (The R Foundation for Statistical Computing).

Results

A total of 228 singleton ongoing pregnancies were included for the analysis, comprising of 135 strictly dated spontaneous pregnancies and 93 IVF/ICSI pregnancies. The median gestational age at recruitment was 7^{+1} weeks and the median number of 3D-US scans per pregnancy was five (range 1-7). From a total of 1162 datasets, 991 were of sufficient quality to perform CRL measurements (85.3%) and 899 to perform EV measurements (77.4%). Baseline characteristics and pregnancy outcomes are listed in Table 1 with comparisons between included and excluded pregnancies.
By using PCA we obtained three uncorrelated dietary patterns explaining 46.8% of the variance of the overall dietary intake of the total study population (Table 2). The first component was labelled ‘high

vegetables, fruits and grain dietary pattern' (18.5% explained variance). The second component was labelled 'high solid fat, snacks and sugars dietary pattern', also associated with a low intake of fruit (17.4% of the variance). The third component was labelled 'high fish and olive oil, low meat dietary pattern' (10.9% explained variance). No differences in the adherence to the three dietary patterns (factor scores) were observed between included and excluded pregnancies, as well as between strictly dated spontaneous and IVF/ICSI pregnancy subgroups. Women with strong adherence to the 'high vegetables, fruits and grain' dietary pattern (defined by factor scores >0) showed significantly higher vitamin B12 concentrations (median values: 331 pmol/l (range 95-713 pmol/l) *versus* 279.5 pmol/l (range 109-953 pmol/l), p=0.01), as well as lower concentrations of tHcy (median values: 6.0 µmol/l (range 4.0-18.0 µmol/l) *versus* 6.6 µmol/l (range 3.0-14.0 µmol/l), p<0.01) compared to women with weak adherence to the same dietary pattern (factor scores <0). In contrast, women with strong adherence to the 'high solid fat, snacks and sugars' dietary pattern showed a lower serum folate compared to women with weak adherence to the same dietary pattern (median values: 38.4 nmol/l (range 11-187 nmol/l) *versus* 37.0 nmol/l (range 12-118 nmol/l), p=0.05). No significant differences in the investigated maternal biomarkers were detected according to the adherence to the 'high fish and olive oil, low meat' dietary pattern.

Linear mixed model analysis showed no significant differences in longitudinal CRL and EV measurements between strictly dated spontaneous and IVF/ICSI pregnancies (fully adjusted model; group effect on CRL analysis: β=0.05 $\sqrt{mm}$ (95% CI: -0.03, 0.12), p=0.27; EV analysis: β=0.02 $\sqrt[3]{cm^3}$ (95% CI: -0.02, 0.06), p=0.29). Table 3 shows the results from linear mixed models. No significant associations were observed between maternal dietary patterns and longitudinal CRL measurements in the total study population and IVF/ICSI pregnancy subgroup. The analysis showed a significant positive association between the 'high fish and olive oil, low meat' dietary pattern and longitudinal CRL measurements in the strictly dated spontaneous pregnancy subgroup, for both crude and fully adjusted models. The transformation to the original scale showed that strong adherence to the 'high fish and olive oil, low meat' dietary pattern (defined as +2 standard deviations (SD) in factor score) increased CRL by 1.9 mm (95% CI: 0.1, 3.63) at 7 weeks (+14.6%) and 3.4 mm (95% CI: 0.2, 7.81) (+6.9%) at

11 weeks compared to weak adherence (-2 SD in factor score). The analysis on EV confirmed no significant results in the total study population and IVF/ICSI pregnancies, while only the ‘high fish and olive oil, low meat’ dietary pattern was significantly associated with increased longitudinal EV measurements in strictly dated spontaneous pregnancies, in both crude and fully adjusted models. The transformation to the original scale showed that strong adherence (+2 SD in factor score) to this dietary pattern increased EV by 0.06 cm^3 (95% CI: 0.01, 0.13) at 7 weeks (+20.4%) and by 1.43 cm^3 (95% CI: 0.99, 1.87) at 11 weeks (+14.4%) compared to weak adherence (-2 SD in factor score).

Figure 2 shows the average regression lines from the fully adjusted model for the ‘high fish and olive oil, low meat’ dietary pattern in strictly dated spontaneous pregnancies.
Finally, linear mixed models showed no associations between the single food groups highly associated with the ‘high fish and olive oil, low meat’ dietary pattern and longitudinal CRL and EV measurements in the total study population and two subgroups.

Table 1. Characteristics of the study subgroups and excluded pregnancies.

	Total study population (n=228)		**Excluded pregnancies (n=124)**		
MATERNAL CHARACTERISTICS		**M**		**M**	**p**
Age, y median (range)	32 (22-44)	0	30 (21-41) *	0	**0.01**
Geographical origin		1		5	0.05
Western, n(%)	205 (89.9)		105 (84.7)		
Non Western, n(%)	22 (9.6)		14 (11.3)		
Educational level		1		5	0.06
High, n(%)	134 (58.8)		66 (53.2)		
Intermediate, n(%)	89 (39.0)		49 (39.5)		
Low, n(%)	4 (1.8)		4 (3.2)		
BMI, kg/m^2 median (range)	24.2 (17.0-42.6)	1	25.8 * (17.8-45.0)	2	**0.02**
Nulliparous, n(%)	74 (32.5)	0	39 (32.5)	4	0.99
Alcohol use, n(%)	84 (37.0)	1	37 (31.9)	8	0.35
Periconceptional smoking, n(%)	33 (14.5)	1	20 (17.2)	8	0.51
Periconceptional folic acid/multivitamin use, n(%)	219 (97.3)	3	113 (94.2)	4	0.43
Chronic diseases, n(%)	26 (11.4)	0	21 (16.9)	0	0.15

Chronic diseases include cardiovascular, autoimmune, endocrinal and metabolic diseases. The comparison among groups was performed using Chi-square or exact tests for ordinal variables and Mann-Whitney U test for continuous variables. M: missing values, BMI: body mass index.

Table 2. Relation between food groups and the identified dietary patterns expressed by factor loadings.

	High vegetables, fruits and grain dietary pattern	High solid fat, snacks and sugars dietary pattern	High fish and olive oil, low meat dietary pattern
Variance explained (%)	18.5	17.4	10.9
Cereals	0.141	-0.269	0.038
Olive Oil	0.231	0.205	0.469*
Solid fat	0.277	0.614*	-0.262
Fish	0.461*	-0.034	0.650*
Fruit	0.580*	-0.365*	-0.007
Grain	0.579*	0.379*	0.018
Vegetables	0.775*	-0.068	0.105
Meat	0.206	0.189	-0.750*
Snacks	-0.075	0.699*	0.029
Sugars	-0.079	0.620*	0.041

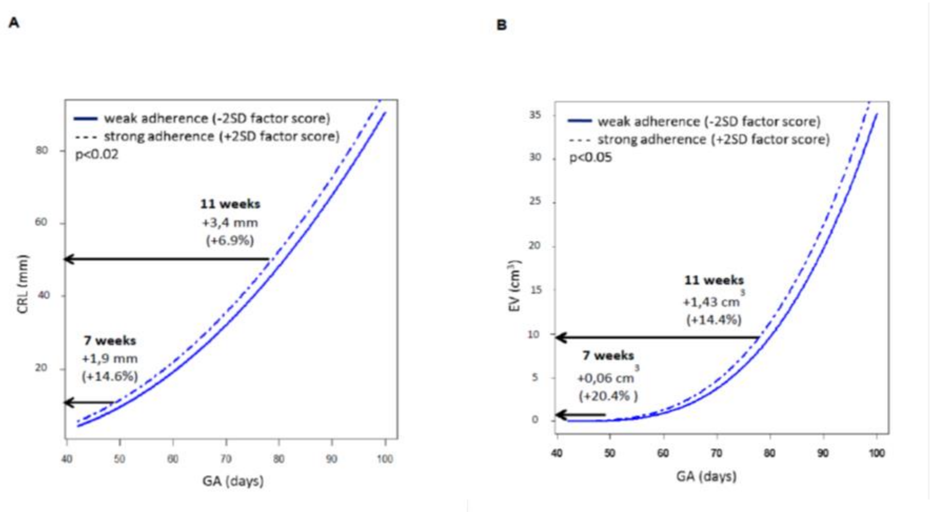
The factor loadings describe the food group contribution to each dietary pattern. The factor loadings with the highest absolute value are presented with an asterisk and were used for labelling (*).

Table 3. Effect estimates from the linear mixed model analysis for associations between maternal dietary patterns and embryonic crown-rump-length (CRL) and volume (EV) in the total study population and in strictly dated spontaneous and IVF/ICSI pregnancies.

DIETARY PATTERN	Effect estimates CRL β (95%CI), √mm		
	TOTAL STUDY POPULATION	STRICTLY DATED SPONTANEOUS PREGNANCIES	IVF/ICSI PREGNANCIES
High vegetables, fruits and grain			
Crude	0.04 (-0.00, 0.08)	0.05 (-0.02, 0.12)	0.02 (-0.02, 0.06)
Fully adjusted	0.04 (-0.01, 0.09)	0.04 (-0.04, 0.12)	0.03 (-0.02, 0.08)
High solid fat, snacks and sugars			
Crude	-0.02 (-0.07, 0.04)	-0.03 (-0.10, 0.05)	-0.00 (-0.06, 0.06)
Fully adjusted	-0.02 (-0.08, 0.04)	-0.03 (-0.11, 0.05)	-0.01 (-0.06, 0.05)
High fish and olive oil, low meat			
Crude	0.03 (-0.01, 0.06)	0.07 (0.01, 0.12) **	-0.03(-0.07, 0.01)
Fully adjusted	0.03 (-0.01, 0.07)	0.07 (0.01, 0.13) **	-0.02(-0.06, 0.02)
	Effect estimates EV β (95%CI), $\sqrt[3]{cm^3}$		
High vegetables, fruits and grain			
Crude	0.01 (-0.01, 0.03)	0.01 (-0.03, 0.05)	0.01 (-0.01, 0.03)
Fully adjusted	0.01 (-0.01, 0.03)	0.01 (-0.03, 0.05)	0.01 (-0.01, 0.03)
High solid fat, snacks, sugars			
Crude	-0.01 (-0.03, 0.01)	-0.02 (-0.06, 0.02)	-0.01 (-0.04, 0.03)
Fully adjusted	-0.02 (-0.04, 0.01)	-0.02 (-0.06, 0.02)	-0.01 (-0.04, 0.02)
High fish and olive oil, low meat			
Crude	0.01 (-0.01, 0.03)	0.03 (0.01, 0.05) *	-0.02 (-0.04, 0.00)
Fully adjusted	0.01 (-0.01, 0.03)	0.03 (0.01, 0.05) *	-0.02 (-0.04, 0.00)

Effect estimates represent the amount of change in square root CRL (√mm) and third root EV ($\sqrt[3]{cm^3}$) per unit of increase of factor score. Crude analysis is adjusted for gestational age and energy intake. Multivariable analysis is adjusted for all potential confounders (parity, alcohol use, smoking habit, folic acid/multivitamin supplement use, maternal age, BMI and comorbidity, fetal gender). *p<0.05**;** ** p<0.02. CI: confidence interval.

Figure 2. Fully adjusted linear mixed models for crown-rump length (CRL, n=614 measurements) (A) and embryonic volume (EV, n=554 measurements) (B) in relation to periconceptional maternal adherence to the ‘high fish and olive oil, low meat’ dietary pattern in the strictly dated spontaneous pregnancy subgroup.



Maternal adherence to the ‘high fish and olive oil, low meat’ dietary pattern is expressed as +2 standard deviations (SD) (dashed line, strong adherence) and -2SD in factor scores (continuous line, weak adherence). Gestational age (GA) is expressed in days. Full adjustment for parity, alcohol use, smoking, folic acid/multivitamin supplement use, maternal age, BMI, comorbidity and fetal gender was performed.

Discussion

We showed significant associations between periconceptional maternal adherence to a ‘high fish and olive oil, low meat’ dietary pattern and increased embryonic growth, depicted by longitudinal CRL and EV measurements, among strictly dated spontaneous pregnancies. Mean CRL measurements were in line with the Robinson curves, while EV measurements were comparable with previous results (Robinson, 1973; Ioannou et al., 2011). Our results point out a greater effect of dietary patterns on EV compared to CRL and in the early compared to the late first trimester embryo. Previous research showed that maternal adherence to an energy rich dietary pattern, resembling our ‘high solid fat, snacks and sugars’ dietary pattern, increased first trimester CRL (Bouwland-Both et al., 2013). Despite a larger sample size, only a single CRL measurement in a routine clinical setting at 12 gestational weeks was performed.
Fish intake, as omega-3 fatty acid rich food group, has been previously related to improved embryo morphology scores in the IVF population, as well as to higher birth weight, but results are controversial (Hammiche et al., 2011; Drouillet et al., 2009; Heppe et al., 2011; Leventakou et al., 2014; Cetin et al., 2009). Controversies are probably due to the beneficial effect of omega-3 fatty acids on cell membrane synthesis, gene expression, and eicosanoid metabolism and the simultaneous adverse effect of contaminants, both present in seafood (Leventakou et al., 2014; Poudyal et al., 2011; Papadopoulou et al., 2013). Our results largely substantiate these findings. Fish intake as a single exposure was not associated with embryonic growth. The effect of a single nutrient is in most cases too small to detect. Moreover, the (un)known interactions and cumulative effects of multiple nutrients included in a dietary pattern are much stronger and therefore can explain our results. The extracted dietary pattern was also related to a very low intake of meat. Recent evidences showed that high processed meat intake is negatively associated with fertilization, implantation and pregnancy rates among couples undergoing conventional IVF (Xia et al., 2015; Braga et al., 2015). Moreover, a dietary pattern high in red meat intake was negatively associated with second and third trimester fetal growth parameters (Knudsen et al., 2008). Recent animal data also showed that maternal olive oil increased piglet birth weight, while reducing plasma IL-

1β and TNF-α levels in the offspring (Shen et al., 2015). We suggest that the lower intake of saturated fats and the increased omega-3/omega-6 fatty acid ratio, both expected in case of strong adherence to the 'high fish and olive oil, low meat' dietary pattern, could impact embryonic growth possibly by modulating inflammation and oxidative stress pathways (Williams et al., 2006; Meher et al., 2016). Our findings suggest that the focus of caregivers should be on the recommendation of a healthy dietary pattern instead of single healthy food intake to (pre)pregnant women (Northstone et al., 2008).

We found no significant associations between dietary patterns and embryonic growth in IVF/ICSI pregnancies, which may also explain the non-significant associations in the total study population. We suggest that the IVF/ICSI technique has a stronger effect on embryonic growth than periconceptional maternal dietary patterns, possibly influencing embryonic responses to maternal exposures. Of interest is to address that recent studies demonstrated an independent effect of culture media on birth weight, showing associations with fetal growth starting as early as the second trimester of pregnancy (Dumoulin et al., 2010; Nelissen et al., 2013). Moreover, when IVF/ICSI is performed, the embryo is not exposed to the natural maternal nutritional environment during the first 3-5 days of development, an essential period when epigenetic reprogramming takes place (Wale et al., 2016). This could explain the missing association with dietary patterns. Another explanation, inherent to observational studies and despite the adjustment for many covariates, is that residual confounding cannot be excluded.

The main strength of our study is the longitudinal evaluation of embryonic growth with a median of five scans per patient, the use of a VR system and three independent CRL measurements per time point, providing an accurate picture of first trimester growth process. Finally we performed the automatic EV measurement on the same datasets with high success rates. A retrospective study recently showed that EV represents a more effective measurement of first trimester growth restriction in aneuploidy fetuses compared to CRL, since all three dimensions are taken into account instead of one dimension (Baken et al., 2013). We minimized confounding of gestational age by including women with strict pregnancy dating only, based on a known LMP, regular cycle and concordant CRL. This means that all ultrasound measurements could be read as response variables and outcome measurements. In order to reduce selection bias, we compared baseline characteristics of included and excluded women and further adjusted the analysis for multiple maternal covariates, including BMI and age. In the present study, a PCA was used with the advantage to take into account the correlation of food groups without a hypothesis-oriented approach (Hoffmann et al., 2004). Moreover, the assessment of biomarkers of one carbon metabolism validates the dietary patterns (Verkleij-Hagoort et al., 2007). Since previous studies demonstrated an overall stability of maternal dietary patterns before and during pregnancy, an early first trimester dietary questionnaire provides a valid representation of maternal diet over the periconceptional period (Crozier et al., 2009). Moreover, the FFQ was validated for the target group (Verkleij-Hagoort et al., 2007).

Inherent to the observational design of the study, some limitations have to be addressed. The associations between the dietary pattern and embryonic growth are statistically significant, but the clinical relevance of small effect sizes has to be further investigated. Moreover dietary surveys could be prone to bias (Beydoun et al., 2007). We tested underreporting using a PAL cutoff of 1.35 and we estimated a FIL mean value of 1.36 in the included population reducing the likelihood of underreporting. This cohort study is embedded in a tertiary hospital, which means by definition that the proportion of maternal comorbidity and pregnancy complications is expected to be higher than in a population-based cohort, reducing the external validity of our findings. Finally, further investigations including the assessment of maternal fatty acid biomarkers are needed to substantiate our findings.

Previous studies showed that first trimester CRL measurements are strongly associated with subsequent fetal growth parameters, the risk of preterm birth, low birth weight and small for gestational age babies and the cardiovascular risk profile in childhood (Mook-Kanamori et al., 2010; Jaddoe et al., 2014; van Uitert et al., 2013). All these results underline that first trimester growth is associated with pregnancy outcome and future health of the offspring. Therefore, improving periconceptional maternal modifiable risk factors seems relevant in order to ameliorate pregnancy outcome and future wellbeing of the offspring.

In conclusion, we have shown that a periconceptional maternal ‘high fish and olive oil, low meat’ dietary pattern is associated with increased embryonic growth in strictly dated spontaneous pregnancies. More research and intervention studies are warranted to investigate the effects in the general population, to reveal underlying mechanisms and to assess the implications for preconceptional and pregnancy care.

Chapter 3

Periconceptional maternal biomarkers of one-carbon metabolism and embryonic growth trajectories: The Rotterdam Periconceptional Cohort (Predict Study)

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Abstract

Objective To study associations between periconceptional maternal biomarkers of one-carbon (I-C) metabolism and embryonic growth.

Design Prospective, periconceptional hospital-based birth cohort.

Setting A tertiary medical care center.

Patients Between 2010 and 2014, we enrolled 236 women with early singleton ongoing pregnancies, resulting in 139 strictly dated spontaneous pregnancies and 97 pregnancies conceived after assisted reproductive technology (ART).

Intervention None.

Main outcome measures Maternal serum vitamin B12 and plasma total homocysteine (tHcy) were assessed at enrollment. Longitudinal first trimester crown-rump length (CRL), embryonic volume (EV) and absolute growth rates were obtained using three-dimensional ultrasound (3D US) and virtual reality.

Results In early pregnancy, we performed a median of five 3D US scans (range 1-7). Vitamin B12 concentrations were positively associated with CRL and EV measurements in the total population (CRL: β 5^10^{-4}(1^10^{-4}- 9^10^{-4}) $\sqrt{}$mm; EV: β 2^10^{-4}(0^10^{-4}- 4^10^{-4}) $\sqrt[3]{}$ cm^3) and in the strictly dated spontaneous pregnancy subgroup. tHcy was negatively associated with embryonic growth in all study groups. High tHcy concentrations (+2 standard deviation (SD), 10.3 µmol/l) were associated with a 1.7 mm smaller CRL (-13.4%) at 7 weeks and a 3.6 mm smaller CRL (-7.1%) at 11 weeks compared to -2SD tHcy (-3.0 µmol/l). High tHcy was also associated with a 0.10 cm^3 smaller EV (-33.3%) at 7 weeks and a 1.65 cm^3 smaller EV (-16.1%) at 11 weeks. Embryonic growth rate was positively associated with vitamin B12 and negatively associated with tHcy.

Conclusions Minor variations in periconceptional maternal concentrations of I-C metabolism biomarkers are associated with human embryonic growth.

Introduction

Over the last decades impaired reproductive health has been associated with poor parental nutrition and lifestyle impacting on the development of gametes, embryo and fetus with long-term implications for health and non-communicable diseases of the offspring (Homan et al., 2007; Kelly et al., 2008; Ashworth et al., 2009; Cetin et al., 2010; Sinclair et al., 2013). Derangements in one-carbon (I-C) metabolism represent one of the causal links between parental poor nutrition, lifestyle and reproductive failures (Steegers-Theunissen et al., 2013). Clinical biomarkers of this metabolic pathway comprise folate, vitamin B12 and total homocysteine (tHcy), with elevated tHcy concentrations representing the most sensitive marker of I-C metabolism derangement (Steegers-Theunissen et al., 2013; Steegers-Theunissen et al., 1991). For many years, researchers studied the associations between folate and reproductive outcome (Steegers-Theunissen et al., 2013). Of interest is that research is focusing now also on the effects of vitamin B12 on perinatal health, showing significant associations with birth defects and weight (Bergen et al., 2012). On the other hand, elevated plasma tHcy has been associated with an increased risk of congenital malformations, small for gestational age fetus, low placental weight, preterm birth, preeclampsia and a poor cardiovascular risk profile in childhood and adulthood (Mook-Kanamori et al., 2010; Bergen et al., 2012; Hogeveen et al., 2012; Steegers-Theunissen et al., 2013; Furness et al., 2013; Yajnik et al., 2014). Moreover, in the last decade the periconceptional period (14 weeks pre-conception to 10 weeks post-conception) has been recognized as one of the most important time windows in life during which gametogenesis, embryogenesis and placentation take place (Steegers-Theunissen et al., 2013). These processes are influenced by genetic and environmental factors affecting mechanisms such as epigenetic programming, further explaining the associations between periconceptional health and outcome, pregnancy outcome and health of the offspring in adult life.

The introduction of high frequency probes and three-dimensional ultrasound (3D US) scans has markedly improved first trimester embryonic evaluation and the precision of crown-rump length (CRL) measurements. Additionally, the use of the Barco I-Space, an immersive virtual reality (VR) system, provides real depth perception and

interaction with 3D US datasets in an intuitive manner. The V-Scope VR application allows offline CRL and embryonic volume (EV) measurements in the I-Space, as important and highly reliable non-invasive biomarkers for embryonic growth (Verwoerd-Dikkeboom et al., 2010; Rousian et al., 2010).
The aim of this study is to evaluate the association between periconceptional maternal vitamin B12 and tHcy concentrations and embryonic growth assessed by longitudinal CRL and EV measurements performed in the Barco I-Space VR system.

Materials and methods

The present study was performed in the Rotterdam Periconception Cohort (Predict study), a prospective periconceptional tertiary hospital-based birth cohort study conducted at the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Centre in Rotterdam, The Netherlands. This ongoing cohort study started in 2009 and aims to investigate periconceptional determinants of first trimester and pregnancy outcome and the biological mechanisms associated with offspring health during the life course (Steegers-Theunissen et al., 2016). The protocol has been approved by the local medical ethics committee and all women signed a written informed consent form before participation.

Study population

Between November 2010 and July 2014 all women of at least 18 years of age with an early first trimester (< 8 weeks of gestation) ongoing singleton pregnancy were eligible for enrollment. Figure 1 shows a flow chart summarizing the excluded and included participants for the current study. Women who conceived spontaneously, after intrauterine insemination (IUI) or assisted reproductive technology (ART), including *in vitro* fertilization (IVF), intracytoplasmatic sperm injection (ICSI) and cryopreserved embryo transfer, were eligible for participation. Among spontaneously conceived pregnancies, exclusion criteria were unknown first day of the last menstrual period (LMP), self-reported irregular cycle or observed CRL ≥ 7 days different from the expected CRL according to the Robinson curves (Robinson et al., 1975). The total study population

included 236 pregnancies defined by reliable pregnancy dating, comprising of 97 ART pregnancies and 139 strictly dated spontaneous pregnancies.
Since the association between maternal blood biomarkers and embryonic growth could eventually be confounded by the conception mode, we adjusted the analysis in the total population for this potential confounder, and we further stratified the analysis in the two subgroups. In this way we could investigate the effect of maternal biomarkers among pregnancies with reliable pregnancy dating only, considering the influence of different conception modes on the resulting associations. Gestational age was defined from LMP for spontaneous pregnancies (adjusted for duration of the menstrual cycle if <25 or >31 days), from LMP or insemination date plus 14 days for IUI pregnancies, from the day of oocyte retrieval plus 14 days for IVF/ICSI pregnancies, and from embryo transfer day plus 17 or 18 days in pregnancies derived from transfer of cryopreserved embryos.

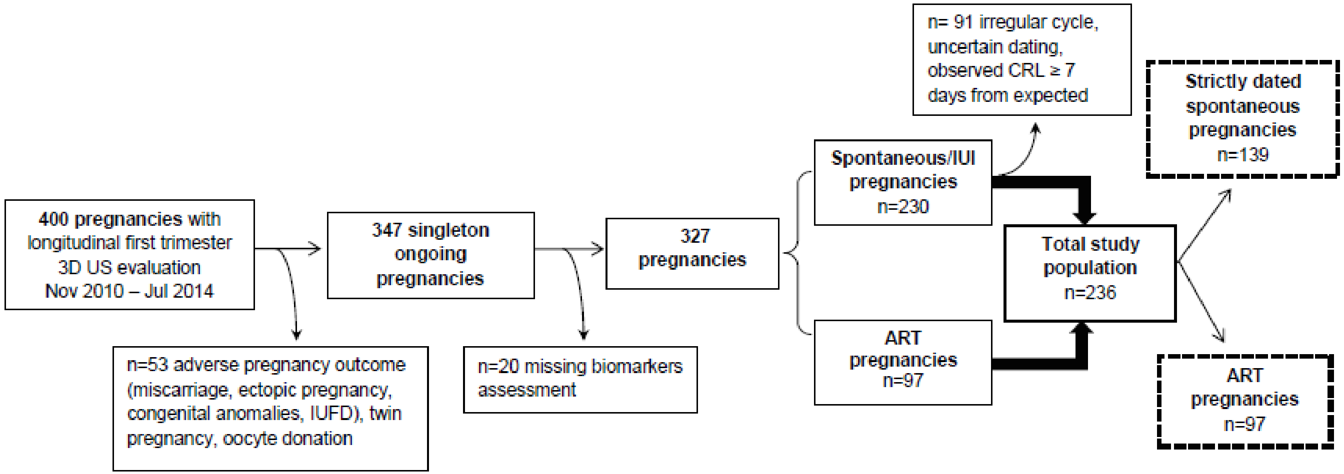
General data and laboratory assays

At enrollment all women completed a self-administered general questionnaire covering details on age, height, weight, ethnicity, education, obstetric and medical history, and periconceptional lifestyle (smoking, alcohol use, folic acid and multivitamin supplements). One fasting venous blood sample per pregnancy was collected at enrollment before 8 weeks of gestation for routine determination of serum vitamin B12 and plasma tHcy amongst others and drawn in a vacutainer ethylenediamine tetraacetate (EDTA) tube and in a dry vacutainer tube (BD diagnostics, Plymouth, UK). The dry vacutainer tubes were centrifuged at 2,000 xg, serum was collected and analyzed for vitamin B12 concentrations using an immunoelectro-chemoluminescence assay (E170; Roche Diagnostics GmbH, Mannheim, Germany). Plasma was separated by centrifugation within one hour for determination of tHcy by using a sensitive liquid chromatography tandem mass spectrum method (HPLC-Tandem MS, Waters Micromass Quattro Premier XE Mass Spectrometer with Acquity UPLC system, Milford, Massachusetts, United States).

Ultrasound data

All women received longitudinal transvaginal 3D US scans from enrollment up to 13^{+0} weeks of pregnancy with a 6 -12 MHz transvaginal probe using GE Voluson E8 equipment and 4D View software (General Electrics Medical Systems, Zipf, Austria). In the pilot study, we performed weekly 3D US scans during the first trimester resulting in a maximum of 7 scans per pregnancy (van Uitert et al., 2013). However, these data showed that an accurate modelling of growth trajectories could be obtained also with three 3D US scans per pregnancy, leading to this reduction after 2013 (Steegers-Theunissen et al., 2016). The 3D US datasets were transformed to Cartesian (rectangular) volumes using 4D View and transferred to the Barco I-Space (Barco N.V., Kortrijk, Belgium) at the Department of Bioinformatics, Erasmus MC, University Medical Centre, Rotterdam, in order to perform offline CRL and EV measurements using the V-Scope software. CRL measurements were performed three times using a length-measuring tool available in V-Scope and the average was used in the analysis. The calipers were placed from crown to caudal rump in a straight line. The correct position in the midsagittal plane was verified by rotating the hologram (Verwoerd-Dikkeboom et al., 2008). A semi-automated volume measuring application, based on gray-scale differences, was used to perform EV measurements as previously validated by Rousian et al. (Rousian et al., 2010). EV measurements were performed once by the investigator on the same dataset selected for CRL measurement. The reliability, technique and methods used for CRL and EV measurements have been extensively studied and described before (Rousian et al., 2010; Verwoerd-Dikkeboom et al., 2008).

Figure 1. Flow chart of the study population.



3D US: three-dimensional ultrasound; IUFD: intrauterine fetal death; ART: assisted reproductive technology; IUI: intrauterine insemination; CRL: crown-rump length.

Statistical analysis

Maternal baseline characteristics and biomarkers were compared between included and excluded pregnancies and between the ART and strictly dated spontaneous pregnancy subgroups using Chi-square or exact tests for ordinal variables and Students t-test or Mann-Whitney U test for continuous variables. Univariable linear regression was performed to evaluate associations between maternal baseline characteristics and biomarker concentrations. Linear mixed models were estimated in the total study population, in the ART and strictly dated spontaneous pregnancy subgroups in order to model longitudinal CRL and EV measurements taking into account the existing correlation between serial measurements within the same pregnancy and to analyze associations with maternal biomarkers with adjustment for potential confounders. Square root transformation of CRL data and third root transformation of EV data were performed to obtain a normal distribution of observations, as required by linear mixed models. This transformation also resulted in approximate linearity with gestational age and an almost constant variance independent from gestational age. Firstly, we performed the analysis with adjustment for gestational age only (model 1) and secondly we adjusted for additional potential confounders (parity, smoking, alcohol and folic acid supplement use, age, BMI, comorbidity and fetal gender) (model 2). Additionally, we investigated the associations between maternal biomarker concentrations and embryonic size parameters separately at enrollment (first available 3D US scan, <8 weeks of gestation) and in late first trimester (last available 3D US scan, >10 weeks of gestation). Finally, linear mixed models were used to study the associations between maternal biomarkers and embryonic absolute growth rate defined as: (CRL1-CRL2)/(GA1-GA2) and (EV1-EV2)/(GA1-GA2), at two consecutive 3D US scans. A random intercept was used to model the within subject correlation. P-values <0.05 were considered statistically significant. All analyses were performed using SPSS Statistics for Windows, Version 21.0 (IBM Corp. Armonk, NY) and R version 3.2.1 (The R Foundation for Statistical Computing).

Results

Baseline characteristics of the study population

Table 1 shows maternal baseline characteristics with comparisons between included and excluded pregnancies and between ART and strictly dated spontaneous pregnancy subgroups. The prevalence of hyperhomocysteinemia in the total study population was 2.1% (> 13 µmol/l). In the univariable linear regression, maternal vitamin B12 showed a positive association with age (β 0.01, 95% confidence interval (CI): 0.002- 0.02, p=0.03) and a negative association with tHcy (β -0.07, 95% CI: -0.10 - -0.04, p<0.001).

Table 1. Maternal baseline characteristics of included and excluded pregnancies.

MATERNAL BASELINE CHARACTERISTICS	Total study population (n=236)		Excluded pregnancies (n=116)			Strictly dated spontaneous pregnancies (n=139)	ART pregnancies (n=97)	
		M		**M**	**p**			**p**
Age, y (median, range)	32 (22-42)	0	30 (21-44)	0	**0.00**	32 (22-42)	32 (24-42)	0.16
Geographical origin		2		4	0.16			0.43
Dutch, n(%)	196 (83.1)		100 (86.2)			119 (85.6)	77 (79.4)	
Other Western, n(%)	11 (4.7)		3 (2.6)			4 (2.9)	7 (7.2)	
Non Western, n(%)	27 (11.4)		9 (7.8)			15 (10.8)	12 (12.4)	
Educational level		2		4	0.36			0.70
High, n(%)	136 (57.6)		64 (55.2)			82 (59)	54 (55.7)	
Intermediate, n(%)	93 (39.4)		45 (38.8)			52 (37.4)	41 (42.3)	
Low, n(%)	5 (2.1)		3 (2.6)			4 (2.9)	1 (1)	
BMI, kg/m^2 (median, range)	24.2 (17.0-42.6)	1	25.8 (17.8-45.0)	2	**0.03**	24.2 (18.6-42.6)	24.4 (17.0-38.4)	0.90
Nulliparous, n(%)	74 (31.4)	2	39 (33.6)	2	0.68	30 (21.6)	44 (45.4)	**0.00**
Alcohol use, n(%)	83 (35.2)	3	38 (32.8)	6	0.09	61 (43.9)	22 (22.7)	**0.00**
Periconceptional smoking, n(%)	33 (14)	2	20 (17.2)	7	**0.01**	21 (15.1)	12 (12.4)	0.81
Folic acid supplementation (<6 wks),n(%)	224 (94.9)	5	108 (93.1)	2	0.57	128 (92.1)	96 (99.0)	**0.05**

Comorbidity, n(%)	27 (11.4)	0	20 (17.2)	0	0.13	22 (15.8)	5 (5.2)	**0.01**
Vitamin B12 (pmol/l) (median, range)	300 (95-953)	0	287.5 (109-915)	25	0.47	290 (95-953)	315 (124-713)	0.12
tHcy (µmol/l) (median, range)	6.4 (3.7-17.6)	4	6.4 (3.4-13.6)	22	0.79	6.6 (4.0-16.3)	6.1 (3.7-17.6)	**0.02**

Excluded pregnancies comprise women with irregular cycle or uncertain pregnancy dating and women without blood samples for biomarkers assessment. Comorbidity includes cardiovascular, autoimmune, endocrine and metabolic diseases. The comparison among groups was performed using Chi-square or exact tests for ordinal variables and Students t-test or Mann-Whitney U test for continuous variables.

M= missing values, ART= assisted reproductive technology, n= number of women, tHcy= plasma total homocysteine, p= p-value.

Embryonic growth trajectories

236 pregnancies with a total of 1207 3D US datasets were included in the analysis. CRL measurements could be performed in 1029 datasets (85.3%) and EV measurements in 941 datasets (78.0%) with good quality. The median gestational age at recruitment was 7^{+1} weeks (range 6^{+0}–11^{+5}) and the median number of 3D US examinations per pregnancy was 5 (range 1-7). The mean absolute CRL and EV growth rates were 1.43 mm/day and 0.46 cm^3/day respectively.

First we compared differences in CRL and EV measurements between the ART and strictly dated spontaneous pregnancy subgroups, showing no significant differences (model 2, CRL analysis: β 0.01 √mm (95% CI -0.03 – 0.05), p=0.65; EV analysis: β 0.01 $\sqrt[3]{cm^3}$ (95% CI -0.01 – 0.03), p=0.60). Table 2 shows the resulting associations between I-C metabolism biomarkers and longitudinal embryonic CRL and volume measurements using linear mixed modelling. Vitamin B12 was positively associated with longitudinal CRL measurements in the total study population and in the strictly dated spontaneous pregnancy subgroup for both model 1 and 2. tHcy was negatively associated with longitudinal CRL measurements in the total study population and in both subgroups for model 1 and 2. In the total study population and after transformation to the original scale, a high tHcy (+2 standard deviation (SD), corresponding to 10.3 µmol/l) was associated with a 1.7 mm smaller CRL (-13.4%) at 7 weeks and a 3.6 mm smaller CRL (-7.1%) at 11 weeks of gestation compared to a low tHcy (-2SD, corresponding to 3.0 µmol/l). Regarding EV analysis, vitamin B12 was significantly associated with increased longitudinal EV measurements in the total study population and in the strictly dated spontaneous pregnancy subgroup for model 1 and 2. tHcy was negatively associated with longitudinal EV measurements in the total study population and ART subgroup for both models, whereas model 2 lost significance in the strictly dated spontaneous pregnancy subgroup. The transformation to the original scale in the total study population showed that high tHcy concentrations (+2 SD) significantly lowered EV measurements with a mean of 0.10 cm^3 (-33.3%) at 7 weeks and 1.65 cm^3 (-16.1%) at 11 weeks of gestation compared to low tHcy (-2 SD).

Table 2. Effect estimates from the linear mixed models for associations between maternal biomarkers of I-C metabolism and longitudinal embryonic crown-rump length (CRL) and volume (EV) measurements.

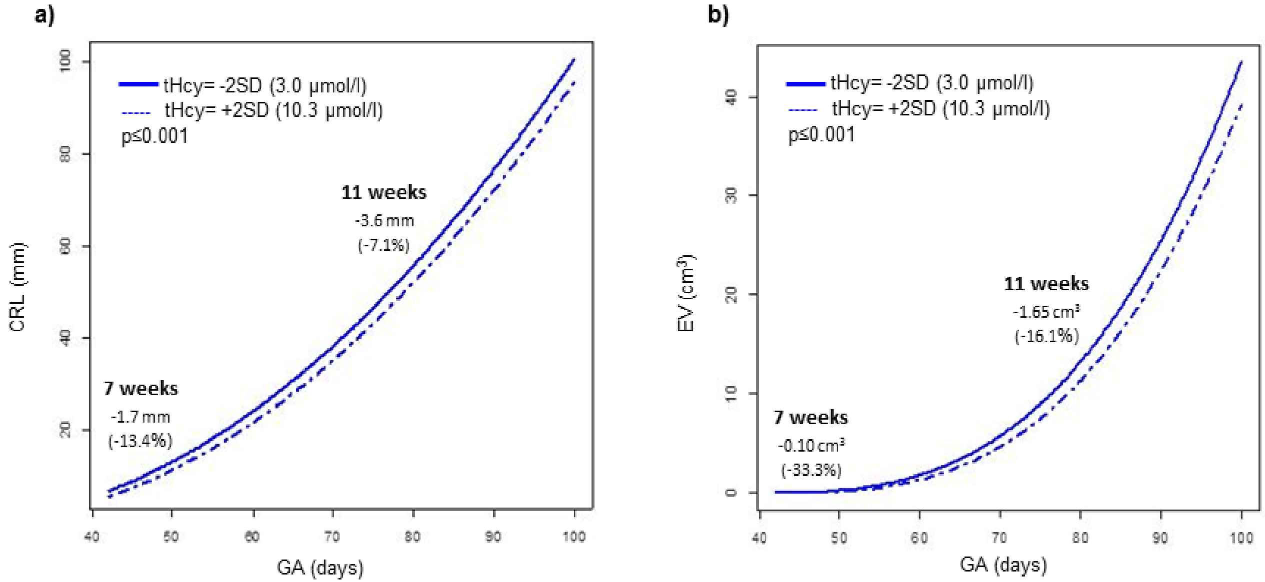
MARKER	Total population	Strictly dated spontaneous pregnancies	ART Pregnancies
Effect estimates CRL β(95%CI), √mm			
B12			
Model 1	4^10^{-4} (1^10^{-4};7^10^{-4})**	7^10^{-4} (3^10^{-4};1^10^{-3})	-1^10^{-4} (-4^10^{-4};3^10^{-4})
Model 2	5^10^{-4} (1^10^{-4};9^10^{-4})**	8^10^{-4} (4^10^{-4};1^10^{-3})	-1^10^{-4} (-5^10^{-4};3^10^{-3})
tHcy			
Model 1	-0.04 (-0.06;-0.02)	-0.04 (-0.08;-0.00)*	-0.04 (-0.05;-0.02)
Model 2	-0.03 (-0.05;-0.01)	-0.03 (-0.07;-0.00)*	-0.03 (-0.05;-0.01)**
Effect estimates EV β(95%CI), $^{3}\sqrt{}$cm^{3}			
B12			
Model 1	2^10^{-4} (0^10^{-4};4^10^{-4})**	3^10^{-4} (1^10^{-4};5^10^{-4})**	-2^10^{-5} (-2^10^{-4};2^10^{-4})
Model 2	2^10^{-4} (0^10^{-4};4^10^{-4})**	3^10^{-4} (1^10^{-4};5^10^{-4})**	-2^10^{-5} (-2^10^{-4};2^10^{-4})
tHcy			
Model 1	-0.02 (-0.03;-0.01)	-0.02 (-0.04;0.00)*	-0.02 (-0.03;-0.01)
Model 2	-0.02 (-0.03;-0.01)	-0.01 (-0.03;0.01)	-0.02 (-0.03;-0.01)

Effect estimates represent the amount of change in square root CRL (√mm) and third root EV ($^{3}\sqrt{}$cm^{3}) per unit increase of biomarker's concentrations. Model 1 is adjusted for gestational age. Model 2 includes adjustment for parity, alcohol use, smoking habit, folic acid supplement use, maternal age, BMI, comorbidity and fetal gender. Vitamin B12 concentrations are measured in serum and tHcy in plasma. tHcy: total homocysteine; CI: confidence interval. *p≤0.05; **p<0.01;   p≤0.001

Figure 2 shows the average regression lines for tHcy in the total study population (model 2).

Supplemental table 1 shows the associations between maternal biomarker concentrations and early or late first trimester embryonic size, confirming the detected associations particularly during the late first trimester. Finally, in the total study population the analysis on embryonic growth rate per day showed significant positive associations for vitamin B12 (model 2, CRL growth rate: β 0.0002 mm/day (95% CI 0.0001 – 0.0003), p=0.02; EV growth rate: β 2.7^10^{-5} cm^3/day (95% CI 5.0^10^{-6} – 4.9^10^{-5}), p=0.02) and negative associations for tHcy (model 2, CRL growth rate: β -0.0001 mm/day (95% CI -0.009 – 0.009), p=0.87; EV growth rate: β -0.001 cm^3/day (95% CI -0.002 - -0.000), p=0.05).

Figure 2. Fully adjusted models for crown-rump length (CRL) (a) and embryonic volume (EV) (b) in relation to total homocysteine (tHcy) concentrations in the total study population.



tHcy is expressed as +2 standard deviations (SD) (dashed line, corresponding to 10.3 µmol/l in the total study population) and -2SD (continuous line, corresponding to 3.0 µmol/l). Gestational age (GA) is expressed in days. Full adjustment for parity, alcohol use, smoking, folic acid use, maternal age, BMI, comorbidity and fetal gender was performed.

Discussion

This study demonstrates that periconceptional maternal tHcy concentrations are negatively and vitamin B12 concentrations are positively associated with embryonic growth depicted by longitudinal CRL and EV measurements and absolute growth rates.

In agreement with previous results showing EV as a more effective measurement of first trimester growth restriction compared to CRL, the strongest effect in our study was observed for EV, with a reduction of more than one third of this volume in case of high tHcy at 7 gestational weeks (Baken et al., 2013). The obtained mean EV measurements in our population were comparable with previous results (Rolo et al., 2009; Ioannou et al., 2011). In particular, a 10-week old embryo showed a mean volume of 5.6 cm^3, which is in agreement with measurements previously reported at the same gestational age (4.2 – 6.2 cm^3) (Rolo et al., 2009; Ioannou et al., 2011). Small variations could be explained by differences in methods (VR versus 3D US), maternal baseline characteristics and pregnancy dating procedures (Rolo et al., 2009; Ioannou et al., 2011). The obtained CRL measurements were also in line with the reference curves provided by the Robinson chart (Robinson et al., 1975).

The effect size of tHcy on CRL is comparable with the estimates previously reported for maternal age, whereas the effect size is 10 times stronger than for alcohol consumption (CRL β -0.0025 √mm 95% CI: -0.0047 - -0.0003) (van Uitert et al., 2013). We previously showed in a smaller study population that maternal periconceptional smoking (>10 cigarettes per day) was associated with a 7 times stronger effect sizes on CRL compared to tHcy (β -0.202 √mm (95% CI: -0.404 - -0.001) (van Uitert et al., 2013). In the Generation R study, maternal age, diastolic blood pressure, hematocrit value, smoking and folic acid supplement use were significantly associated with late first trimester CRL measurements in a multivariable adjusted model, where no use of folic acid supplement revealed the strongest effect (β -1.33 mm, 95% CI: -2.41 - -0.24) (Mook-Kanamori et al., 2010). In our model, the effect estimate of high tHcy (+2 SD) on CRL is almost three times higher. In regard to vitamin B12, associations, albeit significant, showed small effect sizes on CRL and EV (Table 2). The analysis on CRL and EV growth rates confirmed a significant association with maternal I-C

metabolism, pointing out associations not only with embryonic size at a specific step point, but also with growth velocity.
The associations between maternal biomarkers and embryonic growth differ between the two subgroups. We suggest that the ovarian stimulation treatment, the extrauterine development of the preimplantation embryo and the use of culture media can influence embryonic responses to the periconceptional biomarkers exposure. Moreover, despite the adjustments, it is difficult to disentangle the impact of the causes of subfertility as an independent factor affecting embryonic growth (Holst et al., 2016; Jacques et al., 2016).
Our results underline that maternal periconceptional I-C metabolism is significantly associated with embryonic growth, which substantiates previous findings on associations with embryonic development and congenital anomalies (Steegers-Theunissen et al., 1991; Brauer et al., 2004; Verkleij-Hagoort et al., 2007; Steegers-Theunissen et al., 2013). Moreover, our data mean to integrate the known association between an optimal maternal long-term folate status and increased longitudinal CRL measurements finally confirming the importance of maternal I-C metabolism for first trimester embryonic outcome (Van Uitert et al., 2014). Several molecular and biological processes may represent the causal link between maternal I-C metabolism derangements and impaired embryonic growth and development, including direct cytotoxic effects, excessive oxidative stress, impaired DNA synthesis and repair, and increased apoptosis (Di Simone et al., 2003; Bergen et al., 2012; Steegers-Theunissen et al., 2013). Recent data indicate that I-C metabolism is also crucial in the programming and development of mammalian oocytes and preimplantation embryos (Benkhalifa et al., 2010; Kwong et al., 2010). The addition of Hcy to culture medium suppresses the blastocyst development in animal models (Ikeda et al., 2010). In the ART population, we previously demonstrated significant associations between high tHcy in follicular fluid and poor embryo quality, reduced chances of ongoing pregnancy, smaller size of ovarian follicles, and lower number of oocytes retrieved (Boxmeer et al., 2008; Boxmeer et al., 2009). Moreover, the development of mammalian embryos represents a critical stage for epigenetic perturbations, a programming mechanism that can explain the associations shown in animal models between a periconceptional environment deficient in vitamin B12, folate and methionine and offspring exhibiting

hypertension, obesity, insulin resistance and global changes in liver methylation status (Sinclair et al., 2007). These data substantiate again the involvement of maternal I-C metabolism as early as the periconceptional period for growth and development of the human embryo with implications for future health.

The main strengths of this study are the longitudinal prospective design starting very early in pregnancy and the highly precise and repeated measurements of CRL and EV in the same dataset, thereby taking into account embryonic growth and development in three dimensions. By performing a median of 5 scans per pregnancy, we considered first trimester embryonic growth as a continuous and evolving process, providing highly accurate growth curves. Conversely, only two measurements per pregnancy could have not depicted a detailed trajectory of embryonic development, indicating only the starting and ending point of the growth process. Semi-automated volume measurements can be performed using full immersive VR systems (Rousian et al., 2010). Compared to other methods, which reckon on the manually drawing of the contours in different rotational or longitudinal planes and exclude embryonic limb involvement, the V-Scope software enables volume measurements less sensitive to individual variations, further including the whole embryonic body into the measurement (Rousian et al., 2010). The selection of ART pregnancies and spontaneous pregnancies with strict pregnancy dating makes the assessment of gestational age highly reliable and minimizes the risk of confounding of embryonic growth by gestational age. tHcy was significantly lower in the ART subgroup compared to the strictly dated spontaneous pregnancy subgroup which fits with a higher frequency of preconceptional initiation of folic acid supplement use in the ART compared to the spontaneous subgroup. The high frequency of folic acid supplement use in women on ART treatment and a high educational level explain the low prevalence of hyperhomocysteinemia in our population. This makes the results even stronger, since clinically normal tHcy concentrations close to the upper limit already seem to impact embryonic growth. Inherent to the observational design of this study, some limitations also need to be addressed. Despite adjustment for several potential confounders, this cohort study has been conducted in a tertiary hospital population with a high proportion of comorbidity and high-risk pregnancies, which reduces the external validity of our findings.

First trimester embryonic growth has been strongly associated with second and third trimester fetal growth and birth weight (van Uitert et al., 2013). Moreover, a smaller embryo depicted by CRL measurements has been associated with increased risks of preterm birth, low birth weight and small for gestational age babies and poor cardiovascular risk profile in early childhood (Mook-Kanamori et al., 2010; van Uitert et al., 2013; Jaddoe et al., 2014; Nakamura et al., 2015). These data together with our findings illustrate the significance of the periconceptional period and emphasize the importance of an early first trimester antenatal visit in order to improve maternal modifiable conditions involved in I-C metabolism (Steegers-Theunissen et al., 2015; Steegers et al., 2016). In this regard, inadequacies in dietary B vitamins and lifestyle (i.e. smoke, alcohol and coffee consumption) led to an average increase in plasma tHcy concentrations of 1-4 µmol/l over the last decades (Steegers-Theunissen et al., 2013). In clinical settings, a random plasma tHcy represents an overall stable marker, with no seasonal variation, within the same individual (McKinley et al., 2001; López-Alarcón et al., 2015). This is probably due to the general stability of individual exposures highly associated with tHcy concentrations (i.e. nutritional and lifestyle habits). Moreover, serial tHcy measurements have been recently shown to be constant in uncomplicated pregnancies (López-Alarcón et al., 2015). This suggests that a random tHcy measurement is reflective of an individual's average concentration and represents a potential useful predictor of disease, supporting its widespread use as a predictor and prognostic marker of cardiovascular diseases in both high and low-risk populations (Mallikethi-Reddy et al., 2017). Despite our study did not mean to provide a prediction model of embryonic growth, our results suggest that a random maternal tHcy evaluation as early as the preconceptional period could impact and possibly optimize embryonic growth. On the other hand, it seems too early to provide recommendations on periconceptional vitamin B12 assessment for embryonic health, mainly due to the small effect size detected in our results.

Conclusions

We have shown that small variations in biomarkers of periconceptional maternal I-C metabolism are associated with human embryonic growth. Since a smaller embryo is associated with a higher risk of adverse pregnancy outcome and increased risks of non-communicable diseases in later life, periconceptional maternal I-C metabolism may be used as future predictor of embryonic health and maybe health during the life course.

Nevertheless, further research is recommended to elucidate whether the observed associations also apply to the general pregnant population.

Supplemental table 1. Effect estimates from the linear mixed models for associations between maternal biomarkers of I-C metabolism and early or late first trimester crown-rump length (CRL) and embryonic volume (EV) measurements.

		Early first trimester (<8 weeks)		Late first trimester (>10 weeks)	
		CRL β (95%CI), √mm	**EV** β (95%CI), $\sqrt[3]{\text{cm}^3}$	**CRL** β (95%CI), √mm	**EV** β (95%CI), $\sqrt[3]{\text{cm}^3}$
Total population	**B12**	0.0004 (0.0001; 0.0007)*	0.0001 (-0.00002; 0.0002)	0.001 (0.0002; 0.001)**	0.0002 (0.0000; 0.0004)*
	tHcy	-0.02 (-0.04; 0.00)	-0.01 (-0.02; -0.00)*	-0.04 (-0.06; -0.02)	-0.02 (-0.03; -0.01)**
Strictly dated spontaneous pregnancies	**B12**	0.0006 (0.0001; 0.001)*	0.0002 (0.00002; 0.0004)*	0.001 (0.0005; 0.001)	0.0004 (0.0001; 0.001)**
	tHcy	-0.03 (-0.06; 0.00)	-0.01 (-0.03; 0.00)	-0.04 (-0.08; -0.00)*	-0.02 (-0.04; -0.00)*
ART pregnancies	**B12**	0.000 (-0.001; 0.001)	-0.0001 (-0.0003, 0.0001)	-0.0002 (-0.001; 0.000)	-0.000 (-0.000; 0.000)
	tHcy	-0.02 (-0.04, 0.00)	-0.01 (-0.02; 0.00)	-0.04 (-0.07; -0.01)**	-0.03 (-0.04; -0.00)**

Effect estimates represent the amount of change in square root CRL (√mm) and third root EV ($\sqrt[3]{\text{cm}^3}$) per unit increase of biomarker's concentrations. A fully adjusted model, including adjustment for parity, alcohol use, smoking habit, folic acid supplement use, maternal age, BMI, comorbidity and fetal gender, is shown. tHcy: total homocysteine; CI: confidence interval; ART: assisted reproductive technology.

*p≤0.05; **p<0.01;   p≤0.001

Chapter 4

Periconceptional maternal dairy-rich dietary pattern influences prenatal cerebellar growth

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Abstract

Maternal nutrition during pregnancy has been related to intrauterine brain development and neurodevelopmental disabilities in adult life. We aim to investigate associations between periconceptional maternal dietary patterns and prenatal cerebellar growth from the first trimester onwards.

126 women with singleton non-malformed pregnancies were enrolled before 8 weeks of gestation in the Rotterdam periconceptional cohort between 2013 and 2015. Periconceptional maternal dietary patterns were extracted from food frequency questionnaires and associated with blood biomarkers and micronutrient intakes. Serial two-dimensional and three-dimensional ultrasound scans were performed at 9, 11, 22, 26 and 32 weeks of gestation for transcerebellar diameter (TCD) measurement. Linear mixed models were estimated to investigate associations between periconceptional maternal dietary patterns and longitudinal TCD measurements as a function of gestational age.

We performed a median of 4 scans per pregnancy, resulting in 570 total datasets. The success rate of TCD measurements was 87% (range 65-100%), depending on gestational age. The Mediterranean, Western, egg-rich and dairy-rich dietary patterns were extracted, explaining 37.2% of the overall variance of food intake in this population. The dairy-rich dietary pattern was positively associated with cerebellar growth trajectories (β= 0.02 (95%CI: 0.01; 0.03) $\sqrt{}$mm, p=0.01). Maternal strong adherence to this dietary pattern increased TCD measurements by 0.8 standard deviation scores (SDs) compared to weak adherence, reflected in increased TCD estimates of 0.44 mm at 9 weeks (+6.8%), 0.88 mm at 22 weeks (+3.6%), and 1.17 mm at 32 weeks (+2.8%). No significant associations were detected for the Mediterranean, Western and egg-rich dietary patterns.

This study shows a positive association between periconceptional maternal adherence to a dairy-rich dietary pattern and human prenatal TCD measurements as a proxy of cerebellar growth. Next step is the investigation of the impact on neurodevelopmental outcomes in the offspring.

Introduction

The traditional paradigm of the cerebellum exclusively as a part of the motor system has been progressively abandoned in the last decades. It is now clear that the cerebellum plays a crucial role in human acquisition of cognitive and emotional attributes and abilities (Allen et al., 2005; Allin, 2016). Starting in the first weeks of pregnancy with the development of the Purkinje cells, cerebellar development continues during the third trimester of pregnancy and even postnatally when the cerebellum outgrows its dimension more than three times (Knickmeyer et al., 2008; Allin, 2016). This prolonged process explains the wide window of cerebellum sensitivity to environmental insults. Several prenatal exposures have been related to impaired fetal cerebellar growth, including maternal alcohol consumption, smoking and toxin exposures (Cace et al., 2011; de Zeeuw et al., 2012). Moreover, animal models showed significant associations between nutritional exposures during pregnancy and impaired intrauterine cerebellar development, reporting altered growth of Purkinje cells in case of iodine deficiency, modified methylation profiles after folic acid supplementation and increased cerebellar lipoperoxidation among low protein-fed offspring (Bonatto et al., 2006; Yu et al., 2017). No human data are available on the associations between maternal dietary patterns and prenatal cerebellar development, despite the well-known associations with neurodevelopmental outcomes (Nuttall, 2015; Surén et al., 2013; Steegers-Theunissen et al., 2013).

The introduction of high frequency transvaginal and transabdominal probes and three-dimensional ultrasound (3D US) has dramatically improved the chance of evaluating human embryonic and fetal structures, and reference curves of cerebellar growth are now available as early as the embryonic period (Rousian et al., 2013).

In the current study, we aim to investigate associations between periconceptional maternal dietary patterns and prenatal cerebellar growth trajectories measured by longitudinally collected transcerebellar (TCD) diameters from the first trimester onwards.

Materials and methods

This study was conducted at the Department of Obstetrics and Gynecology, Erasmus MC, University Medical Center in Rotterdam, the Netherlands, in the setting of the ongoing Rotterdam Periconception cohort (Predict Study) (Steegers-Theunissen et al., 2016). The protocol was approved by the Medical Ethical and Institutional Review Board at the Erasmus MC, University Medical Center in Rotterdam, the Netherlands, and all participants signed a written informed consent on behalf of themselves and the unborn child (METC Erasmus MC 2004-277).

Eligible for the current study were all women aged at least 18 years old, with an intrauterine singleton viable pregnancy before 8+0 weeks of gestation between November 2013 and March 2015. We selected pregnancies conceived spontaneously, through intrauterine insemination (IUI) or assisted reproductive technologies using homologous oocyte(s). Ongoing pregnancies complicated with congenital anomalies were excluded from the analysis. Missing or unreliable periconceptional maternal nutritional evaluations were additional exclusion criteria. The pregnancy dating procedure was based on crown-rump length (CRL) measurements before 13 weeks of gestation for spontaneous and IUI pregnancies, on the date of oocyte retrieval plus 14 days for in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) derived pregnancies, or from the day of embryo transfer plus 17-18 days for cryopreserved transfers (Robinson, 1973).

At enrolment, baseline anthropometric measurements and general information on age, geographical origin, educational level, obstetric and medical anamnesis and periconceptional exposures (smoking, alcohol use, folic acid or multivitamin supplement use) were collected. Data on birth outcomes were collected from medical records (neonatal gender, birth weight, gestational age at birth).

The semi-quantitative food frequency questionnaire (FFQ) developed by the division of Human Nutrition, Wageningen University, the Netherlands, and validated for women of reproductive age, was used at recruitment to assess maternal nutritional intake over the previous month (Verkleij-Hagoort et al., 2007; Siebelink et al., 2011). Participants were asked to report the frequency of consumption, portion size and preparation method of 196 food items structured according to meal patterns. Average daily nutritional values were calculated using the Dutch food composition table and reports with implausible energy intakes were considered unreliable and therefore excluded from the analysis. Based on similar origin and nutrient contents, the 196 FFQ food items were condensed into 23 food groups and dietary patterns were derived using principal component analysis (PCA) as extensively described by Hoffmann (Slimani et al., 2002; Hoffmann et al., 2004; Netherlands Nutrition Centre, 2011). PCA automatically assigns all participants a component score for each dietary pattern representing their adherence to that specific dietary pattern, meaning that every woman possibly shows high adherence (component scores> 0) for two or more dietary patterns at the same time. One fasting venous blood sample for serum and red blood cell (RBC) folate, serum vitamin B12 and plasma total homocysteine (tHcy) determination was collected at enrolment and the laboratory procedures have been extensively described elsewhere (van Uitert et al., 2014).

All women underwent longitudinal ultrasound examinations performed by one trained sonographer for TCD measurements (IVK). First trimester 3D US examinations were performed using a high-resolution transvaginal probe (6-12 MHz) of the Voluson E8 system (GE Medical Systems, Zipf, Australia). All 3D volumes were stored as Cartesian and 4DView volumes and TCD measurements were performed offline using 4D View Version 5.0 (GE Medical Systems). Due to the low success rate of cerebellar measurements performed before 8 weeks of gestation, only 3D US volumes at 9 weeks of gestation or later were included in the analysis (Rousian et al., 2013). The technique, reliability and protocol used for cerebellar measurements at 9 and 11 weeks of gestation have been previously validated and extensively described by our group (Rousian et al., 2013; Koning et al., 2015). Briefly, first trimester cerebellar measurements were performed manually using a 4D view software in a coronal section of the head through the rhombencephalon, showing the cerebellum, fourth ventricle, and plexus choroideus of the fourth ventricle. An excellent intra- and inter-observer reliability was reported, with intraclass correlation coefficients above 0.98. First trimester cerebellar growth curves and associations with gestational age and CRL were determined in the same population (Rousian et al., 2013). Second and third trimester ultrasound scans were performed at 22, 26 and 32 weeks of gestation using 1-7 MHz transabdominal transducer or a 6-12 MHz transvaginal transducer of the Voluson E8 system. Standardized 2D biometric measurements of the TCD were obtained online by slightly rotating the transducer from the transverse cross-section of the thalamic plane to the posterior fossa, enabling the 'outer-to-outer' measurement of the cerebellum, as recommended by the ISUOG guidelines. All embryonic and fetal TCD measurements were performed three times by the same researcher (IVK) and the mean values were used for the analysis.

Finally, intrauterine growth trajectories according to estimated fetal weight, birth weight and corresponding percentiles were considered in order to detect abnormal fetal growth trajectories.

Statistical analyses

All analyses were performed using IBM SPSS version 21.0 (Armonk, NY: IBM Corp) and R version 3.2.1 (The R Foundation for Statistical Computing). P-values ≤0.05 were considered significant. Maternal characteristics and dietary pattern adherence (component scores) were compared between excluded and included pregnancies using Chi-square or exact tests for ordinal variables and Mann-Whitney U test for continuous variables. The basal metabolic rate (BMR) was calculated using the new Oxford equations stratified by age and gender, as described and validated elsewhere (Ramirez-Zea et al., 2005). Finally, the food intake level (FIL) was obtained as the ratio of energy intake divided by BMR and compared with the physical activity level (PAL) of a sedentary lifestyle (cut off 1.35) to estimate underreporting. We compared serum and RBC folate, vitamin B12, and tHcy concentrations between women with strong (component scores >0) and weak (component scores <0) adherence to each dietary pattern using the Mann-Whitney U test. Univariable linear regression was performed to investigate correlations between nutrient intake and the extracted dietary patterns.

To investigate associations between periconceptional maternal dietary patterns and repeated cerebellar measurements, we estimated linear mixed models using TCD measurements as response variable and gestational age as predictor. This analysis allows the longitudinal modeling of repeated TCD measurements, taking into account the correlation between serial measurements within the same pregnancy. Square root transformation of TCD data resulted in approximate normal distribution of observations with a constant variance, as required by linear mixed models, and linearity with gestational age. A random intercept and slope were used to model the within subject correlation. An interaction with gestational age did not improve the model fit, suggesting a constant and continuous effect of dietary patterns on longitudinal TCD measurements. First, we estimated a mixed model adjusted for energy intake (model 1). Model 2 depicts the estimates including additional adjustment for potential confounding factors (parity, alcohol use, smoking, folic acid/multivitamin supplement use, age, BMI, conception mode and geographical origin). TCD variance expressed as standard deviation score (SDs) over gestation was calculated from model 1 as follows: SDs=TCD(x-mean)/SD to compare the effect of maternal strong versus weak adherence to the dietary patterns significantly associated with cerebellar growth. In order to evaluate the effect of maternal adherence to the dietary patterns in different trimesters of pregnancy and provide a clear interpretation from a clinical perspective, longitudinal TCD measurements were further transformed back to the original scale and plotted against gestational age by comparing maternal strong versus weak adherence to the dietary patterns significantly associated with cerebellar measurements. Finally, due to the association between preterm birth, intrauterine fetal growth and brain development, we performed a subgroup analysis excluding all pregnancies complicated by preterm birth, intrauterine growth restriction and low birth weight.

Results

A total of 126 viable pregnancies out of 227 were included in the analysis, after exclusion of pregnancies achieved after egg cell donation (n=3), missing or unreliable periconceptional FFQs (n=94), fetal or neonatal congenital anomalies (n=3) and missing ultrasound data (n=1). Table 1 shows maternal baseline characteristics and the sensitivity analysis comparing included and excluded pregnancies. Included and excluded pregnancies were comparable for all characteristics, except for folic acid supplement use.

Median gestational age at birth was 272 days (range 182-296), birth weight was 3293 g (range 665-4380) and 50.4% of newborns were males. Preterm births (<37 weeks of gestation) counted a total of 10 pregnancies, including 6 intrauterine growth restricted fetuses, whereas one pregnancy ended with a low birth weight baby (LBW, birth weight <2500 g), thus leading to a total of 115 term pregnancies with normal intrauterine growth trajectory and birth weight. A median of four scans per pregnancy (range 1-5) was performed, resulting in a total of 570 datasets. The success rate of TCD measurements was 86.8%, depending on gestational age (71.1% at 9 weeks, 65% at 11 weeks, 100% at 22 weeks, 98% at 26 weeks and 95% at 32 weeks of gestation). Figure 1 shows cerebellar growth trajectories depicted by longitudinal TCD measurements in the study population.

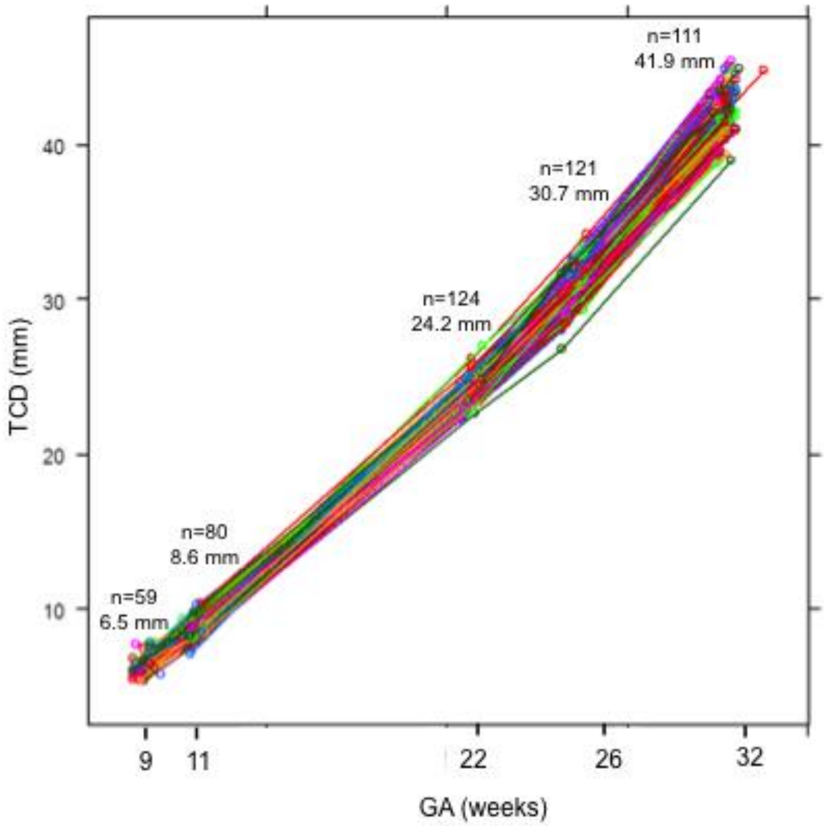
Based on the scree plot and interpretability of the extracted patterns, four major components (dietary patterns) with eigenvalues above 1.5 were identified, explaining 37.2% of the overall variance in dietary intake (Table 2). We labeled the first component ‘Mediterranean’ because of high loadings on vegetables, olive oil, fish, fruit, nuts and legumes, and relatively low loading on meat. The second component included high intakes of meat, soups, snacks, mayonnaise, potatoes and sauces, and low intake of fruit, resembling the ‘Western’ dietary pattern. The third component was highly associated with eggs, nuts, fish, sauces and non-alcoholic beverage consumption and was labeled ‘egg-rich’. The fourth component yielded a dietary pattern high in the consumption of dairy products, fruit, cereals, non-alcoholic beverage and whole grain and was labeled ‘dairy-rich’.

Table 1. Maternal baseline characteristics of the study populationand excluded pregnancies.

Maternal characteristics	Study population (n=126)	M	Excluded pregnancies (n=100)	M	p-value
Maternal age, y median (range)	32 (22-45)	4	31 (21-48)	4	0.45
Nulliparous, n (%)	31 (24.8)	1	34 (35.8)	5	0.08
Geographical origin Western, n (%) Non Western, n (%)	 98 (79.0) 26 (21.0)	2	 79 (82.3) 17 (17.7)	4	0.55
Educational level High, n (%) Intermediate, n (%) Low, n (%)	 60 (48.4) 50 (40.3) 14 (11.3)	2	 46 (49.5) 35 (37.6) 12 (12.9)		0.89
BMI, kg/m^2 median (range)	24.1 (16.3-39.7)	0	23.1 (15.2-43.4)	5	0.83
Alcohol use, n (%)	36 (29.3)	3	29 (30.9)	6	0.80
Periconception smoking, n (%)	18 (14.5)	2	17 (17.2)	1	0.59
Periconception folic acid/multivitamin use, n (%)	115 (92.7)	2	79 (84.0)	6	**0.04**
Mode of conception IVF/ICSI, n (%)	32 (25.4)	0	25 (25.0)	0	0.95

The comparison among groups was performed using Chi-square or exact tests for ordinal variables and Mann-Whitney *U* test for continuous variables.
M=missing values, BMI: body mass index, IVF: in vitro fertilization, ICSI: intracytoplasmic sperm injection.

Figure 1. Longitudinal transcerebellar diameter (TCD, mm) measurements in the study population.



Mean values at 9, 11, 22, 26 and 32 weeks of gestation are reported. Dots and lines represent TCD measurements and trajectories of a single included pregnancy. n: number; GA: gestational age.

Table 2. Relation between food groups and extracted dietary patterns expressed by factor loadings.

Food group	Mediterranean	Western	Egg-rich	Dairy-rich
Variance explained(%)	11.9	10.2	8.2	6.9
Butter	,066	,178	,048	,112
Cereals	-,051	,041	,036	,480
Dairy	-,094	,036	-,049	,832
Eggs	,094	,067	,782	-,095
Fat Liquid	-,448	,188	,209	-,015
Other fats	-,051	,056	-,052	,078
Fish	,279	-,276	,278	-,042
Fruits	,241	-,030	,129	,603
Legumes	,732	,116	,137	-,019
Margarine	-,199	-,013	-,138	,222
Mayonnaise	,147	,702	-,004	-,127
Meat	-,024	,750	-,130	,142
Non-alcoholic	-,238	-,133	,603	,272
Nuts	,378	-,044	,584	,109
Potatoes	-,038	,267	,140	-,027
Refined Grains	,022	-,058	,019	,034
Sauces	,104	,330	,379	-,006
Snacks	,066	,438	,158	-,052
Soup	-,004	,669	,119	-,027
Sugars	-,002	,211	-,053	-,018
Vegetables	,760	,088	,095	,188
Olive oil	,665	-,006	-,061	-,104
Whole grains	,020	-,254	-,096	,395

The factor loadings describe how strong the association between the food groups and each of the extracted dietary patterns is. The factor loadings with the highest absolute value were used for labeling.

Women with strong adherence to the Mediterranean dietary pattern (component scores> 0) showed significantly higher serum and RBC folate concentrations compared to women with weak adherence (component scores< 0) to the same dietary pattern (serum folate: 43.1 (range 16.4-223.0) and 34.6 (range 12.6-451.3) nmol/l, $p<0.001$; RBC folate: 1429.0 (range 634.0-2256.0) and 1244 (range 466-2919) nmol/l, $p=0.02$ respectively). Strong adherence to the egg-rich dietary pattern was associated with significantly higher serum and RBC folate concentrations compared to weak adherence (serum folate: 41.4 nmol/l (range 14.8-223.0) and 34.8 nmol/l (range 12.6-451.3), $p=0.02$; RCB folate: 1468 nmol/l (range 866-2256) and 1258 nmol/l (range 466-2919), $p=0.01$ respectively). Strong adherence to the dairy-rich dietary pattern was associated with significantly lower tHcy concentrations compared to weak adherence (5.9 µmol/l (range 3.4-11.3) and 6.5 µmol/l (range 3.7-31.4), $p=0.02$). Adherence to the Western dietary pattern was not associated with the investigated biomarker concentrations.
S1 table shows maternal baseline characteristics according to high adherence to the extracted dietary patterns. Table 3 shows the correlation coefficients for nutrient intake and the extracted dietary patterns. Maternal intake of all investigated micronutrients was significantly and positively correlated to the dairy-rich dietary pattern, except for the omega-3 fatty acid intake. The mean FIL of the study population was 1.38.
Table 4 shows the effect estimates from linear mixed models in the study population. No significant associations were detected between the Mediterranean, Western, egg-rich dietary patterns and cerebellar growth trajectories, for both model 1 and 2. Conversely, maternal adherence to the dairy-rich dietary pattern was associated with increased TCD measurements for both model 1 ($\beta=0.02$ √mm (95% CI: 0.01; 0.03), $p<0.01$) and 2 ($\beta=0.02$ √mm (95% CI: 0.01; 0.03), $p<0.01$), leading to a difference on TCD variance of about 0.8 SDs over gestation in case of strong *versus* weak adherence to the dietary pattern. Additional adjustment for fetal gender did not modify the detected associations.
The transformation to the original scale showed that strong adherence to this dietary pattern (defined as +2 standard deviation (SD) in component score) increased TCD by 0.44 mm at 9 weeks (+6.8%), 0.52 mm at 11 weeks (+6%), 0.88 mm at 22 weeks (+3.6%), 1.00 mm at 26 weeks (+3.3%), and 1.17 mm at 32 weeks (+2.8%) compared to weak

adherence (-2 SD in component score). Figure 2 shows the average regression lines for the dairy-rich dietary pattern in the study population after transformation to the original scale (model 2).

Table 3. Correlations between nutrient intake and the four extracted dietary patterns expressed by correlation coefficients.

Nutrient intake	Mediterranean	Western	Egg-rich	Dairy-rich
ALA	0.30 **	0.41**	0.32 **	0.20 *
EPA	0.30 **	-0.10	0.41 **	0.02
DHA	0.35 **	-0.13	0.32 **	0.02
Proteins	0.22 *	0.58**	0.15	0.48 **
Fats	0.24 **	0.62 **	0.18 *	0.20 *
Fiber	0.50 **	0.09	0.17	0.53 **
Vitamin B6	0.14	0.35 **	0.21 *	0.55 **
Vitamin B12	0.19 *	0.56 **	0.10	0.27 **
Folate	0.38 **	0.17	0.13	0.46 **
Zink	0.23 **	0.57 **	0.16	0.52 **
Vitamin A	-0.04	0.69 **	-0.05	0.14 *
Vitamin B1	0.16	0.61 **	0.15	0.46 **
Vitamin B2	0.16	0.39 **	0.11	0.72 **
Vitamin C	0.31 **	0.18 *	0.14	0.38 **
Vitamin E	0.28 **	0.40 **	0.24 **	0.18 *

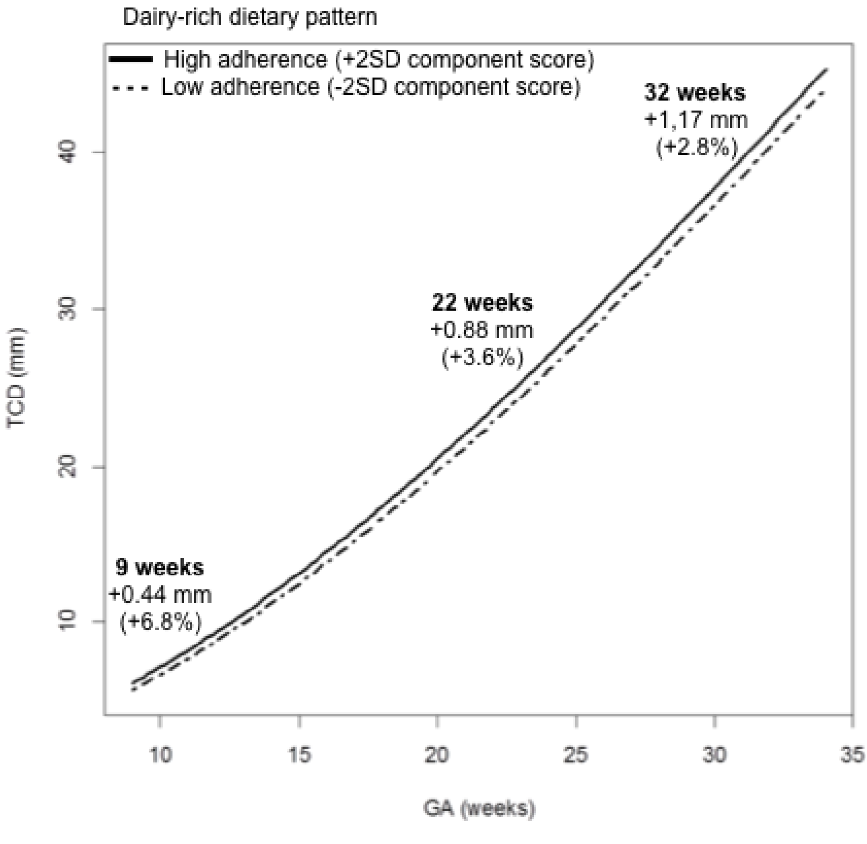
Univariable linear regression was performed to evaluate correlations between nutrient intake and dietary patterns: * $p<0.05$; ** $p<0.01$.
ALA: alpha-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

Table 4. Effect estimates from linear mixed models for the associations between periconceptional maternal dietary patterns and embryonic and fetal cerebellar growth trajectories, as a function of gestational age.

	EFFECT ESTIMATES TCD β (95%CI), √mm			
Dietary pattern	**Model 1**	**p-value**	**Model 2**	**p-value**
Mediterranean	0.00 (-0.01; 0.01)	0.78	0.00 (-0.01; 0.02)	0.94
Western	-0.01 (-0.02; 0.00)	0.17	-0.01 (-0.02; 0.01)	0.44
Egg-rich	-0.00 (-0.01; 0.01)	0.86	0.00 (-0.01; 0.01)	0.99
Dairy-rich	0.02 (0.01; 0.03)	**<0.01**	0.02 (0.01; 0.03)	**<0.01**

Effect estimates represent the amount of change in square root transcerebellar diameter (TCD, √mm) per unit of increase in component score. Model 1 is adjusted for energy intake. Model 2 is adjusted for additional potential confounding factors (energy intake, conception mode, alcohol, smoke, parity, age, BMI, folic acid supplement/multivitamin use, geographical origin).
TCD: transcerebellar diameter; CI: confidence interval.

Figure 2. Associations between the adherence to the periconceptional maternal dairy-rich dietary pattern and longitudinal transcerebellar diameter (TCD) measurements after transformation to the original scale (mm) in the study population using model 2.



The adherence to the dietary pattern is expressed as -2 standard deviation (SD) (weak) or +2SD (strong) in component score. Full adjustment, including energy intake, conception mode, alcohol, smoke, parity, age, BMI, folic acid supplement/multivitamin use and ethnicity, was performed. GA: gestational age; CI: confidence interval.

The subgroup analysis on term newborns with normal intrauterine growth trajectory and birth weight (5^{th} – 95^{th} percentile, n= 115) did not materially change the associations, showing increased TCD measurements in association with the dairy-rich dietary pattern for both model 1 (β=0.02 √mm (95% CI: 0.00; 0.03), $p<0.05$) and 2 (β=0.02 √mm (95% CI: 0.00; 0.03), $p<0.05$).

Discussion

This study shows a positive association between maternal periconceptional adherence to a dairy-rich dietary pattern and prenatal cerebellar growth trajectories assessed by longitudinal TCD measurements from the first trimester onwards. The positive association was confirmed among the subgroup of term pregnancies with normal intrauterine growth trajectory and birth weight. The effect of maternal strong compared to weak adherence to the dairy-rich dietary pattern was higher in the first trimester than later in pregnancy, possibly meaning that additional factors and exposures occurring in the second half of pregnancy could reduce the impact of an early nutritional exposure on cerebellar growth. TCD measurements and trajectories of the study population (figure 1) were comparable to the reference curves previously provided for normal pregnancies (Vinkesteijn et al., 2000). Finally, the association with higher micronutrient intakes and lower tHcy concentrations supports the healthy nature of the dairy-rich dietary pattern. This is also supported by baseline characteristics of women who highly adhered to the dairy-rich dietary pattern. In fact, despite we did not perform comparisons among groups with high adherence to different dietary patterns due to the overlap resulting from the PCA method itself, women with strong adherence to the dairy-rich dietary patterns (component score>0) were generally highly educated, often multiparous, older and with a low percentage of smoking habit.

The major strength of our study is the longitudinal assessment of human embryonic and fetal cerebellar size with a median of four scans per pregnancy. In this study we collected maternal FFQs at enrolment in early pregnancy, covering maternal dietary habits during the periconceptional period with the first and most important stages of cerebellar development. We used PCA in order to extract dietary patterns from FFQs, avoiding hypothesis-driven definitions of dietary patterns (Hoffmann et al., 2004). In this way, this study provides a more complex and comprehensive evaluation of periconceptional maternal nutrition, without focusing on single nutrient or food group intake. Moreover, we checked dietary pattern validity through micronutrient intake and blood biomarkers. To minimize confounding, we adjusted for maternal confounding factors associated with embryonic and cerebellar growth, showing comparable effects in both crude and adjusted models. Finally, the subgroup analysis on term, non-LBW babies allowed to rule out potential cerebellar injuries leading to cerebellar hypoplasia and impaired growth independently on maternal exposures (Pierson et al., 2016). Inherent to the observational design of the study, also some limitations need to be addressed. 3D US scans were performed to obtain first trimester TCD measurements. We only included ultrasound datasets where the TCD measurements could be optimally performed, resulting in lower success rate than what would be expected in clinical practice, where suboptimal measurements are also accepted. Despite 3D US provides highly accurate biometry and volumetry of embryonic structures, a good quality scan of a lying and still embryo is needed in order to obtain accurate offline TCD measurements. Moreover, the entire embryo was targeted during scanning, without specifically focusing on the cerebellum and possibly resulting in lower success rate of the outcome measurements. It could be discussed that the moderate success rate of first trimester cerebellar measurements may compromise the longitudinal setting of the study (Rousian et al., 2013; Koning et al., 2015). Nevertheless, 64% of patients had two first trimester measurements, 98% had at least two measurements during the whole study period and 93% three or more measurements, leading to a good modeling of cerebellar growth trajectories. As outcome measurement, a single cerebellar diameter was taken into account as a proxy of cerebellar growth and development. Despite additional length and volume measurements could certainly improve the study method and the detection rate of growth abnormalities, we decided to focus on the standardized and most used cerebellar measurement in clinical settings, increasing the generalizability of our results. Furthermore, TCD still represents the most representative brain measurement in case of maternal exposures leading to cerebellar dysfunction, being for instance an essential part of early strategies for *in utero* detection of fetal alcohol spectrum disorders (Montag et al., 2016). As dietary surveys could be prone to bias, we compared the mean FIL of the study population with a PAL cutoff for a sedentary lifestyle of 1.35, reducing the likelihood of underreporting. Despite advantages of PCA, the labeling of dietary patterns and the choice of predefined food groups still remain subjective. As we administered FFQs as early as the first trimester, we cannot exclude subsequent variations in maternal dietary habits possibly impacting late cerebellar growth trajectories. Nevertheless, previous studies showed an overall stability of maternal dietary patterns before and during pregnancy, meaning that an early first trimester nutritional assessment may provide a valid representation of maternal diet over pregnancy (Crozier et al., 2009). Finally, the setting of this cohort study in a tertiary hospital, with high educational level and high rates of chronic comorbidity, pregnancy complications and folic acid supplement use, could reduce the external validity of our findings. This setting also justifies the high percentage of IVF/ICSI pregnancies in the study population and our decision to include them, as well as preterm and LBW babies, as a reliable and representative picture of a tertiary hospital population. Adjustment for conception mode and additional exclusion of preterm/LBW babies were performed to reduce selection bias, but further research is warranted to confirm the associations among low-risk pregnancies. Finally, our study showed that a dairy-rich dietary pattern is significantly associated with cerebellar growth trajectory in a multi-adjusted model including geographical origin. Despite adjustment, further research should evaluate how a dairy-rich, north European typical, dietary pattern impacts on cerebellar development among large non-Caucasian population.

Our findings are in line with previous results showing significant associations between maternal preconceptional initiation of folic acid supplements and increased first trimester cerebellar growth as a function of CRL (β = 0.26 mm (95%CI = 0.02-0.49), $p<0.05$) (Koning et al., 2015). Here we show a first trimester TCD increase by 0.44 mm in case of periconceptional strong adherence to the dairy-rich dietary pattern, with long-term effects of early nutritional exposures occurring also during the second and third trimesters of pregnancy. Recently, cerebellar growth trajectories have been shown to be significantly associated with maternal preconceptional BMI in the same population (Koning et al., 2017). Compared to the reference group, overweight women had 4.6%, 3.5%, 1.2%, 1.0% and 0.7% smaller TCD at 9, 11, 22, 26 and 32 weeks respectively. Here we show a double relative difference in case of low *versus* high adherence to a dairy-rich dietary pattern, including adjustment for preconceptional BMI and energy intake. In a prospective study comparing heavy alcohol pregnant consumers to controls, a significant reduction as high as 14% was shown in a single third trimester routine TCD measurement (Handmaker et al., 2006). To our knowledge, no other data are available on the association between prenatal maternal nutrition and human cerebellar growth.

Maternal nutrition has been widely associated with fetal brain development. Animal models of maternal protein and global nutrient restriction showed volume loss, decreased cortical myelination and increased post-mitotic cell death in the cerebellum, with long-term suboptimal performances on behavioral tasks (Ranade et al., 2012; Hunter et al., 2016). Besides proteins, maternal micronutrient intake has also been shown to play an important role in fetal brain development, including vitamins B6, B12, E, A, folate and zinc, all significantly associated with the dairy-rich dietary pattern. Possible mechanisms are direct modulation of cell differentiation, neurotrophic factor expression and neurotransmitter synthesis, as well as regulation of fetal immune system and gut microbiota development (Marques et al., 2015). Finally, a recent animal model pointed out distinct neonatal cerebellar DNA methylation patterns and gene expression in association with high maternal folic acid supplement use during pregnancy (Barua et al., 2016). As the dairy-rich dietary pattern was associated with higher dietary folate intake providing high amounts of substrates and methyl donors, we speculate that the observed associations may be due to epigenetic modifications impacting gene expression and eventually cerebellar growth. Impaired cerebellar methylation patterns and growth have been detected in *post-mortem* human models of autism disorders, however the clinical implications of small increases in prenatal cerebellar growth trajectories need further investigation (Shpyleva et al., 2014).

In conclusion, we showed that maternal periconceptional adherence to a dairy-rich dietary pattern is associated with slightly increased prenatal cerebellar growth trajectories assessed by longitudinal TCD measurements. We are aware that additional cerebellar measurements and the investigation of neurodevelopmental outcomes in the offspring will contribute to gain new insights into pathophysiological mechanisms of cerebellar development. In early future, intrauterine programming of the cerebellar epigenome through maternal nutrition and exposures might be relevant for the pathophysiology and prevention of several neurologic and psychiatric disorders.

S1 table. Maternal baseline characteristics according to dietary pattern high adherence.

Maternal characteristics	High adherence to dietary pattern			
	Mediterranean (n=51)	**Western (n=55)**	**Egg-rich (n=53)**	**Dairy-rich (n=56)**
Maternal age, y median (range)	32 (26-44)	31 (22-45)	33 (22-40)	33 (23-45)
Nulliparous, n(%)	13 (26.0)	17 (29.8)	14 (26.4)	11 (20.0)
Geographical origin, n(%)				
Western	39 (78.0)	50 (87.7)	45 (84.9)	47 (85.5)
Non Western	11 (22.0)	7 (12.3)	8 (15.1)	8 (14.5)
Educational level, n(%)				
High	33 (66.0)	20 (35.1)	29 (54.7)	29 (52.7)
Intermediate	13 (26.0)	29 (50.9)	20 (37.7)	21 (38.2)
Low	4 (8.0)	8 (14.0)	4 (7.5)	5 (9.1)
BMI, kg/m^2 median (range)	22.9 (16.8-39.7)	23.4 (17.7-39.7)	24.3 (17.6-34.9)	22.9 (17.0-39.7)
Alcohol use, n(%)	19 (38.8)	19 (33.9)	17 (32.7)	22 (40.0)
Periconception smoking, n(%)	4 (8.2)	14 (24.6)	10 (18.9)	4 (7.3)
Periconception FA supplement use, n(%)	48 (96.0)	54 (94.7)	52 (98.1)	50 (90.9)
Mode of conception IVF/ICSI, n (%)	16 (31.4)	15 (25.9)	16 (30.2)	17 (30.4)

High adherence to the dietary patters was defined as component score>0. Comparison among group was not performed due to the overlapping between groups as a result of PCA, meaning that a single participant can be highly adherent to two or more dietary patterns at the same time.

BMI: body mass index, IVF: in vitro fertilization, ICSI: intracytoplasmic sperm injection, FA: folic acid.

Part II

The human embryonic morphological development

Chapter 5

Early first trimester maternal 'high fish and olive oil and low meat' dietary pattern is associated with accelerated human embryonic development

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Abstract

Background/Objectives Maternal dietary patterns were associated with embryonic growth and congenital anomalies. We aim to evaluate associations between early first trimester maternal dietary patterns and embryonic morphological development among pregnancies with non-malformed outcome.

Subjects/Methods 228 strictly dated, singleton pregnancies without congenital malformations were enrolled in a periconceptional hospital-based cohort. Principal component analysis was performed to extract early first trimester maternal dietary patterns from food frequency questionnaires. Serial transvaginal three-dimensional ultrasound (3D US) scans were performed between 6^{+0} and 10^{+2} gestational weeks and internal and external morphological criteria were used to define Carnegie stages in a virtual reality system. Associations between dietary patterns and Carnegie stages were investigated using linear mixed models.

Results 726 3D US scans were included (median: three scans per pregnancy). The 'high fish and olive oil and low meat' dietary pattern was associated with accelerated embryonic development in the study population (β=0.12 (95%CI: 0.00; 0.24), $p<0.05$). Weak adherence to this dietary pattern delayed embryonic development by 2.1 days (95%CI: 1.6; 2.6) compared to strong adherence. The 'high vegetables, fruit and grain' dietary pattern accelerated embryonic development in the strictly dated spontaneous pregnancy subgroup without adjustment for energy intake.

Conclusions Early first trimester maternal dietary patterns impacts human embryonic morphological development among pregnancies without congenital malformations. The clinical meaning of delayed embryonic development needs further investigation.

Introduction

Human health is critically dependent on maternal exposures and especially nutrition during pregnancy. Several animal models considered the effect of maternal nutrition during pregnancy, showing interactions with hormonal signaling, placental functioning, fetal growth and metabolism, which further program the foundation for later disease in adult life (Williams et al., 2014; Fleming et al., 2015). More recently, even gametes and early embryos have been recognized to show lasting responses to nutritional programming due to the unique metabolic, epigenetic and developmental events occurring in the periconceptional period (Sinclair et al., 2013; Steegers-Theunissen et al., 2013). Finally, first trimester human embryonic size and growth have been strongly associated with periconceptional maternal dietary patterns and one-carbon biomarkers, supporting the evidence that embryonic growth is not the same in every embryo and pregnancy (Bouwland-Both et al., 2013; van Uitert et al., 2014).

First trimester morphological evaluation provides an extra dimension to gain more insight into embryonic development. The century old Carnegie staging system divides the embryonic period (58 post-conceptional days) into 23 stages of development, taking into account the constant sequence of appearance of internal and external embryonic structures of fixated embryos (O'Rahilly, 1987). Ultrasound techniques additionally enable embryonic staging *in vivo* (Blaas et al., 1998; Blaas, 1999; O'Rahilly et al., 2010). Over the last decade, the combination of transvaginal three-dimensional ultrasound (3D US) and virtual reality (VR) system allows safe, highly precise and automatic crown-rump length (CRL) and embryonic volume (EV) measurements (Verwoerd-Dikkeboom et al., 2008; Rousian et al., 2010). This technology also enables to stage embryonic morphological development according to the Carnegie classification (Verwoerd-Dikkeboom et al., 2008).

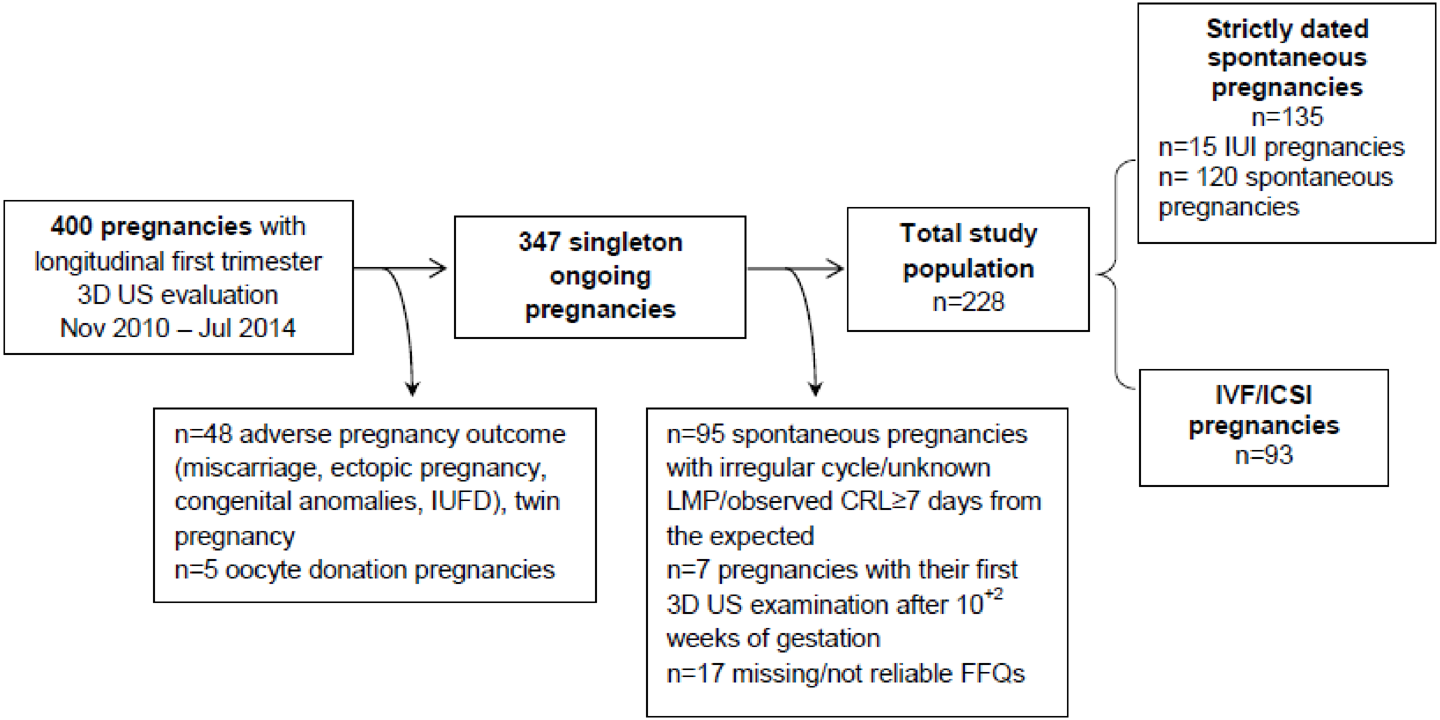
We aim to investigate associations between early first trimester maternal dietary patterns and embryonic morphological development according to the Carnegie stages assessed using 3D US and a VR system.

Subjects and methods

This study was embedded in the ongoing Rotterdam Periconceptional Cohort (Predict Study), a prospective tertiary hospital-based birth cohort, conducted at the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Centre, in Rotterdam, the Netherlands (Steegers-Theunissen et al., 2016). The protocol was approved by the Medical Ethical and Institutional Review Board (METC Erasmus MC 2004-277). All participants provided a written informed consent.

Between November 2010 and July 2014, all women aged at least 18 years old with an intrauterine singleton ongoing pregnancy before 8^{+0} weeks of gestation were eligible for participation. Figure 1 shows the flow chart of the study population. After exclusion of twins, oocyte(s) donation conception, missing or unreliable nutritional evaluations, and pregnancies complicated by miscarriage, ectopic implantation, intrauterine fetal death and congenital anomalies confirmed at birth, we selected spontaneous and intrauterine insemination (IUI) pregnancies with a known first day of last menstrual period (LMP), a self-reported regular cycle and a CRL measurement observed < 7 days different from expected value according to the Robinson curve (strictly dated spontaneous pregnancies) (Robinson, 1973). IUI pregnancies were included in the group of spontaneous pregnancies since the embryo is not exposed to the extrauterine preimplantation environment. Pregnancies achieved after *in vitro* fertilization (IVF), intracytoplasmatic sperm injection (ICSI) and cryo-embryo transfer were also included (IVF/ICSI pregnancies). We further excluded pregnancies with the first 3D US evaluation performed after 10^{+2} weeks of gestation (=58 post-conceptional days, end of embryonic period according to the Carnegie stages). The dating procedure calculates gestational age from LMP for spontaneous pregnancies (with adjustment for cycle duration if <25 or >31 days), from LMP or insemination date plus 14 days for IUI pregnancies, from the day of oocyte retrieval plus 14 days for IVF/ICSI pregnancies and from embryo transfer day plus 17 or 18 days in pregnancies derived from transfer of cryopreserved embryos. In this way, the total study population consisted of strictly dated pregnancies by definition.

Figure 1. Flow chart of the study population.



IUFD: intrauterine fetal death, 3D US: three-dimensional ultrasound, LMP: last menstrual period, CRL: crown-rump length, IUI: intrauterine insemination, IVF: in vitro fertilization, ICSI: intracytoplasmatic sperm injection.

At enrollment, anthropometric measurements (height, weight) and general information on age, ethnicity, educational level, obstetric and medical history and periconceptional exposures (smoking, alcohol use, folic acid or multivitamin supplement use) were collected.
Maternal dietary intake over the previous month was assessed at recruitment using the validated semi-quantitative food frequency questionnaire (FFQ), developed by the division of Human Nutrition, Wageningen University, the Netherlands (Verkleij-Hagoort et al., 2007; Siebelink et al., 2011). The FFQ consists of 196 food items structured according to meal patterns and includes questions on consumption frequency, portion size, and preparation method. Average daily nutritional values were calculated using the Dutch food composition table (Netherlands Nutrition Centre, 2011). The FFQs were finally checked for completeness and consistency. We reduced 196 food items to 11 predefined food groups based on origin and similar nutrient content (cereals, olive oil, solid fat, fish, fruit, grain, vegetables, meat, snacks, sweets, alcohol). Since maternal alcohol consumption impacts embryonic growth and development, we decided to treat it as a potential confounder (van Uitert et al., 2013; Marjonen et al., 2015). Dietary patterns were extracted from the total population with reliable FFQs using the Principal Component Analysis (PCA). This is a standard multivariate statistical technique previously described by Hoffmann et al. that aggregates specific food groups according to the degree in which food items are reciprocally correlated (Hoffmann et al., 2004; Vujkovic et al., 2007; Bouwland-Both et al., 2013). Only the first three dietary patterns (principal components) with eigenvalues ≥1.1 were extracted, reducing bias of multiple testing. By performing PCA, a factor loading is automatically calculated for each food group, showing the extent to which each food group is correlated with the specific dietary pattern. Three factor loadings with the highest absolute value were used to label the dietary patterns. Finally, PCA automatically assigns to all women a component score for each extracted dietary pattern, representing their adherence to that specific dietary pattern.
Since the FFQ was validated for the assessment of folate and vitamin B12 intake, fasting venous blood samples for serum and red blood cell (RBC) folate, serum vitamin B12 and plasma total homocysteine (tHcy) determination were collected at enrollment. The laboratory procedures have been extensively described elsewhere (van Uitert et al., 2014).

Trained examiners performed longitudinal 3D US examinations between 6^{+0} and 10^{+2} weeks of gestation on a weekly or two weekly basis with a high frequency (4.5 – 11.9 MHz) vaginal probe of the GE Voluson E8 (GE, Zipf, Austria). The obtained 3D datasets were stored as Cartesian volumes and transferred to the BARCO I-Space VR system at the Department of Bioinformatics, Erasmus MC, Rotterdam. The I-Space provides real depth perception by projecting stereoscopic images on three walls and the floor, whereas the V-Scope volume rendering application creates a floating hologram of the 3D US image as previously described (Rousian et al., 2011). The Carnegie criteria, including the development of the limbs (arms and legs), the curvature of the embryo and the brain cavity development, were used to stage all embryos by the same experienced observer (O'Rahilly, 1987; O'Rahilly et al., 2010). A complete staging assessment using VR took 1-2 minutes.

Statistical analysis

Maternal characteristics and adherence to the dietary patterns based on component scores were compared between included and excluded pregnancies using Chi-square or exact tests for ordinal variables and Mann-Whitney U test for continuous variables. Serum and RBC folate, vitamin B12, and tHcy concentrations were compared between women with strong (component scores >0) and weak (component scores <0) adherence to each dietary pattern using Mann-Whitney U-tests. The food intake level (FIL) was calculated as the ratio of energy intake divided by basal metabolic rate (BMR) and compared with a physical activity level (PAL) of a sedentary lifestyle (new Oxford equations stratified by age) (Ramirez-Zea, 2005).

To estimate associations between maternal dietary patterns and serial Carnegie stages, we created a continuous score of the stages as response variable that was censored at a maximum value of 23 (which represent the last Carnegie stage ending at 10^{+2} weeks of gestation). Separate linear mixed models were estimated for the total study population and strictly dated spontaneous and IVF/ICSI pregnancy subgroups in order to reduce possible effects of conception mode on embryonic development and responses. This analysis allows to model repeated measurements, further considering potential confounders

(parity, alcohol use, smoking, folic acid/multivitamin supplement use, age, BMI, chronic disease and fetal gender). Model 1 presents the estimates of a crude (gestational age adjusted) and a fully adjusted analysis, without adjustment for energy intake. Model 2 depicts the estimates of the crude and fully adjusted analyses including adjustment for energy intake. Finally, in order to fully understand the results from a clinical perspective, the estimates of embryonic development expressed in days were determined comparing women with strong (+2 standard deviations (SD) in component score) and weak (-2 SD in component score) adherence to the dietary patterns that were significantly associated with the Carnegie stages in the fully adjusted models.
P-values ≤0.05 were considered significant. All analyses were performed using IBM SPSS version 21.0 (Armonk, NY: IBM Corp) and R version 3.2.1 (The R Foundation for Statistical Computing).

Results

A total of 228 pregnancies were included in the analysis, consisting of 135 strictly dated spontaneous pregnancies and 93 IVF/ICSI pregnancies. The median gestational age at enrollment was 7^{+1} weeks (50 days, range: 42-67 days) and a median of three scans per pregnancy (range: 1-5) was performed resulting in a total of 726 3D US datasets. Carnegie stages could be annotated in 589 datasets of good enough quality (81.1%).
Table 1 lists maternal characteristics at baseline and birth outcomes, showing significant differences in maternal age and BMI, folic acid supplement use, chronic diseases, and serum and RBC folate between included and excluded pregnancies. Furthermore, the subgroup of IVF/ICSI pregnancies were older, highly educated, more often nulliparous, with lower BMI and alcohol consumption, and higher serum and RBC folate concentrations compared to the strictly dated spontaneous and excluded pregnancies.

Table 1. Maternal characteristics at enrollment and birth outcomes.

Maternal characteristics	Total study population (n=228)	M	Excluded pregnancies (n=124)	M	p
Age, y, median (range)	32 (22-44)	0	30 (21-41)	0	**0.02**
Nulliparous, n(%)	73 (32.0)	0	40 (32.3)	4	0.80
Geographical origin		1		5	0.55
Western, n(%)	205 (90.3)		105 (88.2)		
Non Western, n(%)	22 (9.7)		14 (11.8)		
Educational level		1		5	0.07
High, n(%)	133 (58.3)		67 (54.0)		
Intermediate, n(%)	90 (39.5)		48 (38.7)		
Low, n(%)	4 (1.8)		4 (3.2)		
BMI, kg/m^2 median (range)	24.2 (17.0-42.3)	1	25.8 (17.8-45.0)	2	**0.02**
Alcohol use, n(%)	84 (37.0)	1	37 (31.9)	8	0.35
Periconception smoking, n(%)	32 (14.1)	1	21 (18.1)	8	0.33
Periconception FA/multivitamin use, n(%)	220 (97.8)	3	112 (93.3)	4	**0.04**
Chronic diseases, n(%)	24 (10.5)	0	23 (18.5)	0	**0.05**
Vitamin B12 (pmol/l) median (range)	297 (95-953)	7	305 (109-915)	13	0.90
Serum folate (nmol/l) median (range)	39.0 (11.0-141.6)	33	34.5 (11.7-187.0)	23	**0.01**
tHcy (µmol/l) median (range)	6.4 (3.7-17.6)	8	6.4 (3.4-13.6)	18	0.58
RBC folate (nmol/l) median (range)	1403 (541-2610)	19	1310 (634-2811)	17	**0.03**
Birth outcomes					
Newborn gender, male n (%)	116 (53.2)	10	50 (43.9)	10	0.11
Birth weight, g median (range)	3370 (1200-5040)	11	3340 (950-4650)	11	0.77
GA at birth, days median (range)	275 (193-295)	11	275.5 (173-292)	20	0.72

The total study population includes strictly dated pregnancies achieved after spontaneous/IUI conception (n=135) or IVF/ICSI (n=93). Excluded pregnancies include oocyte(s) donation (n=5), missing 3D US scans before 10^{+2} weeks of gestation (n=7) or reliable FFQs (n=17) and spontaneous pregnancies with discordant CRL measurements

(≥7 days), unknown LMP or self-reported irregular cycle (n=95). Chronic diseases include cardiovascular, autoimmune, endocrine and metabolic diseases. The comparison was performed using Chi-square or exact tests for ordinal variables and Mann-Whitney U test for continuous variables.
M: missing values, BMI: body mass index, RBC: red blood cell, tHcy: total homocysteine, FA: folic acid, GA: gestational age.

Three dietary patterns were obtained by using PCA, explaining 46.8% of the overall maternal dietary intake variance in the total population (Table 2). We labeled: the first component the ‘high vegetables, fruit and grain’ dietary pattern (explained variance 18.5%); the second component the ‘high calorie solid fat, snacks and sweets’ dietary pattern, also highly associated with low intake of fruit (explained variance 17.4%); the third component the ‘high fish and olive oil and low meat’ dietary pattern (explained variance 10.9%). There were no differences in the adherence (component scores) to the three extracted dietary patterns between included and excluded women. Women with strong adherence (component score>0) to the ‘high vegetables, fruit and grain’ dietary pattern showed significantly higher vitamin B12 concentrations compared to women with weak adherence (component score<0) to the same dietary pattern (334 pmol/l (range 95-713) and 278 pmol/l (range 120-953) respectively, $p<0.01$). Strong adherence to the ‘high calorie solid fat, snacks and sweets’ dietary pattern was associated with significantly lower serum folate concentrations compared to weak adherence (38.0 nmol/l (range 13.2-118.1) and 39.2 nmol/l (range 11.0-141.6) respectively, $p<0.05$). The adherence to the ‘high fish and olive oil and low meat’ dietary pattern was not associated with the investigated blood biomarker concentrations.

Table 2. Factor loadings food groups of the periconceptional maternal dietary patterns.

Dietary pattern	**High vegetables, fruit and grain**	**High calorie solid fat, snacks and sweets**	**High fish and olive oil and low meat**
Variance explained (%)	18.5	17.4	10.9
Cereals	0.141	-0.269	0.038
Olive Oil	0.231	0.205	0.469
Solid fat	0.277	0.614	-0.262
Fish	0.461	-0.034	0.650
Fruit	0.580	-0.365	-0.007
Grain	0.579	0.379	0.018
Vegetables	0.775	-0.068	0.105
Meat	0.206	0.189	-0.750
Snacks	-0.075	0.699	0.029
Sweets	-0.079	0.620	0.041

The factor loadings describe how strong the association between the food groups and each of the extracted dietary patterns is. The factor loadings with the highest absolute values were used for labeling. The three dietary patterns together explained 46.8% of the total variance in maternal dietary intake.

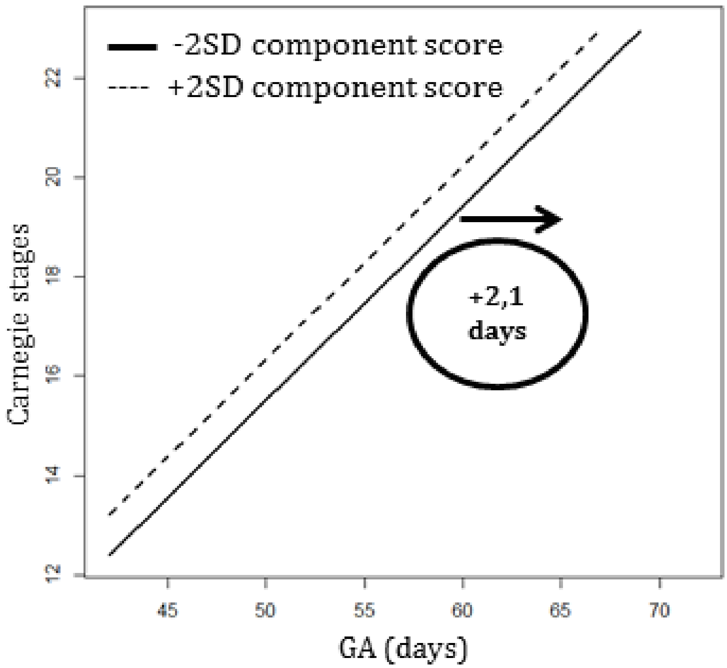
Linear mixed models were firstly estimated to evaluate associations between conception mode and Carnegie stages, showing no significant results (group effect, fully adjusted model: β=0.20 (95% CI: -0.05; 0.46), p=0.12). Energy intake was positively correlated to the 'high vegetables, fruit and grain' and ' high calorie solid fat, snacks and sweets' dietary patterns ($\rho=0.44$, $p<0.001$ and $\rho=0.63$, $p<0.001$, respectively) and significantly associated with the Carnegie stages in the multivariable adjusted model among strictly dated spontaneous pregnancies (β =7.9^10^{-5} (95% CI: 3.3^10^{-6}; 1.5^10^{-4}, p=0.04). Table 3 shows the associations between maternal dietary patterns and Carnegie stages of embryonic development. In the fully adjusted model 1, the 'high vegetables, fruit and grain' dietary pattern was associated with accelerated embryonic development in strictly dated spontaneous pregnancies, whereas the ‘high fish and olive oil and low meat’ dietary pattern was positively associated with embryonic development in the total study population and strictly dated spontaneous pregnancies. After adjustment for energy intake (fully adjusted model 2), the ‘high vegetables, fruit and grain’ dietary pattern attenuated to non-significance, whereas the ‘high fish and olive oil and low meat’ dietary pattern remained significantly associated with accelerated embryonic development in the total study population and strictly dated spontaneous pregnancies. Figure 2 shows the average regression lines for the ‘high fish and olive oil and low meat’ dietary pattern among strictly dated spontaneous pregnancies (fully adjusted model 2). In this subgroup, weak adherence (defined as -2 standard deviations (SD) in component score) to the dietary pattern delayed embryonic development by 2.1 days (CI 95%: 1.6; 2.6) compared to strong adherence (+2 SD in component score). The ‘high calorie solid fat, snacks and sweets’ dietary pattern showed no significant associations with embryonic development.

Table 3. Effect estimates of the associations between periconceptional maternal dietary patterns and the Carnegie stages derived from linear mixed models.

Dietary pattern	EFFECT ESTIMATES CARNEGIE STAGES β (95%CI)			
	MODEL 1		**MODEL 2**	
	Crude	**Full adjusted**	**Crude**	**Full adjusted**
TOTAL POPULATION (n=228)				
High vegetables, fruit and grain	0.08 (-0.04; 0.20)	0.12 (0.00; 0.24)	0.08 (-0.06; 0.21)	0.10 (-0.04; 0.24)
High calorie solid fat, snacks and sweets	0.03 (-0.09; 0.15)	0.03 (-0.12; 0.15)	-0.02 (-0.18; 0.15)	-0.06 (-0.22; 0.10)
High fish and olive oil and low meat	0.09 (-0.04; 0.22)	**0.13 (0.01; 0.26)***	0.08 (-0.04; 0.21)	**0.12 (0.00; 0.24)***
STRICTLY DATED SPONTANEOUS PREGNANCIES (n=135)				
High vegetables, fruit and grain	**0.18 (0.00; 0.36)***	**0.22 (0.04; 0.40)***	0.12 (-0.10; 0.34)	0.15 (-0.07; 0.37)
High calorie solid fat, snacks and sweets	0.10 (-0.08; 0.28)	0.10 (-0.08; 0.28)	-0.04 (-0.29; 0.21)	-0.08 (-0.34; 0.17)
High fish and olive oil and low meat	**0.20 (0.02; 0.38)***	**0.23 (0.05; 0.41)****	**0.19 (0.00; 0.37)***	**0.22 (0.04; 0.40)***
IVF/ICSI PREGNANCIES (n=93)				
High vegetables, fruit and grain	-0.04 (-0.17; 0.09)	0.02 (-0.12; 0.16)	0.01 (-0.14; 0.15)	0.05 (-0.09; 0.19)
High calorie solid fat, snacks and sweets	-0.08 (-0.22; 0.06)	-0.07 (-0.21; 0.07)	0.01 (-0.18; 0.19)	-0.03 (-0.22; 0.14)
High fish and olive oil and low meat	-0.06 (-0.21; 0.09)	-0.05 (-0.20; 0.10)	-0.05 (-0.20; 0.10)	-0.04 (-0.19; 0.10)

Effect estimates represent the change in Carnegie stage per unit of increase of dietary component score. Model 1 excludes adjustment for energy intake and model 2 is adjusted for energy intake. The crude analysis is adjusted for gestational age only. The models with full adjustments include additional confounders (parity, alcohol use, smoking habit, folic acid supplement use, maternal age, BMI and chronic diseases, fetal gender). CI: confidence interval. *p≤0.05, **p≤0.01

Figure 2. Average regression lines for the 'high fish and olive oil and low meat' dietary pattern in strictly dated spontaneous pregnancies.



In the fully adjusted model 2, weak adherence to the 'high fish and olive oil and low meat' dietary pattern (-2 standard deviation (SD) in component score, corresponding to -1.88) delays embryonic development by 2.1 days (95% CI: 1.6-2.6) compared to strong adherence (+2SD in component score, corresponding to 1.89). Full adjustment includes parity, alcohol use, smoking habit, folic acid supplement use, maternal age, BMI and chronic diseases, fetal gender. GA: gestational age (days).

Discussion

This study shows that early first trimester maternal adherence to the 'high fish and olive oil and low meat' dietary pattern accelerates embryonic development according to the Carnegie stages in the total study population and subgroup of strictly dated spontaneous pregnancies. The 'high vegetables, fruit and grain' dietary pattern is associated with embryonic development among strictly dated spontaneous pregnancies, only when adjustment for energy intake is not performed. The latter suggests that energy intake has a stronger effect on embryonic morphological development than the 'high vegetables, fruit and grain' dietary pattern itself. Our findings integrate previous results showing that periconceptional maternal adherence to the 'high fish and olive oil and low meat' dietary pattern is associated with increased serial CRL and EV measurements among strictly dated spontaneous pregnancies (Parisi et al., 2017). Moreover, the association between strong adherence to an energy-rich dietary pattern and late embryonic growth and fetal development are herewith in line (Bouwland-Both et al., 2013).

This study has several strengths. A detailed picture of embryonic development is provided by longitudinal measurements and high success rate of the Carnegie stage annotation using 3D US and VR system. We reduced confounding of gestational age by including women with a strict pregnancy dating. In order to reduce selection bias, we compared baseline characteristics between included and excluded women and further adjusted the analysis for multiple maternal covariates, including BMI, age and chronic disease. Differences in serum and RBC folate concentrations may be related to the inclusion of the IVF/ICSI population highly educated, with lower alcohol consumption and BMI. We used PCA to extract dietary patterns with the advantage of avoiding *a priori* and subjective definition of dietary patterns (Hoffmann et al., 2004). Moreover, the assessment of maternal blood biomarkers checked the extracted dietary patterns for consistency. Inherent to the observational design of the study, also some limitations need to be addressed. Nutritional surveys could be prone to bias. We tested underreporting using a PAL cutoff of 1.35 and we estimated a mean FIL of 1.36 in the total study population, reducing the likelihood of underreporting. Despite advantages of using PCA, the choice of the preselected food groups still remains subjective. Further evaluation of micronutrients associated with the ‘high fish and olive oil and low meat’ dietary pattern, including omega-3 and omega-6 fatty acids, are needed to elucidate mechanisms impacting embryonic development. Finally, this cohort study is embedded in a tertiary hospital, and the high rates of comorbidity and pregnancy complications could reduce the external validity of our findings.

The association between fish and olive oil consumption is a returning issue in reproductive research. Fish intake has been widely associated with intrauterine and long-term child growth (Rogers et al., 2004; Thorsdottir et al., 2004; Stratakis et al., 2016). Recent studies also revealed associations with the likelihood of blastocyst formation and increased embryo morphology scores in the IVF/ICSI population (Hammiche et al., 2011; Braga et al., 2015). Moreover, a one-carbon dietary pattern, also rich in fish and seafood, was associated with a reduction of congenital heart defects (Obermann-Borst et al., 2011). Fish intake may affect embryonic development both directly, by reducing the oxidative burden through increased availability of omega-3 fatty acids in embryonic structures, and indirectly, by altering maternal immune response and tissue remodeling of the endometrium (Ornoy et al., 2007; Waters et al., 2012; Ramaiyan et al., 2016). Nevertheless, controversy still remains about the beneficial effect of fish consumption during pregnancy, mainly attributable to the harmful effect of fish contaminants (Sidhu, 2003). In our population, median fish intake was 247 g/week, which is below the Dutch recommendation and therefore possibly protective against harmful effects of fish contaminants. Moreover, the ‘high fish and olive oil and low meat’ dietary pattern also showed high intake of olive oil and low intake of meat. In diabetic animal models of early organogenesis, maternal diet enriched in olive oil was shown to modulate the expression of genes highly involved in embryonic and endometrial oxidative and inflammatory pathways, leading to a possible reduction of congenital anomalies (Higa et al., 2014). Recent data also showed that processed meat intake is associated with reduced fertilization rates after IVF and increased risk of small for gestational age babies (Knudsen et al., 2008; Xia et al., 2015). The lower exposure to saturated fats and the expected higher omega-3/omega-6 fatty acid ratio in case of strong adherence to the ‘high fish and olive oil and low meat’ dietary pattern may contribute to the improvement in embryonic development (Larqué et al., 2001).

This study showed no significant associations among IVF/ICSI pregnancies. This could be explained by the smaller sample size of this subgroup. Nevertheless, it is plausible that the extrauterine development of the preimplantation embryo, the use of culture media and subfertility itself might have a stronger effect on embryonic development than the adherence to a specific dietary pattern (Mantikou et al., 2013; Zhu et al., 2014; Holst et al., 2016; Jacques et al., 2016).

In conclusion, maternal strong adherence to the ‘high fish and olive oil and low meat’ dietary pattern in early first trimester was shown to slightly but significantly accelerate human embryonic morphological development among pregnancies without congenital malformations. Several studies demonstrated that embryonic growth differs among strictly dated pregnancies and is strongly associated with subsequent pregnancy and health outcomes (Bottomley et al., 2009; Mook-Kanamori et al., 2010; van Uitert et al., 2013; Jaddoe et al., 2014; van Uitert et al., 2014). In particular, increased human embryonic growth assessed by CRL measurements was associated with improved fetal size and birth weight, lower risk of preterm birth, and reduced cardiovascular risk in childhood (Mook-Kanamori et al., 2010; Jaddoe et al., 2014). This emphasizes that the CRL, as single or repeated measurement, represents an important early outcome, positively impacting subsequent health. Here we show that maternal dietary patterns, besides the well-known association with congenital anomalies, are also associated with normal embryonic development according to the Carnegie stages (Vujkovic et al., 2009; Steegers-Theunissen et al., 2013). Our results substantially increase the body of evidence underlining the importance of maternal nutrition not only for fetal growth and pregnancy outcome, but also for first trimester outcome. Moreover, in line with embryonic growth, it could be hypothesized that accelerated embryonic morphological development can impact future outcome as well. However, further research is crucial in order to determine the clinical meaning for birth and long-term outcomes (Gluckman et al., 2004; Skinner, 2008; Steegers-Theunissen et al., 2013; Steegers-Theunissen et al., 2015). These findings could underline that periconceptional care should be part of a health care program aiming not only to ensure a proper reproductive outcome, but also to improve general health of future generations.

Chapter 6

Periconceptional maternal one-carbon biomarkers are associated with embryonic development according to the Carnegie stages

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Abstract

Study question: Is periconceptional maternal one-carbon (I-C) metabolism associated with embryonic morphological development in non-malformed ongoing pregnancies?

Summary answer: Serum vitamin B12, red blood cell (RBC) folate and plasma total homocysteine (tHcy) are associated with embryonic development according to the Carnegie stages.

What is known already: Derangements in maternal I-C metabolism affect reproductive and pregnancy outcomes, as well as future health of the offspring.

Study design, size, duration: Between 2010 and 2014, women with singleton ongoing pregnancies were enrolled in a prospective periconceptional cohort study.

Participants/materials, setting, methods: 234 pregnancies, including 138 spontaneous pregnancies with strict pregnancy dating and 96 pregnancies derived from *in vitro* fertilization (IVF), intracytoplasmatic sperm injection (ICSI) or cryo-embryo transfer (IVF/ICSI pregnancies), underwent longitudinal transvaginal three-dimensional ultrasound (3D US) scans from 6^{+0} up to 10^{+2} weeks of gestation. Carnegie stages were defined using internal and external morphologic criteria in a virtual reality system. Maternal venous blood samples were collected at enrolment for serum vitamin B12, RBC folate and plasma total homocysteine (tHcy) assessment. Associations between biomarker concentrations and longitudinal Carnegie stages were investigated using linear mixed models.

Main results and the role of chance: We performed a median of three 3D US scans per pregnancy (range 1-5) resulting in 600 good quality datasets for the Carnegie stage annotation (80.5%). Vitamin B12 was positively associated with embryonic development in the total study population (β=0.001 (95% CI: 0.000; 0.002), $p<0.05$) and in the subgroup of strictly dated spontaneous pregnancies (β=0.002 (95% CI: 0.001; 0.003), $p<0.05$). Low vitamin B12 concentrations (-2 standard deviation (SD), 73.4 pmol/l) are associated with delayed embryonic development by 1.4 days (95% CI: 1.3-1.4) compared to high concentrations (+2SD, 563.1 pmol/l). RBC folate was positively associated with Carnegie stages only in IVF/ICSI pregnancies (β=0.001 (95% CI: 0.0005; 0.0015), $p<0.05$). Low RBC folate concentrations (-

2SD, 875.4 nmol/l) were associated with a 1.8-day delay (95% CI: 1.7-1.8) in development compared to high concentrations (+2SD, 2119.9 nmol/l). tHcy was negatively associated with embryonic development in the total study population (β = -0.08 (95% CI: -0.14; -0.02), $p<0.01$), as well as in the IVF/ICSI subgroup (β= -0.08 (95% CI: -0.15; -0.01), $p<0.05$). High tHcy concentrations (+2SD, 10.4 μmol/l) were associated with a delay of 1.6 days (95% CI: 1.5-1.7) in embryonic development compared to low concentrations (-2 SD, 3.0 μmol/l).

Limitations, reasons for caution: The study was performed in a tertiary care centre, resulting in high rates of folic acid supplement use and comorbidity that may reduce the external validity of our findings.

Wider implications of the findings: In periconceptional care, maternal I-C biomarkers should be taken into account as predictors of embryonic morphological development. Combining embryonic size measurements with morphological assessment could better define normal embryonic development.

Introduction

One-carbon (I-C) metabolism is known to play a crucial role in cellular metabolism and proliferation, as well as in the regulation of gene expression through epigenetic mechanisms. Useful biomarkers of I-C metabolism for research and clinical practice are serum vitamin B12 and folate, red blood cell (RBC) folate and plasma total homocysteine (tHcy). Several studies linked maternal I-C biomarkers to reproductive, pregnancy and health outcomes in the offspring (Steegers-Theunissen et al., 2013; Kalhan, 2016; Bergen et al., 2012; Yajnik et al., 2014; van Uitert and Steegers-Theunissen, 2013). Most evidence is available on the association between maternal folate deficiency, folic acid supplement use and congenital anomalies (Steegers-Theunissen et al., 2013). Nevertheless, plasma tHcy concentration seems to be a more sensitive marker, with increased concentrations strongly associated with miscarriage, hypertensive disorders, preterm birth and birth defects (Ronnenberg et al., 2007; Steegers-Theunissen et al., 1991; Hogeveen et al., 2012; Vollset et al., 2000). Due to the increased adherence to a vegetarian diet and frequent association with vitamin deficiency, recent research also focused on the associations between vitamin B12, birth defects and birth weight (Finkelstein et al., 2015).

The introduction of high resolution three-dimensional ultrasound (3D US) scans combined with visualization in immersive virtual reality (VR) systems, providing real depth perception and more sensitive embryonic size measurements and morphological evaluations, has markedly improved the opportunity to accurately study the periconceptional period (time window: 14 weeks pre-conception to 10 weeks post-conception) (Rousian et al., 2010; Baken et al., 2014; Steegers-Theunissen et al., 2013). So far these innovative techniques were used to study embryonic crown-rump length (CRL) and volume (EV) trajectories as non-invasive measures of first trimester embryonic growth (Steegers-Theunissen et al., 2016). On the other hand, the Carnegie stages of human embryonic development were introduced as a century old morphological classification of fixated embryos dividing the embryonic period (58 post-conceptional days) into 23 stages (O'Rahilly et al., 1987). The combination of 3D US and VR visualization will allow us to investigate embryonic morphological development *in vivo,* according to the longitudinal annotation of the Carnegie stages (O'Rahilly and Müller,

2010; Blaas et al., 1998; Verwoerd-Dikkeboom et al., 2008). Despite the fact that the normal sequence of developmental events is constant and predictable in every embryo, different times and velocities can occur, making comparisons possible and worthwhile.
Here, we aim to investigate the associations between periconceptional maternal biomarkers of I-C metabolism and first trimester embryonic development, using serial Carnegie stage annotation obtained by 3D US and VR.

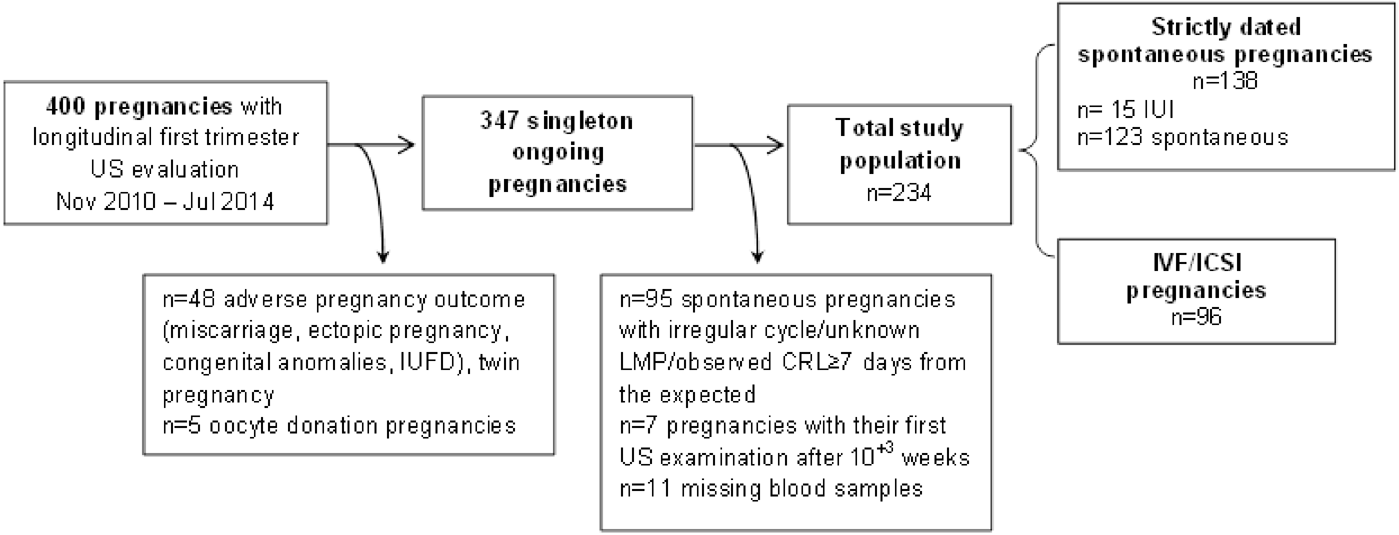
Materials and methods

This study was performed in the setting of the Rotterdam Periconception Cohort (Predict Study), a prospective periconceptional tertiary hospital-based cohort study started in 2009 at the Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Centre, Rotterdam, with the aim to assess periconceptional determinants and predictors of pregnancy outcome and offspring health (Steegers-Theunissen et al., 2016).

Study population and sample

All women before 8^{+0} weeks of gestation who conceived spontaneously, or after intrauterine insemination (IUI), *in vitro* fertilization (IVF), intracytoplasmatic sperm injection (ICSI) or cryopreserved embryo transfer, were eligible for participation between 2010 and 2014 (figure 1). After exclusion for age below 18 years old, twins, miscarriage, ectopic implantation, intrauterine fetal death, congenital anomalies and oocyte(s) donation, 347 singleton ongoing pregnancies were enrolled. Since the Carnegie stages describe embryonic development until the end of the embryonic period (10^{+2} weeks, 58 post-conceptional days), we excluded seven additional pregnancies for missing 3D US scans before 10^{+2} weeks of gestation. Among spontaneously conceived pregnancies, we selected pregnancies with known first day of last menstrual period (LMP), self-reported regular cycle and observed crown-rump length (CRL) measurement corresponding to the expected according to the Robinson curves (<7 days different) (Robinson and Fleming, 1975). The resulting total study population counted 234 pregnancies, consisting of 138 spontaneous or intrauterine insemination (IUI) pregnancies with strict pregnancy dating and 96 IVF/ICSI pregnancies. Gestational age was defined from LMP for spontaneous pregnancies (adjusted for duration of the menstrual cycle if <25 or >31 days), from LMP or insemination date plus 14 days for IUI pregnancies, from the day of oocyte retrieval plus 14 days for IVF/ICSI pregnancies, and from embryo transfer day plus 17 or 18 days in pregnancies derived from transfer of cryopreserved embryos. Therefore, the total study population included only pregnancies with strict and reliable dating by definition. Since a possible influence of conception mode cannot be excluded, we performed the analysis first in the total study population using conception mode as a confounder and we further stratified the analysis to the two subgroups of strictly dated spontaneous and IVF/ICSI pregnancies.

Figure 1. Flow chart of the study population.



IUFD: intrauterine fetal death, US: ultrasound, LMP: last menstrual period, CRL: crown-rump length, IUI: intrauterine insemination, IVF: in vitro fertilization, ICSI: intracytoplasmatic sperm injection.

General data

Self-administered general questionnaires reporting items on age, geographical origin, education, obstetric and medical history, and periconceptional lifestyle (smoking, alcohol consumption, folic acid and multivitamin supplement use) were collected at enrolment. Anthropometric measures were recorded by trained researchers.

Blood sample analysis

One first trimester fasting venous blood sample for serum vitamin B12, RBC folate and plasma tHcy assessment was collected at enrolment and drawn in a vacutainer ethylenediamine tetraacetate (EDTA) tube and in a dry vacutainer tube (BD diagnostics, Plymouth, UK). The dry vacutainer tubes were centrifuged at 2,000 xg, serum was collected and analyzed for vitamin B12 measurement using an immunoelectro-chemoluminescence assay (E170; Roche Diagnostics GmbH, Mannheim, Germany). Plasma was separated by centrifugation within one hour for determination of tHcy by using a sensitive liquid chromatography tandem mass spectrum method (HPLC-Tandem MS, Waters Micromass Quattro Premier XE Mass Spectrometer with Acquity UPLC system, Milford, Massachusetts, United States). EDTA-blood was kept on ice and 0.1 ml EDTA blood was hemolysed with 0.9 ml freshly prepared 1.0% ascorbic acid. The hematocrit was determined with the ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Leverkusen, Germany). RBC folate was calculated with the following formula:

$$(\text{nM hemolysate folate} \times 10/\text{hematocrit}) - [\text{nM serum folate} \times (1-\text{hematocrit})/\text{hematocrit}] = \text{nM RBC folate}.$$

Ultrasound data

From 6^{+0} up to 10^{+2} weeks of gestation, all included women underwent serial 3D US scans performed by trained researchers using the high frequency (4.5 – 11.9 MHz) vaginal probe of a GE Voluson E8 (GE Healthcare, Zipf, Austria). Ultrasound scans were performed on a weekly basis until 2013 and then reduced to a two weekly-basis after the pilot study showed an accurate modelling of growth trajectory obtained with 3 scans per pregnancy (at 7, 9, 11 weeks of gestation) (van Uitert et al., 2013b). The obtained 3D datasets were stored as Cartesian volumes and transferred to the BARCO I-Space VR system at the Department of Bioinformatics, Erasmus University Medical Centre, Rotterdam. This system, running the V-Scope volume rendering application, aims to improve dataset visualization by projecting a hologram in a 4-walled CAVE-like (Cave Automatic Virtual Environment) VR system, allowing full depth perception and intuitive interaction with the volume (Verwoerd-Dikkeboom et al., 2010; Koning et al., 2009). The Carnegie criteria for external and internal morphological characteristics were used by one trained researcher to stage all embryos, as previously described (Verwoerd-Dikkeboom et al., 2008; O'Rahilly and Müller, 2010). As external morphological characteristics we used the Carnegie criteria for the development of limbs (arms and legs) and embryonic curvature. Internal morphological characteristics primarily included the criteria for the brain cavity development. The assessment of Carnegie stages required 1 to 2 minutes per embryo.

Statistical analysis

In order to evaluate selection bias, we compared maternal baseline characteristics and biomarker concentrations between excluded and included pregnancies using Chi-square or exact tests for ordinal variables and Mann-Whitney U test for continuous variables. Univariable linear regression was performed to evaluate associations between maternal baseline characteristics and biomarker concentrations.
To estimate associations between maternal biomarkers of I-C metabolism and embryonic development, we treated the Carnegie stages as a continuous variable that was censored at its maximum value of 23. This was used as the response variable in separate linear mixed models estimated for the total study population and secondly for the subgroups of strictly dated spontaneous and IVF/ICSI pregnancies. This analysis allows the linear modelling of longitudinal measurements, taking into account the existing correlation between serial measurements within the same pregnancy and potential confounders for adjustment (parity, alcohol use, smoking, folic acid/multivitamin supplement use, fetal gender, maternal age, BMI and comorbidity). Firstly, we performed a crude analysis with adjustment for gestational age only (model 1) and secondly we adjusted for additional confounders

(model 2). Finally, the estimates of embryonic developmental change expressed in days were determined comparing women with high (+2 standard deviation (SD)) and low (-2 SD) concentrations of the biomarkers that were significantly associated with the Carnegie stages in model 2. Due to the exclusion of pregnancies with uncertain dating and the possibility of selection bias, we additionally performed a sensitivity analysis including pregnancies with discordant CRL (n=15). P-values ≤0.05 were considered significant. All analyses were performed using IBM SPSS version 21.0 (Armonk, NY: IBM Corp) and R version 3.2.1 (The R Foundation for Statistical Computing).

Ethical approval

The protocol has been approved by the local medical ethics committee and all women signed a written informed consent before participation.

Table I. Maternal baseline characteristics and biomarkers of I-C metabolism.

Maternal characteristics	Total study population (n=234)	M	Excluded population (n=118)	M	p-value
Age, y median (range)	32 (22-42)	0	30 (21-44)	0	**0.00**
Geographical origin		1		5	0.30
Western, n(%)	206 (88.0)		104 (88.1)		
Non Western, n(%)	27 (11.5)		9 (7.6)		
Educational level		1		5	0.08
High, n(%)	135 (57.7)		65 (55.1)		
Intermediate, n(%)	93 (39.7)		45 (38.1)		
Low, n(%)	5 (2.1)		3 (2.5)		
BMI, kg/m^2 median (range)	24.2 (17-42.3)	1	25.8 (17.8-45.0)	2	**0.01**
Nulliparous, n(%)	74 (31.8)	1	39 (33.9)	2	0.69
Alcohol use, n(%)	83 (35.8)	2	38 (34.2)	7	0.78
Periconception smoking, n(%)	32 (13.7)	1	21 (19.1)	8	0.20
Periconception FA multivitamin use, n(%)	224 (97.4)	4	108 (93.9)	3	0.11
Chronic diseases, n(%)	25 (10.7)	0	22 (18.6)		0.05
Vitamin B12 (pmol/l) median (range)	297 (95-953)	0	295.5 (109-915)	20	0.76
RBC folate (nmol/l) median (range)	1408 (541-2811)	12	1294 (634-1942)	23	**0.01**
tHcy (µmol/l) median (range)	6.4 (3.7-17.6)	3	6.2 (3.4-13.6)	23	0.51

The total study population includes strictly dated pregnancies achieved after spontaneous conception (n=138) or IVF/ICSI (n=96). Excluded pregnancies include oocyte(s) donation (n=5), missing 3D US scans before 10^{+2} weeks of gestation (n=7) and spontaneous pregnancies with discordant CRL measurements (≥7 days, n=15), unknown LMP (n=14) or self-reported irregular cycle (n=77). Chronic diseases include cardiovascular, autoimmune, endocrine and metabolic diseases. The comparison was performed using Chi-square or exact tests for ordinal variables and Mann-Whitney U test for continuous variables. M: missing values, BMI: body mass index, FA: folic acid, RBC: red blood cell, tHcy: total homocysteine.

Results

We included 234 pregnancies with a median of three scans per pregnancy (range 1-5), counting for a total of 745 3D US scans. The Carnegie stage annotation was feasible in 600 good quality datasets (success rate 80.5%). Carnegie stage distribution in the total study population ranged from stage 13 to 23 (6^{+0} – 10^{+2} weeks of gestation). Table I shows maternal characteristics and biomarker concentrations at baseline with comparisons between included and excluded ongoing pregnancies. The prevalence of hyperhomocysteinemia in the total study population was 1.3% (> 13 µmol/l). Vitamin B12 was significantly associated with maternal age (β=0.15 pmol/l, (95%CI: 0.13; 0.16), p<0.05), RBC folate (β=0.20 pmol/l, (95% CI: 0.19; 0.20), p<0.01) and tHcy concentrations (β= -0.34 pmol/l, (95% CI: -0.37; -0.32), p<0.001). RBC folate was significantly associated with maternal age (β=0.20 nmol/l, (95% CI: 0.19; 0.21), p<0.01), smoking (β= -0.16 nmol/l, (95% CI: -0.25; -0.07), p<0.05), folic acid supplement use (β=0.23 nmol/l, (95% CI: 0.04; 0.43), p<0.001), comorbidity (β= -0.14 nmol/l, (95% CI: -0.24; -0.04), p<0.05) and tHcy concentrations (β= -0.25 nmol/l, (95% CI: -0.27; -0.24), p<0.001).

Embryonic development

Embryonic development according to the Carnegie stages was comparable between the subgroups of strictly dated spontaneous and IVF/ICSI pregnancies (model 2, group effect: β= -0.20, (95% CI: -0.46; 0.05), p=0.12).

Table II shows the estimates from linear mixed models.

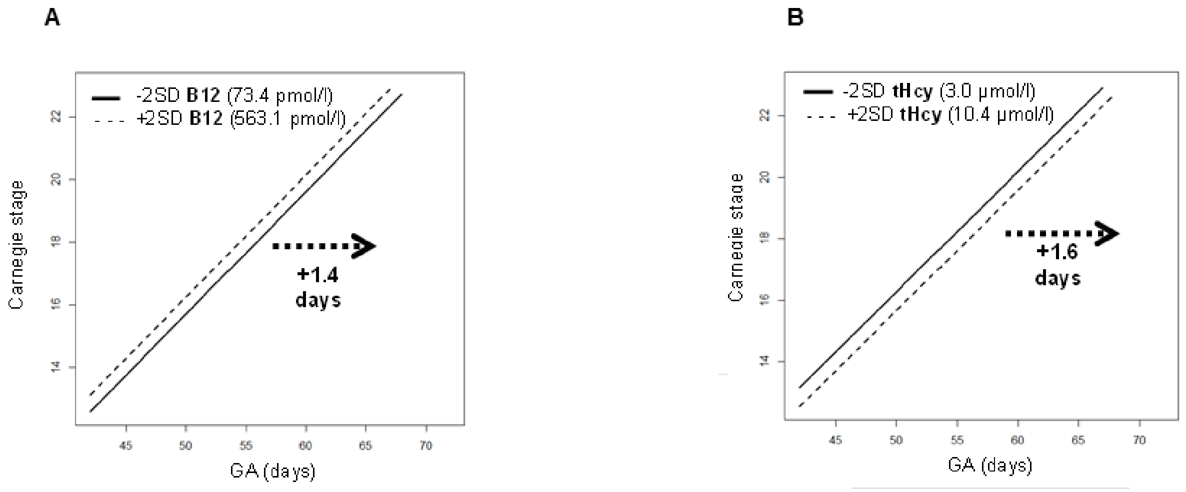
Table II. Maternal biomarker effect estimates for the Carnegie stages of embryonic development derived from linear mixed models.

Biomarkers	EFFECT ESTIMATES CARNEGIE STAGES β (95%CI)	
	Model 1	**Model 2**
Total study population (n=234)		
Vitamin B12	**0.001 (0.000; 0.002) ***	**0.001 (0.000; 0.002) ***
RBC folate	**0.0004 (0.0001; 0.0007) ***	0.000 (0.000; 0.001)
tHcy	**-0.09 (-0.15; -0.03) ****	**-0.08 (-0.14; -0.02) ****
Strictly dated spontaneous pregnancies (n=138)		
Vitamin B12	**0.002 (0.001; 0.003) ***	**0.002 (0.001; 0.003) ***
RBC folate	0.000 (-0.000; 0.001)	0.003 (0.002; 0.004)
tHcy	-0.07 (-0.17; 0.03)	-0.07 (-0.10; 0.02)
IVF/ICSI pregnancies (n=96)		
Vitamin B12	-0.0004 (-0.002; 0.0008)	-0.000 (-0.002; 0.001)
RBC folate	0.000 (-0.000; 0.001)	**0.001 (0.0005; 0.0015) ***
tHcy	**-0.09 (-0.16; -0.02) ****	**-0.08 (-0.15; -0.01) ***

Effect estimates represent the change in Carnegie stage per unit of increase of biomarker concentration. Model 1 shows the crude model with adjustment for gestational age. Model 2 includes adjustment for potential confounders (parity, alcohol use, smoking habit, folic acid use, age, BMI, chronic diseases, fetal gender).
RBC: red blood cell, IVF: *in vitro* fertilization, ICSI: intracytoplasmatic sperm injection, tHcy: total homocysteine; CI: confidence interval. *p<0.05, **p≤0.01.

In model 2, vitamin B12 concentrations were positively associated with embryonic development in the total study population and in strictly dated spontaneous pregnancies, resulting in small, albeit significant estimates. In the total study population, low vitamin B12 concentrations (-2 SD, corresponding to 73.4 pmol/l) were associated with a 1.4-day delay (95% CI: 1.3-1.4) in embryonic development compared to high concentrations (+2SD, corresponding to 563.1 pmol/l) (figure 2A). After full adjustment, RBC folate was positively associated with the Carnegie stages only in the IVF/ICSI subgroup, and low concentrations (-2SD, corresponding to 875.4 nmol/l) were associated with a 1.8-day delay (95% CI: 1.7-1.8) in embryonic development compared to high concentrations (+2SD, corresponding to 2119.9 nmol/l). Finally, tHcy was strongly and negatively associated with the Carnegie stages in the total study population and in the IVF/ICSI subgroup. In the total study population, high tHcy concentrations (+2SD, corresponding to 10.4 µmol/l) were associated with a 1.6-day delay (95% CI: 1.5-1.7) in embryonic development compared to low concentrations (-2SD, corresponding to 3.0 µmol/l) (figure 2B). The sensitivity analysis including pregnancies with discordant CRL (n=15) did not modify the resulting associations (model 2, vitamin B12: β= 0.001, (95% CI: 0.0001 – 0.002), p=0.03; RBC folate: β= 0.000, (95% CI: -0.000 - 0.001), p=0.06; tHcy: β= -0.08, (95% CI: -0.15 - -0.02;), p=0.01).

Figure 2. Average regression lines for vitamin B12 (A) and total homocysteine (tHcy) (B) concentrations in the total study population.



In model 2, a low vitamin B12 (-2 standard deviation (SD), corresponding to 73.4 pmol/l) delays embryonic development by 1.4 days (95% CI: 1.3-1.4) compared to high concentrations (+2SD, 563.1 pmol/l). Conversely, high tHcy concentrations (+2SD, 10.4 µol/L) delay embryonic development by 1.6 days (95% CI: 1.5-1.7) compared to low concentrations (-2SD, 3.0 µmol/l). GA: gestational age.

Discussion

This study shows significant associations between periconceptional maternal biomarkers of I-C metabolism and embryonic morphological development according to the Carnegie classification in ongoing non-malformed pregnancies. Moreover, IVF/ICSI conception did not affect embryonic morphological development compared to spontaneous conception in strictly dated pregnancies. The inclusion of pregnancies with discordant CRL revealed the same associations.

Our results are in line with previous data showing associations between maternal I-C metabolism and several reproductive, pregnancy and health outcomes (Solé-Navais et al., 2016; Yajnik and Deshmukh, 2012). Recently, maternal early pregnancy high tHcy (≥8.31 μmol/L) and low folate concentrations (≤9.10 nmol/L) have been negatively associated with fetal growth parameters, finally affecting birth weight (Bergen et al., 2016). We also showed that an optimal periconceptional RBC folate level is associated with increased first trimester longitudinal CRL measurements compared to the lowest (β= 0.24 √mm (95%CI: 0.04; 0.44), p=0.02) and highest quartile of concentrations (β= 0.29 √mm (95%CI: 0.09; 0.49), p<0.01) (van Uitert et al., 2014). This result emphasizes that CRL accuracy in pregnancy dating is impacted by maternal I-C metabolism, as well as by several maternal characteristics and exposures (van Uitert et al., 2013). Moreover, embryonic volume (EV) has been described as a more sensitive marker of first trimester growth restriction compared to CRL (Baken et al., 2013). We focused on the Carnegie stages as a century old classification that, together with embryonic size measurements, could implement first trimester investigation and better define a proper embryonic development. Since we excluded all pregnancies with congenital anomalies detected both *in utero* and after birth, our results indicate that even the developmental events of normal ongoing pregnancies are impacted by maternal I-C metabolism. This and previous findings indicate that first trimester growth and development are important embryonic outcomes affected by maternal environment. Nevertheless, CRL, EV and Carnegie stages also represent non-invasive reproducible markers with predictable associations with gestational age, leading to their potential use for pregnancy dating and raising the question which biomarker should be the best candidate (Robinson and Fleming, 1975; O'Rahilly and Müller, 2010). Due to the lack of an optimal pregnancy dating strategy and to unavoidable systematic errors related to the recall of the LMP, imprecise ovulation/implantation dates and parental characteristics impacting embryonic ultrasound measurements, we defined gestational age based on a known LMP, regular cycle and concordant CRL. In this way, all ultrasound measurements could be read as response variables and outcome measurements. In order to reduce selection bias, we compared maternal baseline characteristics, showing that excluded women had a higher BMI, lower age and RBC folate concentrations. This may be mainly explained by the inclusion of a large population of subfertile women and pregnancies achieved after IVF/ICSI treatment (higher age, lower BMI, higher use of folic acid supplements). We also compared the subgroup of included and excluded spontaneous pregnancies showing indeed no significant results (data not shown). Moreover, the sensitivity analysis including pregnancies with discordant CRL confirmed the detected associations, reducing the possibility of selection bias.

The mechanisms linking maternal I-C metabolism and embryonic development are not fully understood. Animal data showed that abnormal activations of I-C metabolism were associated with hypermethylation of mitochondrial DNA, mitochondrial malfunction and decreased oocyte quality (Jia et al., 2016). Recently, a suppression of the inflammatory and upregulation of the high-density lipoprotein pathways have been demonstrated in human follicular fluid of preconception folic acid supplement users (Twigt et al., 2015). Cellular apoptosis and protein homocysteinylation, both dependent on tHcy concentrations, have been suggested as contributors to neural tube, orofacial and cardiac defects (Jakubowski, 2006; Taparia et al., 2007). Finally, periconceptional I-C biomarker mediated epigenetic modifications could modify subsequent gene expression in the embryo (Steegers-Theunissen et al., 2013). All these events may finally lead to impaired first trimester development, thereby supporting our results.

Our findings also reveal that conception mode seems to modify the associations between blood biomarkers and Carnegie stages, despite the fact that no differences in embryonic development have been detected between the two subgroups. As expected, biomarker concentrations differed between spontaneous and IVF/ICSI pregnancies. Besides higher and longer preconceptional folic acid supplement use in the IVF/ICSI subgroup, also the ovarian stimulation treatment may affect I-C blood biomarker concentrations (Boxmeer et al., 2008). Moreover, the IVF/ICSI technique has been associated with different epigenetic patterns, gene expression and preimplantation embryo phenotype compared to spontaneous conception, possibly affecting embryonic responses to maternal I-C biomarkers and explaining different associations detected in our results (Kroener et al., 2016; Zandstra et al., 2015; Giritharan et al., 2007; Song et al., 2015).

The major strength of our study is the longitudinal evaluation of embryonic development by using a median of three scans per patient, the use of 3D US with VR visualization and the consequent high success rate of the Carnegie stage assessment. This gives an accurate and precise picture of the course of first trimester development. Confounding by gestational age is minimized by including women with strict pregnancy dating only. The high rate of folic acid supplement use, resulting in an extremely low rate of hyperhomocysteinemia and high RBC folate concentrations, strongly underlines the importance of our results, since even clinically normal values of tHcy and a non-deranged I-C metabolism could impact embryonic development of non-malformed ongoing pregnancies. The most relevant limitation of this study is related to the tertiary care setting, resulting in expected high rates of folic acid supplement use, chronic comorbidity and pregnancy complications. This may reduce the external validity of our findings. Despite it is reassuring that significant associations were confirmed in IVF/ICSI pregnancies where conception date is known by definition, the implantation date is not known and systematic errors in pregnancy dating are expected. Therefore, it is also possible that the small differences detected in embryonic development reflect an impact on the timing of implantation.

Inadequacies in dietary B vitamins and lifestyle (i.e. smoke, alcohol and coffee consumption) have led to increased dangerous plasma tHcy concentrations in the last decades (Steegers-Theunissen et al., 2013). Our results suggest that this may negatively impact first trimester embryonic development resulting in the highest effect estimates in line with previous findings (Blanco et al., 2016; Steegers-Theunissen et al., 2013). Since plasma tHcy is an overall stable marker within the same individual and in uncomplicated pregnancies, a random periconceptional tHcy measurement is reflective of an individual's status and therefore could represent a potential useful predictor of embryonic development in a clinical setting (McKinley et al., 2001; López-Alarcón et al., 2015).

Conversely, the small estimates detected for vitamin B12 and RBC folate may not address for their clinical use as embryonic development predictors. Nevertheless, while reduced CRL measurements have been associated with adverse pregnancy and health outcomes in the offspring (Mook-Kanamori et al., 2010; van Uitert et al., 2013; Jaddoe et al., 2014), nothing is known about the clinical implications of first trimester developmental delay in ongoing pregnancies.

In conclusion, we have shown significant associations between periconceptional maternal biomarkers of I-C metabolism and Carnegie stages of embryonic development. Further research is needed to investigate associations between Carnegie stages and birth outcomes and to evaluate the validity of our results in the general population.

Chapter 7

Impact of human embryonic morphological development on fetal growth parameters: The Rotterdam Periconceptional Cohort (Predict Study)

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ABSTRACT

Research question Is embryonic morphological development according to the Carnegie stages associated with pregnancy outcome?

Design In a tertiary hospital-based cohort, 182 singleton non-malformed pregnancies were selected. Serial transvaginal three-dimensional ultrasound (3D US) scans were performed between 6+0 and 10+2 gestational weeks. Embryonic development was annotated according to the morphological criteria of the Carnegie classification using a virtual reality system. Second trimester biparietal diameter (BPD), head circumference (HC), abdominal circumference (AC) and femur length (FL) measurements were retrieved from medical records. Z-scores were calculated for mid-pregnancy estimated fetal weight (EFW) and newborn birth weight (BW). Associations between longitudinal Carnegie stages and fetal growth parameters were investigated using linear mixed models, with subgroup analysis based on fetal gender.

Results 576 first trimester 3D US scans were analysed (median of three scans per pregnancy). Embryonic development was positively associated with EFW z-score (β=0.69 (95%CI: 0.51; 0.86), $p<0.001$), BPD and FL, but not with HC, AC and BW z-score. After stratification for fetal gender, positive associations for both males and females were confirmed between embryonic development and EFW z-scores. Moreover, opposite gender-specific associations were detected between embryonic development and BW z-scores (males: β=0.37 (95%CI: 0.04; 0.70), $p<0.05$; females: β= -0.36 (95%CI: -0.62; -0.10), $p\leq0.01$).

Conclusions Human embryonic development according to the Carnegie stages is associated with fetal growth parameters with gender-specificity of BW. These results emphasize the importance of the first trimester of pregnancy, raising the morphological staging of the embryo as a new methodology for early risk assessment and improvement of subsequent fetal growth parameters.

INTRODUCTION

The importance of the periconceptional period and care is underestimated by both patients and health care providers (Finer et al., 2016; M'hamdi et al., 2017). Recent research has shown significant associations between maternal exposures, first trimester embryonic growth and subsequent fetal and newborn outcomes (Fleming et al., 2015; Mook-Kanamori et al., 2010; van Uitert et al., 2013; van Uitert et al., 2013). These findings strongly suggest that the assessment of embryonic growth -as opposed to singular use for pregnancy dating- is an important determinant for pregnancy outcome and health in later life. The preconceptional and embryonic period, i.e. periconceptional period of 14 weeks before to 10 weeks after conception, should receive increasing attention in research, as well as patient care (Steegers-Theunissen et al., 2013).

In the last decades, the extraordinary improvement in high resolution three-dimensional ultrasound (3D US) techniques and the development of immersive virtual reality (VR) systems dramatically increased the possibilities of embryonic examination. Safe, reliable and efficient length and volume measurements, as well as accurate morphological evaluations are now feasible as early as the first trimester of pregnancy (Baken et al., 2014; Rousian et al., 2011; Verwoerd-Dikkeboom et al., 2010). Embryonic morphological development according to the Carnegie classification can be assessed using VR, classifying the first 58 post-conceptional days into 23 stages of development (O'Rahilly, 1987; O'Rahilly and Müller, 2010; Verwoerd-Dikkeboom et al., 2008). Recently, we showed significant associations between periconceptional maternal one-carbon biomarkers and longitudinal Carnegie stages in non-malformed viable pregnancies, depicting embryonic developmental delays of 1.4 days (95% CI: 1.3-1.4) and 1.6 days (95% CI: 1.5-1.7) in mothers with low vitamin B12 and high total homocysteine concentrations, respectively (Parisi et al., 2017). So far, the clinical implications of delayed embryonic development remain unclear. We aim to investigate associations between first trimester embryonic development according to the Carnegie stages and subsequent fetal growth parameters, depicted by mid-pregnancy estimated fetal weight

(EFW) and birth weight (BW), as the main features of pregnancy outcome.

MATERIALS AND METHODS

Data for this study were collected in the ongoing Rotterdam Periconceptional Cohort (Predict Study), a prospective tertiary hospital-based birth cohort study, conducted at the Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands (Steegers-Theunissen et al., 2016). The study protocol was approved by the Medical Ethical and Institutional Review Board of the Erasmus MC (METC Erasmus MC 2004-277, 15/10/2004). A written informed consent form was obtained at enrollment from all participants. Financial funding was provided by the Department of Obstetrics and Gynaecology of the Erasmus MC.

Study population

Between 2010 and 2014, all women before 10^{+2} weeks of gestation, with a singleton viable pregnancy achieved after spontaneous conception, intrauterine insemination (IUI) or assisted reproductive technology with homologous oocytes, were eligible for participation. Twins, egg(s) donation, ectopic implantation, miscarriage, intrauterine fetal death or any congenital anomaly were exclusion criteria. Among spontaneously and IUI conceived pregnancies, we excluded all women with unknown first day of last menstrual period (LMP), self-reported irregular cycle and CRL differing seven or more days from the expected for gestational age (Robinson, 1973). IUI pregnancies were included in the subgroup of spontaneous pregnancies as the embryo is not exposed to the extrauterine preimplantation environment. Gestational age was calculated from LMP for spontaneous pregnancies (with adjustment for cycle duration if <25 or >31 days), from LMP or insemination date plus 14 days for IUI pregnancies, from the day of oocyte retrieval plus 14 days for pregnancies derived from *in vitro* fertilization (IVF) or intracytoplasmatic sperm injection (ICSI) and from embryo transfer day plus 17 or 18 days in pregnancies derived from transfer of cryopreserved embryos. The resulting study population included pregnancies with reliable dating by definition, further confirmed by CRL measurements within seven days from the expected.

Data collection

At enrollment, all women completed a self-administered general questionnaire providing details on age, anthropometrics, geographical origin, education, periconceptional exposures, obstetric and medical history.

Serial 3D US examinations were performed by trained examiners using the high frequency (4.5 – 11.9 MHz) vaginal probe of a GE Voluson E8 (GE, Zipf, Austria). As the Carnegie stages classify embryonic development until the end of the embryonic period (58 postconceptional days, 10^{+2} weeks), serial 3D US scans were performed on a weekly or two weekly basis starting at 6^{+0} weeks of gestation until 10^{+2} weeks of gestation. The obtained 3D datasets were stored as Cartesian volumes and transferred to the BARCO I-Space VR system at the Department of Bioinformatics, Erasmus MC. In the I-Space, the researcher is surrounded by computer-generated stereo images allowing full depth perception and three-dimensional interaction. The V-Scope volume rendering application creates a floating 'hologram' of the embryo, which can be manipulated and measured in three dimensions. The specifications and possibilities of the I-Space VR system and V-Scope have been described elsewhere (Verwoerd-Dikkeboom et al., 2010). The Carnegie criteria were used by the same trained observer (M.R.) to stage all embryos as previously described in detail (Verwoerd-Dikkeboom et al., 2008). As external morphological characteristics, we used the Carnegie criteria for the development of the limbs (arms and legs) and the curvature of the embryo. Internal morphological characteristics primarily included criteria for the brain cavity development. A complete staging assessment using VR took 1-2 minutes per embryo. The Carnegie stage was longitudinally annotated for all first trimester ultrasound scans in order to obtain a detailed trajectory of embryonic morphological development for all included pregnancies.

Routine 20-week ultrasound scan measurements (range 18 – 22 weeks), including biparietal diameter (BPD), head circumference (HC), abdominal circumference (AC) and femur length (FL) measurements, were retrieved from medical records. Mid-pregnancy estimated fetal weight (EFW) was calculated using the Hadlock formula: 10 * (1.3596 - 0.00386 * AC * FL + 0.0064 * HC + 0.00061 * BPD * AC + 0.0424 * AC + 0.174 * FL) (Hadlock et al., 1985). Information on date of birth, gender, BW and congenital anomalies were also obtained from medical records. Gestational age at the time of mid-pregnancy ultrasound examination and birth was calculated using the dating procedure as described above.

Statistical analysis

Appropriate descriptive statistics were calculated for baseline maternal characteristics and newborn outcomes, including median and range for quantitative variables and absolute frequencies and percentages for categorical variables. Gestational age-adjusted z-scores for mid-pregnancy EFW and BW were constructed, based on the unadjusted reference curves provided by Gaillard et al (2011). We created a score of the Carnegie stages as a continuous variable that was censored at its maximum value of 23 (9^{+1}- 10^{+2} weeks of gestation). Linear mixed models were estimated to investigate associations between serial Carnegie stages and fetal growth parameters. This analysis allows the linear modeling of repeated measurements, taking into account the correlation between serial measurements within the same pregnancy. We firstly performed a crude analysis with adjustment for gestational age only and secondly we included adjustment for potential confounding factors based on previous literature (parity, alcohol use, smoking, folic acid/multivitamin supplement use, pregestational BMI, age, geographical origin, conception mode, fetal gender). As we cannot exclude influences of fetal gender on embryonic development, fetal growth parameters and the resulting associations, we additionally stratified the analysis into subgroups of fetal gender and tested the resulting estimates. In addition, an interaction model with fetal gender and conception mode was tested. Finally, the analysis was performed in the subgroup of uncomplicated term pregnancies, after excluding pregnancies with low birth weight babies (LBW, BW < 2500 g) (n=4) and preterm birth (PTB) (n=14).

P-values ≤0.05 were considered significant. All analyses were performed using IBM SPSS version 21.0 (Armonk, NY: IBM Corp) and R version 3.2.1 (The R Foundation for Statistical Computing).

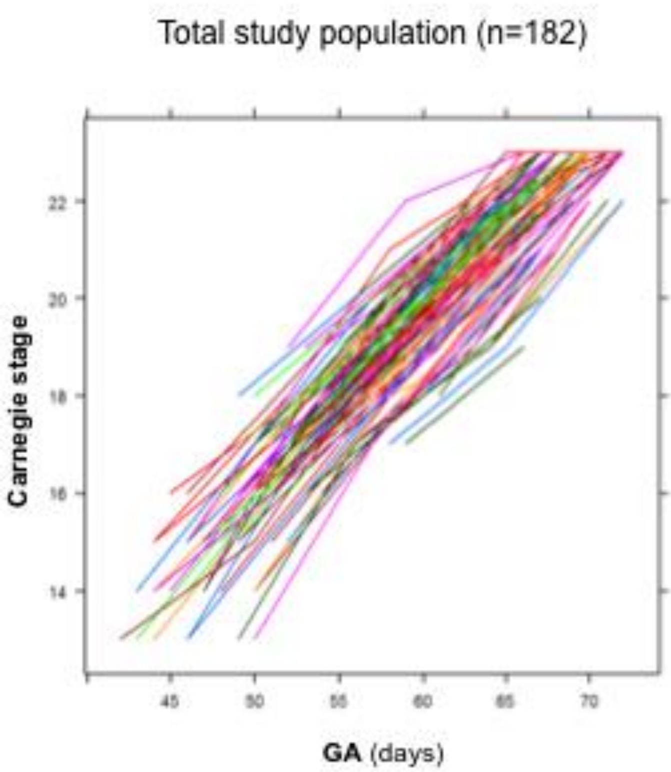
RESULTS

After exclusion of egg donation (n=5), unreliable pregnancy dating (n=60), and pregnancies complicated by twins, miscarriage, ectopic implantation, congenital anomalies and intrauterine fetal death (n=57), a total of 182 pregnancies were included in the analysis, resulting in 114 strictly dated spontaneous/IUI pregnancies and 68 IVF/ICSI pregnancies. Table 1 shows maternal characteristics and newborn outcomes of the study population. The median gestational age at enrollment was 8^{+3} (range 7^{+0}–10^{+0}) weeks, and a median of three (range 1–5) first trimester 3D US scans per pregnancy were performed. 471 out of 576 datasets were of sufficient quality to determine the Carnegie stage (success rate 81.7%).

In figure 1, embryonic development according to longitudinal Carnegie stages is depicted for the study population, showing a linear curve ranging from stage 13 to 23 (6^{+0} – 10^{+2} weeks).

Conception mode and fetal gender showed no significant associations with embryonic development (fully adjusted models: β= 0.17 (95% CI: -0.14; 0.48) p=0.27 and β= -0.03 (95%CI: -0.32; 0.26), p=0.84 respectively) and interaction models did not significantly modify the detected associations. Routine mid-pregnancy ultrasound data and EFW computation could be obtained for all included pregnancies. Birth records were retrieved for 179 (98.4%) pregnancies and BW z-scores were calculated. Table 2 shows the results from linear mixed models in the total study population and subgroups of male and female fetuses. After full adjustment, embryonic development was positively associated with mid-pregnancy EFW z-score (β=0.69 (95% CI: 0.51; 0.86), p<0.001), BPD (β=0.71 mm (95% CI: 0.21; 1.21), p<0.01) and FL (β=0.37 mm (95% CI: 0.00; 0.74), p<0.05) in the total study population. Embryonic development according to the Carnegie stages was not associated with BW in the total study population. Significantly positive associations were confirmed between first trimester embryonic development and mid-pregnancy fetal growth for both male and female subgroups. Embryonic development was positively associated with BW z-score in male fetuses (β= 0.37 (95% CI: 0.04; 0.70), p<0.05), meaning an increase by 165 grams in BW for every unit increase in Carnegie stage in a multi-adjusted model. Interestingly, embryonic development showed a negative association with female BW z-score (β= -0.36 (95% CI: -0.62; -0.10), p≤0.01). Significant differences were detected between male and female β values at birth (p<0.01). Figure 2 shows the associations between Carnegie stages, EFW and BW in the subgroups of male and female fetuses. The exclusion of LBW pregnancies (n=4) and PTB (n=14) did not modify the detected associations in the fully adjusted model and confirmed the positive associations between embryonic development, EFW (β= 0.71 (95% CI: 0.53; 0.90), p<0.001) and BW z-scores (β= 0.41 (95% CI: 0.09; 0.74), p<0.05) in males and negative associations with female BW z-score (β= -0.27 (95% CI: -0.51; -0.02), p<0.05).

Figure 1. Embryonic morphological development according to longitudinal Carnegie stages in the total study population.



GA: gestational age; n:number.

Table 1. General maternal characteristics and newborn outcomes of the study population.

Maternal characteristics	**Included pregnancies (n=182)**	**M**
Age, years, median (range)	32 (22-44)	0
Nulliparous, n(%)	51 (28.0)	1
Geographical origin Western, n(%) Non Western,n(%)	 165 (91.2) 16 (8.8)	1
Educational level High, n(%) Intermediate, n(%) Low, n(%)	 119 (65.4) 60 (33.0) 2 (1.1)	1
BMI, kg/m^2 median (range)	24.4 (18.3-42.3)	1
Alcohol use, n(%)	65 (35.7)	0
Periconception smoking, n(%)	22 (12.2)	1
Periconception folic acid/multivitamin use, n(%)	176 (97.8)	2
IVF/ICSI conception, n (%)	68 (37.4)	0
Birth Outcomes		
Birth weight, g, median (range)	3350 (2040-4540)	3
Gestational age at birth, days, median (range)	274.5 (193-294)	2
Gender male, n (%)	90 (50.0)	2
Small for gestational age babies, n (%)	4 (2.2)	0
Preterm birth, n (%)	14 (7.7)	0

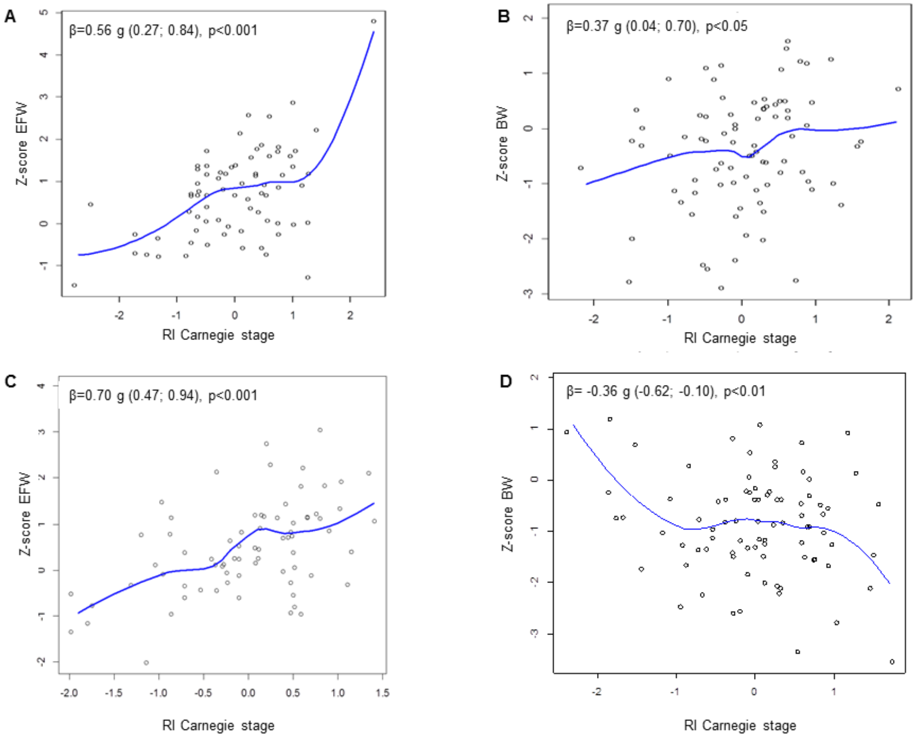
The study population includes singleton strictly dated pregnancies achieved after spontaneous conception, intrauterine insemination (IUI) or assisted reproductive technology with homologous oocytes. IVF: *in vitro* fertilization, ICSI: intracytoplasmatic sperm injection, M: missing values, BMI: body mass index.

Table 2. Effect estimates from linear mixed models for the associations between first trimester embryonic Carnegie stages, mid-pregnancy growth parameters and newborn birth weight in the total study population and after stratification for fetal gender.

EFFECT ESTIMATES FETAL GROWTH PARAMETERS mm, β (95%CI)						
	Total study population (n=182)		**Male fetus (n=90)**		**Female fetus (n=90)**	
	Crude	**Fully adjusted**	**Crude**	**Fully adjusted**	**Crude**	**Fully adjusted**
BPD	**0.75 (0.25; 1.25)****	**0.71 (0.21; 1.21)****	0.62 (-0.13; 1.38)	0.37 (-0.44; 1.19)	**0.79 (0.16; 1.42) ****	**0.89 (0.28; 1.51) ****
HC	1.11 -0.47; 2.69)	0.81 (-0.73; 2.35)	1.41 (-0.88; 3.71)	0.50 (-1.86; 2.86)	0.63 (-1.43; 2.69)	0.44 (-1.61; 2.49)
AC	1.31 (-0.39; 3.01)	1.14 (-0.64; 2.92)	0.85 (-1.54; 3.24)	0.32 (-2.24; 2.87)	1.64 (-0.76; 4.04)	1.32 (-1.20; 3.84)
FL	**0.40 (0.03; 0.77)***	**0.37 (0.00; 0.74)***	**0.58 (0.04; 1.11) ***	0.48 (-0.08; 1.04)	0.23 (-0.28; 0.74)	0.20 (-0.33; 0.73)
TCD	0.16 (-0.02; 0.34)	0.13 (-0.06; 0.32)	0.14 (-0.14; 0.43)	0.07 (-0.22; 0.37)	0.18 (-0.04; 0.39)	0.14 (-0.09; 0.37)
EFFECT ESTIMATES EFW and BW z-scores β (95%CI)						
EFW	**0.66 (0.50; 0.83)■**	**0.69 (0.51; 0.86)■**	**0.56 (0.34; 0.82)■**	**0.56 (0.27; 0.84)■**	**0.66 (0.44; 0.89)■**	**0.70 (0.47; 0.94)■**
BW	-0.03 (-0.24; 0.18)	-0.04 (-0.25; 0.17)	0.27 (-0.03; 0.57)	**0.37 (0.04; 0.70)***	**-0.32 (-0.59; -0.06)***	**-0.36 (-0.62; -0.10)****

Effect estimates represent the change in fetal growth parameter and z-scores of EFW and BW per unit of increase of Carnegie stage. The crude analysis is adj◆usted for gestational age only. The fully adjusted model includes additional confounders (parity, alcohol use, smoking, folic acid/multivitamin supplement use, pregestational BMI, age, ethnicity, conception mode, fetal gender). Newborn gender was missing for two included pregnancies. *p≤0.05, **p≤0.01, ■ p<0.001. CI: confidence interval; BPD: biparietal diameter; HC: head circumference; AC: abdominal circumference; FL: femur length; TCD: transcerebellar diameter; EFW: estimated fetal weight; BW: birth weight.

Figure 2. Associations between Carnegie stages of embryonic morphological development, estimated fetal weight (EFW) and birth weight (BW) Z-scores expressed by weighted regression lines in the subgroups of male (A, B) and female fetuses (C, D).



Full adjustment for parity, alcohol use, smoking, folic acid/multivitamin supplement use, BMI, age, geographical origin and conception mode was performed. Embryonic development is defined by subject specific random intercept (RI) of the Carnegie stage, which indicates the position of the line of a certain subject relative to the average development.

DISCUSSION

This study shows a significantly positive association between first trimester embryonic morphological development according to the Carnegie stages and mid-pregnancy fetal growth. Accelerated embryonic development is additionally associated with a subsequent significant increase of BW in males, but with a decrease in females, not distorted by IVF/ICSI treatment. Both conception mode and fetal gender are not associated with first trimester embryonic development. We previously showed that periconceptional maternal one-carbon metabolism is significantly associated with first trimester longitudinal Carnegie stages of embryonic development in the same population (Parisi et al., 2017). Here we contribute to the interpretation of this finding, suggesting that a delay in embryonic morphological development can negatively impact mid-pregnancy fetal growth and the endpoint of growth according to fetal gender.

The major strength of our study is the evaluation of the embryonic developmental course through repeated measurements (median three 3D US scans/pregnancy), the use of 3D US with VR visualization, the highly standardized measurement method and the high success rate of the Carnegie stage assessment. Due to the close relationship between embryonic growth and morphological development, objectified by the previously reported linear association between CRL and Carnegie stage measurements, we decided to include only pregnancies strictly dated on LMP, further confirmed by CRL measurements within seven days from the expected (Rousian et al., 2013). This allowed to use embryonic measurements as study variables and to minimize confounding by gestational age. This may also explain the differences detected between included and excluded pregnancies, as irregular cycle can be associated with age, smoking habit and educational status. The most relevant limitation of our study is related to the setting in a tertiary care center with high rates of folic acid supplement use, IVF/ICSI conception, chronic comorbidity and pregnancy complications. Further research is warranted to confirm the detected associations among low-risk pregnancies.

Our findings integrate previous results showing a significant correlation between first trimester embryonic growth, depicted by longitudinal CRL measurements, and EFW and BW (Gaillard et al., 2014; van Uitert et al., 2013). Together, these results strongly underline that first trimester embryonic morphological development and growth impact subsequent fetal development, leading to significant modifications of growth trajectories in a multi-adjusted model. Moreover, several first trimester maternal characteristics have been studied in association with birth weight and outcomes (Boucoiran et al., 2013). A recent meta-analysis confirmed the well-known association between maternal smoking and fetal size, with a significant reduction of EFW only during the third trimester of pregnancy (Abraham et al., 2017). Here we show a significant impact of embryonic morphological development on EFW starting as early as the second trimester of pregnancy. The opposite associations in gender may explain the non-significant associations between embryonic development and BW in the total study population for both crude and adjusted models. In line with these gender-specific results, human chorionic gonadotropin concentrations during the late first trimester have been positively associated with BW (β=0.194 (95% CI 0.063, 0.326), $p<0.001$), and fetal size in female, but not in male fetuses (Barjaktarovic et al., 2017). Here, we show a twofold higher effect of embryonic morphological development on male and female BW in a fully adjusted model. This result is in line with recent literature showing gender specific strategies to cope with the same maternal environment (Mandò et al., 2016; Reichetzeder et al., 2016). Gene expression has been shown to be sex-specific already in the preimplantation embryo (Bermejo-Alvarez et al., 2011). Dimorphic transcriptome differences, including glucose and protein metabolism, and sex-specific epigenetic mechanisms may finally impact subsequent fetal growth, supporting our results (Gabory et al., 2009; Bermejo-Alvarez et al., 2010; Reichetzeder et al., 2016; Tan et al., 2016). Sexual dimorphic placental responses, leading to different placental growth rates, efficiency and adaptive responses, represent an additional crucial mediator of sex-specific fetal growth effects (Bermejo-Alvarez et al., 2011; Gabory et al., 2013; Mandò et al., 2016). In this regard, previous models of low-grade chronic inflammation, including asthma and preeclampsia, reported sexually dimorphic responses, with higher risks of a severe phenotype and growth restriction in female fetuses (Clifton, 2010; Schalekamp-Timmermans et al., 2017).

In the last decade, embryonic morphological assessment has become a critical part of first trimester evaluation, aiming to improve pregnancy outcome through early screening and diagnosis (Karim et al., 2017). While it is well-defined its role for early detection and management of abnormal development and congenital anomalies, very little is known about the clinical meaning of developmental delays in otherwise normal embryos. According to our results, the staging of morphological development firstly represents an embryonic outcome potentially modified by maternal nutrition and it secondly impacts pregnancy outcome according to fetal growth parameters (Parisi et al., 2017; Parisi et al., 2018). Moreover, human development according to the Carnegie stages is constant and predefined, with every embryo developing continuously through different morphological stages, at predictable gestational ages (O'Rahilly and Müller, 2010). This feature connects the Carnegie stages to other embryonic size measurements, including CRL and volume, as useful, non-invasive and reproducible predictors of gestational age. This raises the question which marker should be the best candidate for dating procedures, taking into consideration that both embryonic growth and development are impacted by maternal environment and exposures. Due to the low number of preterm and LBW newborns in our cohort, additional subgroup analyses were not performed. Neverthless, it will be of great interest to elucidate whether embryonic morphological development could represent a useful predictor of pregnancy outcome in this group of high-risk pregnancies.

In conclusion, we showed that human embryonic development according to the Carnegie stages is associated with mid-pregnancy fetal growth and newborn weight, with gender-specificity only at birth. These results underline the importance of first trimester care for subsequent fetal outcome and raise embryonic morphological staging as a new methodology for early risk assessment and improvement of subsequent fetal growth parameters. Further research is needed to investigate associations between embryonic morphological development and future health of the offspring, whereas intervention trials are warranted to investigate the effect of early optimization of maternal nutrition and lifestyle on embryonic morphological development and pregnancy outcome.

Part III

Chapter 8

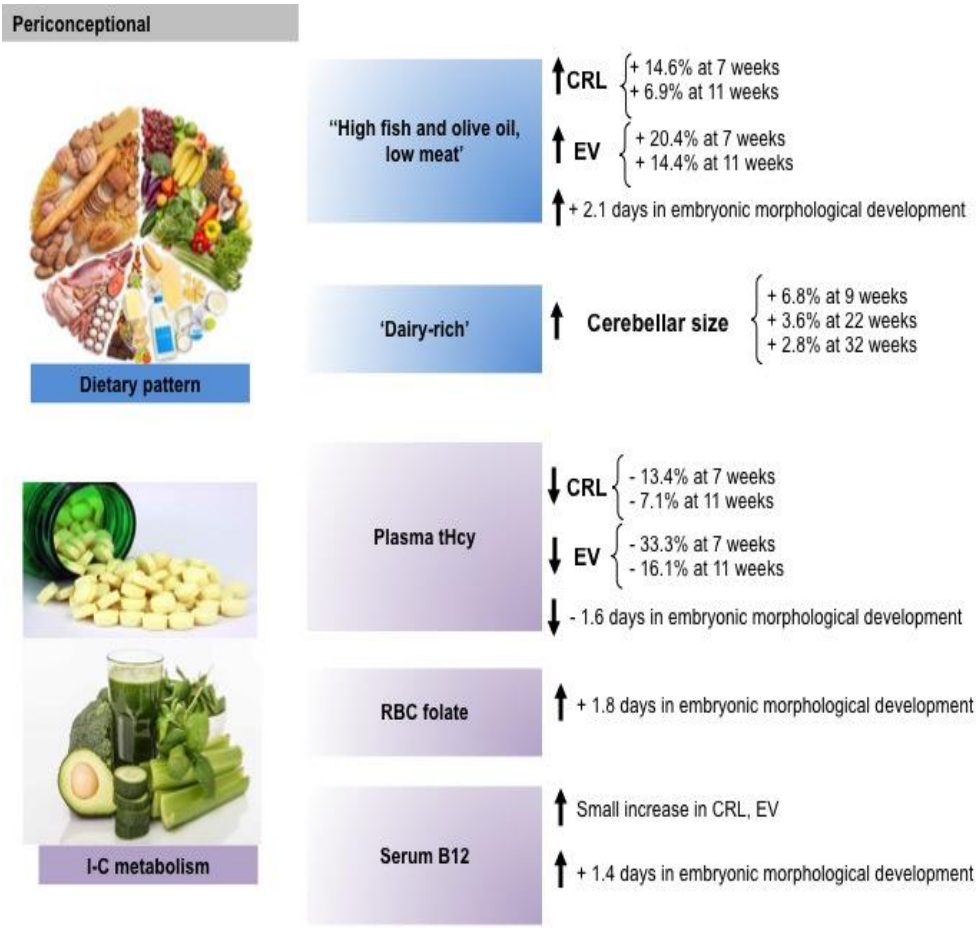
General discussion

In this thesis I have investigated longitudinal human embryonic growth and morphological development *in vivo* by using the combination of 3D US scans and visualization in a VR system. The associations with periconceptional maternal dietary patterns and I-C metabolism were investigated among ongoing, non-malformed, strictly dated pregnancies by using linear mixed models adjusted for confounding factors. In addition, the clinical meaning of a delayed embryonic morphological development was investigated by studying the associations with subsequent fetal growth parameters as the main feature of pregnancy outcome (EFW and birth weight). Finally, to gain more insights into maternal nutrition and human neurodevelopment, associations were studied between periconceptional maternal dietary patterns and longitudinal prenatal cerebellar growth depicted by repeated ultrasound measurements starting from the first trimester of pregnancy onwards.
In this chapter, I discuss the main findings and their clinical implications, some methodological considerations and future perspectives.

Main findings and clinical implications

Figure 4 summarizes the main findings of this thesis.

Figure 4. Overview of the main findings in non-malformed strictly dated pregnancies.



CRL: crown-rump length; EV: embryonic volume; tHcy: total homocysteine; RBC: red blood cell. Embryonic morphological development is defined according to the Carnegie stages.

Periconceptional maternal dietary patterns

This thesis shows significant associations between maternal periconceptional adherence to a ‘high fish and olive oil, low meat’ dietary pattern and increased embryonic growth, depicted by longitudinal CRL and EV measurements, among non-malformed strictly dated spontaneous pregnancies (Chapter 2). In chapter 5 and in the same study subgroup, maternal adherence to the same dietary pattern is shown to be positively associated with embryonic morphological development according to the Carnegie stages. These results are in line with previous data showing significant associations between maternal use of a Western diet and increased risks of congenital anomalies, thus supporting the hypothesis that dietary patterns as composite exposure of food groups can consistently impact complex embryonic morphological development leading to both evident malformations and altered trajectory of otherwise normal morphological development (Vujkovic et al., 2007). In particular, after full adjustment for confounding factors, periconceptional weak adherence to the ‘high fish and olive oil, low meat’ dietary pattern reduces the early first trimester CRL measurement by 14.6%, EV by 20.4% and further delays embryonic morphological development by 2.1 days compared to strong adherence to the same dietary pattern. If a single food group intake is taken into consideration, no significant associations are detected, supporting previous controversies regarding the association between fish intake and pregnancy outcome (Cetin et al., 2009; Drouillet et al., 2009; Heppe et al., 2011; Papadopoulou et al., 2013; Leventakou et al., 2014). As nutrition represents a complex exposure, I suggest that the effect of a single nutrient or food group is in most cases too small to detect. The cumulative effects of multiple nutrients and food groups included in a complex dietary pattern, however, are much stronger and therefore can explain our results. In particular, the low intake of saturated fats and the increased omega-3/omega-6 fatty acid ratio expected in case of strong adherence to the ‘high fish and olive oil, low meat’ dietary pattern are possible explanations for the detected associations, leading to increased embryonic growth and accelerated morphological development possibly by modulated eicosanoid metabolism, gene expression, inflammation and oxidative stress pathways (Meher et al., 2016). Our results suggest that caregivers should recommend a healthy, balanced dietary pattern, instead of a single healthy nutrient or food group, to (pre)pregnant women and couples who are trying to conceive (Northstone et al., 2008; Oostingh et al., 2017).

Periconceptional maternal I-C metabolism

Chapter 3 and 6 focus on periconceptional maternal I-C metabolism. Once again, the two processes of embryonic growth and morphological development show the same direction and comparable associations with maternal I-C environment. Early first trimester serum vitamin B12 concentrations are positively associated with longitudinal CRL, EV and Carnegie stage measurements. However, the small effect estimates limit the clinical implications and utility of this biomarker as embryonic health predictor. This is in contrast to plasma tHcy, whose even clinically normal concentrations strongly impact embryonic growth and development. High maternal tHcy concentrations (corresponding to 10.3 µmol/l in our highly supplemented population) reduce early first trimester CRL by 13.4%, EV by 33.3% and delay embryonic development by 1.6 days compared to low concentrations (3.0 µmol/l) in a fully adjusted model. Mitochondrial hypermethylation and malfunction, increased cellular apoptosis, up-regulation of the inflammatory pathway, protein homocysteinylation and epigenetic modifications of growth-related genes are possible explanations for I-C metabolism-mediated effects on embryonic growth and development (Jakubowski et al., 2006; Taparia et al., 2007; Steegers-Theunissen et al., 2013; van Mil et al., 2014; Twigt et al, 2015; Jia et al., 2016). In clinical settings, these findings could have a strong impact. The high frequency of inadequacies in dietary B vitamins and lifestyle (i.e. smoking, alcohol and coffee consumption) led to an average increase in plasma tHcy concentrations by 1-4 µmol/l (Steegers-Theunissen et al., 2013). Elevated tHcy concentrations during pregnancy have been independently associated with the risk of recurrent pregnancy loss, preeclampsia, preterm and low birth weight neonates (Vollset et al., 2000; Nelen, 2001; Ronnenberg et al., 2002). Our results suggest that high tHcy concentrations might additionally have a dramatic effect on embryonic growth, leading to an impressive reduction in embryonic volume measurements in the early first trimester. Moreover, the associations between increased maternal plasma tHcy concentrations, embryonic development and congenital anomalies have been repeatedly addressed by animal and human studies (Czeizel et al., 1992; van Driel et al., 2008; Steegers-Theunissen et al., 2013; Blanco et al., 2016; Lubinsky, 2018). Slightly higher tHcy concentrations were found in mothers with NTDs offspring compared to controls, but results are controversial maybe due to the influence of confounders (e.g. sampling time, serum folate concentration) (Yang et al., 2017). Here, in a multi-adjusted model and using a standardized sampling protocol, we show a significant impact of periconceptional maternal tHcy concentrations on embryonic morphological development of otherwise normal embryos without congenital malformations. Chapter 7 further explains the clinical meaning of this result, showing that a delayed first trimester morphological development significantly affects pregnancy outcome leading to reduced EFW and gender-specific changes in birth weight. Finally, associations between a deranged maternal I-C metabolism during pregnancy and future health outcomes in the offspring are a returning issue in recent research, with unfavorable effects demonstrated on cardiovascular risk profile, kidney development and insulin resistance in offspring adult life (Sinclair et al., 2007; Miliku et al., 2017; McGee et al., 2018). Derangements in maternal I-C metabolism, in fact, may alter the intrauterine bioavailability of methyl groups, possibly resulting in permanent changes to the fetal epigenome with subsequent adverse health outcomes (Sinclair et al., 2007; Steegers-Theunissen et al., 2013).

In routine clinical care, plasma tHcy has been shown to be an overall stable marker in uncomplicated pregnancies, with no seasonal variations and a high reliability coefficient (McKinley et al., 2001; Cikot et al., 2001; López-Alarcón et al., 2015). This suggests that a single periconceptional measurement, picturing an individual's average concentration, could be used as a predictor of embryonic health. Nevertheless, the other question raised by our results addresses the optimal maternal tHcy concentrations for embryonic health. We showed a linear negative association between tHcy concentrations, embryonic growth and morphological development, contrary to what previously shown for RBC folate concentrations (van Uitert et al., 213). Since impressive negative associations have been detected for tHcy concentrations corresponding to the clinically normal value of 10.3 µmol/l, we could hypothesize that a lower tHcy cutoff is desirable in pregnant women compared to the non-pregnant state. Moreover, our results suggest a revision of the proposed plasma tHcy cutoff of 11.3 µmol/l for congenital anomalies in order to ensure a proper embryonic growth and morphological development (Wong et al., 1999; Verkleij-Hagoort et al., 2006).

Embryonic morphological development according to the Carnegie stages

We firstly show that embryonic morphological development is not affected by ART, as previously shown for first trimester embryonic growth, nor by fetal gender (Eindhoven et al., 2014). While clinical implications of a reduced first trimester CRL measurement for pregnancy and health outcomes were already known, chapter 7 further clarifies the clinical meaning of a delay in first trimester embryonic development in ongoing pregnancies without congenital malformations (Mook-Kanamori et al., 2010; van Uitert et al., 2013; Nakamura et al., 2015; Jaddoe et al., 2014). We show a positive association between embryonic development according to the Carnegie stages and mid-pregnancy fetal growth parameters, finally resulting in significant changes in birth weight according to fetal gender. Sex-specific epigenetic mechanisms, variations in cellular transcriptomes and placental responses may all represent crucial mediators of sex-dimorphic growth effects (Gabory et al., 2009; Clifton et al., 2010; Bermejo-Alvarez et al., 2011; Reichetzeder et al., 2016). In the setting of the Generation R study and in line with our findings, significant fetal sex-specific differences in placental biomarkers were observed as early as the first trimester of pregnancy (Brown et al., 2014). Our findings suggest that accurate first trimester morphological evaluations and embryonic staging could positively impact subsequent fetal outcomes, with gender specificity at birth. This also provides further evidence of the crucial role of the -still neglected- first trimester pregnancy period in antenatal care. The Carnegie classification represents a validated, reproducible and reliable system of embryonic staging, which would provide the opportunity to better define proper and abnormal embryonic development in clinical settings, in combination with first trimester size measurements and aneuploidy screening (Blaas et al., 1998; Verwoerd-Dikkeboom et al., 2008; O'Rahilly et al., 2010). Moreover, it would be of interest for further research to evaluate the associations between the Carnegie stages of embryonic development and future health outcomes in the offspring.

Another crucial issue arising from our research relates to the importance of very strict pregnancy dating procedures. Two premises need to be clarified: first, gestational age estimates based on menstrual dates can be unreliable as a result of poor anamnestic quality and delayed ovulation/implantation; second, imprecise dating may suggest abnormalities of growth being unrecognized later in pregnancy, as well as to inappropriate intervention. Therefore, gestational age based on LMP usually needs to be further confirmed in clinical settings using reliable and reproducible embryonic markers with a predictable association with gestational age. For a long time, this role has been assigned to the CRL measurement (Robinson, 1973). Nevertheless, our and previous studies demonstrated that CRL is dependent on maternal conditions (e.g. age, parity) and environmental exposures (e.g. folic acid, smoking, alcohol, dietary patterns) in very strictly dated pregnancies. This suggests that CRL accuracy in pregnancy dating is questionable and that it should be firstly taken into account as an important embryonic health outcome. Therefore, we raise the question: *Which embryonic measurement represents the best available candidate for pregnancy dating?* For both maternal dietary pattern and I-C biomarker exposures, the Carnegie stages are overall less impacted compared to the CRL and EV measurements. This could make embryonic staging a more stable predictor of gestational age compared to size measurements, but further research is warranted to increase the precision of pregnancy dating. On the other hand, customized embryonic growth curves based on the characteristics of that specific individual/population may represent another important solution.

Prenatal cerebellar growth trajectory

Finally, to gain more insights on periconceptional maternal exposures and intrauterine development, chapter 4 focuses on the associations between maternal periconceptional dietary patterns and prenatal cerebellar growth trajectories. Aside from the body of evidence associating maternal nutrition with fetal brain development and outcome (e.g. neural tube defects), nothing was known about the association between maternal dietary patterns and human prenatal cerebellum development. It is now well established that cerebellar development undertakes a crucial role in human acquisition of cognitive and emotional abilities, making this research topic crucial in order to ameliorate future neurodevelopmental outcome (Steegers-Theunissen et al., 2013; D'Mello et al., 2015; Portugal et al., 2016). Several animal models showed that the cerebellum is vulnerable to maternal malnutrition. A recent study demonstrated that malnourished rats with a multi-deficient diet particularly low in omega-6/omega-3 fatty acid ratio showed an irreversible reduction in cerebellar growth, despite a relative protection of the cerebellar redox status (Augusto et al., 2017). Also a folate and vitamin B12 deficient diet during pregnancy and lactation was shown to impair cerebellar proteomics in rats, in line with our previous findings showing significant associations between maternal preconceptional folic acid supplement use and increased human first trimester cerebellar growth as a function of CRL (Koning et al., 2015; Pourié et al., 2015). Here we show a positive association between maternal periconceptional adherence to a dairy-rich dietary pattern and prenatal cerebellar growth trajectories from the first trimester of pregnancy onwards. This result is in line with previous studies showing significant associations between maternal milk consumption, increased fetal weight gain and lower risks of small for gestational age babies among north-European populations (Olsen et al., 2007; Heppe et al., 2011). This also supports the idea of a non-tissue specific effect of dairy products, with a general positive impact on both fetal and cerebellar tissues. Higher protein, glucose and micronutrient intakes, as well as lower tHcy concentrations in case of strong adherence to the dairy-rich dietary pattern could explain our result on cerebellar growth, through direct modulation of cell differentiation, neurotrophic factor expression, neurotransmitter synthesis, cerebellar DNA methylation patterns and gene expression (Marques et al., 2015; Barua et al., 2016). Nevertheless, it is crucial to determine whether the detected associations are valuable also in non-Nordic populations. In future, early programming of the cerebellar epigenome through maternal nutrition and exposures might be relevant for the pathophysiology and prevention of several neurologic and psychiatric disorders.

Methodological considerations

The longitudinal evaluation of embryonic growth and development by using repeated measurements, 3D US and VR systems, and highly standardized measurement procedures, represents the main strength of our studies. The reliability, technique and methods used for CRL, EV and Carnegie stage measurements have been extensively described before and high success rates were provided in our studies (Verwoerd-Dikkeboom et al., 2008; Rousian et al., 2010). The result is an accurate and precise picture of the course of first trimester embryonic growth and development. On the other hand, the use of a VR system could also compromise the external validity of our findings, as the access to this technology is far from being common in research, as well as in clinical practice.

Our study population is represented by ongoing, non-malformed, strictly dated pregnancies by definition. The exclusion of women reporting uncertain LMP or irregular cycle, and pregnancies showing discrepancies between the observed and expected CRL for gestational age (> 6 days), minimizes the confounding by gestational age. Since the studied population was heterogeneous, including strictly dated spontaneous as well as IVF/ICSI pregnancies, conception mode was always taken into account for both adjustment and further stratification to subgroup analysis.

We performed maternal nutritional evaluations based on food frequency questionnaire data by using a principle component analysis (PCA) and avoiding *a priori* and subjective definition of dietary patterns. A blood biomarker validation of the extracted dietary patterns has always been carried out. Nevertheless, underreporting of intake, subjective labeling of dietary patterns and the choice of preselected food groups are still issues to be considered in the interpretation of the results. The studies have been performed in a tertiary care center, which gives rise to a limited external validity due to relatively higher rates of folic acid supplement use, educational level, chronic comorbidity and risks of pregnancy complications compared to the general population.

Future perspectives

This thesis demonstrated the crucial role of periconceptional maternal dietary patterns and I-C metabolism for first trimester embryonic growth and morphological development, as well as for prenatal cerebellar growth. I additionally addressed the positive association between first trimester morphological development and fetal growth among non-malformed embryos. These results further increase the body of observational studies emphasizing the role of the periconceptional maternal environment to improve pregnancy outcome, with consequences for offspring disease risk over their lifetime (Stephenson et al., 2018). Several human and animal models have repeatedly addressed associations between maternal diet and metabolic status, and embryonic potential, moving the focus of the DOHaD hypothesis to the time around conception (Fleming et al., 2018). Therefore, targeting health improvement in women with child desire should represent the goal of periconceptional care (Barker et al., 2018). Nutritional and supplementation strategies have been universally proposed in order to improve preconception health. Recently, a multi-level model of intervention strategies starting as early as childhood has been proposed in order to promote parental preconception health and increase the adherence to behavioral changes (Barker et al., 2018). This thesis suggests that early modifications of maternal dietary patterns and optimization of I-C biomarkers significantly improve embryonic development and cerebellar growth, but intervention studies are needed to define their effectiveness for mother and baby outcomes on a large scale of low-risk pregnancies. This thesis additionally provides evidence on the role of I-C biomarkers as early markers of embryonic health. While the role of vitamin B12 does not seem to be clinically relevant due to low effect estimates, maternal plasma tHcy could represent a cost-effective marker to optimize embryonic growth, besides the well-known effect on morphological development and congenital anomalies. More research is still warranted to reveal the underlying mechanisms of the detected associations and to evaluate the implementation and (cost)effectiveness of periconceptional lifestyle interventions.

Chapter 9

Summary & Samenvatting

The periconceptional period has gained increasing attention in recent research in order to improve pregnancy and future health outcomes. Several maternal characteristics and exposures were known to affect fetal outcome, and a growing body of evidence has recently demonstrated effects on embryonic health as early as the first trimester of pregnancy. This thesis focuses on periconceptional maternal nutrition and I-C metabolism in association with embryonic health, assessed by longitudinal growth and morphological development measurements, with further investigations on the effects on subsequent fetal growth parameters. Moreover, we addressed the associations between periconceptional maternal dietary patterns and human prenatal cerebellar growth as a proxy of future neurodevelopmental outcome.

General Introduction

This section presents the background information on topics that are addressed in this thesis: the DOHaD hypothesis and the need to shift the time window of this research topic to the periconceptional period, the importance of maternal nutrition and I-C metabolism for embryonic, fetal and pregnancy outcomes, and the methodological and technical achievements providing impressive opportunities to investigate small embryonic structures with high accuracy and precision. The aim, objectives and methodology of this thesis are further addressed here.

Part I - Human embryonic and prenatal cerebellar growth

In chapter 2, associations between periconceptional maternal dietary patterns and first trimester embryonic growth are investigated. Longitudinal CRL and embryonic volume (EV) measurements were performed using 3D US scans and visualization in a virtual reality system from 6^{+0} up to 13^{+0} weeks of gestation. Periconceptional maternal dietary patterns were extracted *via* principal component analysis from food frequency questionnaires. We showed positive associations between a ‘high fish and olive oil, low meat’ dietary pattern and embryonic growth trajectories among strictly dated spontaneous pregnancies. From a clinical perspective, maternal strong adherence to the ‘high fish and olive oil, low meat’ dietary pattern increased CRL and EV measurements at 7 weeks (+14.6% and +20.4% respectively) and 11 weeks (+6.9% and +14.4% respectively) compared to weak adherence to the same dietary pattern.

In Chapter 3 we focus on the associations between periconceptional maternal biomarkers of I-C metabolism, including serum vitamin B12 and plasma total homocysteine (tHcy), and embryonic growth trajectories. Maternal vitamin B12 concentrations were shown to be positively associated with CRL, EV measurements, and growth rate. However, the small effect estimates did not potentially support a role in clinical care. Conversely, tHcy was negatively associated with embryonic growth, showing impressive effects on growth trajectories. In particular, in case of high tHcy concentrations (10.3 µmol/l in our population), CRL and EV measurements were decreased respectively by 13.4% and 33.3% at 7 weeks, and by 7.1% and 16.1% at 11 weeks compared to low tHcy (3.0 µmol/l in our population). Since a smaller embryo is associated with a higher risk of adverse pregnancy outcome and an increased risk of non-communicable diseases in later life, both chapter 2 and 3 underline that periconceptional maternal dietary patterns and I-C metabolism may be used, and eventually optimized, as predictors of embryonic health and later outcomes in clinical settings. In particular, new tHcy cutoff should be investigated in order to optimize embryonic growth.

In chapter 4, associations between periconceptional maternal dietary patterns and prenatal cerebellar growth from the first trimester onwards are further addressed. Serial ultrasound scans were performed from 9 up to 32 weeks of gestation for transcerebellar diameter (TCD) measurement. We showed that maternal periconceptional adherence to a ‘dairy-rich’ dietary pattern was positively associated with cerebellar growth trajectories, reporting increased TCD estimates of 0.44 mm at 9 weeks (+6.8%), 0.88 mm at 22 weeks (+3.6%), and 1.17 mm at 32 weeks (+2.8%). As cerebellar development has been widely associated with future neurodevelopmental outcome, we could hypothesize that intrauterine programming of the cerebellar epigenome through maternal nutritional exposures might be relevant for the pathophysiology and prevention of several neurologic and psychiatric disorders in early future.

Part II - Human embryonic morphological development

Chapter 5 focuses on the associations between early first trimester maternal dietary patterns and embryonic morphological development according to the Carnegie stages among pregnancies with non-malformed outcome. Serial transvaginal 3D US scans were performed between 6^{+0} and 10^{+2} gestational weeks to define the Carnegie stage of morphological development in a virtual reality system. We demonstrated that maternal adherence to the 'high fish and olive oil and low meat' dietary pattern was positively associated with embryonic development, showing a delayed embryonic development by 2.1 days (95%CI: 1.6; 2.6) in case of weak compared to strong adherence to this dietary pattern. Chapter 6 further addresses the associations between periconceptional maternal I-C metabolism and embryonic morphological development in the same population. Significant positive associations were detected between vitamin B12 and red blood cell (RBC) folate concentrations and embryonic morphological development. In particular, low vitamin B12 and RBC folate concentrations delayed embryonic development by 1.4 and 1.8 days respectively compared to high concentrations. Conversely, tHcy was negatively associated with embryonic development, with high concentrations delaying embryonic development by 1.6 days compared to low concentrations. Chapter 7 clarifies the clinical meaning and implications of these results. We showed positive associations between first trimester embryonic morphological development according to the Carnegie stages and second trimester fetal growth parameters, including EFW, biparietal diameter and femur length. After stratification for fetal gender, gender-specific associations were detected between embryonic development and birth weight. We suggest that an accurate embryonic morphological evaluation by means of a century old classification system could represent a useful methodology for early risk assessment and improvement of subsequent growth trajectories in periconceptional care.

Part III

In Chapter 8 we discuss the main findings, clinical implications, methodological considerations and future perspectives. The results of these studies support the evidence that an early optimization of maternal dietary patterns and I-C metabolism could represent a useful instrument to improve embryonic health, with additional impact on pregnancy outcome and fetal growth parameters. Moreover prenatal cerebellar growth could be implemented by modifying maternal dietary patterns as early as the periconceptional period and further research should investigate the effects on later neurodevelopmental outcome.

Intervention trials are needed to verify the (cost)effectiveness of periconceptional implementation of maternal dietary patterns and I-C biomarkers.

De laatste decennia is de belangstelling voor de periconceptionele periode enorm toegenomen. Steeds meer studies hebben aangetoond dat de periconceptionele gezondheid van de aanstaande moeder bijdraagt aan een gezonde zwangerschap en de geboorte van een gezond kind. Het is bekend dat verschillende maternale karakteristieken en blootstellingen een effect hebben op foetale uitkomsten. Echter, er is nu steeds meer bewijs voorhanden dat de effecten van deze blootstellingen reeds aantoonbaar zijn in het eerste trimester van de zwangerschap.

Dit proefschrift bestudeert voor het eerst de associaties tussen maternale periconceptionele voedingspatronen, biomarkers van het 1-koolstofmetabolisme en embryonale gezondheid. De voedingspatronen en biomarkers werden gerelateerd aan longitudinale embryonale groei en morfologische parameters in het eerste trimester, en aan foetale groeiparameters in het tweede en derde trimester van de zwangerschap. Bovendien hebben we de associaties tussen periconceptionele maternale voedingspatronen en humane prenatale cerebellaire groei ook bestudeerd als proxy voor toekomstige neurologische ontwikkelingsuitkomsten.

Algemene Introductie

In dit onderdeel van het proefschrift wordt de achtergrond van het onderwerp van dit proefschrift beschreven: 1) de DOHAD-hypothese en de noodzaak tot het verrichten van onderzoek in juist de periconceptionele periode, 2) het belang van maternale voedingspatronen en 1-koolstofmetabolisme voor embryonale, foetale en zwangerschapsuitkomsten, 3) de methodologische en technische ontwikkelingen waardoor unieke mogelijkheden zijn ontstaan om kleine embryonale structuren accuraat en precies te bestuderen.

Deel I – Humane embryonale en prenatale cerebellaire groei

In hoofdstuk 2 zijn de associaties tussen periconceptionele maternale voedingspatronen en eerste trimester embryonale groei bestudeerd. Longitudinale kop-stuit lengte (CRL) en embryonale volume (EV) metingen zijn uitgevoerd door gebruik te maken van 3D echoscopie datasets en virtual reality tussen 6^{+0} en 13^{+0} weken amenorroe. Informatie met betrekking tot periconceptionele maternale voedingspatronen werd verkregen via voedingsvragenlijsten en bestudeerd door gebruik te maken van principale component analyse.

Wij hebben aangetoond dat er een positieve associatie aanwezig is tussen een ‘vis en olijfolie-rijk en vlees-arm’ voedingspatroon met longitudinale embryonale groeitrajecten in spontane eenlingzwangerschappen. De embryo’s van zwangeren die een uitgesproken vis- en olijfolierijk en vleesarm voedingspatroon gebruikten, waren gemiddeld groter - de CRL (+14.6% bij 7 weken en +6.9% bij 11 weken zwangerschap) en EV (+20.4% bij 7 en +14.4% bij 11 weken zwangerschap) - in vergelijking met vrouwen die dit voedingspatroon niet of in mindere mate gebruikten.
In hoofdstuk 3 werden associaties tussen periconceptionele maternale biomarkers van het 1-koolstofmetabolisme – vitamine B12 en totaal homocysteïne – bestudeerd en de longitudinale embryonale groeitrajecten. Maternale vitamine B12 concentraties hadden een positieve associatie met de CRL, het EV en het groeipercentage. Echter deze effecten waren zo klein, dat dit waarschijnlijk geen klinische relevante betekenis zal hebben. Homocysteïne was negatief geassocieerd met embryonale groei en dit effect was veel meer uitgesproken. Een hoge totaal homocysteïne concentratie (+2 standaarddeviatie, >10.3 µmol/l) was geassocieerd met een 13.4% kleinere CRL en 33.3% kleinere EV bij 7 weken als ook een 7.1% kleinere CRL en 16.1% kleinere EV bij 11 weken amenorroe in vergelijking met de groep vrouwen met een lage homocysteïne concentratie (-2 standaarddeviatie, <3.0 µmol/l). De literatuur tot nu toe laat zien dat een klein embryo geassocieerd is met een hoger risico op negatieve zwangerschapsuitkomsten en een verhoogd risico op chronische ziekten in het latere leven. Hoofdstuk 2 en 3 onderbouwen dat de hypothese dat periconceptionele maternale voedingspatronen en het 1-koolstofmetabolisme gebruikt zouden kunnen worden als voorspellers van embryonale gezondheid en zwangerschapsuitkomst. In het bijzonder zouden nieuwe homocysteïne grenswaarden voor optimale embryonale groei moeten worden bestudeerd.
Associaties tussen periconceptionele maternale voedingspatronen en prenatale cerebellaire groei gemeten vanaf het eerste trimester van de zwangerschap worden bestudeerd in hoofdstuk 4. Longitudinale echoscopische onderzoeken werden uitgevoerd tussen 9 en 32 weken amenorroe. In de verkregen datasets werd de transversale cerebellaire diameter (TCD) gemeten. Het periconceptioneel gebruik van een ‘zuivel-rijk’ voedingspatroon door de zwangeren, was positief geassocieerd met

TCD-groeitrajecten. De gemeten TCD van het embryo/de foetus in zwangeren die een ‘granen-rijk’ voedingspatroon gebruikten was gemiddeld 0.44 mm groter bij 9 weken (+6.8%), 0.88 mm groter bij 22 weken (+3.6%), en 1.17 mm groter bij 32 weken (+2.8%) in vergelijking met vrouwen die dit voedingspatroon minder sterk gebruikten. Gezien het feit dat cerebellaire ontwikkeling geassocieerd is met de toekomstige neurologische ontwikkeling is onze hypothese dat de intra-uteriene programmering van het cerebellaire epigenoom mede wordt beïnvloed door maternale voedingspatronen. Dit is dientengevolge relevant voor de pathofysiologie en preventie van verschillende toekomstige neurologische en psychiatrische aandoeningen.

Deel II – Morfologische ontwikkeling van het humane embryo

In Hoofdstuk 5 worden associaties bestudeerd tussen periconceptionele maternale voedingspatronen en de morfologische ontwikkeling van het embryo. Voor de beschrijving van de morfologie is gebruik gemaakt van de Carnegie stadia die bepaald zijn in zwangerschappen zonder aangeboren afwijkingen. Seriële transvaginale 3D echoscopiedatasets werden gegenereerd tussen 6^{+0} en 10^{+2} weken amenorroe. Met behulp van een virtual reality systeem werd de morfologische ontwikkeling beschreven op basis van de Carnegie criteria. Het gebruik van een ‘vis-rijk’ voedingspatroon was positief geassocieerd met de embryonale ontwikkeling. De embryo’s van zwangeren die een minder ‘vis-rijk’ voedingspatroon gebruikten, toonden een tragere embryonale ontwikkeling van 2.1 dagen (95%CI: 1.6; 2.6) in vergelijking met het embryo van vrouwen die een sterk ‘vis-rijk’ voedingspatroon gebruikten.

De associatie tussen het periconceptionele 1-koolstofmetabolisme en de embryonale morfologische ontwikkeling werd in dezelfde populatie onderzocht in hoofdstuk 6. Positieve associaties werden gevonden tussen vitamine B12 en foliumzuur concentraties en de embryonale morfologische ontwikkeling. Een lage vitamine B12 en foliumzuur concentratie, resulteert in een vertraagde embryonale ontwikkeling van respectievelijk 1.4 en 1.8 dag. Anderzijds toonden we een negatieve associatie aan tussen de totaal homocysteïne concentratie en de embryonale ontwikkeling; hoge concentraties waren geassocieerd met een vertraagde embryonale ontwikkeling van 1.6 dag.

Tenslotte werd in het laatste hoofdstuk 7 van dit deel van het proefschrift onderzoek beschreven naar de klinische toepasbaarheid van de embryonale morfologische resultaten. Een positieve associatie werd gevonden tussen eerste trimester embryonale morfologische ontwikkeling gebaseerd op de Carnegie stadia en tweede trimester foetale groeiparameters (EFW, bipariëtale diameter, femurlengte). Na additionele stratificatie voor foetaal geslacht, werden geslachts-specifieke associaties vastgesteld tussen embryonale ontwikkeling en geboortegewicht. De conclusie is dat een embryonale morfologische evaluatie kan bijdrage aan een vroege risicoselectie waardoor eerder gestart kan worden met diagnostiek en behandeling .

Deel III

In hoofdstuk 8 worden de belangrijkste bevindingen, klinische toepasbaarheid, methodologische overwegingen en toekomstperspectieven bediscussieerd. De resultaten van de studies uitgevoerd in dit proefschrift ondersteunen de aanname dat een vroege optimalisering van maternale voedingspatronen en het 1-koolstofmetabolisme kunnen bijdragen aan een betere embryonale gezondheid. Uiteindelijk zal dit ook een impact kunnen hebben op foetale groeiparameters en de zwangerschapsuitkomsten. Het veranderen van het maternale voedingspatroon kan tevens resulteren in de beïnvloeding van de prenatale cerebellaire groei vanaf het eerste trimester van de zwangerschap. Toekomstig onderzoek zal moeten uitwijzen of dit tevens een effect heeft op latere neurologische ontwikkeling van het kind en volwassenen. Tot slot zijn interventie onderzoeken noodzakelijk om de uiteindelijke (kosten)effectiviteit van periconceptionele aanpassing van maternale voedingspatronen en 1-koolstofmetabolisme biomarkers aan te tonen.

Addendum

Abbreviations
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ABBREVIATIONS

I-C: one-carbon
3D US: three-dimensional ultrasound
AC: abdominal circumference
ART: assisted reproductive technology
BMR: basal metabolic rate
BMI: body mass index
BPD: biparietal diameter
BW: birth weight
CRL: crown-rump length
EFV: estimated fetal weight
EV: embryonic volume
FIL: food intake level
FFQ: food frequency questionnaire
FL: femur length
GA: gestational age
HC: head circumference
IUI: intrauterine insemination
IVF: *in vitro* fertilization
ICSI: intracytoplasmic sperm injection
LMP: last menstrual period
LBW: low birth weight
PAL: physical activity level
PCA: principal component analysis
RBC: red blood cell
SD: standard deviation
SGA: small for gestational age
TCD: transcerebellar diameter
tHcy: total homocysteine
VR: virtual reality

REFERENCES

Abraham M, Alramadhan S, Iniguez C, Duijts L, Jaddoe VW, Den Dekker HT, Crozier S, Godfrey KM, Hindmarsh P, Vik T, Jacobsen GW, Hanke W, Sobala W, Devereux G, Turner S. A systematic review of maternal smoking during pregnancy and fetal measurements with meta-analysis. PLoS One. 2017;12:e0170946.

Allen G, McColl R, Barnard H, Ringe WK, Fleckenstein J, Cullum CM. Magnetic resonance imaging of cerebellar-prefrontal and cerebellar-parietal functional connectivity. Neuroimage. 2005;28:39-48.

Allin MP. Novel insights from quantitative imaging of the developing cerebellum. Semin Fetal Neonatal Med. 2016;21:333-338.

Ashworth CJ, Toma LM, Hunter MG. Nutritional effects on oocyte and embryo development in mammals: implications for reproductive efficiency and environmental sustainability. Philos Trans R Soc Lond B Biol Sci. 2009;364:3351-3361.

Augusto RL, Isaac AR, Silva-Júnior II, Santana DF, Ferreira DJ, Lagranha CJ, Gonçalves-Pimentel C, Rodrigues MC, Andrade-da-Costa BL. Fighting Oxidative Stress: Increased Resistance of Male Rat Cerebellum at Weaning Induced by Low Omega 6/Omega 3 Ratio in a Protein-Deficient Diet. Cerebellum. 2017;16:103-117.

Baken L, van Heesch PN, Wildschut HI, Koning AH, van der Spek PJ, Steegers EA, Exalto N. First-trimester crown-rump length and embryonic volume of aneuploid fetuses measured in virtual reality. Ultrasound Obstet Gynecol. 2013;41:521-525.

Baken L, Rousian M, Koning AH, Bonsel GJ, Eggink AJ, Cornette JM, Schoonderwaldt EM, Husen-Ebbinge M, Teunissen KK, van der Spek PJ, Steegers EA, Exalto N. First-Trimester Detection of Surface Abnormalities: A Comparison of 2- and 3-Dimensional Ultrasound and 3-Dimensional Virtual Reality Ultrasound. Reprod Sci. 2014;21:993-999.

Barjaktarovic M, Korevaar TI, Jaddoe VW, de Rijke YB, Visser TJ, Peeters RP, Steegers EA. Human chorionic gonadotropin (hCG) concentrations during the late first trimester are associated with fetal growth in a fetal sex-specific manner. Eur J Epidemiol. 2017;32:135-144.

Barker DJ. The origins of the developmental origins theory. J Intern Med. 2007;261:412-417.

Barker M, Dombrowski SU, Colbourn T, Fall CHD, Kriznik NM, Lawrence WT, Norris SA, Ngaiza G, Patel D, Skordis-Worrall J, Sniehotta FF, Steegers-Theunissen R, Vogel C, Woods-Townsend K, Stephenson J. Intervention strategies to improve nutrition and health behaviours before conception. Lancet. 2018;391:1853-1864.

Barua S, Kuizon S, Brown WT, Junaid MA. DNA Methylation Profiling at Single-Base Resolution Reveals Gestational Folic Acid Supplementation Influences the Epigenome of Mouse Offspring Cerebellum. Front Neurosci. 2016;10:168.

Benkhalifa M, Montjean D, Cohen-Bacrie P, Ménézo Y. Imprinting: RNA expression for homocysteine recycling in the human oocyte. Fertil Steril. 2010;93:1585-1590.

Bergen NE, Jaddoe VW, Timmermans S, Hofman A, Lindemans J, Russcher H, Raat H, Steegers-Theunissen RP, Steegers EA. Homocysteine and folate concentrations in early pregnancy and the risk of adverse pregnancy outcomes: the Generation R Study. BJOG. 2012;119:739-751.

Bergen NE, Schalekamp-Timmermans S, Jaddoe VW, Hofman A, Lindemans J, Russcher H, Tiemeier H, Steegers-Theunissen RP, Steegers EA. Maternal and Neonatal Markers of the Homocysteine Pathway and Fetal Growth: The Generation R Study. Paediatr Perinat Epidemiol. 2016;30:386-396.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proc Natl Acad Sci USA. 2010;107:3394-3399.

Bermejo-Alvarez P, Rizos D, Lonergan P, Gutierrez-Adan A. Transcriptional sexual dimorphism during preimplantation embryo development and its consequences for developmental competence and adult health and disease. Reproduction. 2011;141:563-570.

Beydoun MA, Kaufman JS, Ibrahim J, Satia JA, Heiss G. Measurement error adjustment in essential fatty acid intake from a food frequency questionnaire: alternative approaches and methods. BMC Med Res Methodol. 2007;7:41.

Blaas HG, Eik-Nes SH, Berg S, Torp H. In-vivo three-dimensional ultrasound reconstructions of embryos and early fetuses. Lancet. 1998;352:1182-1186.

Blaas HG. The examination of the embryo and early fetus: how and by whom? Ultrasound Obstet Gynecol. 1999;14:153-158.

Blanco R, Colombo A, Pardo R, Suazo J. Maternal biomarkers of methylation status and non-syndromic orofacial cleft risk: a meta-analysis. Int J Oral Maxillofac Surg. 2016;45:1323-1332.

Bonatto F, Polydoro M, Andrades ME, Conte da Frota ML Jr, Dal-Pizzol F, Rotta LN, Souza DO, Perry ML, Fonseca Moreira JC. Effects of maternal protein malnutrition on oxidative markers in the young rat cortex and cerebellum. Neurosci Lett. 2006;406:281-284.

Bottomley C, Daemen A, Mukri F, Papageorghiou AT, Kirk E, Pexsters A, De Moor B, Timmerman D, Bourne T. Assessing first trimester growth: the influence of ethnic background and maternal age. Hum Reprod. 2009;24:284-290.

Boucoiran I, Djemli A, Taillefer C, Rypens F, Delvin E, Audibert F. First-trimester prediction of birth weight. Am J Perinatol. 2013;30:665-672.

Bouwland-Both MI, Steegers-Theunissen RP, Vujkovic M, Lesaffre EM, Mook-Kanamori DO, Hofman A, Lindemans J, Russcher H, Jaddoe VW, Steegers EA. A periconceptional energy-rich dietary pattern is associated with early fetal growth: the Generation R study. BJOG. 2013;120:435-445.

Bouwland-Both MI, van Mil NH, Stolk L, Eilers PH, Verbiest MM, Heijmans BT, Tiemeier H, Hofman A, Steegers EA, Jaddoe VW, Steegers-Theunissen RP. DNA methylation of IGF2DMR and H19 is associated with fetal and infant growth: the generation R study. PLoS One. 2013;8:e81731.

Bouwland-Both MI, van Mil NH, Tolhoek CP, Stolk L, Eilers PH, Verbiest MM, Heijmans BT, Uitterlinden AG, Hofman A, van Ijzendoorn MH, Duijts L, de Jongste JC, Tiemeier H, Steegers EA, Jaddoe VW, Steegers-Theunissen RP. Prenatal parental tobacco smoking, gene specific DNA methylation, and newborns size: the Generation R study. Clin Epigenetics. 2015;7:83.

Boxmeer JC, Brouns RM, Lindemans J, Steegers EA, Martini E, Macklon NS, Steegers-Theunissen RP. Preconception folic acid treatment affects the microenvironment of the maturing oocyte in humans. Fertil Steril. 2008;89:1766-1770.

Boxmeer JC, Steegers-Theunissen RP, Lindemans J, Wildhagen MF, Martini E, Steegers EA, Macklon NS. Homocysteine metabolism in the pre-ovulatory follicle during ovarian stimulation. Hum Reprod. 2008;23:2570-2576.

Boxmeer JC, Macklon NS, Lindemans J, Beckers NG, Eijkemans MJ, Laven JS, Steegers EA, Steegers-Theunissen RP. IVF outcomes are associated with biomarkers of the homocysteine pathway in monofollicular fluid. Hum Reprod. 2009;24:1059-1066.

Braga DP, Halpern G, Setti AS, Figueira RC, Iaconelli A Jr, Borges E Jr. The impact of food intake and social habits on embryo quality and the likelihood of blastocyst formation. Reprod Biomed Online. 2015;31:30-38.

Brauer PR, Tierney BJ. Consequences of elevated homocysteine during embryonic development and possible modes of action. Curr Pharm Des. 2004;10:2719-2732.

Brown ZA, Schalekamp-Timmermans S, Tiemeier HW, Hofman A, Jaddoe VW, Steegers EA. Fetal sex specific differences in human placentation: a prospective cohort study. Placenta. 2014;35:359-364.

Cace IB, Milardovic A, Prpic I, Krajina R, Petrovic O, Vukelic P, Spiric Z, Horvat M, Mazej D, Snoj J. Relationship between the prenatal exposure to low-level of mercury and the size of a newborn's cerebellum. Med Hypotheses. 2011;76:514-516.

Cetin I, Alvino G, Cardellicchio M. Long chain fatty acids and dietary fats in fetal nutrition. J Physiol. 2009;587:3441-3451.

Cetin I, Berti C, Calabrese S. Role of micronutrients in the periconceptional period. Hum Reprod Update. 2010;16:80-95.

Cikot RJ, Steegers-Theunissen RP, Thomas CM, de Boo TM, Merkus HM, Steegers EA. Longitudinal vitamin and homocysteine levels in normal pregnancy. Br J Nutr. 2001;85:49-58.

Clifton VL. Review: Sex and the human placenta: mediating differential strategies of fetal growth and survival. Placenta. 2010;31 Suppl:S33-S39.

Crozier SR, Robinson SM, Godfrey KM, Cooper C, Inskip HM. Women's dietary patterns change little from before to during pregnancy. J Nutr. 2009;139:1956-1963.

Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supple- mentation. NEJM. 1992;327:1832-1835.

D'Mello AM, Crocetti D, Mostofsky SH, Stoodley CJ. Cerebellar gray matter and lobular volumes correlate with core autism symptoms. Neuroimage Clin. 2015;7:631-639.

de Zeeuw P, Zwart F, Schrama R, van Engeland H, Durston S. Prenatal exposure to cigarette smoke or alcohol and cerebellum volume in attention-deficit/hyperactivity disorder and typical development. Transl Psychiatry. 2012;2:e84.

Di Simone N, Maggiano N, Caliandro D, Riccardi P, Evangelista A, Carducci B, Caruso A. Homocysteine induces trophoblast cell death with apoptotic features. Biol Reprod. 2003;69:1129-1134.

Drouillet P, Kaminski M, De Lauzon-Guillain B, Forhan A, Ducimetière P, Schweitzer M, Magnin G, Goua V, Thiébaugeorges O, Charles MA. Association between maternal seafood consumption before pregnancy and fetal growth: evidence for an association in overweight women. The EDEN mother-child cohort. Paediatr Perinat Epidemiol. 2009;23:76-86.

Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, Schreurs IL, Dunselman GA, Kester AD, Geraedts JP, Evers JL. Effect of in vitro culture of human embryos on birthweight of newborns. Hum Reprod. 2010;25:605-612.

Eindhoven SC, van Uitert EM, Laven JS, Willemsen SP, Koning AH, Eilers PH, Exalto N, Steegers EA, Steegers-Theunissen RP. The influence of IVF/ICSI treatment on human embryonic growth trajectories. Hum Reprod. 2014;29:2628-2636.

Finer LB, Zolna MR. Declines in Unintended Pregnancy in the United States, 2008-2011. NEJM. 2016;374:843-852.

Finkelstein JL, Layden AJ, Stover PJ. Vitamin B-12 and Perinatal Health. Adv Nutr. 2015;6:552-563.

Fleming TP, Velazquez MA, Eckert JJ. Embryos, DOHaD and David Barker. J Dev Orig Health Dis. 2015;6:377-383.

Fleming TP, Watkins AJ, Sun C, Velazquez MA, Smyth NR, Eckert JJ. Do little embryos make big decisions? How maternal dietary protein restriction can permanently change an embryo's potential, affecting adult health. Reprod Fertil Dev. 2015;27:684-692.

Fleming TP, Watkins AJ, Velazquez MA, Mathers JC, Prentice AM, Stephenson J, Barker M, Saffery R, Yajnik CS, Eckert JJ, Hanson MA, Forrester T, Gluckman PD, Godfrey KM. Origins of lifetime health around the time of conception: causes and consequences. Lancet. 2018;391:1842-1852.

Furness D, Fenech M, Dekker G, Khong TY, Roberst C, Hague W. Folate, vitamin B12, vitamin B6 and homocysteine: impact on pregnancy outcome. Matern Child Nutr. 2013;9:155-166.

Gabory A, Attig L, Junien C. Sexual dimorphism in environmental epigenetic programming. Mol Cell Endocrinol. 2009;304:8-18.

Gabory A, Roseboom TJ, Moore T, Moore LG, Junien C. Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics. Biol Sex Differ. 2013;4:5.

Gaillard R, de Ridder MA, Verburg BO, Witteman JC, Mackenbach JP, Moll HA, Hofman A, Steegers EA, Jaddoe VW. Individually customized fetal weight charts derived from ultrasound measurements: the Generation R Study. Eur J Epidemiol. 2011;26:919-926.

Gaillard R, Steegers EA, de Jongste JC, Hofman A, Jaddoe VW. Tracking of fetal growth characteristics during different trimesters and the risks of adverse birth outcomes. Int J Epidemiol. 2014;43:1140-1153.

Giritharan G, Talbi S, Donjacour A, Di Sebastiano F, Dobson AT, Rinaudo PF. Effect of in vitro fertilization on gene expression and development of mouse preimplantation embryos. Reproduction. 2007;134:63-72.

Gluckman PD, Hanson MA. Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. Pediatr Res. 2004;56:311-317.

Gluckman PD, Hanson MA, Spencer HG. Predictive adaptive responses and human evolution. Trends Ecol Evol. 2005;20:527-533.

Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. NEJM. 2008;359:61-73.

Gluckman PD, Hanson MA, Mitchell MD. Developmental origins of health and disease: reducing the burden of chronic disease in the next generation. Genome Med. 2010;2:14.

Gresham E, Byles JE, Bisquera A, Hure AJ. Effects of dietary interventions on neonatal and infant outcomes: a systematic review and meta-analysis. Am J Clin Nutr. 2014;100:1298-1321.

Grieger JA, Clifton VL. A review of the impact of dietary intakes in human pregnancy on infant birthweight. Nutrients. 2014;7:153-178.

Hadlock FP, Harrist RB, Sharman RS, Deter RL, Park SK. Estimation of fetal weight with the use of head, body, and femur measurements – a prospective study. Am J Obstet Gynecol. 1985;151:333-337.

Hales CN, Barker DJ. The thrifty phenotype hypothesis. Br Med Bull. 2001;60:5-20.

Hammiche F, Vujkovic M, Wijburg W, de Vries JH, Macklon NS, Laven JS, Steegers-Theunissen RP. Increased preconception omega-3 polyunsaturated fatty acid intake improves embryo morphology. Fertil Steril. 2011;95:1820-1823.

Handmaker NS, Rayburn WF, Meng C, Bell JB, Rayburn BB, Rappaport VJ. Impact of alcohol exposure after pregnancy recognition on ultrasonographic fetal growth measures. Alcohol Clin Exp Res. 2006;30:892-898.

Hart R, Norman RJ. The longer-term health outcomes for children born as a result of IVF treatment: Part I–General health outcomes. Hum Reprod Update. 2013;19:232-43.

Heppe DH, Steegers EA, Timmermans S, Breeijen Hd, Tiemeier H, Hofman A, Jaddoe VW. Maternal fish consumption, fetal growth and the risks of neonatal complications: the Generation R Study. Br J Nutr. 2011;105:938-949.

Heppe DH, van Dam RM, Willemsen SP, den Breeijen H, Raat H, Hofman A, Steegers EA, Jaddoe VW. Maternal milk consumption, fetal growth, and the risks of neonatal complications: the Generation R Study. Am J Clin Nutr. 2011;94:501-509.

Higa R, Roberti SL, Musikant D, Mazzucco MB, White V, Jawerbaum A. Effects of maternal dietary olive oil on pathways involved in diabetic embryopathy. Reprod Toxicol. 2014;49:185-195.

Hoffmann K, Schulze MB, Achienkiewitz A, Nothlings U, Boeing H. Application of a new statistical method to derive dietary patterns in nutritional epidemiology. Am J Epidemiol. 2004;159:935-944.

Hogeveen M, Blom HJ, den Heijer M. Maternal homocysteine and small-for-gestational-age offspring: systematic review and meta-analysis. Am J Clin Nutr. 2012;1:130-136.

Hoeijmakers L, Kempe H, Verschure PJ. Epigenetic imprinting during assisted reproductive technologies: The effect of temporal and cumulative fluctuations in methionine cycling on the DNA methylation state. Mol Reprod Dev. 2016;83:94-107.

Holst S, Kjær SK, Jørgensen ME, Damm P, Jensen A. Fertility problems and risk of gestational diabetes mellitus: a nationwide cohort study. Fertil Steril. 2016;106:427-434.e1.

Homan GF, Davies M, Norman R. The impact of lifestyle factors on reproductive performance in the general population and those undergoing infertility treatment: a review. Hum Reprod Update. 2007;13:209-233.

Hu FB, Rimm EB, Smith-Warner SA, Feskanich D, Stampfer MJ, Ascherio A, Sampson L, Willet WC. Reproducibility and validity of dietary patterns assessed with a food-frequency questionnaire. Am J Clin Nutr. 1999;69:243-249.

Hunter DS, Hazel SJ, Kind KL, Owens JA, Pitcher JB, Gatford KL. Programming the brain: Common outcomes and gaps in knowledge from animal studies of IUGR. Physiol Behav. 2016;164:233-248.

Ikeda S, Namekawa T, Sugimoto M, Kume S. Expression of methylation pathway enzymes in bovine oocytes and preimplantation embryos. J Exp Zool A Ecol Genet Physiol. 2010;313:129-136.

Ioannou C, Sarris I, Salomon LJ, Papageorghiou AT. A review of fetal volumetry: the need for standardization and definitions in measurement methodology. Ultrasound Obstet Gynecol. 2011;38:613-619.

Jacques M, Freour T, Barriere P, Ploteau S. Adverse pregnancy and neonatal outcomes after assisted reproductive treatment in patients with pelvic endometriosis: a case-control study. Reprod Biomed Online. 2016;32:626-634.

Jaddoe VW, de Jonge LL, Hofman A, Franco OH, Steegers EA, Gaillard R. First trimester fetal growth restriction and cardiovascular risk factors in school age children: population based cohort study. BMJ. 2014;348:g14.

Jakubowski H. Pathophysiological consequences of homocysteine excess. J Nutr. 2006;136:1741S-1749S.

Jia L, Li J, He B, Jia Y, Niu Y, Wang C, Zhao R. Abnormally activated one-carbon metabolic pathway is associated with mtDNA hypermethylation and mitochondrial malfunction in the oocytes of polycystic gilt ovaries. Sci Rep. 2016;6:19436.

Kalhan SC. One carbon metabolism in pregnancy: Impact on maternal, fetal and neonatal health. Mol Cell Endocrinol. 2016;435:48-60.

Karim JN, Roberts NW, Salomon LJ, Papageorghiou AT. Systematic review of first trimester ultrasound screening in detecting fetal structural anomalies and factors affecting screening performance. Ultrasound Obstet Gynecol. 2017;50:429-441.

Kelly T, Yang W, Chen CS, Reynolds K, He J. Global burden of obesity in 2005 and projections to 2030. Int J Obes (Lond). 2008;32:1431-1437.

Knickmeyer RC, Gouttard S, Kang C, Evans D, Wilber K, Smith JK, Hamer RM, Lin W, Gerig G, Gilmore JH. A structural MRI study of human brain development from birth to 2 years. J Neurosci. 2008;28:12176-12182.

Knudsen VK, Orozova-Bekkevold IM, Mikkelsen TB, Wolff S, Olsen SF. Major dietary patterns in pregnancy and fetal growth. Eur J Clin Nutr. 2008;62:463-470.

Koning AH, Rousian M, Verwoerd-Dikkeboom CM, Goedknegt L, Steegers EA, van der Spek PJ. V-scope: design and implementation of an immersive and desktop virtual reality volume visualization system. Stud Health Technol Inform. 2009;142:136-138.

Koning IV, Groenenberg IA, Gotink AW, Willemsen SP, Gijtenbeek M, Dudink J, Go AT, Reiss IK, Steegers EA, Steegers-Theunissen RP. Periconception Maternal Folate Status and Human Embryonic Cerebellar growth Trajectories: The Rotterdam Predict Study. PLoS One. 2015;10:e0141089.

Koning IV, Dudink J, Groenenberg IAL, Willemsen SP, Reiss IKM, Steegers-Theunissen RPM. Prenatal cerebellar growth trajectories and the impact of periconceptional maternal and fetal factors. Hum Reprod. 2017;32:1230-1237.

Koning IV, van Graafeiland AW, Groenenberg IAL, Husen SC, Go ATJI, Dudink J, Willemsen SP, Cornette JMJ, Steegers-Theunissen RPM. Prenatal influence of congenital heart defects on trajectories of cortical folding of the fetal brain using three-dimensional ultrasound. Prenat Diagn. 2017;37:1008-1016.

Kramer MS. Determinants of low birth weight: methodological assessment and meta-analysis. Bull World Health Organ. 1987;65:663-737.

Kroener L, Wang ET, Pisarska MD. Predisposing factors to abnormal first trimester placentation and the impact on fetal outcomes. Semin Reprod Med. 2016;34:27-35.

Kwong WY, Adamiak SJ, Gwynn A, Singh R, Sinclair KD. Endogenous folates and single-carbon metabolism in the ovarian follicle, oocyte and pre-implantation embryo. Reproduction. 2010;139:705-715.

Larqué E, Zamora S, Gil A. Dietary trans fatty acids in early life: a review. Early Hum Dev. 2001;65 Suppl:S31-S41.

Leventakou V, Roumeliotaki T, Martinez D, Barros H, Brantsaeter AL, Casas M, Charles MA, Cordier S, Eggesbø M, van Eijsden M, Forastiere F, Gehring U, Govarts E, Halldórsson TI, Hanke W, Haugen M, Heppe DH, Heude B, Inskip HM, Jaddoe VW, Jansen M, Kelleher C, Meltzer HM, Merletti F, Moltó-Puigmartí C, Mommers M, Murcia M, Oliveira A, Olsen SF, Pele F, Polanska K, Porta D, Richiardi L, Robinson SM, Stigum H, Strøm M, Sunyer J, Thijs C, Viljoen K, Vrijkotte TG, Wijga AH, Kogevinas M, Vrijheid M, Chatzi L. Fish intake during pregnancy, fetal growth, and gestational length in 19 European birth cohort studies. Am J Clin Nutr. 2014;99:506-516.

López-Alarcón M, Montalvo-Velarde I, Vital-Reyes VS, Hinojosa-Cruz JC, Leaños-Miranda A, Martínez-Basila A. Serial determinations of asymmetric dimethylarginine and homocysteine during pregnancy to predict pre-eclampsia: a longitudinal study. BJOG. 2015;122:1586-1592.

Lubinsky M. An epigenetic association of malformations, adverse reproductive outcomes, and fetal origins hypothesis related effects. J Assist Reprod Genet. 2018;35:953-964.

M'hamdi HI, van Voorst SF, Pinxten W, Hilhorst MT, Steegers EA. Barriers in the uptake and delivery of preconception care: Exploring the views of care providers. Matern Child Health J. 2017;21:21-28

Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. Hum Reprod Update. 2002;8:333-343.

Mallikethi-Reddy S, Briasoulis A, Akintoye E, Afonso L. Novel biomarkers with potential for cardiovascular risk reclassification. Biomarkers. 2017;22:189-199.

Mandò C, Calabrese S, Mazzocco MI, Novielli C, Anelli GM, Antonazzo P, Cetin I. Sex specific adaptations in placental biometry of overweight and obese women. Placenta. 2016;38:1-7.

Mantikou E, Youssef MA, van Wely M, van der Veen F, Al-Inany HG, Repping S, Mastenbroek S. Embryo culture media and IVF/ICSI success rates: a systematic review. Hum Reprod Update. 2013;19:210-220.

Marjonen H, Sierra A, Nyman A, Rogojin V, Gröhn O, Linden AM, Hautaniemi S, Kaminen-Ahola N. Early maternal alcohol consumption alters hippocampal DNA methylation, gene expression and volume in a mouse model. PLoS One. 2015;10:e0124931.

Marques AH, Bjørke-Monsen AL, Teixeira AL, Silverman MN. Maternal stress, nutrition and physical activity: Impact on immune function, CNS development and psychopathology. Brain Res. 2015;1617:28-46.

McGee M, Bainbridge S, Fontaine-Bisson B. A crucial role for maternal dietary methyl donor intake in epigenetic programming and fetal growth outcomes. Nutr Rev. 2018;76:469-478.

McKinley MC, Strain JJ, McPartlin J, Scott JM, McNulty H. Plasma homocysteine is not subject to seasonal variation. Clin Chem. 2001;47:1430-1436.

Meher A, Randhir K, Mehendale S, Wagh G, Joshi S. Maternal Fatty Acids and Their Association with Birth Outcome: A Prospective Study. PLoS One. 2016;11:e0147359.

Miliku K, Mesu A, Franco OH, Hofman A, Steegers EAP, Jaddoe VWV. Maternal and Fetal Folate, Vitamin B12, and Homocysteine Concentrations and Childhood Kidney Outcomes. Am J Kidney Dis. 2017;69:521-530.

Montag AC, Hull AD, Yevtushok L, Zymak-Zakutnya N, Sosyniuk Z, Dolhov V, Jones KL, Wertelecki W, Chambers CD; CIFASD. Second-Trimester ultrasound as a tool for early detection of fetal alcohol spectrum disorders. Alcohol Clin Exp Res. 2016;40:2418-2425.

Mook-Kanamori DO, Steegers EA, Eilers PH, Raat H, Hofman A, Jaddoe VW. Risk factors and outcomes associated with first-trimester fetal growth restriction. JAMA. 2010;303:527-534.

Nakamura M, Hasegawa J, Arakaki T, Takita H, Hamada S, Ichizuka K, Sekizawa A. Repeated measurement of crown-rump length at 9 and 11-13 Weeks' gestation: association with adverse pregnancy outcome. Fetal Diagn Ther. 2015;38:262-268.

Nelen WL. Hyperhomocysteinaemia and human reproduction. Clin Chem Lab Med. 2001;39:758-763.

Nelissen EC, Van Montfoort AP, Smits LJ, Menheere PP, Evers JL, Coonen E, Derhaag JG, Peeters LL, Coumans AB, Dumoulin JC. IVF culture medium affects human intrauterine growth as early as the second trimester of pregnancy. Hum Reprod. 2013;28:2067-2074.

Netherlands Nutrition Centre. NEVO-tabel; Nederlands Voedingsstoffenbestand 2011, RIVM/Voedingscentrum, Den Haag. 2011.

Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr. 2002;132:2333S-2335S.

Northstone K, Emmett PM, Rogers I. Dietary patterns in pregnancy and associations with nutrient intakes. Br J Nutr. 2008;99:406-415.

Nuttall JR. The plausibility of maternal toxicant exposure and nutritional status as contributing factors to the risk of autism spectrum disorders. Nutr Neurosc. 2015;20:209-218

O'Rahilly R. Human embryo. Nature. 1987;329:385.

O'Rahilly R, Müller F. Developmental stages in human embryos: revised and new measurements. Cells Tissues Organs. 2010;192:73-84.

Obermann-Borst SA, Vujkovic M, de Vries JH, Wildhagen MF, Looman CW, de Jonge R, Steegers EA, Steegers-Theunissen RP. A maternal dietary pattern characterised by fish and seafood in association with the risk of congenital heart defects in the offspring. BJOG. 2011;118:1205-1215.

Olsen SF, Halldorsson TI, Willett WC, Knudsen VK, Gillman MW, Mikkelsen TB, Olsen J; NUTRIX Consortium. Milk consumption during pregnancy is associated with increased infant size at birth: prospective cohort study. Am J Clin Nutr. 2007;86:1104-1110.

Oostingh EC, Steegers-Theunissen RP, de Vries JH, Laven JS, Koster MP. Strong adherence to a healthy dietary pattern is associated with better semen quality, especially in men with poor semen quality. Fertil Steril. 2017;107:916-923.e2.

Ornoy A. Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. Reprod Toxicol. 2007;24:31-41.

Papadopoulou E, Caspersen IH, Kvalem HE, Knutsen HK, Duarte-Salles T, Alexander J, Meltzer HM, Kogevinas M, Brantsæter AL, Haugen M. Maternal dietary intake of dioxins and polychlorinated biphenyls and birth size in the Norwegian Mother and Child Cohort Study (MoBa). Environ Int. 2013;60:209-216.

Parisi F, Rousian M, Huijgen NA, Koning AH, Willemsen SP, de Vries JH, Cetin I, Steegers EAP, Steegers-Theunissen RPM. Periconceptional maternal 'high fish and olive oil, low meat' dietary pattern is associated with increased embryonic growth: The Rotterdam Periconceptional Cohort (Predict Study). Ultrasound Obstet Gynecol. 2017; 50:709-716.

Parisi F, Rousian M, Koning AH, Willemsen SP, Cetin I, Steegers-Theunissen RP. Periconceptional maternal one-carbon biomarkers are associated with embryonic development according to the Carnegie stages. Hum Reprod. 2017;32:523-530.

Parisi F, Rousian M, Steegers-Theunissen RPM, Koning AHJ, Willemsen SP, de Vries JHM, Cetin I, Steegers EAP. Early first trimester maternal 'high fish and olive oil and low meat' dietary pattern is associated with accelerated human embryonic development. Eur J Clin Nutr. 2018. In press.

Pierson CR, Al Sufiani F. Preterm birth and cerebellar neuropathology. Semin Fetal Neonatal Med. 2016;21:305-311.

Portugal LC, Rosa MJ, Rao A, Bebko G, Bertocci MA, Hinze AK, Bonar L, Almeida JR, Perlman SB, Versace A, Schirda C, Travis M, Gill MK, Demeter C, Diwadkar VA, Ciuffetelli G, Rodriguez E, Forbes EE, Sunshine JL, Holland SK, Kowatch RA, Birmaher B, Axelson D, Horwitz SM, Arnold EL, Fristad MA, Youngstrom EA, Findling RL, Pereira M, Oliveira L, Phillips ML, Mourao-Miranda J. Can emotional and behavioral dysregulation in youth be decoded from functional neuroimaging? PLoS One. 2016;11:e0117603.

Poudyal H, Panchal SK, Diwan V, Brown L. Omega-3 fatty acids and metabolic syndrome: effects and emerging mechanisms of action. Prog Lipid Res. 2011;50:372-387.

Pourié G, Martin N, Bossenmeyer-Pourié C, Akchiche N, Guéant-Rodriguez RM, Geoffroy A, Jeannesson E, El Hajj Chehadeh S, Mimoun K, Brachet P, Koziel V, Alberto JM, Helle D, Debard R, Leininger B, Daval JL, Guéant JL. Folate- and vitamin B12-deficient diet during gestation and lactation alters cerebellar synapsin expression via impaired influence of estrogen nuclear receptor α. FASEB J. 2015;29:3713-3725.

Ramaiyan B, Bettadahalli S, Talahalli RR. Dietary omega-3 but not omega-6 fatty acids down-regulate maternal dyslipidemia induced oxidative stress: A three generation study in rats. Biochem Biophys Res Commun. 2016;477:887-894.

Ramirez-Zea M. Validation of three predictive equations for basal metabolic rate in adults. Public Health Nutr. 2005;8:1213-1228.

Ranade SC, Sarfaraz Nawaz M, Kumar Rambtla P, Rose AJ, Gressens P, Mani S. Early protein malnutrition disrupts cerebellar development and impairs motor coordination. Br J Nutr. 2012;107:1167-75.

Reichetzeder C, Dwi Putra SE, Li J, Hocher B. Developmental Origins of Disease - crisis precipitates change. Cell Physiol Biochem. 2016;39:919-938.

Robinson HP. Sonar measurement of fetal crown rump length as means of assessing maturity in first trimester of pregnancy. BMJ. 1973;4:28-31.

Robinson HP, Fleming JE. A critical evaluation of sonar ‘crown-rump length’ measurements. BJOG. 1975;82:702–710.

Rogers I, Emmett P, Ness A, Golding J. Maternal fish intake in late pregnancy and the frequency of low birth weight and intrauterine growth retardation in a cohort of British infants. J Epidemiol Community Health. 2004;58:486-492.

Rolo LC, Nardozza LM, Araujo Júnior E, Nowak PM, Bortoletti Filho J, Moron AF. Measurement of embryo volume at 7-10 weeks' gestation by 3D-sonography. J Obstet Gynaecol. 2009;29:188-191.

Ronnenberg AG, Goldman MB, Chen D, Aitken IW, Willett WC, Selhub J, Xu X. Preconception homocysteine and B vitamin status and birth outcomes in Chinese women. Am J Clin Nutr. 2002;76:1385-1391.

Ronnenberg AG, Venners SA, Xu X, Chen C, Wang L, Guang W, Huang A, Wang X. Preconception B-vitamin and homocysteine status, conception, and early pregnancy loss. Am J Epidemiol. 2007;166:304-312.

Rousian M, Koning AH, van Oppenraaij RH, Hop WC, Verwoerd-Dikkeboom CM, van der Spek PJ, Exalto N, Steegers EA. An innovative virtual reality technique for automated human embryonic volume measurements. Hum Reprod. 2010;25:2210-2216.

Rousian M, Koning AH, van der Spek PJ, Steegers EA, Exalto N. Virtual reality for embryonic measurements requiring depth perception. Fertil Steril. 2011;95:773-774.

Rousian M, Groenenberg IA, Hop WC, Koning AH, van der Spek PJ, Exalto N, Steegers EA. Human embryonic growth and development of the cerebellum using 3-dimensional ultrasound and virtual reality. Reprod Sci. 2013;20:899-908.

Rousian M, Hop WC, Koning AH, van der Spek PJ, Exalto N, Steegers EA. First trimester brain ventricle fluid and embryonic volumes measured by three-dimensional ultrasound with the use of I-Space virtual reality. Hum Reprod. 2013;28:1181-1189.

Rousian M, Koster MPH, Mulders AGMGJ, Koning AHJ, Steegers-Theunissen RPM, Steegers EAP. Virtual reality imaging techniques in the study of embryonic and early placental health. Placenta. 2018;64 Suppl 1:S29-S35.

Schalekamp-Timmermans S, Arends LR, Alsaker E, Chappell L, Hansson S, Harsem NK, Jälmby M, Jeyabalan A, Laivuori H, Lawlor DA, Macdonald-Wallis C, Magnus P, Myers J, Olsen J, Poston L, Redman CW, Staff AC, Villa P, Roberts JM, Steegers EA. Global Pregnancy Collaboration. Fetal sex-specific differences in gestational age at delivery in pre-eclampsia: a meta-analysis. Int J Epidemiol. 2017;46:632-642.

Shen Y, Wan H, Zhu J, Fang Z, Che L, Xu S, Lin Y, Li J, Wu D. Fish oil and olive oil supplementation in late pregnancy and lactation differentially affect oxidative stress and inflammation in sows and piglets. Lipids. 2015;50:647-658.

Sidhu KS. Health benefits and potential risks related to consumption of fish or fish oil. Regul Toxicol Pharmacol. 2003;38:336-344.

Siebelink E, Geelen A, de Vries JH. Self-reported energy intake by FFQ compared with actual energy intake to maintain body weight in 516 adults. Br J Nutr. 2011;106:274-281.

Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA, Lea RG, Craigon J, McEvoy TG, Young LE. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci USA. 2007;104:19351-19356.

Sinclair KD, Watkins AJ. Parental diet, pregnancy outcomes and offspring health: metabolic determinants in developing oocytes and embryos. Reprod Fertil Dev. 2013;26:99-114.

Skinner MK. What is an epigenetic transgenerational phenotype? F3 or F2. Reprod Toxicol. 2008;25:2-6.

Slimani N, Fahey M, Welch AA, Wirfält E, Stripp C, Bergström E, Linseisen J, Schulze MB, Bamia C, Chloptsios Y, Veglia F, Panico S, Bueno-de-Mesquita HB, Ocké MC, Brustad M, Lund E, González CA, Barcos A, Berglund G, Winkvist A, Mulligan A, Appleby P, Overvad K, Tjønneland A, Clavel-Chapelon F, Kesse E, Ferrari P, Van Staveren WA, Riboli E. Diversity of dietary patterns observed in the European Prospective Investigation into Cancer and Nutrition (EPIC) project. Public Health Nutr. 2002;5:1311-1328.

Shpyleva S, Ivanovsky S, de Conti A, Melnyk S, Tryndyak V, Beland FA, James SJ, Pogribny IP. Cerebellar oxidative DNA damage and altered DNA methylation in the BTBR T+tf/J mouse model of autism and similarities with human post mortem cerebellum. PLoS One. 2014;9:e113712.

Solé-Navais P, Cavallé-Busquets P, Fernandez-Ballart JD, Murphy MM. Early pregnancy B vitamin status, one carbon metabolism, pregnancy outcome and child development. Biochimie. 2016;126:91-96.

Song S, Ghosh J, Mainigi M, Turan N, Weinerman R, Truongcao M, Coutifaris C, Sapienza C. DNA methylation differences between in vitro- and in vivo-conceived children are associated with ART procedures rather than infertility. Clin Epigenetics. 2015;7:41.

Steegers EA, Barker ME, Steegers-Theunissen RP, Williams MA. Societal valorisation of new knowledge to improve perinatal health: Time to act. Paediatr Perinat Epidemiol. 2016;30:201-204.

Steegers-Theunissen RP, Boers GH, Trijbels FJ, Eskes TK. Neural-tube defects and derangement of homocysteine metabolism. NEJM. 1991;324:199-200.

Steegers-Theunissen RP, Twigt J, Pestinger V, Sinclair KD. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. Hum Reprod Update. 2013;19:640-655.

Steegers-Theunissen RP, Steegers EA. Embryonic health: new insights, mHealth and personalised patient care. Reprod Fertil Dev. 2015;27:712-715.

Steegers-Theunissen RP, Verheijden-Paulissen JJ, van Uitert EM, Wildhagen MF, Exalto N, Koning AH, Eggink AJ, Duvekot JJ, Laven JS, Tibboel D, Reiss I, Steegers EA. Cohort Profile: The Rotterdam Periconceptional Cohort (Predict Study). Int J Epidemiol. 2016;45:374-381.

Stephenson J, Heslehurst N, Hall J, Schoenaker DAJM, Hutchinson J, Cade JE, Poston L, Barrett G, Crozier SR, Barker M, Kumaran K, Yajnik CS, Baird J, Mishra GD. Before the beginning: nutrition and lifestyle in the preconception period and its importance for future health. Lancet. 2018;391:1830-1841.

Stratakis N, Roumeliotaki T, Oken E, Barros H, Basterrechea M, Charles MA, Eggesbø M, Forastiere F, Gaillard R, Gehring U, Govarts E, Hanke W, Heude B, Iszatt N, Jaddoe VW, Kelleher C, Mommers M, Murcia M, Oliveira A, Pizzi C, Polańska K, Porta D, Richiardi L, Rifas-Shiman SL, Schoeters G, Sunyer J, Thijs C, Viljoen K, Vrijheid M, Vrijkotte TG, Wijga AH, Zeegers MP, Kogevinas M, Chatzi L. Fish intake in pregnancy and child growth: A pooled analysis of 15 European and US birth cohorts. JAMA Pediatr. 2016;170:381-390.

Surén P, Roth C, Bresnahan M, Haugen M, Hornig M, Hirtz D, Lie KK, Lipkin WI, Magnus P, Reichborn-Kjennerud T, Schjølberg S, Davey Smith G, Øyen AS, Susser E, Stoltenberg C. Association between maternal use of folic acid supplements and risk of autism spectrum disorders in children. JAMA. 2013;309:570-577.

Tan K, Wang Z, Zhang Z, An L, Tian J. IVF affects embryonic development in a sex-biased manner in mice. Reproduction. 2016;151:443-453.

Taparia S, Gelineau-van Waes J, Rosenquist TH, Finnell RH. Importance of folate-homocysteine homeostasis during early embryonic development. Clin Chem Lab Med. 2007;45:1717-1727.

Thorsdottir I, Birgisdottir BE, Halldorsdottir S, Geirsson RT. Association of fish and fish liver oil intake in pregnancy with infant size at birth among women of normal weight before pregnancy in a fishing community. Am J Epidemiol. 2004;160:460-465.

Timmermans S, Jaddoe VW, Hofman A, Steegers-Theunissen RP, Steegers EA. Periconception folic acid supplementation, fetal growth and the risks of low birth weight and preterm birth: the Generation R Study. Br J Nutr. 2009;102:777-785.

Twigt JM, Bezstarosti K, Demmers J, Lindemans J, Laven JS, Steegers-Theunissen RP. Preconception folic acid use influences the follicle fluid proteome. Eur J Clin Invest. 2015;45:833-841.

Uauy R, Kain J, Corvalan C. How can the Developmental Origins of Health and Disease (DOHaD) hypothesis contribute to improving health in developing countries? Am J Clin Nutr. 2011;94:1759S-1764S.

van den Berg CB, Chaves I, Herzog EM, Willemsen SP, van der Horst GTJ, Steegers-Theunissen RPM. Early- and late-onset preeclampsia and the DNA methylation of circadian clock and clock-controlled genes in placental and newborn tissues. Chronobiol Int. 2017;34:921-932.

van Driel LM, de Jonge R, Helbing WA, van Zelst BD, Ottenkamp J, Steegers EA, Steegers-Theunissen RP. Maternal global methylation status and risk of congenital heart diseases. Obstet Gynecol. 2008;112:277-283.

van Uitert EM, van der Elst-Otte N, Wilbers JJ, Exalto N, Willemsen SP, Eilers PH, Koning AH, Steegers EA, Steegers-Theunissen RP. Periconception maternal characteristics and embryonic growth trajectories: the Rotterdam Predict study. Hum Reprod. 2013;28:3188-3196.

van Uitert EM, Exalto N, Burton GJ, Willemsen SP, Koning AH, Eilers PH, Laven JS, Steegers EA, Steegers-Theunissen RP. Human embryonic growth trajectories and associations with fetal growth and birthweight. Hum Reprod. 2013;28:1753-1761.

van Uitert EM, Steegers-Theunissen RP. Influence of maternal folate status on human fetal growth parameters. Mol Nutr Food Res. 2013;57:582-595.

van Uitert EM, van Ginkel S, Willemsen SP, Lindemans J, Koning AH, Eilers PH, Exalto N, Laven JS, Steegers EA, Steegers-Theunissen RP. An optimal periconception maternal folate status for embryonic size: the Rotterdam Predict study. BJOG. 2014;121:821-829.

Verkleij-Hagoort AC, Verlinde M, Ursem NT, Lindemans J, Helbing WA, Ottenkamp J, Siebel FM, Gittenberger-de Groot AC, de Jonge R, Bartelings MM, Steegers EA, Steegers-Theunissen RP. Maternal hyperhomocysteinaemia is a risk factor for congenital heart disease. BJOG. 2006;113:1412-1418.

van Mil NH, Bouwland-Both MI, Stolk L, Verbiest MM, Hofman A, Jaddoe VW, Verhulst FC, Eilers PH, Uitterlinden AG, Steegers EA, Tiemeier H, Steegers-Theunissen RP. Determinants of maternal pregnancy one-carbon metabolism and newborn human DNA methylation profiles. Reproduction. 2014;148:581-592.

Verkleij-Hagoort A, Bliek J, Sayed-Tabatabaei F, Ursem N, Steegers E, Steegers-Theunissen R. Hyperhomocysteinemia and MTHFR polymorphisms in association with orofacial clefts and congenital heart defects: a meta-analysis. Am J Med Genet A. 2007;143A:952-960.

Verkleij-Hagoort AC, de Vries JH, Stegers MP, Lindemans J, Ursem NT, Steegers-Theunissen RP. Validation of the assessment of folate and vitamin B12 intake in women of reproductive age: the method of triads. Eur J Clin Nutr. 2007;61:610-615.

Verwoerd-Dikkeboom CM, Koning AH, Hop WC, Rousian M, Van Der Spek PJ, Exalto N, Steegers EA. Reliability of three-dimensional sonographic measurements in early pregnancy using virtual reality. Ultrasound Obstet Gynecol. 2008;32:910-916.

Verwoerd-Dikkeboom CM, Koning AH, van der Spek PJ, Exalto N, Steegers EA. Embryonic staging using a 3D virtual reality system. Hum Reprod. 2008;23:1479-1484.

Verwoerd-Dikkeboom CM, Koning AH, Hop WC, van der Spek PJ, Exalto N, Steegers EA. Innovative virtual reality measurements for embryonic growth and development. Hum Reprod. 2010;25:1404-1410.

Vinkesteijn AS, Mulder PG, Wladimiroff JW. Fetal transverse cerebellar diameter measurements in normal and reduced fetal growth. Ultrasound Obstet Gynecol. 2000;15:47-51.

Vollset SE, Refsum H, Irgens LM, Emblem BM, Tverdal A, Gjessing HK, Monsen AL, Ueland PM. Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine study. Am J Clin Nutr. 2000;71:962-968.

Vujkovic M, Ocke MC, van der Spek PJ, Yazdanpanah N, Steegers EA, Steegers-Theunissen RP. Maternal Western dietary patterns and the risk of developing a cleft lip with or without a cleft palate. Obstet Gynecol. 2007;110:378-384.

Vujkovic M, Steegers EA, Looman CW, Ocké MC, van der Spek PJ, Steegers-Theunissen RP. The maternal Mediterranean dietary pattern is associated with a reduced risk of spina bifida in the offspring. BJOG. 2009;116:408-415.

Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. Hum Reprod Update. 2016;22:2-22.

Waterland RA, Jirtle RL. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition. 2004;20:63-68.

Waters SM, Coyne GS, Kenny DA, MacHugh DE, Morris DG. Dietary n-3 polyunsaturated fatty acid supplementation alters the expression of genes involved in the control of fertility in the bovine uterine endometrium. Physiol Genomics. 2012;44:878-888.

Watkins AJ, Ursell E, Panton R, Papenbrock T, Hollis L, Cunningham C, Wilkins A, Perry VH, Sheth B, Kwong WY, Eckert JJ, Wild AE, Hanson MA, Osmond C, Fleming TP. Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. Biol Reprod. 2008;78:299-306.

Wijnands KP, van Uitert EM, Roeters van Lennep JE, Koning AH, Mulders AG, Laven JS, Steegers EA, Steegers-Theunissen RP. The periconception maternal cardiovascular risk profile influences human embryonic growth trajectories in IVF/ICSI pregnancies. Hum Reprod. 2016;31:1173-1181.

Williams MA, Frederick IO, Qiu C, Meryman LJ, King IB, Walsh SW, Sorensen TK. Maternal erythrocyte omega-3 and omega-6 fatty acids, and plasma lipid concentrations, are associated with habitual dietary fish consumption in early pregnancy. Clin Biochem. 2006;39:1063-1070.

Williams L, Seki Y, Vuguin PM, Charron MJ. Animal models of in utero exposure to a high fat diet: a review. Biochim Biophys Acta. 2014;1842:507-519.

Wong WY, Eskes TK, Kuijpers-Jagtman AM, Spauwen PH, Steegers EA, Thomas CM, Hamel BC, Blom HJ, Steegers-Theunissen RP. Nonsyndromic orofacial clefts: association with maternal hyperhomocysteinemia. Teratology. 1999;60:253-257.

Xia W, Chiu YH, Williams PL, Gaskins AJ, Toth TL, Tanrikut C, Hauser R, Chavarro JE. Men's meat intake and treatment outcomes among couples undergoing assisted reproduction. Fertil Steril. 2015;104:972-979.

Yajnik CS, Deshmukh US. Fetal programming: maternal nutrition and role of one-carbon metabolism. Rev Endocr Metab Disord. 2012;13:121-127.

Yajnik CS, Chandak GR, Joglekar C, Katre P, Bhat DS, Singh SN, Janipalli CS, Refsum H, Krishnaveni G, Veena S, Osmond C, Fall CH. Maternal homocysteine in pregnancy and offspring birthweight: epidemiological associations and Mendelian randomization analysis. Int J Epidemiol. 2014;43:1487-1497.

Yang M, Li W, Wan Z, Du Y. Elevated homocysteine levels in mothers with neural tube defects: a systematic review and meta-analysis. J Matern Fetal Neonatal Med. 2017;30:2051-2057.

Yu Y, Dong J, Wang Y, Wang Y, Min H, Shan Z, Teng W, Chen J. Maternal marginal iodine deficiency limits dendritic growth of cerebellar purkinje cells in rat offspring by NF-κB signaling and MAP1B. Environ Toxicol. 2017;32:1241-1251.

Zandstra H, Van Montfoort AP, Dumoulin JC. Does the type of culture medium used influence birthweight of children born after IVF? Hum Reprod. 2015;30:530-542.

Zhu J, Li M, Chen L, Liu P, Qiao J. The protein source in embryo culture media influences birthweight: a comparative study between G1 v5 and G1-PLUS v5. Hum Reprod. 2014;29:1387-1392.

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BIBLIOGRAPHY

- **This thesis**

Parisi F, Rousian M, Koning IV, Willemsen SP, de Vries JHM, Steegers EAP, Steegers-Theunissen RPM
Periconceptional maternal dairy-rich dietary pattern influences prenatal cerebellar growth
PLoS One. 2018;13:e0197901

Parisi F, Rousian M, Koning AHJ, Willemsen SP, Steegers EAP, Steegers-Theunissen RPM
Impact of human morphological embryonic development on fetal growth: The Rotterdam Periconceptional Cohort (Predict Study)
Submitted

Parisi F, Rousian M, Huijgen NA, Koning AHJ, Willemsen SP, de Vries JHM, Cetin I, Steegers EAP, Steegers-Theunissen RPM.
Periconceptional maternal ‘high fish and olive oil, low meat’ dietary pattern is associated with increased embryonic growth: The Rotterdam Periconceptional Cohort (Predict Study).
Ultrasound Obstet Gynecol. 2017;50:709-716

Parisi F, Rousian M, Koning AH, Willemsen SP, Cetin I, Steegers EA, Steegers-Theunissen RP.
Periconceptional maternal biomarkers of one-carbon metabolism and embryonic growth trajectories: The Rotterdam Periconceptional Cohort (Predict Study).
Fertil Steril. 2017;107:691-698.e1

Parisi F, Rousian M, Steegers-Theunissen RPM, Koning AHJ, Willemsen SP, de Vries JHM, Cetin I, Steegers EAP.
Early first trimester maternal ‘high fish and olive oil and low meat’ dietary pattern is associated with accelerated human embryonic development.
Eur J Clin Nutr. 2018. In press.

Parisi F, Rousian M, Koning AH, Willemsen SP, Cetin I, Steegers-Theunissen RP.
Periconceptional maternal one-carbon biomarkers are associated with embryonic development according to the Carnegie stages.
Hum Reprod. 2017;32:523-530

- **Other manuscripts**

Parisi F, di Bartolo I, Savasi VM, Cetin I.
Micronutrient supplementation in pregnancy: who, what and how much?
Obstet Med. 2018. In press.

Antonazzo P, Di Bartolo I, **Parisi F**, Cetin I, Savasi VM.
Preoperative and postoperative ultrasound assessment of stress urinary incontinence.
Minerva Ginecol. 2018. In press.

Savasi V, Oneta M, Laoreti A, **Parisi F**, Parrilla B, Duca P, Cetin I.
Effects of Antiretroviral Therapy on Sperm DNA Integrity of HIV-1-Infected Men.
Am J Mens Health. 2018. In press.

Parisi F, Berti C, Mandò C, Martinelli A, Mazzali C, Cetin I.
Effects of different regimens of iron prophylaxis on maternal iron status and pregnancy outcome: a randomized control trial.
J Matern Fetal Neonatal Med. 2017;30:1787-1792.

Mandò C, De Palma C, Stampalija T, Anelli GM, Figus M, Novielli C, **Parisi F**, Clementi E, Ferrazzi E, Cetin I.
Placental mitochondrial content and function in intrauterine growth restriction and preeclampsia.
Am J Physiol Endocrinol Metab. 2014;306:E404-413.

Parisi F, Laoreti A, Cetin I.
Multiple micronutrient needs in pregnancy in industrialized countries.
Ann Nutr Metab. 2014;65:13-21.

Savasi V, Leone FP, Fusè F, **Parisi F**, Cetin I.
Assisted reproductive technologies and uterine factors influencing their success. *Minerva Ginecol. 2013;65:505-524.*

Cetin I, **Parisi F**, Berti C, Mandò C, Desoye G.
Placental fatty acid transport in maternal obesity.
J Dev Orig Health Dis. 2012;3:409-414.

Cetin I, Berti C, Mandò F, **Parisi F**.
Placental iron transport and maternal absorption.
Ann Nutr Metab. 2011;59:55-58.

- **Book chapters**

Cetin I, Massari M, **Parisi F**.
Role of micronutrients in the periconceptional period.
Book title: Handbook of diet and nutrition in the menstrual cycle, periconception and fertility. Human Health Handbooks no. 7. doi: 10.3920/978-90-8686-767-7

Cetin I, Mandó C, **Parisi F**.
Maternal characteristics predisposing to fetal growth restriction.
Book title: Diet, nutrition and fetal programming. Chapter 5, pg 54-66.

Cetin I, **Parisi F**, Mazzocco MI.
Diet and the obese pregnant patient.
Book title: Pregnancy and obesity. Chapter 9, pg 111-122. doi:10.1515/9783110487817-009

- **Others**

Collaboration in the drafting of the Italian guidelines: “Nutrizione in gravidanza e durante l’allattamento” (Nutrition during pregnancy and breastfeeding). 2018.

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PHD TRAINING

Biostatistical methods (MED01, University of Milan, Italy)	2012
Genetics (MED03, University of Milan, Italy)	2012
Biology (BIO/13, University of Milan, Italy)	2012
Scientific Integrity (Erasmus MC)	2015
TOEFL certification (total score 103, Amsterdam)	2016

LOCAL SEMINARS AND WORKSHOPS

- Annual Wladimiroff Award Meeting (RCOG), Rotterdam, the Netherlands (2015)
- Annual Sophia Research day (2015)

- Annual PhD day (2015)
- Weekly research meeting of the department of Obstetrics and Gynecology (2014-2015)

PRESENTATIONS AT CONFERENCES

- 9° Congresso Nazionale Società Italiana Endoscopia Ginecologica, Milan, Italy (2014) [oral]
- Congresso Nazionale Di Medicina Della Riproduzione, Riccione, Italy (2014) [oral]
- 18° Congresso Nazionale Societá Italiana di Medicina Perinatale (SIMP), Assisi, Italy (2015) [oral & poster]
- X Congresso Regionale AOGOI Sicilia: Stili Di Vita E Salute Della Donna Mediterranea, Palermo, Italy (2015) [oral]
- Mini-symposium on developmental programming for human disease: preconception nutrition and lifelong health. Grasmere, England (2016) [oral]
- XI Congresso Regione Sicilia AOGOI, Catania, Italy (2016) [oral]
- Approccio a differenti tematiche della salute della donna: carenza di ferro, disfunzione genito-urinaria, infezioni vaginali, patologia emorroidaria, Verona, Italy (2016) [oral]
- Preis school, Nutrition in pregnancy. Florence (2017) [oral]
- 19° Congresso Nazionale Societá Italiana di Medicina Perinatale (SIMP), Naples, Italy, (2017) [oral & poster]
- 64th Annual meeting of the Society for Reproductive Investigation, Orlando (2017) [poster]
- VI Edizione Le Giornate Di Carlo Cannella: Micronutrienti E Composti Bioattivi, Roma, Italy (2017) [oral]
- Aggiornamenti in medicina materno-fetale, Assisi, Italy (2017) [oral]

TEACHING ACTIVITIES

Supervising theses of medical student
Cristina Melinte, Università degli Studi di Milano, Italy (2017)
Teaching programs and exam commission
Course of Obstetrics and Gynecology, Università degli Studi di Milano, Milan, Italy (2016-2018)

AWARDS

- Best oral presentation (Giorgio Pardi Foundation Prize): Maternal one carbon metabolism and first trimester embryonic growth using virtual reality: The Rotterdam Periconceptional Cohort (Predict Study). National Congress of Perinatal medicine (Congresso Nazionale Societá Italiana di Medicina Perinatale (SIMP)), Assisi, Italy, December 3-5, 2015.

- Best contributed paper (The Rank Prize Funds, Nutrition Committee): Periconceptional maternal dietary patterns, one-carbon biomarkers and embryonic growth trajectories. Mini-symposium on developmental programming for human disease: preconception nutrition and lifelong health. Grasmere, England, February 22-25, 2016.

- Best oral presentation (SIFES and MR prize): Associations between first trimester embryonic development and mid-pregnancy fetal size: The Rotterdam Periconceptional Cohort (Predict Study). National Congress of the Italian Society of Fertility and Sterility and Reproductive Medicine (Congresso Nazionale Società Italiana di fertilità e Sterilità e Medicina della Riproduzione (SIFES e MR)). Riccione, Italy, May 25-27, 2017.

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ABOUT THE AUTHOR

Francesca Parisi was born on August 28th, 1985 in Varese, Italy.
She had always wanted to be a doctor since her childhood, when she looked at the doctors who changed her brother's life as heroes. In 2004 she started her medical study in Milan and graduated in 2010. In the same year, she started to work as a resident at the department of Obstetrics and Gynecology at the "L. Sacco" University Hospital in Milan, Italy, following both clinical and research activities under the guidance of prof.dr. Irene Cetin. With her support and help, she experienced a wonderful year of research at the department of Obstetrics and Gynaecology of the Erasmus MC in Rotterdam under the supervision of prof.dr. Régine P.M. Steegers-Theunissen, resulting in this PhD thesis. In 2016, she completed her residency in Milan and she started to work as a gynecologist at the department of Obstetrics and Gynecology at the "L. Sacco" University Hospital, Milan.
Francesca became mother of a wonderful little girl named Giorgia on April 8th, 2018.

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