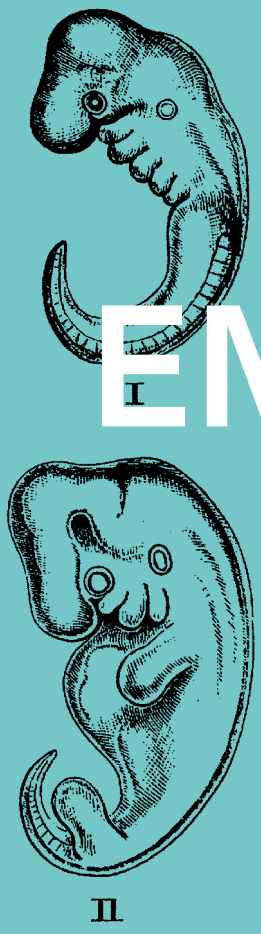

HUMAN EMBRYONIC GROWTH

Periconception parental and environmental factors

Evelyne M. van Uitert

STELLINGEN

Behorende bij het proefschrift
Human embryonic growth - Periconception parental and environmental factors

1 Embryonale groei is niet uniform en is geassocieerd met groei later in de zwangerschap en geboortegewicht (dit proefschrift).

2 Zowel maternale als paternale factoren zijn van invloed op de embryonale groei van het kind (dit proefschrift).

3 Er bestaat een optimale periconceptionele maternale foliumzuurstatus met betrekking tot de embryonale groei (dit proefschrift).

4 Een IVF/ ICSI behandeling heeft geen invloed op de embryonale groei (dit proefschrift).

5 Met driedimensionaal echoscopisch onderzoek en virtual reality technieken kan de kromming van een embryo betrouwbaar in vivo worden gemeten (dit proefschrift).

6 Driedimensionale echografie geeft mogelijkheden voor het zeer nauwkeurig meten van driedimensionale structuren en voor het verkrijgen van meer inzicht in de embryonale ontwikkeling in vivo.

7 Preconceptie- en prenatale zorg zijn van groot belang, niet alleen voor de gezondheid van de eerstvolgende generatie maar ook voor die van de generaties daarna.

8 Met het ontwikkelen van customized groeicurves waarbij rekening wordt gehouden met ouderlijke en omgevingskarakteristieken, kan de datering en daarmee de diagnostiek van afwijkende groei verder worden verbeterd.

9 Het is belangrijk te weten wat men weet, maar nog vele malen belangrijker is het besef van wat men niet weet.

10 Nothing is a waste of time if you use the experience wisely. (Auguste Rodin, geciteerd in Heads and Tales (1936) door Malvina Hoffman)

11 Omnium rerum principia parva sunt. (Marcus Tullius Cicero, De Finibus Bonorum et Malorum)

Evelyne van Uitert, 25 april 2014

HUMAN EMBRYONIC GROWTH

Periconception parental and environmental factors

Evelyne M. van Uitert

Human embryonic growth
Periconception parental and environmental factors
Thesis, Erasmus MC, University Medical Centre, Rotterdam, The Netherlands

The research presented in this dissertation was performed at the department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Centre, Rotterdam, The Netherlands, and within the framework of the Erasmus Postgraduate School Molecular Medicine.

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HUMAN EMBRYONIC GROWTH

Periconception parental and environmental factors

Humane embryonale groei
Periconceptionele ouderlijke en omgevingsfactoren

Proefschrift

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

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
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door

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Geboren te Stanford (CA), USA

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	Prof.dr. F.M. Helmerhorst

Paranimfen	Dr. A.M. Oosterbaan
	Drs. B.W. Prick

CONTENTS

INTRODUCTION

PRENATAL GROWTH

After fertilisation, the conceptus migrates to the uterus while cellular divisions rapidly take place. In the following week implantation ensues and the embryo nests into the endometrium. At 6 weeks of gestation, defined as the time that has passed since the first day of the last menstrual period, the greatest length of the human embryo measures approximately 5mm and using ultrasound a heartbeat can be visualized. At 7 weeks limb buds are visible, and between 7 and 8 weeks the first jerky movements can be observed. At 10 weeks of gestation, the embryo measures approximately 30mm from crown to rump, and the embryonic period draws to an end as organogenesis is completed [1]. In essence all organ systems are now present, although functionally these will have to develop further throughout the remainder of pregnancy [1].

Prenatal growth in the second half of pregnancy and subsequent birth weight have been studied for decades and have been shown to be associated not only with pregnancy outcome but also with health and disease throughout adult life [2-4]. The periconception period is highly important with respect to cell multiplication, differentiation and epigenetic programming of the gametes, embryo and placenta by DNA methylation of genes implicated in growth and development [3, 5]. During the preconceptional period, both members of the couple constitute the environment of the gametes providing the DNA of the future embryo. After conception has taken place, during pregnancy the mother-to-be is the main environment of the developing embryo and fetus, whereas paternal influence during pregnancy is restricted to indirect and passive exposures of the mother-to-be. Many parental and environmental factors during pregnancy have been shown to influence birth weight [6-11]. While maternal characteristics in association with fetal growth and birth outcome have been studied extensively over the years, paternal characteristics have received considerably less attention and those studies that have addressed the father-to-be have focused mainly on semen quality and fertility outcome [12, 13]. Although the embryonic period is perhaps the most important period of prenatal development as this is the period in which organogenesis is completed [1], first trimester embryonic growth has received far less attention [14-17]. Moreover, longitudinal studies are scarce.

In current day clinical practice embryonic crown-rump length (CRL) is commonly used to date pregnancies, as it is more precise than using the first day of the last menstrual period (LMP). An important underlying assumption is that contrary to birth weight, the CRL is believed to be uniform.

One of the most widespread exposures in the preconception and embryonic period is the use of folic acid supplements, shown to prevent neural tube defects and therefore recommended by the World Health Organisation to use from the periconception period up to 12 weeks of pregnancy [18, 19]. Folate is an important substrate of the one-carbon

metabolism, in which one-carbon groups are provided for DNA methylation and the synthesis of DNA, RNA, proteins and lipids [20]. Although folic acid supplement use has been investigated thoroughly with regard to birth weight, the influence of other folate determinants and on other growth parameters remains less clear. Therefore, a review of studies on maternal folate status during pregnancy may yield comprehensive insights into associations with embryonic and fetal growth parameters. Furthermore, if folate and other parental and environmental factors would affect embryonic growth, more insight into these associations may create opportunities to improve periconception and prenatal care.

Recent developments in ultrasound and virtual reality techniques have tremendously improved the possibilities of visualisation of the human embryo. The use of three-dimensional (3D) ultrasound in combination with virtual reality leads to the ultimate benefit of the third dimension by enabling depth perception and thus an actual view of the third dimension [21]. Through intuitive interaction with the projected images the optimum plane can be obtained, resulting in precise and reliable measurement of the human embryo in vivo [22-24]. CRL measurements using 3D ultrasound and virtual reality techniques have shown show excellent agreement with 2D ultrasound measurements and good inter- and intraobserver agreement [23]. To obtain measurements of maximum precision these techniques will be used to perform embryonic measurements in the studies presented throughout this thesis.

AIMS OF THE THESIS

In this thesis we aim to study human embryonic growth and its associations with periconception parental and environmental factors. The main objectives of this thesis are as follows:

1. How do human embryonic growth trajectories and embryonic curvature evolve in the first trimester and is human embryonic growth associated with subsequent prenatal growth and birth weight?

2. Are parental characteristics, lifestyle factors and environmental exposures associated with differences in embryonic growth?

METHODOLOGY

Data for the studies presented in this thesis were collected in the Rotterdam Predict study, a prospective periconception cohort study at the Department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Centre Rotterdam, the Netherlands.

All women of at least 18 years old with ongoing singleton pregnancies of 6 to 8 weeks gestation and their partners were eligible for participation and included in 2009 and 2010. Women received weekly transvaginal three-dimensional ultrasound scans from enrolment up to the 13th week of pregnancy. Ultrasound scans were performed with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D View software (General Electrics Medical Systems, Zipf, Australia). Afterwards the obtained 3D-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. This is a four-walled CAVE^{TM}-like (Cave Automatic Virtual Environment) virtual reality system, allowing depth perception and interaction with the projected images using stereoscopic imaging (**Figure 1**) [25]. CRL measurements were performed offline using the I-Space and V-Scope software [21], and by placing the callipers at the outer side of the crown and rump in the mid-sagittal plane (**Figure 2**).

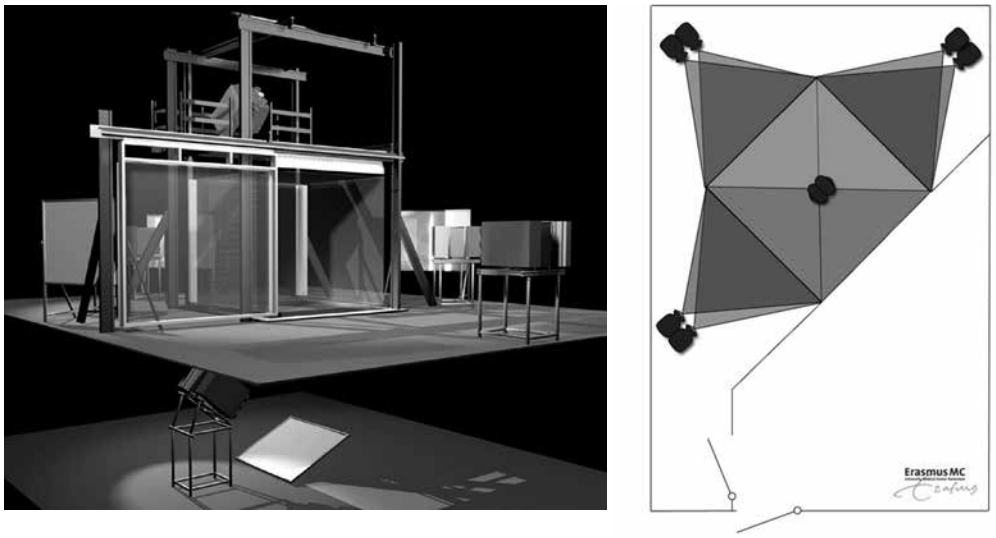


Figure 1 Barco I-space multi-walled stereoscopic environment (copyright Barco N.V., Kortrijk, Belgium www.Barco.com; Erasmus MC)

Information on the routine structural ultrasound examination at approximately 20 weeks gestation, the infant's date of birth, gender, birth weight and presence of one or multiple congenital anomalies were obtained from medical records.

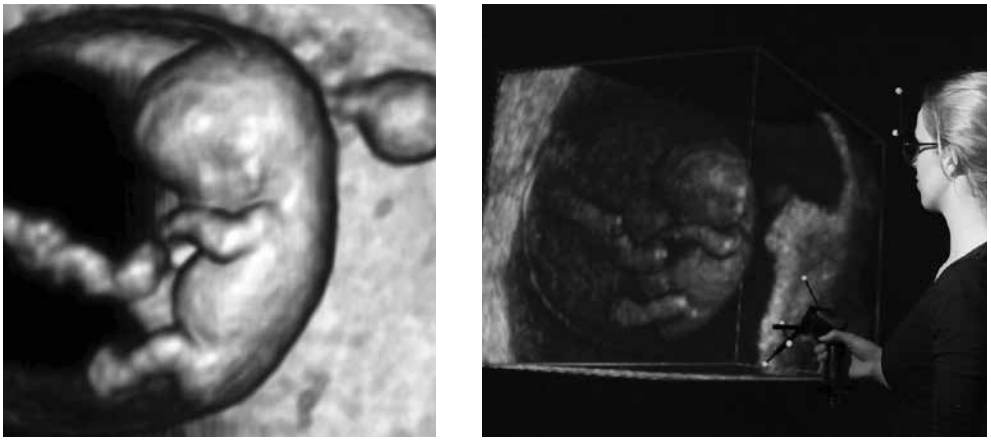


Figure 2 An embryo at 9^{+1} weeks of gestation on a 3D ultrasound image (left), and in the I-Space (right).

CHAPTER OUTLINE

In Part One we study longitudinal human embryonic growth in vivo and the association between embryonic growth and subsequent growth in mid-pregnancy and at birth. In the second chapter we evaluate whether embryonic curvature can be measured reliably using three-dimensional ultrasound and virtual reality, and whether curvature is different in embryos resulting in a miscarriage.

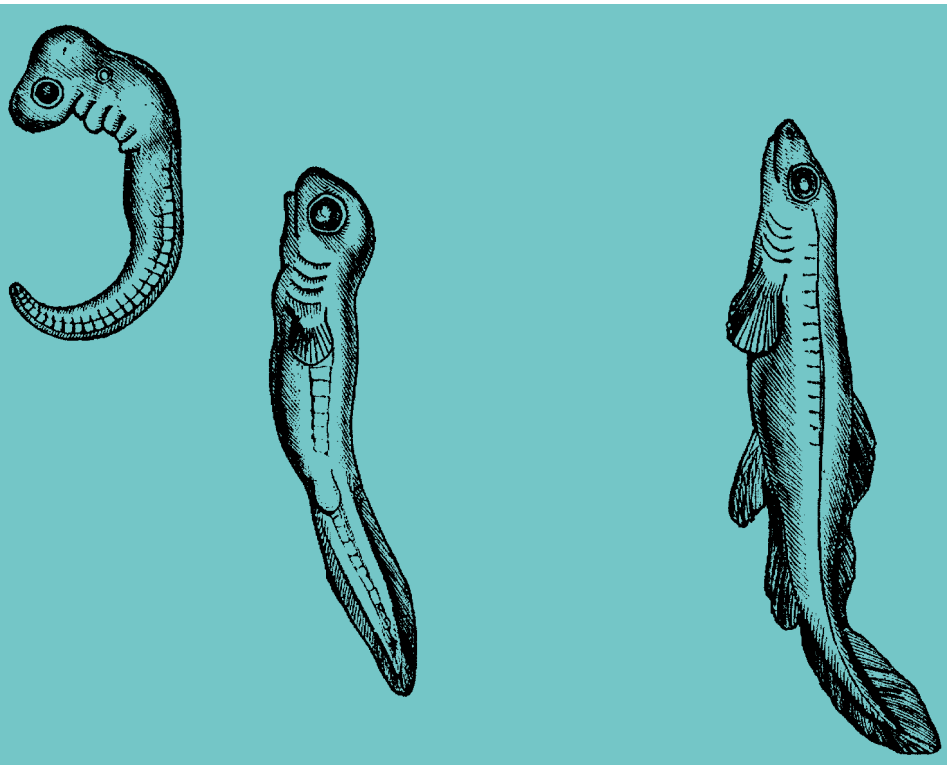
Part Two comprises a review of the literature on folate and prenatal growth, followed by a chapter on the association between maternal red blood cell folate and embryonic growth. In the second half of Part Two the associations between other parental characteristics, lifestyle factors and environmental exposures and embryonic growth are addressed.

PART

ONE

Human embryonic growth

CHAPTER



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Human embryonic growth trajectories and associations with fetal growth and birth weight

Evelyne M van Uitert, Niek Exalto, Graham J Burton, Sten P Willemsen, Anton HJ Koning, Paul HC Eilers, Joop SE Laven, Eric AP Steegers and Régine PM Steegers-Theunissen

Hum Reprod, 2013. 28(7): p. 1753-61.

ABSTRACT

Background Fetal growth is associated with health and disease risks in later life. Until recently, prenatal care and research were focused predominantly on fetal growth in the second and third trimesters of pregnancy. Longitudinal first trimester data remains scarce. In this study we assess human embryonic growth trajectories in the first trimester, and whether first trimester embryonic growth is associated with fetal growth and birth weight.

Methods We recruited 201 pregnancies before 8 weeks gestation in a prospective periconception cohort study conducted in a tertiary centre and performed weekly three-dimensional ultrasound scans from enrolment up to 13 weeks of gestation. To create embryonic growth trajectories, serial crown-rump length (CRL) measurements were performed using V-Scope software in the Barco I-Space. Mid-pregnancy fetal growth parameters and birth weight were obtained from medical records. *Z*-scores were calculated for CRL, mid-pregnancy estimated fetal weight (EFW) and birth weight. Associations between embryonic and fetal growth parameters were investigated using Pearson's correlation coefficients.

Results During early first trimester (up to 9 weeks gestation) we observed a constant absolute mean embryonic CRL growth rate of 0.99 mm/day (standard deviation (SD) 0.10), while relative growth rate decreased. Between 9 and 10 weeks gestation the absolute growth rate increased, and during late first trimester (from 10 weeks gestation onwards) we observed a constant mean relative growth rate of 4.1% (SD 0.006) per day. Overall, early and late first trimester median CRL *Z*-score were strongly correlated with mid-pregnancy EFW ($r_{overall/early/late}$ = 0.57/ 0.57/ 0.54, $P < .001$) but only overall and late CRL with birth weight ($r_{overall}$ = 0.15, $P = .04$; r_{early} = 0.10, $P = .17$; r_{late} = 0.17, $P = .02$).

Conclusions This study shows differences between early and late first trimester embryonic growth coinciding with changes in intrauterine nourishment. The established associations between first trimester embryonic growth and fetal size in mid-pregnancy and at birth emphasize that more research is warranted to establish the importance of these results for preconceptional and early pregnancy care.

INTRODUCTION

The Barker hypothesis states that intrauterine conditions can affect fetal and newborn weight and subsequent disease risks in later life [2, 4]. Although the first trimester of pregnancy is critical for growth and development of major embryonic organ systems and the placenta [1], approximately 20-25% of native Dutch pregnant women in the Netherlands and 18% of pregnant women in the United States (range across different states:17-31%) do not enter obstetric care before 14 and 12 weeks gestation, respectively [26, 27]. Until recently, prenatal care and research were focused predominantly on fetal growth in the second and third trimester of pregnancy. The high growth rates in the first trimester renders this one of the most vulnerable periods in life, in which poor health conditions and lifestyles may have permanent consequences for fetal and postnatal growth, development and health. This is well illustrated by the study of Mook-Kanamori et al. who demonstrated an inverse association between late first trimester embryonic size, measured as crown-rump-length (CRL), and the risk of adverse birth outcomes [16]. This study also showed that maternal smoking and no use of folic acid supplements are associated with a smaller late first trimester CRL [16]. Although this first trimester cross-sectional data is fascinating, longitudinal data on first trimester embryonic growth remain scarce [14].

As a result of recent developments of transvaginal three-dimensional ultrasound techniques visualization of the embryo during the first trimester has improved tremendously. The combination of these novel ultrasound techniques with the virtual reality technology of the Barco I-Space and V-scope visualization software leads to the ultimate benefit of the third dimension by enabling depth perception and thus an actual view of the third dimension [21]. Together, these technological developments have enabled the performance of highly precise and reliable early first trimester embryonic measurements in vivo [22-24] and have improved the means to assess embryonic growth longitudinally from the early first trimester of pregnancy onwards.

The aim of this study is to investigate first trimester embryonic growth trajectories using longitudinal CRL measurements, and associations between embryonic growth in early and late first trimester and fetal size in mid-pregnancy and at birth.

METHODS

Data for this study were collected in a prospective periconception cohort study at the Department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Centre Rotterdam, the Netherlands. At enrolment, all participants signed a written informed consent form.

All women of at least 18 years old with ongoing singleton pregnancies of 6 to 8 weeks gestation were eligible for participation and included in 2009 and 2010. The majority of participating women were derived from the outpatient clinic of the Department of Obstetrics and Gynaecology at the Erasmus MC, and a small group (25%) was derived from outside the hospital. The latter group heard of the study from midwives and Erasmus MC staff. Women were informed about the study through study brochures and posters, available throughout the outpatient clinics of Obstetrics and Fertility, and actively had to contact the research team to sign up for participation.

Ultrasound data

Women received weekly transvaginal three-dimensional ultrasound scans from enrolment up to the 13th week of pregnancy. Scans were generally performed every 7 days, however, for logistic reasons the number of days between ultrasounds occasionally varied from 6 to 8 days, or 13 to 15 days when women missed an appointment. Ultrasound scans were performed with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D View software (General Electrics Medical Systems, Zipf, Austria). Afterwards the obtained 3D-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. This is a four-walled CAVE^{TM}-like (Cave Automatic Virtual Environment) virtual reality system, allowing depth perception and interaction with the projected images [25]. CRL measurements were performed offline using the I-Space and V-Scope software [21], and by placing the callipers at the outer side of crown and rump in the mid-sagittal plane. CRL measurements performed in the I-Space show good agreement with 2D measurements and good inter- and intraobserver agreement [23]. All CRL measurements were performed three times by the same researcher, and the mean of these three measurements was used in the analyses.

Questionnaires

At enrolment participants completed a self-administered general questionnaire covering details on maternal age, anthropometrics, ethnicity, education, obstetric history, and periconception exposures.

Pregnancy dating

Data on the first day of the last menstrual period (LMP) and of regularity and duration of the menstrual cycle were obtained in a personal interview by the researcher performing the ultrasound at the first visit. We calculated the gestational age from the LMP in spontaneously conceived pregnancies, from the date of oocyte pick-up plus 14 days in pregnancies conceived through in vitro fertilization with or without intracytoplasmic sperm injection (IVF/ ICSI) procedures, from the LMP or insemination date plus 14 days in pregnancies conceived through intra-uterine insemination (IUI), and from the day of embryo transfer plus 17 or 18 days in pregnancies originating from transfer of cryopreserved embryos, depending on the number of days between oocyte pickup and cryopreservation of the embryo. When the menstrual cycle was regular but more than

three days different from 28 (28±>3 days), we adjusted the gestational age for the duration of the menstrual cycle.

Study population

If the first day of the LMP was missing in spontaneously conceived pregnancies, or if the observed CRL differed by more than six days from the expected CRL according to the Robinson curve [28], pregnancies were excluded from the analysis. Furthermore, we selected spontaneously conceived pregnancies and pregnancies conceived through assisted reproductive techniques using biological oocytes from the participating mother-to-be only. In addition, ectopic pregnancies and pregnancies that ended in a miscarriage before 16 weeks gestation were excluded.

Follow-up

In The Netherlands all pregnant women are offered a routine structural ultrasound examination at approximately 20 weeks gestation. Biparietal diameter (BPD), head circumference (HC), abdominal circumference (AC) and femur length (FL) measurements performed during this ultrasound examination were retrieved from the medical records. Mid-pregnancy estimated fetal weight (EFW) was calculated using the Hadlock formula: EFW = 10**(1.326 - 0.00326*AC*FL + 0.0107*HC + 0.0438*AC + 0.158*FL) [29].

Information on the infant's date of birth, gender, birth weight (BW) and presence of one or multiple congenital anomalies were obtained from medical records. One pregnancy was terminated at 14 weeks after diagnosis of trisomy 21, and therefore no birth record was completed.

Gestational age at the time of mid-pregnancy ultrasound examination and at birth was calculated from the dating procedure used in the first trimester, as described above.

Statistical analysis

First trimester embryonic growth trajectories and embryonic growth rates were studied using CRL, absolute CRL growth rate per day and relative CRL growth rate per day of measurements performed between 6^{+0} and 12^{+6} weeks. CRL growth rate per day was calculated for the longest strain of consecutive measurements no more than seven days apart using the following formula: $(CRL_i - CRL_{i-1})/(GA_i - GA_{i-1})$, with CRL_i representing the CRL at the i^{th} visit in this pregnancy, GA_i the gestational age at that time, and $GA_i - GA_{i-1}$ less or equal to seven days. The gestational age for the value thus obtained was calculated as the mean of the gestational ages at which the two measurements were performed: $(GA_i + GA_{i-1})/2$. In pregnancies with two strains of an equal number of consecutive measurements, the strain with the largest gestational age was included. Relative CRL growth rate was calculated as the CRL growth rate divided by the mean of the two CRL measurements from which the absolute growth rate was calculated. Group means were calculated using the median from all growth rates per pregnancy.

We constructed gestational age adjusted *Z*-scores for CRL, mid-pregnancy EFW and BW based on our own data using R software (version 2.15.1, GAMLSS package version 4.5.1; a more detailed description of the *Z*-score calculations is provided in the supplementary material available online). *Z*-scores for mid-pregnancy ultrasound data were calculated for all structural ultrasounds performed between 18 and 22 weeks gestation. Because of a small number of preterm births (n=16), we calculated BW *Z*-scores for term births only (gestational age ≥37 weeks).

Analyses were performed in the total group and in the subgroup of IVF/ ICSI pregnancies. In addition, we repeated the analyses in subgroups of: 1) pregnancies with a reliable gestational age based on a very strictly regular menstrual cycle of 28±3 days and a certain LMP or conception date, 2) spontaneously conceived pregnancies, and 3) uncomplicated pregnancies. The subgroup of complicated pregnancies in our study population was too small and heterogeneous and therefore not investigated separately.

To assess associations between embryonic growth and fetal growth parameters, and BW we used the medians of all available first trimester CRL *Z*-scores per pregnancy.

We used Pearson correlation coefficients to assess the associations between CRL and fetal growth parameters, all expressed as *Z*-scores.

All analyses were performed using IBM SPSS Statistics Version 20.0 for Windows software (IBM, Armonk, NY, USA).

Ethical approval

This study has been approved by the Central Committee on Research in The Hague and the local Medical Ethical and Institutional Review Board of the Erasmus MC.

RESULTS

Of 259 enrolled pregnancies we excluded 2 pregnancies conceived by oocyte donation, 44 pregnancies that ended in a miscarriage or ectopic pregnancy, and 12 pregnancies dated by CRL, resulting in 201 pregnancies available for the analysis of first trimester embryonic growth trajectories.

The median gestational age at enrolment was 6^{+5} (range 6^{+0}-8^{+6}) weeks, and the median number of ultrasound visits per pregnancy was 6 (range 4-8). From a total of 1,262 datasets, 1,144 (90.6%) were of sufficient quality to perform CRL measurements. We performed a median of 6 (range 1-8) CRL measurements per pregnancy.

Routine mid-pregnancy ultrasound data could be obtained for 177 pregnancies (88.1%), and fetal parameters were measured in 86.1% to 88.1% of pregnancies (BPD: 173 (86.1%);

Table 1 General characteristics of study population (n=201).

Characteristics	n	Missing
Maternal (at enrolment)[a]		
Age, y	32.2±4.8	9
Ethnicity		7
Dutch	149 (76.8)	
Other western	16 (8.2)	
Non western	29 (14.9)	
Education		16
Low	18 (9.7)	
Middle	56 (30.3)	
High	111 (55.2)	
BMI (median (range)), kg/m^2	23.8 (18.6-48.9)	6
Nulliparous	122 (62.2)	5
Periconception smoking	31 (15.8)	5
Pregnancy and outcome		
Conception through IVF/ICSI	61 (30.3)	0
Reliable gestational age[b]	157 (78.1)	0
Infant gender male	95 (47.5)	1
Birth weight, g	3276±636	1
Gestational age at delivery (median (range)), wk^{+d}	39^{+3} (14^{+3}-42^{+0})	1
Pregnancy complications	43 (21.4)	0
Maternal	17 (8.5)	1
Hypertensive disorder	14 (7.0)	1
Gestational diabetes	4 (2.0)	1
Fetal	28 (13.9)	0
Major congenital anomaly	6 (3.0)	1
Fetal/neonatal death	5 (2.5)	0
Low birth weight (less than 2500g)	15 (7.5)	1
Premature delivery (before 37 wk)	16 (8.0)	1
SGA (less than 10^{th} customized centile)[c]	12 (6.3)	9

Data are presented as mean ± standard deviation or n (%) unless otherwise specified.

BMI, body-mass index; IVF/ICSI In vitro fertilisation with or without intracytoplasmic sperm injection; SGA, small for gestational age.

[a] Of 5 women all maternal characteristics are missing because of unreturned questionnaires. [b] Gestational age based on a menstrual cycle of 28±3 days or conception date. [c] Defined as weight under the 10^{th} centile for gestational age, gender and parity according to Dutch reference charts [39].

FL: 175 (87.1%); HC and AC: 177 (88.1%)). EFW could be computed for 175 (87.1%) pregnancies. Birth records were obtained for 200 (99.5%) pregnancies and BW *Z*-scores were calculated for all 184 (91.5%) pregnancies with term deliveries.

Maternal and pregnancy characteristics are shown in **Table 1**. Mean maternal age was 32.2 (standard deviation (SD) 4.8) years and women mainly had a high education (55.2%) and were of Dutch descent (76.8%). In 157 (78.1%) pregnancies gestational age was based on a strictly regular menstrual period of 28±3 days or conception date, including 61 (30.3% of 201 included pregnancies) pregnancies that were conceived after IVF/ ICSI treatment. Pregnancy complications occurred in 43 (21.4%) pregnancies.

First trimester embryonic growth trajectories

In **Figure 1** CRL growth trajectories are depicted for the total group and for IVF/ ICSI pregnancies. Growth trajectories demonstrated a smooth curve, although the distribution in the total group was wider than in IVF/ ICSI pregnancies only. Growth trajectories in spontaneously conceived pregnancies and in those with a reliable gestational age were comparable to the total group (data not shown).

In 177 (88.1%) pregnancies of the total group and 57 (93.4%) of the IVF/ ICSI pregnancies, at least two CRL measurements had been performed no more than seven days apart, and in both groups a median of 3 (range 2-6) embryonic CRL growth rates could be computed. **Figure 2** displays the mean absolute and relative growth rates for the total group and for IVF/ ICSI pregnancies only. In both groups, the mean absolute growth rate was constant up to 9 weeks gestation at 0.99 (SD 0.10) and 1.01 (SD 0.09) mm/day (computed from n=112 and n=34 pregnancies with embryonic growth rate measurements

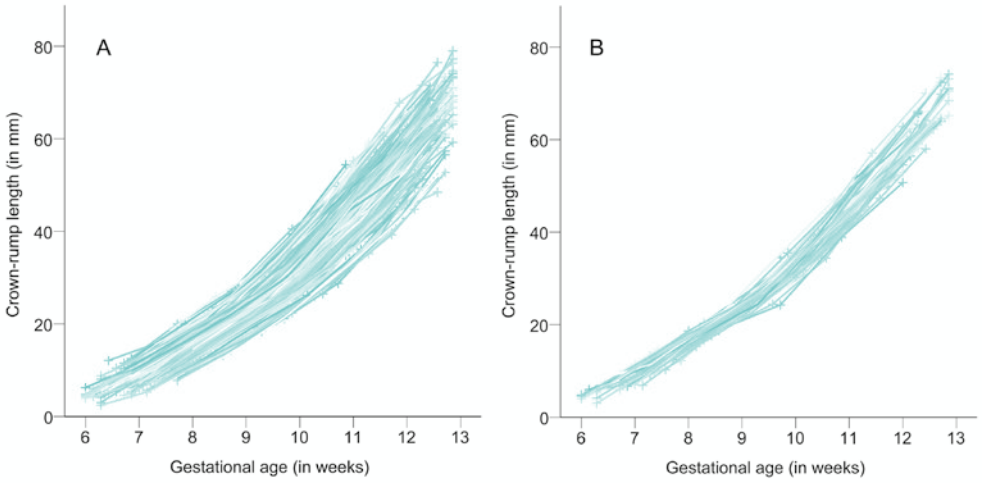


Figure 1 Longitudinal first trimester embryonic growth trajectories, measured by weekly crown-rump length (CRL) measurements for the total group of pregnancies (A) and IVF/ ICSI pregnancies only (B).

up to 9 weeks, respectively). After 9 weeks gestation embryonic growth displayed a substantial increase in growth rate, the first onset of which varied approximately between 9 and 10 weeks gestation. After 10 weeks gestation the steady increase in absolute growth rate translated to a constant mean relative growth rate of 4.1% (SD 0.006) and 3.9% (SD 0.004) per day in spontaneously conceived and IVF/ ICSI pregnancies, respectively (computed from n=146 and n=46 pregnancies with embryonic growth rate measurements from 10 weeks onwards). Mean absolute and relative growth rates in subgroups of pregnancies with a reliable gestational age and in spontaneously conceived pregnancies were comparable to those observed in the total group (data not shown).

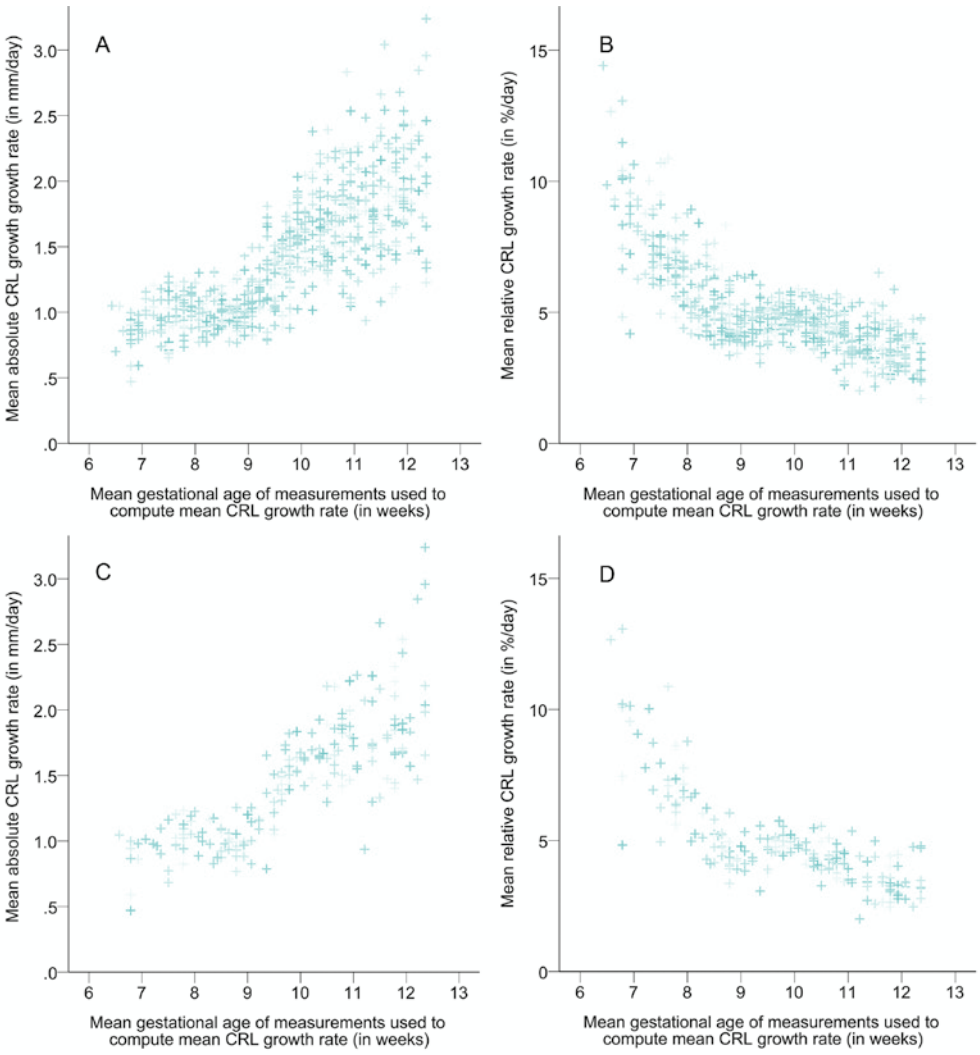


Figure 2 Mean absolute and relative embryonic crown-rump length (CRL) growth rates, for the total group (A, B) and IVF/ ICSI pregnancies only (C, D).

Associations between first trimester embryonic growth and subsequent fetal growth

In **Figure 3** associations between median first trimester CRL and mid-pregnancy EFW and BW *Z*-scores are presented for the total group and for IVF/ ICSI pregnancies. In the total group median CRL *Z*-score was significantly correlated with all fetal growth parameters including mid-pregnancy EFW *Z*-score (EFW: r = 0.57, HC: r = 0.58, BPD: r = 0.41, AC: r = 0.50, FL: r = 0.41; all *P*-values < .001) and BW *Z*-score (r = 0.15, *P* = .04), explaining about 33% and 2.3% of the variance, respectively. In IVF/ ICSI pregnancies

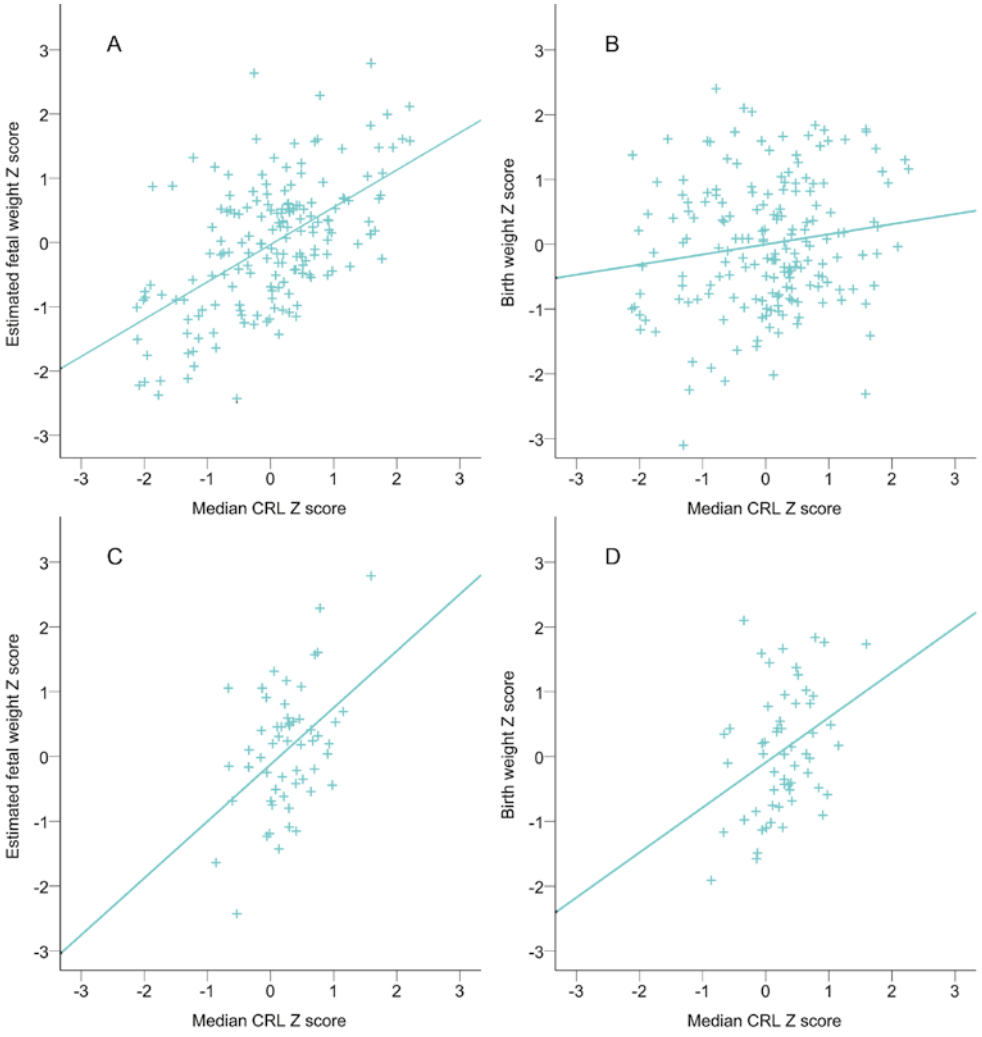


Figure 3 Correlations of first trimester median crown-rump length (CRL) and mid-pregnancy estimated fetal weight and birth weight *Z*-scores for the total group (A: Pearson's correlation coefficient (r) = 0.57, r^2 = 0.33, *P* < .001; and B: r = 0.15, r^2 = 0.02, *P* = .04) and IVF/ ICSI pregnancies only (C: r = 0.45, r^2 = 0.20, *P* = .001; and D: r = 0.35, r^2 = 0.12, *P* = .008).

the estimates attenuated for mid-pregnancy parameters (EFW: r = 0.45, *P* = .001, HC: r = 0.28, *P* = .04; BPD: r = 0.16, *P* = .26; AC: r = 0.42, *P* = .001; FL: r = 0.25, *P* = .06) but became stronger for BW *Z*-score (r = 0.35, *P* = .008). The explained variance was 20% for EFW and 12% for BW.

We repeated the analyses after stratification in early and late first trimester CRL. Because of the variation in the timing of the onset of the increase in embryonic growth rate as described before, we defined early trimester as the period up to and including 9 weeks gestation, and late first trimester as the period from 10 up to 13 weeks gestation. Early and late first trimester median CRL *Z*-scores were calculated as the median of all measurements up to and including 9 weeks and from 10 weeks gestation onwards, respectively. In the total group associations with mid-pregnancy fetal growth parameters were comparable for both early and late CRL to those established with overall first trimester CRL. However, only late first trimester CRL was correlated with BW (r_{late} = 0.17, *P* = .02; r_{early} = 0.11, *P* = .17), explaining 2.9% of the variance.

In IVF/ ICSI pregnancies, early and late first trimester CRL remained correlated with mid-pregnancy EFW (r_{early} = 0.37, *P* = .008; r_{late} = 0.28; *P* = .04) with explained variances of 14% and 8%. The correlations between late CRL and AC attenuated (AC: r_{late} = 0.22, *P* = .12) whereas a correlation between early CRL and BPD emerged (BPD: r_{early} = 0.28, *P* = .05). Early median CRL *Z*-score was not significantly correlated with BW, whereas late median CRL *Z*-score was significantly correlated with BW (r = 0.33, *P* = .01), explaining 11% of the variance.

Subgroup analyses

The analysis of associations between first trimester embryonic growth and fetal growth were repeated in a subgroup of 157 pregnancies with a reliable age based on a very strictly regular menstrual cycle of 28±3 days, 140 spontaneously conceived pregnancies, and 158 uncomplicated pregnancies. Pearson's correlation coefficients for all these subgroups and growth parameters are provided in the supplementary material available online (**Table S1**).

In the three subgroups the correlation coefficients for overall, early and late first trimester CRL and mid-pregnancy fetal growth parameters and BW were of similar size and significance to those observed in the total group. However, exceptions were pregnancies with a reliable gestational age, in which the correlation between CRL and BW emerged in early first trimester (r_{early} = 0.18, *P* = .04) and became stronger in late first trimester (r_{late} = .25, *P* = .003), and in spontaneously conceived pregnancies in which both early and late median CRL *Z*-score were not significantly correlated with BW.

DISCUSSION

In this prospective study from early pregnancy onwards we observed that first trimester embryonic growth rate is approximately constant up to 9 weeks of gestation and substantially increases after 9 to 10 weeks gestation. Furthermore, first trimester embryonic growth appears to be strongly correlated with mid-pregnancy fetal growth parameters and less strongly with BW. Correlations with BW were stronger and more consistently observed for late than for early first trimester embryonic growth. Finally, associations were comparable in pregnancies with the most reliable pregnancy dating and in IVF/ ICSI pregnancies.

This study has several strengths. We acquired weekly ultrasound data from the earliest stages of pregnancy up to 13 weeks gestation in more than 200 pregnancies. Furthermore, we performed all CRL measurements using true three-dimensional holograms, offering a high degree of precision and reliability [23], which is even further increased by using the mean of three CRL measurements per time point per pregnancy for the analyses. In addition, fetal growth data from mid-pregnancy and birth were obtained from medical records rather than from questionnaires. An important issue which we considered in the design and analysis of the study is the dependency of embryonic growth on gestational age. While in IVF/ ICSI pregnancies the moment of implantation is the only determinant of gestational age, in spontaneously conceived pregnancies variations in timing of ovulation and implantation and recollection of LMP result in a less precise determination of gestational age. For that reason, we excluded pregnancies with a discrepancy between observed and expected CRL of more than 6 days. Furthermore, we repeated the analyses in a subgroup of pregnancies with the most reliable gestational age, which did not substantially alter the results. Finally, we stratified the analysis by mode of conception. In all groups a significant correlation of embryonic growth with mid-pregnancy EFW was observed. In the total group of pregnancies we showed a correlation between embryonic growth and BW, which was stronger in pregnancies with a reliable gestational age and IVF/ ICSI pregnancies, but absent in spontaneously conceived pregnancies. These data support the internal validity of our results, but also show some confounding by a less precise pregnancy dating in spontaneously conceived pregnancies. In addition, embryonic growth seems not to be uniform but is also influenced by maternal conditions, endometrial receptivity and exposures, such as maternal age, ethnicity and smoking [16, 30]. Therefore, we dated pregnancies using LMP rather than CRL. Maternal influences on embryonic growth is a very important and interesting issue to be further investigated in large periconception cohort studies in the future.

There are some limitations that have to be addressed as well. This study was carried out in a tertiary hospital and therefore its external validity is expected to be limited. The proportion of high risk pregnancies and pregnancy complications is likely to be higher than in a population-based cohort study. However, after repeating the analyses in a subgroup of

uncomplicated pregnancies, the results were comparable to the total group. Because our study population also contains a relatively high proportion of women with a higher education and pregnancies conceived after IVF/ ICSI treatment, our results will have to be confirmed in other populations. Unfortunately the current study population was too small to study the association between first trimester embryonic growth and adverse pregnancy outcome.

Individual embryonic growth trajectories displayed a smooth curve and an increasing growth rate in late first trimester. The onset of the observed increase in embryonic growth rate between 9 and 10 weeks gestation is in line with Deter et al., who showed an increase in growth rate at 9^{+1} weeks gestation [31]. This period coincides with the transition from histiotrophic to haemotrophic nutrition. In the early first trimester of pregnancy nourishment of the embryo is characterized by the transport of carbohydrate-rich proteinaceous secretions from the uterine glands into the intervillous space of the developing placenta, i.e., histiotrophic nutrition [32]. The secretions are phagocytosed by the trophoblast, and nutrients pass into the coelomic cavity, from where they may be transported to the embryo via the yolk sac. Eight to nine weeks after conception, trophoblast plugs originally blocking the spiral arteries gradually dissipate, and maternal blood begins to enter the marginal zone of the placenta. This leads to a transition from a histiotrophic to an increasingly haemotrophic nutrient and oxygen supply of the embryo and thus the initiation of the haemochorial function of the placenta [32-34]. As a result, there is a three-fold increase in the intraplacental oxygen concentration between the end of the first, and the start of the second, trimester. Thus, if the increase in embryonic growth rate occurs as a consequence of this transition, factors influencing dissipation of the plugs may also change the timing of the increase in embryonic growth rate itself. Pregnancy complications such as pre-eclampsia and fetal growth restriction have been associated with premature loosening of these trophoblast plugs, which gives rise to excessive placental oxidative stress [35].

We demonstrated a strong correlation between embryonic growth and mid-pregnancy fetal growth parameters which is in line with data from a large population-based prospective cohort study showing correlations of similar magnitude between late first trimester CRL and HC, FL and mid-pregnancy EFW [16]. Although the observed estimates were small, we were able to demonstrate an association between first trimester embryonic growth and BW. The presence of this association despite the time interval of approximately seven months, and the fact that most fetal weight gain occurs in the last trimester of pregnancy, stresses the importance of pre- and periconception maternal conditions, lifestyles and care. One of the potential mechanisms underlying the association between first trimester growth and BW is the programming of the embryonic genome by epigenetic mechanisms with consequences for subsequent fetal growth. This is in line with the developmental origin of health and diseases, in which the prenatal environment of the fetus is an important determinant of future health and disease. The weaker association between embryonic growth and BW is supported by the majority of studies conducted in pregnancies conceived through artificially reproductive techniques and spontaneous pregnancies.

In pregnancies conceived through artificial reproductive techniques, embryonic CRL has been positively associated with BW and inversely with risk of having a small for gestational age (SGA) infant [15, 36, 37]. In two large prospective cohorts of spontaneously conceived pregnancies, a small CRL was associated with an increased risk of low birth weight and SGA [16, 17]. In contrast, in a large cohort study no association was observed between a small CRL and SGA risk [38]. However, in the same study an association was observed between first trimester BPD below the tenth centile and increased SGA risk [38].

To our knowledge, this is the first study to show associations between BW and early and late first trimester embryonic growth separately. Whereas most studies on late first trimester CRL support our findings and reported positive associations between CRL measurements after 10 weeks gestation and BW, early CRL was measured from 6^{+4} weeks onwards in only one other study [36]. In the analysis, however, all measurements up to 10^{+6} weeks were included and early embryonic growth was not investigated separately [36].

In conclusion, we have shown in a prospective periconception cohort study that first trimester embryonic growth trajectories and growth rates show individual variation. First trimester embryonic growth, particularly from 10 weeks gestation onwards, appears to be associated with fetal and newborn growth parameters, emphasizing the need for more research to establish the implications of these results for preconceptional and early pregnancy care. Further investigation and extension of this cohort will enable an estimation of the onset of the increase in embryonic growth rate in the first trimester, the extent to which this moment influences pregnancy course and outcome, and whether it is influenced by periconception constitutional factors and exposures, and ultimately assessment of the predictive value of first trimester CRL for normal and adverse pregnancy outcome.

CHAPTER

2

First trimester human embryonic curvature measurements using 3D ultrasound

Hein Bogers, Evelyne M van Uitert, Sharon van Ginkel, Elisabeth DH van der Mooren, Irene AL Groenenberg, Paul HC Eilers, Niek Exalto, Eric AP Steegers and Régine PM Steegers-Theunissen

Submitted

ABSTRACT

Background Small crown-rump length (CRL) measurements predict spontaneous miscarriage and neural tube defects are frequently seen in miscarriages. An increased curvature, as in the curly tail mutated mouse, is associated with neural tube defects. Is it possible to measure the embryonic curvature using three-dimensional ultrasonographic techniques in the first trimester and is there a difference in embryonic curvature between ongoing pregnancies and pregnancies ending in a miscarriage?

Methods Pregnant women were recruited from our tertiary care outpatient clinic and enrolled in a prospective periconception cohort study, the Rotterdam Predict study. Miscarriages were derived from this cohort and from an additional recurrent miscarriage cohort. In 202 ongoing pregnancies and 21 miscarriages CRL and total arc length (TAL) measurements of the embryo could be performed. In the ongoing pregnancies there was an excellent reliability of the measurements between and within the two operators with interclass correlation coefficients (ICC) values above 0.997.

Results TAL increased and showed more variation with advancing gestation. The CRL/TAL ratio showed a strong increase from 8^{+0} to 10^{+0} weeks of gestational age, after which the curve flattens. The curvature measurements of embryos resulting in a miscarriage were not different from ongoing pregnancies.

Conclusions A reference curve for first trimester curvature was constructed by measuring CRL and the TAL of the embryo and calculating the CRL/TAL ratio, which can be measured reliably. No differences were observed between ongoing pregnancies and pregnancies ending in a miscarriage. Due to our relatively small study population and a low prevalence of neural tube defects we were unable to investigate the association between curvature and neural tube defects.

INTRODUCTION

Ultrasonographic parameters, such as crown rump length (CRL), embryonic volume, and description of Carnegie Stages are available for clinical and scientific evaluation of embryonic growth and development [24, 40, 41]. Recently, we demonstrated that human embryonic growth trajectories are associated with estimated fetal weight and birth weight [41]. Birth weight has been demonstrated to be influenced by the use of folic acid initiated in the preconception period for the prevention of neural tube defects (NTDs) [42]. In miscarriages, impaired embryonic growth is frequently observed and the frequency of NTDs is 10-fold higher [43, 44]. Therefore, it is of interest to compare embryonic curvature between pregnancies resulting in a miscarriage and ongoing pregnancies.

In attempts to understand the mechanism behind the origin of neural tube defects and the protective effect of folic acid, one may speculate about the influence of embryonic changes from a curved to an upright position during organogenesis, illustrated by the Carnegie Stage pictures [45], leading to a decrease in embryonic curvature.

Since the embryonic curvature has not been measured before, the aim of this study was to develop a reliable measurement technique for the embryonic curvature using three-dimensional (3D) ultrasound and to develop reference charts for the curvature in relation to gestational age and CRL. A secondary aim was to study whether embryos from pregnancies resulting in a miscarriage demonstrated differences in curvature compared to ongoing pregnancies.

METHODS

This study was embedded in the Rotterdam Predict Study, an ongoing prospective periconception cohort study conducted at the Erasmus MC, University Medical Centre, in Rotterdam, the Netherlands [41]. All participants signed a written informed consent and the local medical ethics committee approved the study protocol. Pregnant women who participated in this study in 2009 and 2010 were enrolled via the outpatient clinic of the department of Obstetrics and Gynaecology at the Erasmus MC and local midwifery practices. In the same outpatient clinic between 2008 and 2012 an additional cohort of pregnant women with a history of recurrent miscarriages was also included for analysis. All women with ongoing pregnancies and those that resulted in a miscarriage received weekly 3D ultrasound scans between 6^{+0} and 12^{+6} weeks of gestational age. Only women less than eight weeks pregnant with a singleton pregnancy were eligible for participation in this study.

Ultrasound data

The ultrasonographic volumes were obtained with Voluson E8 ultrasound equipment (GE Medical Systems, Zipf, Austria) using a transvaginal probe (GE-probe RIC-6-12-D; 4.5–11.9 MHz). The 3D volumes were evaluated off-line by projecting these on the screen of the ultrasound machine and were displayed in the multiplanar mode for analysis. The images were rotated in order to obtain a very precise midsagittal view of the embryo in the A-plane resulting in an axial view in the B- and a coronal view in the C-plane. All data were stored and measurements performed offline. The following distances in the midsagittal plane were measured (**Figure 1**): CRL, known as the greatest length of the embryo [45], and the total arc length (TAL) defined as the dorsal contour traced from the cranial calliper of the CRL to the caudal calliper. Two observers randomly performed these measurements in triplicate and the mean of these three measurements was used for further analysis.

In the ongoing pregnancies data on pregnancy course and outcome was obtained from medical records.

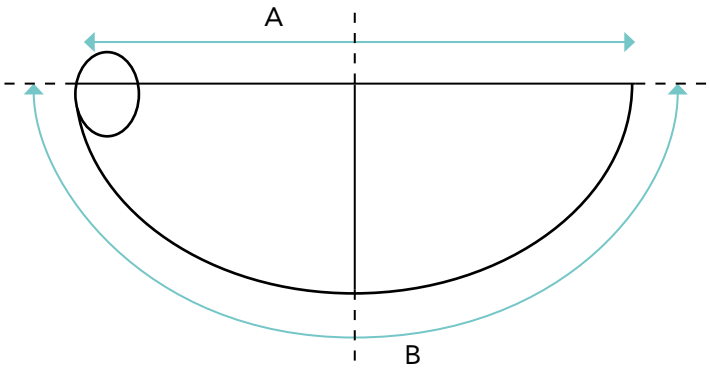


Figure 1 Schematic representation of measurements: A, crown-rump length (CRL), and B, total arc length (TAL).

Pregnancy dating

Gestational age was calculated according to the first day of the last menstrual period (LMP) if the woman had a regular cycle of 28±3 days and adjusted for a longer or shorter cycle. In case of assisted reproductive technology (in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI)), gestational age was determined by the date of oocyte retrieval plus 14 days in pregnancies conceived through IVF with or without ICSI (IVF/ ICSI) procedures, from the LMP or insemination date plus 14 days in pregnancies conceived through intrauterine insemination, and from the day of embryo transfer plus 17 or 18 days in pregnancies originating from the transfer of cryopreserved embryos, depending on the number of days between oocyte retrieval and cryopreservation of the embryo.

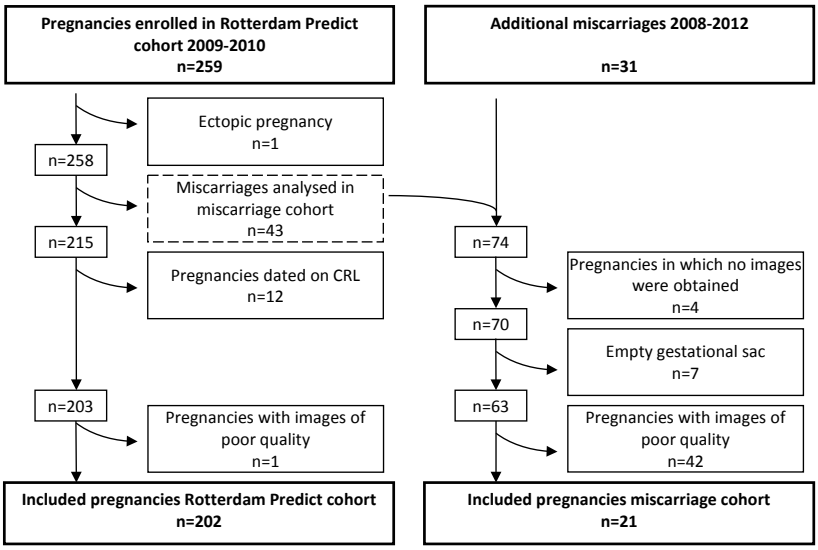


Figure 2 Flowchart of the study population

Study population

From the Rotterdam Predict study, we included 259 pregnancies enrolled in 2009 and 2010 (**Figure 2**). We excluded one ectopic pregnancy, 12 pregnancies with a discrepancy in gestational age of >6 days between CRL and the LMP based on the Robinson curve or in case of an unknown LMP (n=12) and one pregnancy in which we could not obtain volumes of sufficient quality to perform measurements. In the 245 pregnancies included in the analyses we observed 43 miscarriages, defined as fetal death until 16 weeks of gestational age. Enrolled pregnancies included pregnancies ending in an intrauterine foetal demise (n=1), congenital (n=4) and genetic (n=2, both trisomy 21) anomalies diagnosed before and after birth.

The 43 miscarriages in the Rotterdam Predict study were analysed together with 31 miscarriages derived from the additional miscarriage cohort resulting in a total group of 74 miscarriages (**Figure 2**). From this cohort we excluded four pregnancies in which no images could be obtained, seven in which an empty gestational sac was diagnosed and 42 pregnancies in which no images or only images of poor quality were obtained. Of the resulting 21 miscarriages available for further analysis, 12 occurred in women with a history of recurrent miscarriages defined as three or more miscarriages.

Statistical analysis

Maternal and pregnancy characteristics were summarised for the Rotterdam Predict cohort and miscarriage cohort and compared between groups using the χ^2-test for categorical data, Student's *t*-test for normal distributions and the Mann-Whitney U-test for nonparametric continuous data.

Computations were performed with SPSS 20 (IBM inc., Armonk, NY, USA) and R (R Foundation for Statistical Computing, Vienna, Austria). Standard deviation (SD) curves were computed with the R package gamlss [46], assuming a model with a normal distribution, no transformation, a spline for the mean curve and a linear relationship between the logarithm of the SD and gestational age. The graph in **Figure 3D** was constructed with the package ggplot2.

Reproducibility

The CRL and TAL measurements in one volume were independently repeated three times and the mean values were used for analysis. To assess intra- and interobserver reproducibility, a randomly selected subset of 30 volumes from 30 randomly selected pregnancies was measured a second time by the same examiner (SvG) and independently by another examiner (EvdM). For this purpose, five volumes were selected of each gestational week. Both examiners were blinded to the results of each other's measurements, each volume was unadjusted (raw data) and each measurement required manual adjustment of the volume to obtain the right image. To assess inter- and intraobserver agreement, intraclass correlation coefficients (ICC) were calculated.

RESULTS

General characteristics of the ongoing pregnancy and miscarriage cohorts are shown in **Table 1**. The mean age (years ± SD) in both groups was comparable (32.1 ± 4.8 vs 33.5 ± 4.7; $P = 0.47$). Approximately half of all women were nulliparous (62.9% vs 47.6%; $P = 0.17$) and the majority of pregnancies was conceived spontaneously (30.7% vs 23.8%; $P = 0.51$). In the Predict cohort, 5 (2.5%) pregnancies ended in foetal or neonatal demise and of the remaining 197 pregnancies three (1.5%) resulted in a congenital anomaly. Median gestational age at enrolment was 7^{+0} (6^{+0} – 9^{+1}) and 7^{+1} (6^{+0} – 9^{+4}) weeks in ongoing pregnancies and miscarriages, respectively, with a median of 6 (4-8) and 3 (1-7) visits per pregnancy. A total of 1294 and 71 3D scans were performed in both groups, of which in 1010 (78.1%) and 45 (63%) scans image quality was sufficient to perform curvature measurements. Median number of measurements per patient was 5 (2-7) and 1 (1-5).

The reproducibility of the curvature measurements is shown in the supplementary data (supplementary **Table S1**). All ICC values of inter- and intraobserver agreement were above 0.997, representing excellent reliability of the measurements between and within the two operators. The Bland-Altman plots showed good agreement between the measurements as well (supplementary **Figure S1**).

The percentages of ultrasound volumes in which curvature measurements could be performed varied with gestational age (**Table 2**). Between 8 and 12 weeks more than 90% of volumes could be measured, whereas at 6 and 7 weeks this was achieved in only 24% and 64%, respectively. After 12 weeks, the percentage dropped to 86%.

Table 1 General characteristics of the Predict cohort and cohort of miscarriages included for the analysis.

	Predict cohort		Miscarriages		
	n=202	Missing	n=21	Missing	*P*
Maternal age, years	32.1 ± 4.8	9	33.5 ± 4.7	0	0.24
Nulliparous	124 (62.9)	5	10 (47.6)	0	0.17
Conception via IVF/ICSI	62 (30.7)		5 (23.8)	0	0.51
Pregnancy outcome		0		0	
Miscarriage	-		21 (100.0)		
Termination of pregnancy	2 (1.0)		-		
Intra-uterine foetal death (>16wks)	2 (1.0)		-		
Neonatal death	1 (0.5)		-		
Livebirth	197 (97.5)		-		
Gestational age at delivery, weeks^{+days} (median (range))					
All pregnancies	39^{+3} (14^{+3} – 42^{+0})	1	8^{+6} (6^{+4} – 16^{+0})	1	
>24 weeks	39^{+3} (27^{+0} – 42^{+0})	0	-		
Birth weight (>24weeks), g	3305 ± 554	0	-		
Infant sex, male	95 (47.3)	1	-		
Congenital anomaly			-		
All pregnancies	6 (2.5)	1			
Live births	3 (1.5)	0			

Numbers are n (%) or mean ± standard deviation unless otherwise specified. IVF/ICSI, in vitro fertilisation with or without intracytoplasmic sperm injection

Table 2 Success percentages of total arc length measurements by gestational age (measurements/ number of images).

Week	All pregnancies	%	On-going pregnancies	%	Miscarriages	%
6	33/138	23.9	32/127	25.2	1/11	9.1
7	125/196	63.8	117/178	65.7	8/18	44.4
8	191/208	91.8	175/190	92.1	16/18	88.9
9	183/197	92.9	174/186	93.5	9/11	81.8
10	178/190	93.7	174/185	94.1	4/5	80.0
11	165/191	86.4	161/187	86.1	4/4	100.0
12	138/182	75.8	136/179	76.0	2/3	66.7

[a] Week 6 defined as 6^{+0} weeks up to and including 6^{+6} weeks

Figure 3 displays the relation of the TAL with gestational age and CRL. The TAL increased and showed more variation with increasing gestational age and CRL (**Figure 3**). The CRL/TAL ratio represents the embryonic curvature and shows a strong increase at the beginning of the curve from 8^{+0} to 10^{+0} weeks, after which the curve flattens (**Figure 3**). Embryos from pregnancies that resulted in a miscarriage showed no differences in curvature compared to the ongoing pregnancies (**Figure 3**).

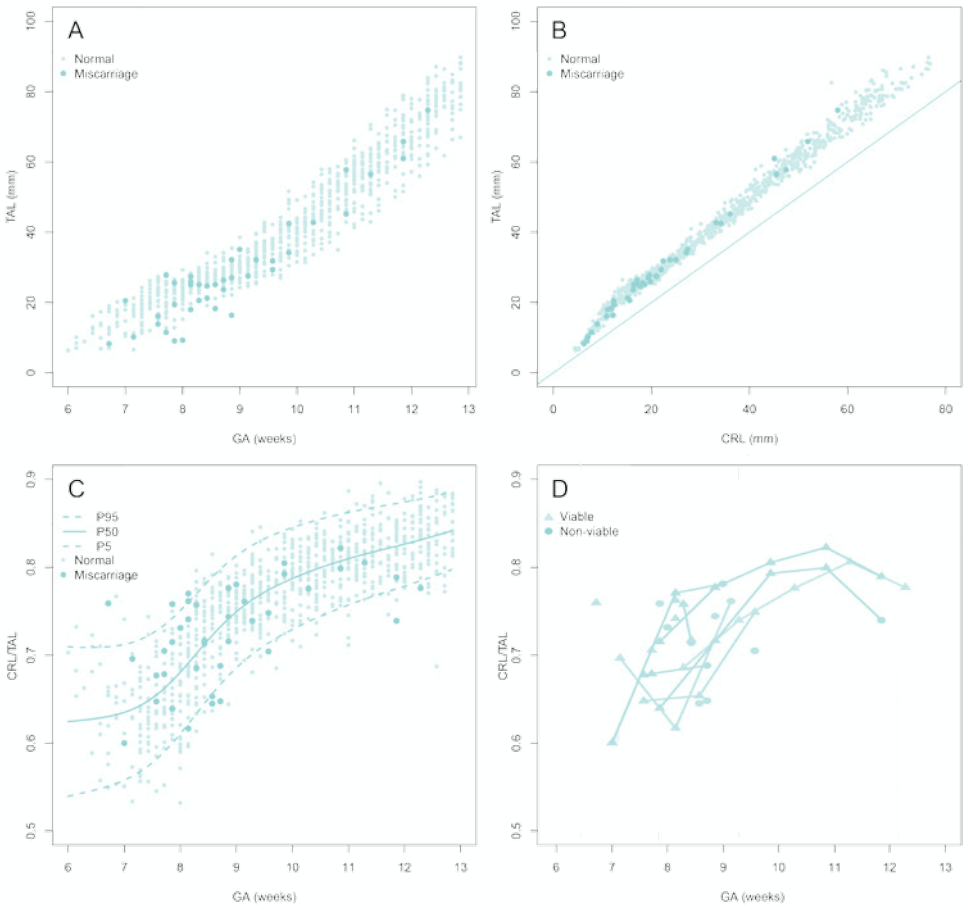


Figure 3 Total arc length (TAL) versus gestational age (GA; A) and crown-rump length (CRL; B), and reference chart with percentile lines of the CRL/TAL ratio versus GA (C) in ongoing pregnancies and miscarriages, and longitudinal CRL/TAL measurements in miscarriages (D).

DISCUSSION

In this prospective periconception cohort study we have created for the first time reference charts of the human embryonic curvature in the first trimester of pregnancy in a tertiary

hospital based study population. We demonstrated that the first trimester curvature of the human embryo can be measured reliably by means of the TAL and CRL/TAL ratio. Embryonic curvature was positively associated with CRL and gestational age and decreased towards the end of the first trimester. There were no differences observed in curvature between miscarriages and ongoing pregnancies.

First trimester embryonic measurements are strongly determined by gestational age. In order to reduce confounding by imprecise pregnancy dating we excluded pregnancies with a discrepancy in gestational age of more than 6 days between CRL and the LMP. Another strength of the study is that repeated measurements were performed on ultrasound scans obtained between 6^{+0} to 12^{+6} weeks of gestational age. The feasibility of the measurements appeared to be optimal between 8 to 11 weeks of gestational age. The problems of imprecise measurements before 8 weeks and after 12 weeks are most probably due to the small size of the embryos and the increasing number of artefacts due to fetal movements, respectively. No difference in embryonic curvature could be observed between the ongoing pregnancies and pregnancies ending in a miscarriage, which might be due to the small number of miscarriages which is a weak point of this study.

In the upcoming years we expect prenatal detection of congenital anomalies to gradually shift to the first trimester. Therefore, as a first step the feasibility of embryonic curvature measurements is demonstrated. This may be of particular importance for the screening of specific anomalies of the spinal column, including NTDs such as spina bifida. NTDs are a major congenital anomaly with a prevalence of approximately 1 in 1000 births in Europe [47]. Prenatal detection of NTDs depends on the specific type of NTD, e.g., anencephaly can usually be detected by ultrasound in the first trimester, and on the precision of the estimation of the gestational age. We can only speculate about the question whether the CRL/TAL ratio in the future may contribute to an earlier detection of spina bifida, which in current clinical practise is diagnosed mostly in the second trimester of pregnancy [48]. In mice, the *curly tail* mutation is associated with spina bifida. It is not clear whether the increased curvature associated with closure failure of the posterior neuropore is the cause or consequence of spina bifida aperta [49]. In other species, including man, decreased curvature has been related to an increase in closure of the posterior neuropore [50]. Closure of the vertebral arches may also be facilitated by changing from a curved to a straightened position. We were unable to demonstrate changes in curvature in fetuses with NTDs, because of our relatively small study population and the low NTD prevalence rate. Moreover, although NTDs are more prevalent in miscarriages, miscarriage tissue was not collected and could therefore not be investigated for the presence of these and other anomalies. It would be interesting to further investigate the embryonic curvature in spina bifida and other spinal column related anomalies in a large first trimester birth cohort with the pathological investigation of miscarriage tissue and stillbirths in the future. This would also present opportunities to investigate associations between periconceptional exposures such as folic acid supplement use and the embryonic curvature.

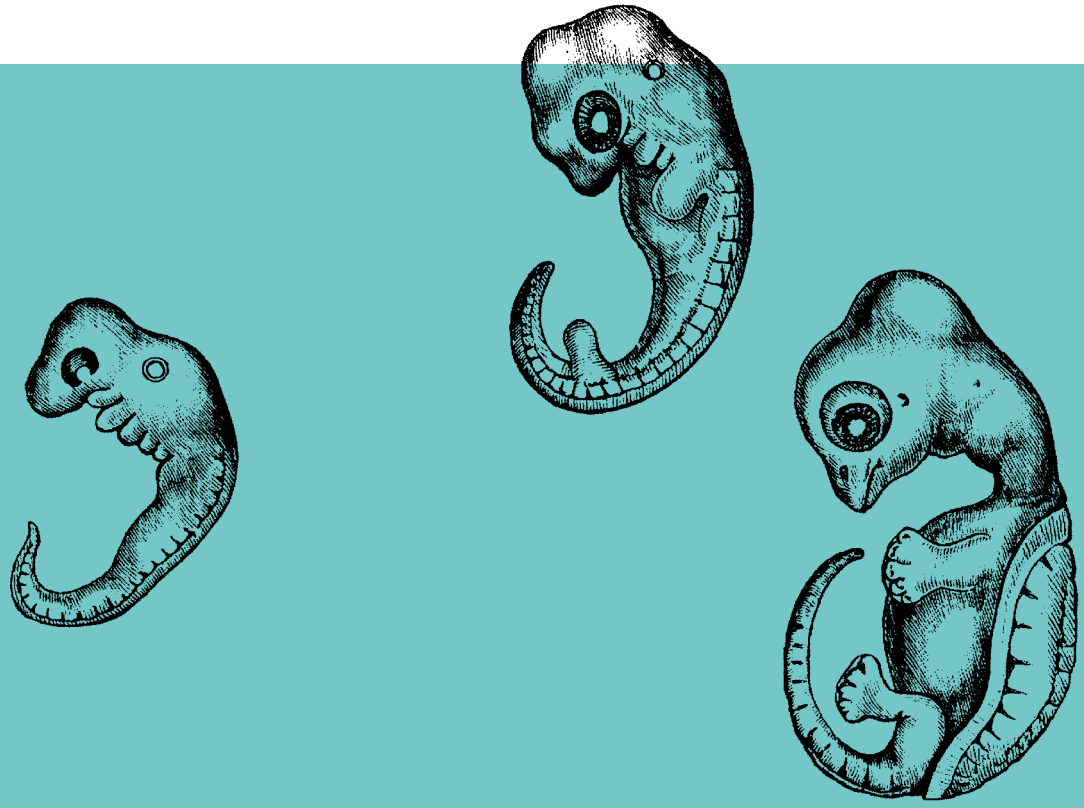
In conclusion, first trimester trajectories of the curvature of the human embryo can be measured reliably using 3D ultrasound by means of the TAL and CRL/TAL ratio with an optimal time window of 8 to 12 weeks of gestation. This new embryonic measurement provides several opportunities for future research and hopefully for further development of first trimester prenatal diagnosis of anomalies related to the spinal column.

PART

TWO

Parental and environmental exposures

CHAPTER



3

Influence of maternal folate status on human fetal growth parameters

Evelyne M van Uitert and Régine PM Steegers-Theunissen

Mol Nutr Food Res, 2013. 57(4): p. 582-95

ABSTRACT

Background Worldwide periconceptional folic acid supplement use is recommended to prevent neural tube defects. This also stimulated research on maternal folate status in association with fetal growth, an important predictor of perinatal and future development and health. We provide an overview of literature on associations between maternal folate status during pregnancy determined by folate biomarker concentrations in blood, folic acid supplement use and dietary folate intake, and fetal growth parameters.

Methods Literature was searched in PubMed up to November 2011.

Results Some studies suggest inverse associations between serum folate, folic acid supplement use and dietary folate intake and risk of a low birth weight or small for gestational age infant. The strongest evidence, however, revealed positive associations between birth weight and red blood cell (RBC) folate, folic acid supplement use and dietary folate intake. RBC folate appeared to be most consistently associated with other fetal growth parameters.

Conclusions These findings contribute to the knowledge of the impact of maternal folate status on fetal growth, and subsequently perinatal health and disease risks in later life. Future research is recommended to examine effects of windows, duration and dose of folic acid supplement use and use of folate-rich dietary patterns in different populations on fetal growth parameters.

INTRODUCTION

Deficiency of the essential B-vitamin folate is widespread and constitutes a major global burden of morbidity, which also affects women during the reproductive period [51]. The main cause of folate deficiency is poor dietary intake. Folate is an important substrate of the one-carbon metabolism, in which one-carbon groups are provided for DNA methylation and the synthesis of DNA, RNA, proteins and lipids [20]. It is clear that these folate-dependent processes are essential during time windows of rapid cell division and growth. Therefore, folate requirement during pregnancy is markedly increased to cover the needs of embryonic and fetal growth and development.

In the past decades the interest in folate received new attention when two large randomized controlled trials showed that periconceptional use of folic acid supplements, i.e. the synthetic form of folate, prevented recurrences [52] and first occurrences of neural tube defects [53]. These findings have been further substantiated by a Cochrane review that has led to the recommendation by the World Health Organization for women planning pregnancy to use 400µg folic acid daily from the preconception period up to twelve weeks of pregnancy (http://www.who.int/reproductivehealth/publications/maternal_perinatal_health/neural_tube_defects.pdf) [19, 54].

The evidence that the use of folic acid supplements can prevent neural tube defects has also stimulated research on associations between maternal folate status and other pregnancy outcomes. In the most recent meta-analysis, a combination of re-analysed data from the Aberdeen Folate Supplementation Trial 1966–67 and a Cochrane review [55], it was concluded that maternal folic acid supplement use does not affect mean birth weight, a parameter often used as proxy for fetal growth, but may lower the risk of having a low birth weight (LBW) infant, particularly at high doses [55, 56].

Maternal folate status is strongly determined by folic acid supplement use due to the high stability and bioavailability of the synthetic folic acid form. However, dietary folate intake, metabolism, the use of medication, certain lifestyles and health conditions, and genetic variations in folate genes, such as the gene encoding the enzyme methylenetetrahydrofolate reductase, also affect folate status [57, 58]. Red blood cell (RBC) and serum or plasma folate are biomarkers used for the assessment of folate status and should be considered endpoints of the determinants of folate status. RBC folate is a biomarker of long-term folate status reflecting the previous 2-4 months, since RBCs only accumulate folate during erythropoiesis and have a life span of approximately 120 days [59]. In contrast, serum and plasma folate represent the short-term folate status of the previous 1-3 days and are therefore often used to assess current folic acid supplement use.

Fetal growth is an important predictor of perinatal morbidity and mortality as well as of future health and disease risks. It has been suggested that fetal growth is influenced by

maternal folate status during pregnancy. Although folic acid supplement use has been investigated thoroughly, the influence of other folate determinants remains less clear. Therefore, a review of studies on maternal folate status during pregnancy may yield comprehensive insights into associations with fetal growth parameters.

In this review, we provide an overview of the literature on associations between maternal folate status during pregnancy determined by folate biomarker concentrations in blood, folic acid supplement use or dietary folate intake, and fetal growth parameters.

We will use the term folic acid for the synthetic form of folate used in supplements and fortified foods. All other forms of folate will be referred to as folate.

METHODS

We evaluated relevant literature up to November 2011 using the following search strategy in PubMed:

((“Folic Acid”[Mesh]) OR (“Folic Acid”) OR (“Folate”)) AND (“Pregnancy”) AND ((“Crown-Rump Length”[Mesh]) OR (“Crown-rump length*”) OR (“Embryonic growth*”) OR (“Fetal Weight”[Mesh]) OR (“Fetal weight*”) OR (“Fetal Growth*”) OR (“Abdominal circumference*”) OR (“Biparietal diameter*”) OR (“Head circumference*”) OR (“Femur length*”) OR (“Birth Weight”[Mesh]) OR (“Birth Weight*”) OR (“Infant, small for gestational age, ”[Mesh]) OR (“Small for gestational age”)))

Limits were set to include only human studies written in English and to exclude review articles.

All abstracts retrieved using this search strategy were checked and if in an abstract the authors addressed both folate or folic acid and any parameter of human fetal growth, the full text article was checked for relevance, i.e. whether the study actually investigated this particular relationship.

We subsequently included studies that addressed folate biomarkers in maternal blood, folic acid supplement use or dietary folate intake combined with at least one parameter of fetal growth. Fetal growth parameters included crown-rump length, biparietal diameter, head circumference, abdominal circumference, femur length, birth weight, and the risk or prevalence of having a LBW or small for gestational age (SGA) infant. We did not include studies on intra-uterine growth restriction unless validated by SGA at birth, because of substantial measurement errors in the estimates.

Studies examining associations between supplements containing both folic acid and iron or multivitamin supplements, or a combination of supplements were included only when

within the study the groups were compared to a control group using the same supplements but without folic acid.

In this review, we did not include studies on the effects of folic acid food fortification, because of the lack of accuracy of the total dose of folic acid exposure.

In all studies, dietary intake was assessed using food frequency questionnaires, 24 hour recall interviews, or weighing and recording of all foods consumed. Biomarker concentrations were determined using folate binding protein or microbiological assays in all studies. Details of dietary assessment and biomarker determination are provided in the supplementary material (**Table S1**).

Units and levels of serum/ plasma and RBC folate levels and dietary intake varied across studies. We recalculated all biomarker levels to nmol/L (1 ng/ml = 2.265 nmol/L) and dietary intake to µg/ day where possible. Mean/ median RBC folate levels ranged from 290 to 1686 nmol/L, serum folate levels from 11.8 to 52 nmol/L, and plasma folate levels from 7.3 to 32 nmol/L. Mean/ median dietary intake ranged from 206 to 669 µg/ day. In the presentation of overall ranges of folate levels we excluded 1 study because of incorrect biomarker summary measures [60], and 4 studies on biomarker levels and 2 on dietary folate intake because summary measures were not provided [61-66].

In most studies LBW is defined as birth weight <2500 g. In the studies by Baumslag et al. and Hogeveen et al. LBW is defined as <2270 g (5 lbs) and <3075 g (lowest quintile in study population), respectively, and Neggers et al. do not provide a definition [67-69]. In the majority of studies, SGA is defined according to local 10th percentiles of birth weight adjusted for gestational age at birth and in some studies additionally adjusted for gender [70, 71, 73-78]. Rolschau et al. define SGA as birth weight for gestational age of -2 standard deviations under the mean, Martin et al. as sex-specific birth weight for gestational age under the 2.5th percentile and Timmermans et al. under the 5th percentile [10, 63, 72]. Tamura et al. define SGA as birth weight for gestational age under the 15th percentile based on Alabama standards for race, gender and parity [79], and Hibbard et al. do not provide a definition of SGA [61].

When results of subgroup analyses are used, the number of study participants mentioned in this review comprises the number of participants included in these analyses only.

We judged the quality of each study from highest (A) to lowest quality (D) according to study design, population and confounder handling. Confounder handling includes randomization, selection and matching of participants and adjustment of statistical analysis. One randomized trial in which a significant difference in gestational age between groups was described was considered not to have taken into account gestational age sufficiently [80]. By definition, SGA is adjusted for gestational age, and in some studies additionally for gender, parity and/ or race, and LBW is independent of gestational age

and gender. Therefore, we judged SGA and LBW studies as if analyses were adjusted for gestational age, and where applicable additionally for gender, parity and/ or race. Quality codes were assigned according to the following criteria: A: study population n ≥100, randomized or prospective cohort study, adjustment for gestational age, gender, smoking, maternal age, maternal weight/ BMI and parity; B: study population n≥100, randomized or prospective cohort study, adjustment for gestational age, gender and smoking; C: study population n≥100, randomized or prospective cohort study, adjustment for smoking; D: all other studies with either study population n<100, non-randomized or retrospective cohort or case-control study, and/ or no (complete) adjustment for confounders.

Where in studies on supplement use associations with fetal growth parameters are generally described as dichotomous effects of folic acid use, i.e. use or no use, the associations of folate biomarkers and dietary intake can be estimated as either dichotomous or continuous effects of decreasing and increasing folate levels or intake. In this review, we report the associations according to the original analysis and description by the authors. For clarity reasons we describe studies on the lowest percentile and decreasing levels or intake as studies on low or decreasing folate levels or intake and studies on highest percentile and increasing levels or intake as studies on high or increasing folate levels or intake. We report effect sizes for those studies in which they are described explicitly. More details on the effect sizes are provided in the supplementary **Table S1**.

RESULTS

Our search strategy resulted in a total of 285 hits. Screening of all titles and abstracts revealed 105 eligible studies of which 58 studies fulfilled our inclusion criteria. We defined 'study' as a unique analysis of data, which could be derived from the same study population. In two studies the same study population was analysed twice in a similar manner. Therefore, only the 2 most relevant studies were included, leading to a total number of 56 studies for evaluation in this review.

In none of the included studies associations between maternal folate status during pregnancy and fetal biparietal diameter were investigated.

In the next paragraphs we evaluate associations between each determinant of maternal folate status and various fetal growth parameters and refer to tables 1-5 in which study results are summarized. Study results are outlined in more detail in the supplementary **Table S1**.

Maternal folate status determined by folate biomarkers

From the 56 included studies, 33 addressed associations between maternal folate status and fetal growth parameters by folate biomarkers determined in blood. We elaborate on studies with assessments of long-term RBC folate status and short-term serum or plasma folate status separately.

Table 1 Overview of studies on associations between long-term maternal red blood cell (RBC) folate status and fetal growth parameters assessed at birth.

Author	Year	Study type	n	Study moment	Birth weight	Head circum-ference	LBW	SGA	Quality[a)]
High or increasing RBC folate status									
Rolschau [62]	1979	cR	36	Birth	Increased				D
Ek [82]	1982	CS	139	Birth	Increased				D
Tamura [109]	1994	pC	76	Wk 17	<>				D
Frelut [71]	1995	CC	21	Wk 32	Increased			<>	D
				Birth	<>			<>	
Rondo [76]	1995	CC	712	Birth				<>	D
Rondo [77]	2000	CC	636	Birth				<>	D
Weerd [110]	2003	pC	194	Preconception	<>				D
				Wk 6	<>				
				Wk 10	<>				
Relton [8]	2005	pC	683	Wk 12	Increased				D
Takimoto [83]	2007	pC	94	Wk 7-14	<>	<>			D
				Wk 26-29	<>	Increased			
				Wk 34-36	Increased	<>			
Brough [84]	2010	cR	353	Wk 12	<>	Increased			D
				Wk 24	<>	<>			
				Wk 36	<>	<>			
Schlotz [85]	2010	rC	100	Wk 13	<>	Increased			D
Parazzini [111]	2011	pC	244	Wk 8-10	<>				D
				Wk 16	<>				
				Wk 22	<>				
Low or decreasing RBC folate status									
Chanarin [86]	1968	cR	206	Wk 37		<>			D
Hibbard [61]	1975	pC	723	<wk 16			<>		D
Relton [60]	2005	pC	998	Wk 12	Decreased				D
Yajnik [112]	2005	nCC	80	Wk 18				<>	D
				Wk 28				<>	
Martin [72]	2007	CC	82	Birth				Increased	D
Baker [81]	2009	pC	263	Wk 30				Increased	C

a) Quality: C: study population n≥100, randomized or prospective cohort study, adjustment for smoking; D: all other studies with either study population n<100, non-randomized or retrospective cohort or case-control study, and/ or no (complete) adjustment for confounders. <> No significant association; LBW low birth weight; RBC Red blood cell; SGA small for gestational age; R Randomized Controlled Trial, cR cohort evaluation within randomized trial, pC prospective cohort, C cohort, rC retrospective cohort, CC case-control, nCC nested case-control, CS cross-sectional study

Maternal RBC folate status

In **Table 1** an overview is given of 18 studies on associations between RBC folate and fetal growth parameters assessed at birth, of which 12 studies focused on associations with high or increasing, and 6 studies on associations with low or decreasing RBC folate. One study was judged to be of moderate (C) [81], and all other studies of low (D) quality.

5 out of 10 studies investigating associations between high or increasing RBC folate established a significantly positive association with birth weight [8, 62, 71, 82, 83]. This is supported by 1 study which showed a corresponding significant association of low RBC folate levels with decreased birth weight [60]. Association estimates were described as a correlation coefficient of 0.18 [82] and 0.53 [62], effect estimates of 0.36 grams increase in birth weight [83] and a 0.11 increase in birth weight *Z*-score [8] for every nmol/L increase in RBC folate, and a -0.31 decrease in birth weight *Z*-score in the lowest compared to the highest RBC quintile (RBC levels of quintiles cannot be given due to an error in the units used in this study) [60]. In the studies that observed this significant association, RBC folate was determined in 2 studies in the third trimester, in 2 studies at birth, and in 2 studies at the end of the first trimester.

All 3 studies examining head circumference at birth revealed a significantly positive association with RBC folate determined in the first and late first trimester in 2 studies, and in the second trimester in 1 study [83-85]. Estimates for the associations were reported as a correlation coefficient of 0.11 [84] and effect sizes of 0.05 mm [83] and 0.75 mm [85] for every nmol/L increase in RBC folate.

In only 1 study the risk of having a LBW infant was assessed, and results did not show a significant association with low or decreasing RBC folate at the end of the third trimester [86].

A significantly increased risk of having a SGA infant with low or decreasing RBC folate was observed in 2 out of 4 studies, in which RBC folate was assessed in the third trimester or at birth [72, 81]. Association estimates were described as a 125 [81] to 300 nmol/L [72] decrease in RBC folate level in SGA mothers compared to mothers of an appropriate weight for gestational age infant. None of the other studies on low or decreasing RBC folate in the second and third trimester of pregnancy, and of 3 studies on high or increasing RBC folate in the third trimester or at birth showed significant associations with SGA risk.

In conclusion, 2 large prospective studies substantiated by smaller cohort and case-control studies suggest that high or increasing maternal RBC folate measured at different times during pregnancy or at birth is positively associated with birth weight. This can be concluded from significant positive associations observed in just over half of the studies, and also in half of all prospective and nearly half of all larger (n≥100) studies. Moreover, none of the other studies observed associations in the opposite direction. This is further

supported by significant positive associations with head circumference observed in all 3 studies concerned, although associations were not observed consistently across all trimesters of pregnancy. However, of all studies on head circumference at birth and birth weight, only 1 of the studies took into account potential confounding of gestational age at birth, infant gender and smoking [85]. Although some studies considered potential confounding factors, in none of those associations were additionally adjusted for maternal age, body-mass index or weight, and parity together.

RBC folate appears not to be strongly associated with the risk of SGA, because a significant association was observed in only 1 small case-control study and 1 large (n≥100) prospective cohort study compared with non-significant results of 5 other studies, including another large prospective cohort study. However, in only 1 out of 7 studies analysis was adjusted for gestational age at birth, infant gender and smoking simultaneously [81] and none of the studies additionally adjusted for the confounders maternal age, BMI or weight, and parity. Due to the small number of studies performed so far, we cannot draw conclusions about associations with the risk of having a LBW infant.

Maternal serum and plasma folate status

In **Table 2** we depict 20 studies on associations between fetal growth parameters and serum or plasma folate. In 17 studies associations with high or increasing and in 6 studies associations with low or decreasing short-term folate status were investigated, including 3 studies that investigated associations in both directions. 3 studies were judged to be of excellent (A) [68, 74, 87], 1 of moderate (C) [81], and all others of low (D) quality.

In 3 out of 15 studies on high or increasing serum or plasma folate during pregnancy significant associations were observed with birth weight, including a significant increase in birth weight in 2 studies [79, 88] and a significant decrease in 1 study [89]. In 1 out of 2 studies a significant association was shown between low or decreasing folate at birth and decreased birth weight [82]. Associations and effect sizes were described as a correlation coefficient of 0.78 [88], a 2.4 nmol/L lower plasma folate in infants with birth weights up to 3,000 grams compared to infants of 3,001 to 4,000 grams [82], a 97 to 330 grams increase in birth weight in women with serum folate above 44 nmol/L compared to lower levels [79], and a 4 grams decrease in birth weight for every nmol/L increase in plasma folate [89]. No significant associations were observed between high or increasing folate during pregnancy and head circumference in 3 studies.

In contrast to the non-significant results of 2 studies evaluating high or increasing folate preconceptionally or in the third trimester, 1 study showed a significantly decreased risk of having a LBW infant with increasing folate levels in the second trimester [64]. The effect size of the association was estimated as an odds ratio (OR) for LBW of 0.985, which translates to a 1.5% decrease in LBW risk for every nmol/L increase in serum folate [64]. Both studies that assessed low or decreasing folate during the third trimester and at birth showed no significant associations with the risk of having a LBW infant.

Table 2 Overview of studies on associations between short-term serum or plasma folate status and fetal growth parameters assessed at birth.

Author	Year	Study type	n	Study moment	Serum/ Plasma	Birth weight	Head circum-ference	LBW	SGA	Qua-lity[a)]
High or increasing serum or plasma folate										
Whiteside [66]	1968	pC	60	Wk 12	Serum	<>				D
				Wk 26		<>				
				Wk 38		<>				
Rolschau [62]	1979	cR	36	Birth	Plasma	<>				D
Ek [82]	1982	CS	139	Birth	Plasma	<>				D
Mukherjee [89]	1984	pC	264	Unknown	Plasma	Decreased	<>			D
Tamura [79]	1992	nCCp	285	Wk 18	Serum	<>			<>	D
				Wk 30		Increased			Decreased	
Tamura [109]	1994	pC	76	Wk 17	Serum	<>				D
Frelut [71]	1995	CC	21	Wk 32	Plasma	<>			<>	D
				Birth		<>			<>	
Scholl [64]	1996	pC	832	Wk 28	Serum			Decreased		D
Stefanidis [113]	1999	CS	101	Birth	Serum	<>				D
Ronnenberg [90]	2002	CC	434	Preconception	Plasma			<>	<>	D
Weerd [110]	2003	pC	194	Preconception	Serum	<>				D
				Wk 6		<>				
				Wk 10		<>				
Sram [78]	2005	nCC	766	Birth	Plasma	<>[b)]			Decreased	D
Takimoto [83]	2007	pC	94	At 7-14	Serum	<>	<>			D
				Wk 26-29		<>	<>			
				Wk 34-36		<>	<>			
Faintuch [88]	2009	rC	13	2nd trimester	Serum	Increased				D
Hogeveen [68]	2010	pC	366	Wk 30-34	Plasma	<>		<>		A
Nilsen [74]	2010	pC	2934	Wk 18	Plasma	<>	<>			D
Parazzini [111]	2011	pC	244	Wk 8-10	Plasma	<>				D
				Wk 16		<>				
				Wk 22		<>				
Low or decreasing serum or plasma folate										
Baker [114]	1977	CC	100	Birth	Plasma			<>		D
Ek [82]	1982	CS	139	Birth	Plasma	Decreased				D
Baker [81]	2009	pC	288	Wk 30	Serum				<>	C
Dijk [87]	2010	pC	4044	Wk 13	Serum	<>				A
Hogeveen [68]	2010	pC	366	Wk 30-34	Plasma			<>		A
Nilsen [74]	2010	pC	2934	Wk 18	Plasma				<>	A

a) Quality: A: study population n ≥100, randomized or prospective cohort study, adjustment for gestational age, gender, smoking, maternal age, maternal weight/ BMI and parity; C: study population n≥100, randomized or prospective cohort study, adjustment for smoking; D: all other studies with either study population n<100, non-randomized or retrospective cohort or case-control study, and/ or no (complete) adjustment for confounders; b) increased in Prague smokers subgroup only. <> No significant association; LBW low birth weight; SGA small for gestational age; R Randomized Controlled Trial, pC prospective cohort, C cohort, rC retrospective cohort, CC case-control, nCCp nested case-control within prospective cohort, CS cross-sectional study;

High or increasing folate in the third trimester and at birth was associated with a significantly decreased risk of having a SGA infant in 2 out of 4 studies [78, 79]. A plasma folate level above 28.8 nmol/L was associated with ORs for SGA risk of 0.38 to 0.44 [78]. Results of both studies on low or decreasing folate at birth demonstrate no significant associations of low or decreasing folate in the second and third trimester with the risk of having a SGA infant.

In conclusion, despite the significant associations of a cross-sectional and large case-control study [79, 82], further supported by a small retrospective study [88], the contradictive significant results of a large prospective cohort study [89] and non-significant results of 8 other prospective cohorts indicate that short-term maternal folate status during pregnancy or at birth is not associated with birth weight. The only 2 large prospective studies that take into account confounders (A scores), show no significant associations of first and third trimester folate with birth weight [68, 87]. Results from 2 large nested case-control studies suggest that short-term maternal folate status in the third trimester and at birth may be negatively associated with the risk of having a SGA infant [78, 79], but in 2 other case-control studies and two large prospective cohort studies with excellent and good quality scores, no associations are observed [71, 74, 81, 90]. Short-term maternal folate status during pregnancy or at birth seems not to be associated with head circumference at birth and the risk of having a LBW infant. However, these associations have been investigated in only 3 and 4 studies, respectively and with the exception of one prospective cohort study of excellent quality (A score) [68] none of the studies effectively accounted for potential confounding factors.

Maternal folic acid supplement use

24 of the 56 included studies on maternal folate status reported on fetal growth parameters and maternal folic acid supplement use during pregnancy.

In **Table 3**, 23 studies are presented on maternal folic acid supplement use and fetal growth parameters assessed at birth. Study quality was judged excellent (A) in 10 studies [10, 56, 63, 67, 70, 74, 91-94], high (B) in 1 study [75], moderate (C) in 1 study [81] and low (D) in all other studies.

From 18 studies on folic acid use and birth weight, 5 studies showed significantly positive associations with assessment of folic acid use during the second or third trimester [62, 80, 94-96], and another study with folic acid use during the periconception period [10]. The effect size for folic acid supplement use ranged from a 41 to 200 grams increase in birth weight [10, 62, 80, 94-96]. In none of 4 studies, significant associations with head circumference at birth were observed. However, in 3 out of 8 studies folic acid use in the periconception period or from the first or second trimester onwards was significantly associated with a reduced risk of having a LBW infant [10, 63, 67]. The effect sizes for folic acid supplement use were estimated as decrease in LBW risk from 6.0 to 4.9% [63] and from 30.2 to 6.2% [67], and as ORs for LBW of 0.43 to 0.61 [10].

Table 3 Overview of studies on associations between maternal folic acid supplement use and fetal growth parameters assessed at birth.

Author	Year	Study type	n	Daily dosage (in µg)	Folic acid use	Birth weight	Head circum-ference	LBW	SGA	Quality[a)]
Baumslag [67]	1970	R	242	5000	From 24-28 wks			Decreased		A
Giles [92]	1971	R	620	5000	From <10, 10-20, 20-30, >30 wks	<>				A
Iyengar [96]	1971	R	95	100/ 200/ 300	From 20-24 wks	Increased				D
Hamilton [93]	1972	R	685	5000	Not specified	<>				A
Fleming [115]	1974	R	89	500	From 20 wks	<>				D
Iyengar [94]	1975	R	189	500	From 20-28 wks to end	Increased				A
Rolschau [62]	1979	R	36	500	From 21-28 wks to end	Increased				D
Tchernia [80]	1982	R	1982	350	From 6 months to end	Increased	<>			D
Fleming [91]	1986	R	200	1000	From 18wks	<>				A
Shaw [116]	1997	C	734	Any	Any use within -4 to +12 wks and use throughout -4 to +12 wks			<>		D
Rolschau [63]	1999	R	13860	1000 or 2500[b)]	From preconception or <19 wks	<>		Decreased	Decreased	A
Christian [70]	2003	R	1313	400	From pre/ periconception period	<>	<>	<>	<>	A
Mitchell [73]	2004	CC	1076	Use ≥1/wk	Use during 1st trimester				Decreased	D
			1019		Use during 3rd trimester				<>	
Charles [56]	2005	R	2928	200 or 5000[a]	From mean wk 17	<>		<>		A
Katz [117]	2006	pC	4696	400	From 1st trimester	<>				D
Palma [118]	2008	CC	112	Not specified	Use for at least 1 wk at any time in pregnancy			<>		D
Baker [81]	2009	pC	498	Not specified	Any use in first 20wks				<>	C
Timmermans [10]	2009	pC	6353	400-500	From preconception period	Increased		Decreased	Decreased	A
					Use from <8wks	Increased		Decreased	<>	
					Use from wk 8	<>		<>	<>	
Czeizel [95]	2010	C	13612	5600	Use in 1st trimester only	<>		<>		D
					Use in 2nd trimester only	<>		<>		
					Use in 3rd trimester only	<>		<>		
					Use in 1st and 2nd trimester	<>		<>		
					Use in 2nd and 3rd trimester	Increased		<>		
					Use in all trimesters	<>		<>		
					Use in 3rd trimester regardless of use in other trimesters	<>		<>		
Hogeveen [68]	2010	pC	366	Not specified	Early pregnancy (no specifics)	<>				D
Nilsen [74]	2010	pC	2934	None vs. <400 vs. ≥ 400	Any use up to median 18 wk	<>	<>		<>	D/A[-/]
Hossein [103]	2011	pC	113	1000	Use in 1st and 2nd trimester only vs. to end	<>	<>			D
Pastor-Valero [75]	2011	pC	786	No use vs. moderate (≤1000) vs. high use (>1000)	Use for ≥1 month between -12 and +4 wks	<>			Increased	B

a) Quality: A: study population n ≥100, randomized or prospective cohort study, adjustment for gestational age, gender, smoking, maternal age, maternal weight/ BMI and parity; B: study population n≥100, randomized or prospective cohort study, adjustment for gestational age, gender and smoking; C: study population n≥100, randomized or prospective cohort study, adjustment for smoking; D: all other studies with either study population n<100, non-randomized or retrospective cohort or case-control study, and/ or no (complete) adjustment for confounders; b) no difference between dosages; c) birth weight and head circumference D score, SGA A score. <> No significant association; R Randomized Controlled Trial, pC prospective cohort, C cohort, CC case-control study

Of 7 studies assessing the risk of having a SGA infant, the results of a large randomized controlled trial, a prospective cohort study and a case-control study revealed a significantly decreased risk as a consequence of maternal folic acid use initiated in the periconception period [10, 73] or before 20 weeks gestation [63]. Another prospective cohort study, however, observed a trend in the opposite direction towards an increased risk of a SGA infant with no, moderate and high dose folic acid supplement use [75]. Effect sizes were reported as ORs for SGA of 0.70 [73] and 0.40 [10], a decrease in incidence of SGA from 3.7% to 1.7% with earlier initiation of folic acid use [63] and an increase in incidence from 9.5% (no folic acid supplement use) to 15.8% (moderate folic supplement use) and 16.5% (high dose folic acid supplement use) [75].

In **Table 4** the results are shown of 2 studies performed in a large prospective birth cohort study in the Netherlands, in which fetal growth parameters were assessed during pregnancy rather than at birth, of excellent (A) and good (B) quality [10, 16]. Results revealed that maternal folic acid use during the first trimester was significantly positively associated with crown-rump length, although the significance of the effect estimate attenuated after adjustment for multiple testing [16].The reported effect size of folic acid supplement use was a 0.17 increase in CRL standard deviation score [16]. In contrast to femur length, abdominal circumference assessed in the second and third trimester, and head circumference in the third trimester of pregnancy were significantly associated with preconceptional initiation of maternal folic acid use [10]. Effect sizes of folic acid supplement use were estimated at 0.61 mm and 1.34 mm for second and third trimester head circumference and 1.71 mm for third trimester abdominal circumference [10].

In summary, we conclude from the associations observed in 4 out of 11 randomized controlled trials and 2 large cohort studies that maternal folic acid supplement use during pregnancy may be positively associated with birth weight, especially when used from the second trimester onwards. However, in 7 out of 9 studies of excellent or good quality (A or B score) no significant associations were observed [10, 56, 63, 70, 75, 91-94], although no associations were observed in the opposite direction either. The potential positive association with birth weight is however in line with the beneficial effect of maternal folic acid use on the risk of having a LBW infant observed in 3 out of 5 studies of excellent quality (A scores) [10, 63, 67]. Beneficial effects on the risk of having a SGA infant are less clear, where a significant negative association is observed in only 2 out of 6 excellent to moderate studies (A-C scores) [10, 63], of which one study of good quality (B score) even observes a significant association in the opposite direction [75]. Although in 2 large randomized controlled trials and 2 large prospective cohort studies no significant associations were established with head circumference at birth, the only study on fetal growth during pregnancy showed a significant association for preconception initiation of maternal folic acid use with fetal head and abdominal circumference in the second and third trimester [10]. However, associations with fetal growth during pregnancy rather than at birth have been assessed in this prospective cohort study only and therefore need replication in other birth cohorts and populations.

Table 4 Overview of studies on associations between maternal folic acid supplement use and fetal growth parameters assessed during pregnancy.

Author	Year	Study type	n	Daily dosage (in µg)	Folic acid use	CRL	Head circum-ference	Abdominal circum-ference	Femur length	Quality[a)]
First trimester										
Mook [16]	2010	pC	1631	400-500	1st trimester	Increased [b)]				B
Second trimester										
Timmermans [10][c)]	2009	pC	6353	400-500	Precon-ception		Increased	<>	<>	A
					1st trimester		**<>**	**<>**	**<>**	
Third trimester										
Timmermans [10][c)]	2009	pC	6353	400-500	Precon-ception		Increased	Increased	<>	A
					1st trimester		<>	<>	<>	

a) Quality: A: study population n ≥100, randomized or prospective cohort study, adjustment for gestational age, gender, smoking, maternal age, maternal weight/ BMI and parity; B: study population n≥100, randomized or prospective cohort study, adjustment for gestational age, gender and smoking; b) NS after adjustment for multiple testing; c) These lines refer to the same study. CRL Crown-rump length; <> No significant association; pC prospective cohort study

Maternal folate status determined by dietary folate intake

In 14 out of 56 included studies in this review maternal dietary folate intake and fetal growth parameters are addressed, of which the results are presented in **Table 5**. 12 studies evaluated high or increasing intake and 4 studies evaluated low or decreasing intake, including 2 studies that evaluated maternal dietary folate intake in both directions. 3 studies were judged to be of high (B) [69, 75, 97], 1 of moderate (C) [81], and the others of low (D) quality.

Maternal dietary folate intake in the first trimester or throughout pregnancy was significantly positively associated with birth weight in 3 out of 12 studies [65, 69, 75]. In none of the other 9 studies significant associations were observed. Reported effect sizes were a 4 [65] to 5 [69] grams increase in birth weight for every 100 µg/day increase in folate intake, and increasing quintiles of folate intake were associated with 99 to 169 grams increase in birth weight [75].

In none of 5 studies maternal dietary folate intake was significantly associated with head circumference.

In contrast to the non-significant results of a study evaluating high or increasing maternal dietary folate intake and the risk of having a LBW infant [98], 1 out of 2 studies on associations with low or decreasing intake in the second and third trimester of pregnancy showed a significantly increased risk of having a LBW infant [64]. The effect size was reported as an OR for LBW of 3.33 with intake <240 µg/day compared to intake >400 µg/day [64]. In 1 out of 2 studies on high or increasing maternal dietary folate intake, increased first trimester folate intake was significantly associated with a decreased risk of having a SGA infant [75]. In addition, 1 of 2 studies reporting on low or decreasing maternal dietary folate intake established a corresponding increased SGA risk with decreasing third trimester dietary folate intake [81]. Reported effect sizes were ORs for SGA of 0.21 to 0.86 with increasing quintiles of dietary folate intake [75] and an OR for SGA of 3.13 with intakes under 187 µg/day [81].

In conclusion, the results of 3 substantial prospective studies and 1 retrospective cohort study, including 3 out of 4 studies of good to moderate quality (B or C score), suggest maternal dietary folate intake to be associated with birth weight and the risk of having a SGA infant. Few studies effectively adjust for confounders, however, as only 4 out of 14 studies on birth weight or SGA are of good to moderate quality (B or C score). Dietary folate intake appears not to be associated with head circumference at birth, and current evidence is too limited to suggest associations with the risk of having a LBW infant.

DISCUSSION

In this review we present the results of studies ranging from excellent to low quality in

Table 5 Overview of studies on associations between maternal dietary folate intake and fetal growth parameters assessed at birth.

Author	Year	Study type	n	Study moment	Birth weight	Head circum-ference	LBW	SGA	Qua-lity[a)]
High or increasing intake									
Whiteside [66]	1968	pC	60	Wk 12	<>				D
				Wk 26	<>				
				Wk 38	<>				
Johnson [98]	1994	pC	332	Mean intake throughout pregnancy	<>		<>		D
Frelut [71]	1995	CC	21	Wk 27	<>	<>		<>	D
Neggers [69]	1997	pC	289	Mean intake of wk 18 & 30	Increased				B
Mathews [97]	1999	pC	624	Wk 9-20	<>				B
				Wk 28	<>				
Takimoto [83]	2007	pC	94	Wk 7-14,	<>	<>			D
				Wk 26-29	<>	<>			
				Wk 34-36	<>	<>			
Watanabe [119]	2008	pC	197	Wk 12	<>				D
				Wk 20	<>				
				Wk 32	<>				
Kordas [65]	2009	rC	474	Birth, on entire pregnancy	Increased	<>			D
Bawadi [120]	2010	rC	700	Birth	<>				D
Nilsen [74]	2010	pC	2934	Wk 18	<>	<>			D
Schlotz [85]	2010	rC	100	Wk 13	<>	<>			D
				Wk 29	<>	<>			
Pastor-Valero [75]	2011	pC	786	1st trimester intake	Increased			Decreased	B
Low or decreasing intake									
Scholl [64]	1996	pC	832	20 & 28 wks combined			Increased		D
Neggers [69]	1997	pC	289	Mean intake of wk 18 & 30			<>		B
Baker [81]	2009	pC	290	3rd trimester				Increased	C
Nilsen [74]	2010	pC	2934	Wk 18				<>	D

a) Quality: A: study population n ≥100, randomized or prospective cohort study, adjustment for gestational age, gender, smoking, maternal age, maternal weight/ BMI and parity; B: study population n≥100, randomized or prospective cohort study, adjustment for gestational age, gender and smoking; C: study population n≥100, randomized or prospective cohort study, adjustment for smoking; D: all other studies with either study population n<100, non-randomized or retrospective cohort or case-control study, and/ or no (complete) adjustment for confounders. <> No significant association; pC prospective cohort, rC retrospective cohort, CC case-control study

design and analysis that were directed to find associations between several determinants of maternal folate status assessed during pregnancy and at birth, and fetal growth parameters. We draw our conclusions according to the quality of the studies.

The strongest evidence reveals the positive influence on birth weight by maternal folate status, determined by long-term RBC folate and to a lesser extent by folic acid supplement use and dietary folate intake. This finding is further supported by associations between SGA risk and maternal folic acid supplement use and dietary folate intake. So far, evidence is too limited to draw firm conclusions on associations between maternal folate status and LBW, and crown-rump length, head and abdominal circumference and femur length in pregnancy. In addition, data were insufficient to define the most sensitive period to maternal folate status before or during pregnancy with regard to birth weight and other fetal growth parameters.

Maternal RBC folate appears to yield the most consistent results with regard to the association with birth weight. Although few studies adequately handled confounding variables, none of the studies observed associations in the opposite direction, further supporting the association with birth weight. RBC folate is a biomarker which unlike folic acid use or dietary folate intake is not affected by recall bias and reflects long-term folate status and thus is more stable compared to the short-term biomarkers serum and plasma folate. Therefore, RBC folate may be the best determinant in order to assess maternal folate status and might be used as one of the predictors of birth weight. The relatively small effect sizes however emphasize that there are stronger predictors of birth weight.

The strongest association of RBC folate with birth weight is supported by the possible association between periconceptional folic acid use and decreased SGA risk. This is in line with the association between low dietary folate intake and increased SGA risk as reported in two studies. These findings are supported by a large prospective study, in which low maternal adherence to a folate-rich dietary pattern was associated with increased SGA risk [99]. The comparability of dietary intake studies is however hampered, due to variation between studies in computation of dietary folate intake by including or excluding intake from folic acid supplements. In general maternal dietary folate intake based on the calculation of nutrient levels appears to be the weakest determinant of folate status in association with fetal growth. This can be explained by the overall very low intake as well as bioavailability and stability of dietary folate compared to folic acid from supplements [100].

The main challenge in comparing the results of the various studies was the tremendous heterogeneity in study designs and populations and the different study windows before and during pregnancy to assess folic acid use, dietary folate intake and biomarkers. In addition, there were many differences in dosages of folic acid supplements used as well as definitions and analysis of high or low folate status that varied across studies. In several studies, the amount of detailed information on the duration of maternal folic acid use was limited. Folate biomarker levels and moment of determination of biomarkers and dietary folate

intake in pregnancy or at birth differed substantially across studies, rendering numbers too small to draw more specific conclusions on the most folate sensitive period of fetal growth. Statistical analyses differed substantially across studies and where one study did not adjust for confounders at all, another adjusted for an entire range of covariates. A clear example is the potential confounding of dietary intake and folic acid supplement use by education, ethnicity, and unplanned pregnancy which often needs adjustments [101, 102]. Furthermore, studies carried out in third world countries among populations with severe undernourishment including folate deficiency cannot be compared with populations in Western countries where qualitative malnutrition is the main cause of folate deficiency and where folate fortification and folic acid supplement use is widespread. However, as most studies are confined to a specific geographical and cultural area, subjects are often rather homogeneous in ethnicity, education and social economic class within study populations, and so it is rather the comparison between studies in which these factors substantially affect the interpretation of the results.

Another important issue is the evident association of fetal growth with gestational age. We came across a study in which fetal growth parameters at birth were compared between folic acid use in the first two versus all three trimesters, and statistical analysis was not adjusted for gestational age at birth [103]. In another study a significant difference in gestational age between treatment groups is described, but subsequently not adjusted for in the analysis [80]. Thus, residual confounding may account for a considerable amount of noise and explain differences in or absence of findings across studies.

Although we do not expect that differences in the techniques used for the measurement of folate biomarker levels explain findings within studies, we address this issue with regard to the interpretation of the results across studies. In all included studies folate biomarkers were determined using either microbiological or folate binding protein assays. The microbiological method can measure all folate moieties, whereas folate binding protein assays bind predominantly the main folate moiety in the blood, i.e., 5- methyl tetrahydrofolate [59]. Therefore, folate binding protein assays may slightly underestimate folate concentrations as compared to microbiological assays. However, intracellular folate is stored in the polyglutamate form, which has an increased affinity for folate binding proteins used in the determination of especially RBC folate [59]. This on the other hand can lead to an overestimation of RBC folate by folate binding protein assays, in particular as different polyglutamate degrading buffers and protocols are used across studies. Although absolute levels thus may slightly differ, we do not expect relative differences to be substantially affected, and therefore consider it unlikely that the different findings across studies are substantially confounded by differences in measurement techniques.

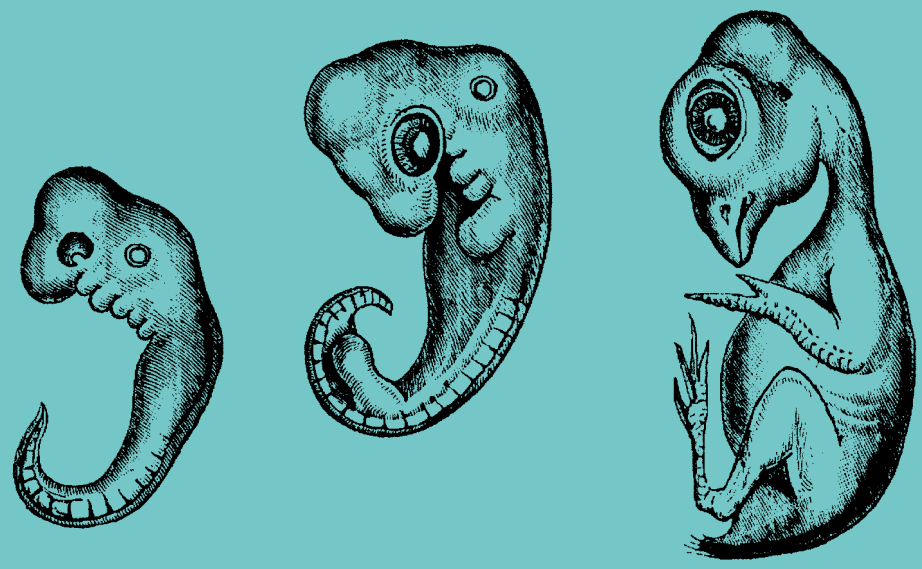
Of all studies included in this review, two studies observed a negative influence of folate status on fetal growth [75, 89]. Of interest in this respect are the reported epigenetic effects of prenatal exposure to similar dosages of folic acid and dietary folate content in animal studies and first human studies [104-106], suggesting the possibility of both positive

and negative influences of folate in higher amounts. These findings emphasize the need to identify both lower and upper limits for maternal folate status during pregnancy in association with fetal growth and long term health outcomes [107].

We retrieved the studies for this review after an extensive search of the PubMed database and therefore believe that all important studies have been included. However, we cannot exclude that we may have missed some studies. To balance clarity and completeness of this review, we decided to omit some details, which can be found however in the supplementary **Table S1**.

This review reveals evidence of maternal folate status influencing birth weight, in particular during pregnancy. This finding considerably contributes to the knowledge of the significant impact of prenatal nutrition on fetal growth, for which birth weight is often used as proxy, and the associations with health and disease risks in later life [108]. Because poor maternal folate status is a global health problem, preconceptional and antenatal care should be stimulated and tailored on improvement of maternal folate status, of which RBC folate seems to be the best predictor and biomarker to assess. Maternal folate status can be modified and improved by stimulating dietary folate intake and using low-dose folic acid supplements. However, the implementation of these recommendations is not very easy. Studies in which fetal growth parameters are assessed during pregnancy suggest that maternal folic acid use may exert its effects as early as in the first trimester of pregnancy. In addition, other determinants of maternal folate status suggest influences on fetal growth parameters in particular during mid and late pregnancy. To identify the most folate-sensitive period of fetal growth and to examine beneficial and harmful effects of maternal folate status, periconceptional prospective birth cohorts and randomized controlled trials are warranted. In those studies detailed effects of the window, duration and dose of folic acid supplement use, and the use of folate-rich dietary patterns in different countries and populations should also be addressed. We hope that this review will stimulate further research and support preconceptional care and preventive strategies.

CHAPTER



4

An optimal periconception maternal folate status for embryonic size: The Rotterdam Predict study

Evelyne M van Uitert, Sharon van Ginkel, Sten P Willemsen, Jan Lindemans, Anton HJ Koning, Paul HC Eilers, Niek Exalto, Joop SE Laven, Eric AP Steegers and Régine PM Steegers-Theunissen

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ABSTRACT

Background First-trimester maternal red blood cell (RBC) folate has been positively associated with weight and head circumference in newborns. Although folic acid supplements are recommended to be used predominantly in the periconception period and all major organ systems are developed within the first 10 weeks of gestation, little is known on the influence of folate on embryonic growth.

Methods We recruited women before 8 weeks of gestation and performed weekly three-dimensional ultrasound scans from enrolment up to 13 weeks of gestation. As measure of embryonic growth crown-rump length (CRL) measurements were performed using V-Scope software in the Barco I-Space. Maternal blood was collected to determine first-trimester long-term RBC folate status. Non-malformed live births were included in the analysis. We calculated quartiles of RBC folate, square-root transformed CRL-data and performed multivariable linear mixed model analyses.

Results In total 484 ultrasound scans were performed in 77 women, of which 440 (90.7%) CRLs could be measured. RBC folate in the third quartile (1513-1812 nmol/L) was significantly associated with an increased CRL compared to the first two quartiles (814-1512 nmol/L) and the upper quartile (1813-2936 nmol/L; $P_{overall}$ = 0.03; adjusted for gestational age, smoking, BMI and fetal sex). Compared to the third quartile, embryos in the upper quartile were 24.2% smaller at 6^{+0} weeks (4.1 mm (95%CI 3.5, 4.7) versus 5.4 mm (95%CI 4.8, 6.1)) and 7.6% smaller at 12^{+0} weeks (55.1 mm (95%CI 52.9, 57.3) versus 59.6 mm (95%CI 57.4, 62.0)) gestation, respectively.

Conclusions This study suggests that a very high maternal periconception folate status is associated with reduced embryonic size. Whether these effects are beneficial or harmful requires further investigation.

INTRODUCTION

Fetal growth is associated with health and disease risks in later life [4]. Fetal growth is influenced by a multitude of genetic and environmental factors, including maternal folate status [42]. Folate is an important substrate of the one-carbon metabolism, in which one-carbon groups are provided for essential cellular processes such as the synthesis of proteins, lipids, DNA, RNA, and methylation of chromatin [3]. As pregnancy is a period of rapid growth and numerous cell divisions, folate requirements are increased throughout pregnancy. In addition, periconceptional use of folic acid (FA) supplements has been shown to prevent neural tube defects (NTDs), which has lead the World Health Organisation to recommend FA supplement use from the periconception period up to 12 weeks of pregnancy [18, 19]. Due to the high stability and bioavailability of the synthetic FA form, maternal folate status is strongly determined by FA supplement use. However, dietary folate intake, metabolism, the use of medication, certain lifestyles and health conditions, and genetic variations in folate genes, such as the gene encoding the enzyme methylenetetrahydrofolate reductase, also affect folate status [57, 58]. Therefore, folate biomarkers provide a more precise estimation of folate status. Whereas serum or plasma folate levels are subject to daily fluctuations in FA and dietary folate intake and represent short term folate status, red blood cell (RBC) folate represents long-term folate status reflecting the previous 2-4 months since RBCs only accumulate folate during erythropoiesis and have a life span of approximately 120 days [59]. Thus, in early pregnancy maternal RBC folate reflects the folate status in the periconception period.

First-trimester maternal RBC folate has been positively associated with weight and head circumference in newborns [8, 60, 84, 85]. Although FA supplements are recommended to be used predominantly in the periconception period and all major organ systems are developed within the first 10 weeks of gestation, to date no studies have been performed on associations between maternal RBC folate status and embryonic growth trajectories in early pregnancy.

Therefore, in this study we investigated whether maternal RBC folate levels are positively associated with first-trimester embryonic growth, as determined by crown-rump length (CRL).

METHODS

Data for this study were collected in the Rotterdam Predict study, a prospective periconception cohort study conducted at the Department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Centre Rotterdam, the Netherlands. This study has been approved by the Central Committee on Research in The Hague and the local Medical Ethical and Institutional Review Board of the Erasmus MC. At enrolment, all participants signed a written informed consent form before participation.

All women of at least 18 years old with ongoing intrauterine singleton pregnancies of 6 to 8 weeks gestation were eligible for participation and recruited in 2009 and 2010. In a subgroup first-trimester maternal RBC folate was determined at enrolment. The majority of participating women were recruited from the outpatient clinic of the Department of Obstetrics and Gynaecology at the Erasmus MC and a smaller group (23%) was recruited from outside the hospital. For the current study, we only included those pregnancies in which first-trimester maternal RBC folate was determined.

Ultrasound data

Women received weekly transvaginal three-dimensional (3D) ultrasound scans from enrolment up to the 13th week of pregnancy. Ultrasound scans were performed with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D View software (General Electrics Medical Systems, Zipf, Austria). Scanning time per visit was as short as possible (less than 20 minutes) and the thermal as well as mechanical indices were kept below one, in line with the international recommendations for safe scanning [121-123]. The obtained 3D-datasets were stored and transformed to Cartesian (rectangular) volumes afterwards, to be transferred to the Barco I-Space (Barco N.V., Kortrijk, Belgium) at the Department of Bioinformatics, Erasmus MC, University Medical Centre Rotterdam. This is a four-walled CAVE^{TM}-like (Cave Automatic Virtual Environment) virtual reality system, allowing depth perception and interaction with the projected images [25]. CRL measurements were performed offline using the I-Space and V-Scope software [21] and by placing virtual callipers at the outer side of crown and rump in the midsagittal plane. CRL measurements performed in the I-Space show good agreement with 2D measurements and good inter- and intraobserver agreement [23]. All CRL measurements were performed three times by the same researcher, and the mean of these three measurements was used in the analyses.

Questionnaires

At enrolment participants completed a self-administered general questionnaire covering details on maternal age, anthropometrics, ethnicity, education, obstetric history, and periconception exposures.

Blood samples

In addition, a venous blood sample was collected to determine first-trimester maternal RBC folate levels. Blood was collected in an 8.5ml Vacutainer ethylenediaminetetraacetic acid (EDTA) tube (BD Diagnostics, Plymouth, UK). Directly after blood sampling, the haemolysate was prepared by diluting 0.1 ml full blood in 0.9 ml freshly prepared 1.0% ascorbic acid. Subsequently, the haematocrit of the remaining EDTA full blood was determined on a Sysmex XE-2100 Haematology Analyser (Sysmex, Europe GmbH, Norderstedt, Germany). In serum, folate was measured using electrochemiluminescence immunoassay (Modular E170, Roche GmbH, Mannheim, Germany). The haemolysate was centrifuged at 1000 *g* for 5 min at 18°C, just before the folate measurement. The haemolysate folate concentration was recalculated into RBC folate concentration using

the following formula: (nM haemolysate folate * 10/haematocrit) - (nM serum folate * (1-haematocrit)/haematocrit) = nM RBC folate.

Pregnancy dating

Data on the first day of the last menstrual period (LMP) and of regularity and duration of the menstrual cycle were obtained in a personal interview by the researcher performing the ultrasound at the first visit. We calculated the gestational age from the LMP in spontaneously conceived pregnancies, from the date of oocyte pick-up plus 14 days in pregnancies conceived through in vitro fertilisation with or without intracytoplasmic sperm injection (IVF/ ICSI) procedures, from the LMP or insemination date plus 14 days in pregnancies conceived through intra-uterine insemination (IUI), and from the day of embryo transfer plus 17 or 18 days in pregnancies originating from transfer of cryopreserved embryos, depending on the number of days between oocyte pickup and cryopreservation of the embryo. When the menstrual cycle was regular but more than three days different from 28 (28±>3 days), we adjusted the gestational age for the duration of the menstrual cycle.

Study population

From the total number of 102 pregnancies in whom first-trimester maternal RBC folate was determined, we excluded pregnancies conceived by oocyte donation (n=2), pregnancies ending in a miscarriage (n=12), pregnancies in which the first day of the LMP was missing or the observed CRL differed more than six days from the expected CRL according to the Robinson curve [28] (n=6) and pregnancies that ended in a major malformation with (n=2) or without (n=2) subsequent termination. Of the remaining women only one woman reported no FA supplement use and was therefore additionally excluded, resulting in a total of 77 pregnancies available for first-trimester analysis.

Follow-up

Information on the infants' date of birth, sex, birth weight and presence of one or multiple congenital anomalies were obtained from medical records. Gestational age at birth was calculated from the dating procedure used in the first-trimester.

Statistical analysis

Embryonic growth was studied using CRL measurements performed between 6^{+0} and 12^{+6} weeks.

Maternal characteristics were summarized for the total group and stratified by RBC folate quartiles. Distribution across quartiles was tested using Students *t*-test for normal distributions, Kruskal-Wallis test for non-parametric distributions, and Chi-square or exact tests for categorical data depending on the number of cells with an expected value below five. Birth weight was compared between groups by taking into account gestational age at birth using linear regression.

Potential confounders were identified using ANOVA with ethnicity and education as explanatory variables, and by calculating Spearman correlation coefficients for the other maternal characteristics listed in **Table 1**.

To assess the association between maternal RBC folate levels and embryonic growth trajectories we performed multivariable linear mixed model analyses. By using a mixed model we take into account that there is correlation between the observations that belong to the same pregnancy. RBC folate levels were divided into quartiles. Square root transformation of CRL data resulted in linearity with gestational age and a constant variance independent of gestational age and was therefore used in the analysis. First, we performed a univariate analysis in which we adjusted for gestational age only, and tested for time-interaction of RBC folate. In the second or fully adjusted model, we additionally entered fetal sex and all covariates that were significantly correlated to RBC folate levels with and without time interaction into the model. The third and final model was derived from the fully adjusted model after stepwise elimination of all covariates with *P*-values above the 20^{th} percentile.

Analyses were performed in the total group and repeated in a subgroup restricted to pregnancies with the most reliable gestational age, defined as those pregnancies dated on a strictly regular menstrual cycle of 28±3 days and a certain LMP, or conception date. In addition, we repeated the analyses in the subgroups of IVF/ ICSI pregnancies only, in spontaneous pregnancies only, and in spontaneous pregnancies with a reliable gestational age based on a strictly regular menstrual cycle of 28±3 days only.

Linear mixed model analyses were performed using PROC MIXED in SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). All other analyses were performed using IBM SPSS Statistics Version 20.0 for Windows software (IBM, Armonk, NY, USA).

RESULTS

The median gestational age at the first ultrasound scan was 6^{+5} (range 6^{+0}-9^{+1}, interquartile range (IQR) 6^{+3}-7^{+0}) weeks, and the median number of visits per pregnancy was 6 (range 4-7, IQR 6-7). From a total of 484 datasets, 440 (90.9%) were of sufficient quality to perform CRL measurements (**Table 1**). We performed a median of 6 (range 3-7) CRL measurements per pregnancy.

Maternal and pregnancy characteristics are shown in **Table 2**. Mean maternal age was 32.7 (standard deviation (SD) 4.5) years and women had predominantly had a high education (60.0%) and were of Dutch descent (78.9%). In 63 (81.8%) pregnancies gestational age was based on a regular menstrual period of 28±3 days or conception date, including 27 (35.1% of 77 included pregnancies) pregnancies that were conceived after IVF/ ICSI treatment. Pregnancy complications occurred in 11 (14.3%) pregnancies. Maternal

Table 1 Ultrasound scans and crown-rump length (CRL) measurements obtained in each gestational week.

Gestational week*	Number of ultrasound scans		Number of CRL measurements		
	n (2 scans)**	%***	n (2 scans)**	%***	%****
6	54 (1)	68.8	38 (1)	48.1	70.4
7	68 (1)	87.0	59 (1)	75.3	86.8
8	75 (1)	96.1	72 (1)	92.2	96.0
9	74 (2)	93.5	71 (2)	89.6	95.9
10	69 (2)	87.0	67 (2)	84.4	97.1
11	76 (4)	93.5	72 (4)	88.3	94.7
12	68 (0)	88.3	61 (0)	79.2	89.7

* gestational week defined as week $^{+0}$ to $^{+6}$ days (week 6 = 6^{+0} to 6^{+6} weeks gestation); ** between brackets the number of pregnancies with two ultrasound scans within the same gestational week; *** percentage of pregnancies with at least one ultrasound scan/ CRL measurement (n_{total}=77); **** successpercentage of CRL measurements

Table 2 General characteristics of study population for the total group and stratified by red blood cell (RBC) folate quartiles.

			RBC folate in quartiles (nmol/L)				
	All (n=77)	**Missings**	**Q1** (814-1223)	**Q2** (1224-1512)	**Q3** (1513-1812)	**Q4** (1813-2696)	***P***
Maternal (at enrolment)							
Maternal age, years (mean ± SD)	32.7 ± 4.5	3	31.4 ± 3.6	32.2 ± 4.4	33.9 ± 4.9	33.4 ± 4.1	.357
Ethnicity		1					.331
Dutch	60 (78.9%)		13 (68.4)	16 (80.0)	14 (77.8)	17 (89.5)	
Other western	9 (11.8%)		5 (26.3)	2 (10.0)	1 (5.6)	1 (5.3)	
Non western	7 (9.2%)		1 (5.3)	2 (10.0)	3 (16.7)	1 (5.3)	
Education		2					.951
Low	7 (9.3%)		2 (10.5)	2 (10.5)	2 (11.1)	1 (5.3)	
Middle	23 (30.7%)		4 (21.1)	6 (31.6)	6 (33.3)	7 (36.8)	
High	45 (60.0%)		13 (68.4)	11 (57.9)	10 (55.6)	11 (57.9)	
BMI, kg/m^2	23.3 (18.6-38.3)	1	24.9 (20.7-30.5)	24.4 (19.5-31.0)	22.6 (18.6-29.7)	23.5 (19.4-38.3)	.053
Primiparous	49 (63.6%)	0	12 (63.2)	13 (65.0)	11 (57.9)	13 (68.4)	.924
Periconception smoking	13 (16.9%)	0	7 (36.8)	4 (20.0)	1 (5.3)	1 (5.3)	.024
Pregnancy and outcome							
Conception through IVF/ICSI	27 (35.1%)	0	6 (31.6)	8 (40.0)	6 (31.6)	7 (36.8)	.931
Reliable gestational age*	63 (81.8%)	0	14 (73.7)	17 (85.0)	16 (84.2)	16 (84.2)	.817
Gestational age at RBC folate determination, week^{+d}	7^{+4} (4^{+1}-11^{+0})	0	7^{+2} (4^{+1}-10^{+3})	7^{+4} (5^{+2}-10^{+0})	7^{+4} (5^{+5}-11^{+0})	8^{+2} (6^{+4}-10^{+6})	.169
Infant sex male	36 (46.8%)	0	11 (57.9)	10 (50.0)	7 (36.8)	8 (42.1)	.584
Birth weight, g (mean ± SD)	3327 ± 484	0	3157 ± 559	3576 ± 381	3349 ± 432	3214 ± 472	.187***
Gestational age at delivery, week^{+d}	39^{+2} (34^{+2}-41^{+3})	0	39^{+4} (35^{+4}-41^{+2})	40^{+0} (37^{+6}-41^{+3})	39^{+2} (37^{+1}-41^{+3})	38^{+6} (34^{+2}-41^{+1})	.071
Complications	11 (14.3%)	0	3 (15.8)	2 (10.0)	4 (21.1)	2 (10.5)	.772
Maternal	5 (6.5%)		0 (0.0)	2 (10.0)	2 (10.5)	1 (5.3%)	.747
Hypertensive complication	3 (3.9%)		0 (0.0)	1 (5.0)	1 (5.3)	1 (5.3)	1.00
Gestational diabetes	2 (2.6%)		0 (0.0)	1 (5.0)	1 (5.3)	0 (0.0)	1.00
Fetal	6 (7.8%)		3 (15.8)	0 (0.0)	2 (10.5)	1 (5.3)	.232
Low birth weight (less than 2500g)	4 (5.2%)		2 (10.5)	0 (0.0)	1 (5.3)	1 (5.3)	.458
Premature delivery (before 37wk)	4 (5.2%)		2 (10.5)	0 (0.0)	0 (0.0)	2 (10.5)	.250
SGA**	4 (5.2%)		2 (10.5)	0 (0.0)	2 (10.5)	0 (0.0)	.250

Data are presented as median (range) or n (%) unless otherwise specified. SD, standard deviation; BMI, body-mass index; RBC, red blood cell; SGA, small for gestational age.
* Defined as gestational age based on a menstrual cycle of 28±3 days or conception date. ** Defined as weight under the tenth percentile for gestational age, sex and parity according to Dutch reference charts.[44] *** Adjusted for gestational age at delivery.

characteristics were not significantly different across RBC folate quartiles with the exception of periconception smoking, which was more common in lower quartiles (P = 0.24; **Table 2**).

Maternal RBC folate levels were significantly correlated to body mass index (r = -0.26, P = 0.02), and periconception smoking (smokers: mean 1257 (sd 239) nmol/L; non-smokers mean 1627 (sd 475) nmol/L; P < 0.01).

Testing for time interaction showed no significant interaction of RBC folate with gestational age (P = 0.94) and we therefore assumed a linear relation between RBC folate and embryonic size for the remainder of the analyses. The effect estimates from the linear mixed model analyses are displayed in **Table 3**. Univariate linear mixed model analysis showed that RBC folate in the third quartile (1513-1812 nmol/L) was associated with significantly increased embryonic growth compared to all other quartiles ($P_{overall}$ = 0.02), including the highest quartile (1813-2969 nmol/L; PQ3-Q4 < 0.01). The estimates for the first two and highest RBC folate quartiles did not significantly differ from each other (P-values not shown). Results from the fully adjusted model and the final model derived after backward elimination showed effects of comparable size and significance (**Table 3**). In **Figure 1** regression lines for the two upper RBC folate quartiles derived from the final model are displayed using square root transformed CRL and after retransformation to the original scale. Compared to the third quartile, RBC folate in the highest quartile was associated with a 1.1 mm (23.5%) and 4.5 mm (7.4%) smaller embryo at 6^{+0} and 12^{+0} weeks gestation, respectively. Estimated differences between RBC folate in the two lowest quartiles and the third quartile were comparable, with embryos that were 1.1 mm (19.4%) and 1.0 mm (17.6%) smaller at 6^{+0} weeks and 3.6 mm (6.0%) and 3.2 mm (5.4%) smaller at 12^{+0} weeks gestation, respectively.

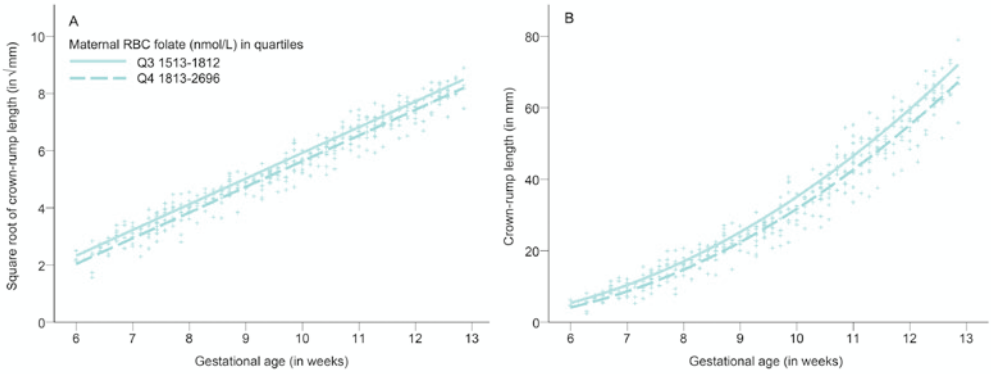


Figure 1 Regression lines for crown-rump length (CRL) growth conditional on red blood cell (RBC) folate quartiles derived from the final model adjusted for fetal sex, displayed using square root CRL data (A) and after retransformation to the original CRL scale (B). Regression lines are shown for the upper two quartiles only, as the regression lines for both lower quartiles are very close to the upper quartile.

Table 3 Effect estimates of maternal red blood cell (RBC) folate levels in quartiles for crown-rump length (CRL) from the univariate, fully adjusted and final models, using square-root transformed CRL.

Model	$n_{subjects}$	$n_{observations}$	Effect estimate (95% CI), √mm	*P*	$P_{overall}$
Univariate*	77	440			
Q1			-0.26 (-0.45, -0.05)	0.01	0.02
Q2			-0.22 (-0.42, -0.03)	0.03	
Q3			0 [Reference]	-	
Q4			-0.30 (-0.50, -0.10)	< 0.01	
Fully adjusted**	76	437			
Q1			-0.24 (-0.46, -0.02)	0.03	0.03
Q2			-0.24 (-0.45, -0.03)	0.02	
Q3			0 [Reference]	-	
Q4			-0.30 (-0.50, -0.10)	< 0.01	
Final***	77	440			
Q1			-0.24 (-0.44, -0.04)	0.02	0.02
Q2			-0.21 (-0.41, -0.02)	0.03	
Q3			0 [Reference]	-	
Q4			-0.29 (-0.49, -0.09)	< 0.01	

$P_{overall}$: *P*-value for RBC folate in quartiles; CI: confidence interval. *Adjusted for gestational age. **Adjusted for gestational age, periconception smoking, body mass index, and fetal sex with and without interaction with gestational age. *** Derived from the fully adjusted model after stepwise elimination, adjusted for gestational age and fetal sex.

Table 4 Effect estimates of maternal red blood cell (RBC) folate levels in quartiles for crown-rump length from the univariate models for different subgroups of pregnancies, using square-root transformed CRL and adjusted for gestational age.

	$n_{subjects}$	$n_{observations}$	Effect estimate (95% CI), √mm	*P*	$P_{overall}$
IVF/ ICSI *or* spontaneous regular*	63	367			
Q1			-0.23 (-0.43, -0.03)	0.03	0.03
Q2			-0.22 (-0.41, -0.03)	0.03	
Q3			0 [Reference]	-	
Q4			-0.28 (-0.47, -0.08)	< 0.01	
IVF/ ICSI	27	163			
Q1			-0.18 (-0.35, -0.01)	0.04	0.21
Q2			-0.07 (-0.23, 0.09)	0.02	
Q3			0 [Reference]	-	
Q4			-0.13 (-0.29, 0.04)	0.14	
Spontaneous	50	277			
Q1			-0.29 (-0.57, -0.01)	0.04	0.04
Q2			-0.32 (-0.60, -0.03)	0.03	
Q3			0 [Reference]	-	
Q4			-0.39 (-0.68, -0.10)	< 0.01	
Spontaneous regular*	35	197			
Q1			-0.27 (-0.59, -0.05)	0.11	0.07
Q2			-0.35 (-0.66, -0.03)	0.03	
Q3			0 [Reference]	-	
Q4			-0.39 (-0.71, -0.06)	0.02	

$P_{overall}$, *P*-value for RBC folate in quartiles; CI, confidence interval. * strictly regular menstrual cycle of 28±3 days.

We repeated the analysis restricted to specific subgroups of pregnancies (**Table 4**). In the subgroups of pregnancies with a reliable gestational age (i.e. IVF/ ICSI pregnancies or spontaneous pregnancies with a regular menstrual cycle of 28±3 days), spontaneous pregnancies, and spontaneous pregnancies with a regular menstrual cycle of 28±3 days only, effect estimates and significance from the univariate model were similar to those observed in the total group, with the exception that in the latter group the overall *P*-value and the *P*-value of the first quartile were no longer significant ($P_{overall}$ = 0.07, P_{Q1} = 0.11). In the subgroup of IVF/ ICSI pregnancies effect estimates pointed in the same direction, but were smaller and did not reach significance except for the first quartile, presumably due to small numbers ($P_{overall}$ = 0.21, $P_{Q1/2/4\text{-}Q3}$ = 0.04/ 0.37/ 0.14).

DISCUSSION

Maternal first-trimester RBC folate appears to follow an optimum curve in which both lower (<50th percentile, 814-1513 nmol/L) and very high levels (>75th percentile, 1813-2936 nmol/L) are associated with reduced embryonic size.

In this first study on maternal RBC folate and embryonic growth trajectories, CRL measurements were of excellent quality as a result of the use of 3D ultrasound scans with a virtual reality environment [23]. High precision was achieved by using the means of in triplo performed measurements. Furthermore, the assessment of long-term RBC folate reflects the periconception maternal folate status.

We excluded pregnancies with major congenital malformations and pregnancies resulting in fetal or neonatal demise. Compared to the general population, our study population was well educated, more often conceived using IVF/ ICSI, and more often used FA supplements and was likely to be at a higher risk for pregnancy complications These factors may explain the overall high RBC folate levels and absence of folate deficiencies. Future research has to elucidate whether the observed association also applies to the general population and whether it is in addition associated with pregnancy outcome. We are aware that the inclusion of both spontaneously conceived pregnancies and IVF/ ICSI pregnancies may have decreased the precision of the determination of gestational age. We therefore excluded pregnancies with a difference of more than 6 days between pregnancy dating by using the LMP compared to CRL. Although we cannot exclude the possibility that folate status influences menstrual cycle regularity and endometrial receptivity important for implantation, the direction of misdating is likely to be random and randomly distributed across RBC folate quartiles. This is supported by the results from the subgroup analyses restricted to pregnancies with the most reliable gestational age. Our numbers were too small to observe significant results in IVF/ ICSI pregnancies only, although effect estimates point in the same direction.

Our results suggest a difference in embryonic growth, however, we were unable to demonstrate a significant interaction between RBC folate and gestational age on the square root scale. Results are therefore discussed as a difference in embryonic size rather than embryonic growth.

Our data support the results from animal studies in which high folate levels have been investigated. In rodents, a 20-fold enriched folate diet was associated with a smaller embryo and decreased newborn length and weight, but also with an increase in embryonic development at 10.5 days post coitum [124, 125]. However, in these studies synthetic FA and plasma folate were studied compared to long-term RBC folate in our study.

In previous human studies, associations between first-trimester RBC folate and prenatal growth have been assessed at birth only. Results show positive associations with birth weight and head circumference [8, 60, 84, 85]. This substantiates our positive association between embryonic size and RBC folate up to 1812 nmol/L as the previously reported levels of first-trimester RBC folate were below this level. Only Takimoto et al. reported a high first-trimester mean RBC folate of 1317 (SD 824) nmol/L, but they observed no associations with birth weight or head circumference [83], which may be due to a small sample size (n=51) and performance of continuous rather than stratified analysis for different cut-offs for RBC folate.

Mechanisms by which very high maternal periconception RBC folate could affect embryonic size remain to be elucidated. The periconception period is highly important with regard to cell multiplication, differentiation and epigenetic programming of the embryo and placenta by DNA methylation of genes implicated in growth and development [5]. One-carbon metabolism provides one-carbon groups for these processes, of which folate is an important substrate. High folate levels also require high levels of cofactors such as other B-vitamins, a shortage of which can also derange these biological processes implicated in embryonic growth. We previously showed that maternal periconception FA supplementation was associated with increased DNA methylation of the maternally imprinted embryonic growth gene insulin-like growth factor-2 (IGF2) in the very young child [104]. In addition, increased methylation of IGF2 was associated with a lower birth weight. This epigenetic effect of FA supplementation is in line with the results of another study showing that periconception exposure to famine was associated with decreased IGF2 methylation [126]. These findings are further supported by a study in mice demonstrating widespread hypomethylation and adverse effects on health in later life after preconception exposure to a folate deficient diet [127]. Thus, increased periconception RBC folate may lead to increased DNA methylation and consequently silencing of imprinted genes implicated in early prenatal growth with long-term health consequences.

Another potential mechanism stems from the observation that high folate levels can inhibit folate-dependent enzymes [128, 129]. In human intestinal and renal epithelial cells, long-

term excessive FA supplementation leads to a specific and significant down-regulation of folate uptake [130]. FA supplementation has been associated with elevated folate levels in amniotic fluid [131]. In late first-trimester the maternal-to-fetal exchange of nutrients begins and high folate levels reach the fetus and placenta. Umbilical folate concentrations exceed maternal concentrations [132], and although investigated at birth only, embryonic concentrations may well begin to rise towards the end of the first trimester, thus potentially leading to down-regulation of folate-dependent enzymes and uptake resulting in the inhibition of DNA synthesis and growth.

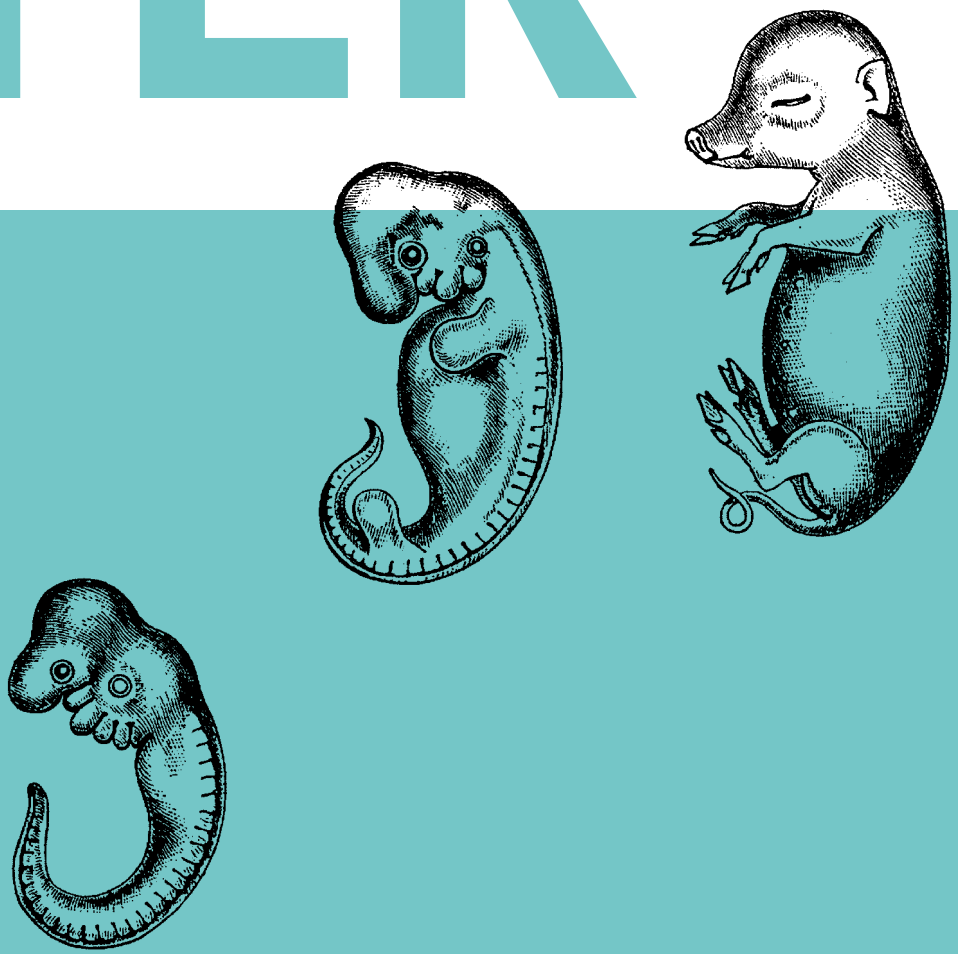
Finally, the high RBC folate levels in our study population are likely due to long-term FA supplement intake. FA in dosages above 200 μg cannot completely be reduced and bound to proteins in the plasma and thus enter the blood plasma in the unmetabolised form [133]. Although unmetabolised FA does not appear to accumulate in the fetus [134], the effects of repeated exposure over a substantial period of time remain unclear.

The mean absolute embryonic sizes in our study population was comparable to data by Pexsters et al. and Robinson et al., except for size at 6 weeks where our embryos appeared to be larger compared to data by Pexsters (mean 4.6 (95% reference interval (RI) 2.2, 7.9) vs 1.9 (95% RI 0.4, 4.5); supplementary **Table S1**) [28, 135]. This difference may be explained by differences in precision of pregnancy dating and measurements using 3D ultrasound and virtual reality.

Although we are often inclined to view embryonic size as the larger the better, the consequences of increased embryonic size are not yet clarified. Embryonic growth has been positively associated with birth weight [16, 36, 37, 41], and as birth weight is an important determinant for health in later life this indeed suggests a positive quality. However, long-term consequences of potential epigenetic modifications, and determination of the optimum of RBC folate regarding embryonic growth are issues still to be unravelled. Given the vast amount of influences on prenatal growth in the second and third trimesters, our study population is too small to assess the implications of the association between RBC folate and embryonic size for subsequent fetal growth and pregnancy outcome. Finally, because the implications of a smaller embryo are unclear and high dose FA supplementation is unequivocally effective in the prevention of NTD recurrences [52, 54, 136], our results have to be interpreted with caution.

Periconceptional maternal RBC folate levels above 906 nmol/L prevent NTDs in the offspring [137]. In our study 95% of all women demonstrated RBC folate levels exceeding this threshold. Beneficial and harmful effects of long-term use of high doses of FA supplements resulting in very high periconception RBC folate levels are not yet clarified [138], whereas with the increasing use of FA, multivitamins and fortified foods its safety is becoming increasingly important. Therefore, more research is needed on the optimal folate level regarding embryonic size and growth, including effects on subsequent fetal growth and pregnancy outcome with potential implications for current folate recommendations.

CHAPTER



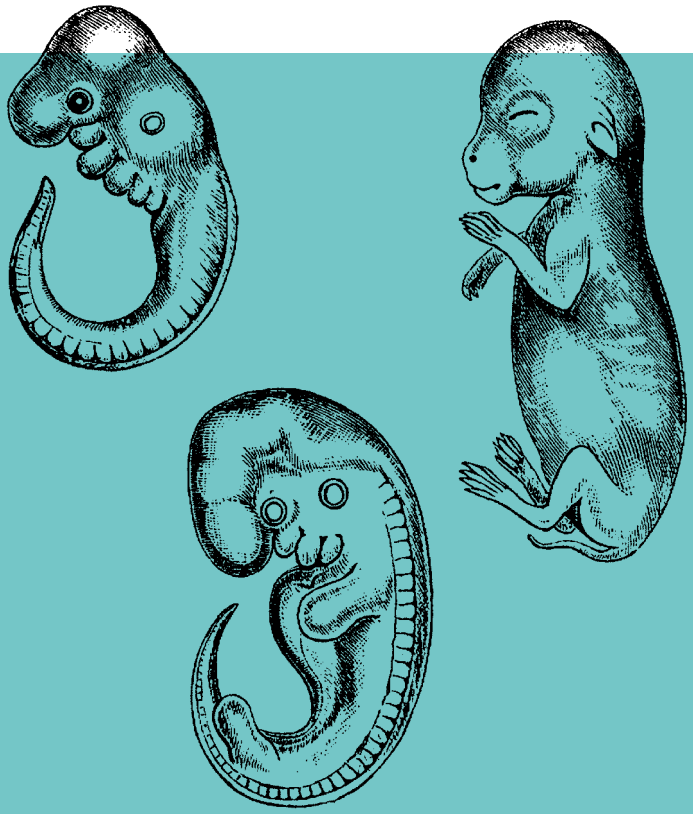
5

Periconception maternal characteristics and embryonic growth trajectories: the Rotterdam Predict study

Evelyne M van Uitert, Nikki van der Elst-Otte, Jet J Wilbers, Niek Exalto, Sten P Willemsen, Paul HC Eilers, Anton HJ Koning, Eric AP Steegers and Régine PM Steegers-Theunissen

Human Reprod, 2013. 28(12): p. 3188-96.

CHAPTER



6

Human embryonic growth trajectories: does the father matter? The Rotterdam Predict study

Evelyne M van Uitert, Eric AP Steegers, Gouke J Bonsel, Gerard JJM Borsboom, Anton HJ Koning, Joop SE Laven, Niek Exalto and Régine PM Steegers-Theunissen

Submitted

ABSTRACT

Background In this study we aimed to investigate associations between periconceptional paternal characteristics and lifestyle factors and first trimester human embryonic growth trajectories.

Methods In a prospective periconception cohort study in a tertiary centre we recruited pregnant couples before 8 weeks gestation. We performed weekly three-dimensional ultrasound scans from enrolment up to 13 weeks of gestation. Crown-rump length (CRL) measurements were performed using virtual reality. At enrolment a questionnaire was completed. For the current study we selected 81 spontaneously conceived pregnancies that ended in non-malformed live birth. Associations between paternal characteristics and embryonic growth were assessed using square root transformed CRL as response in linear mixed model analyses. Paternal characteristics considered were age, height, weight, body mass index, ethnicity, education, birth weight *Z*-score, smoking and alcohol use.

Results In the univariable analysis, paternal height was negatively associated with embryonic growth ($P = 0.02$). In the multivariable analyses this association attenuated and a new association between paternal birth weight *Z*-score and embryonic growth emerged ($P = 0.04$). After additional adjustment for maternal characteristics and fetal gender, a one-point increase in paternal birth weight *Z*-score was associated with a 0.0019 √mm/day (95%CI 0.0004, 0.0034) increase in CRL ($P = 0.01$). Retransformed to the original scale, the difference in embryonic CRL between a paternal birth weight *Z*-score of +2 compared to -2 was 0.5 mm (12.2%) and 6.5 mm (12.3%) at 6 and 12 weeks gestational age, respectively.

Conclusions Paternal birth weight is positively associated with embryonic growth in spontaneously conceived pregnancies. Future studies will have to confirm our results, elucidate underlying mechanisms and address the clinical implications for pregnancy dating based on CRL.

INTRODUCTION

Fetal and newborn size are important determinants of both newborn and adult health and disease [2, 4]. The periconception period is highly important with respect to cell multiplication, differentiation and epigenetic programming of the gametes, embryo and placenta by DNA methylation of genes implicated in growth and development [3, 5].

During the preconceptional period, the couple serves as the environment of the gametes providing the DNA of the future embryo and placenta. During pregnancy the woman is the main environment of the developing embryo and fetus, whereas paternal influence during pregnancy is restricted to indirect and passive exposures of the mother-to-be. While maternal characteristics in association with fetal growth and birth outcome have been studied extensively over the years, paternal characteristics have received considerably less attention. Studies that have addressed the father-to-be have focused mainly on semen quality and fertility outcome [12, 13].

Developments in ultrasound techniques and three-dimensional (3D) software including virtual reality, have provided the opportunity to study in detail human embryonic growth trajectories in vivo [21, 23]. Previous studies demonstrated associations of embryonic growth with subsequent fetal growth and pregnancy outcome [15-17, 41]. Moreover, maternal characteristics, such as age, smoking and alcohol use, have been shown to have small but significant effects on growth as early as in the embryonic period [16, 30, 159]. Up to date, paternal influences on human embryonic growth have not been studied.

Therefore, in this study we investigated associations between paternal characteristics including lifestyle factors and first trimester embryonic growth trajectories.

METHODS

Data for this study were collected in The Rotterdam Predict study, an ongoing prospective periconception cohort study conducted at the Department of Obstetrics and Gynaecology at the Erasmus MC, University Medical Centre Rotterdam, the Netherlands. This study has been approved by the Central Committee on Research in The Hague and the local Medical Ethical and Institutional Review Board of the Erasmus MC. At enrolment, all participants signed a written informed consent form.

The design of this study has previously been described elsewhere [41]. Briefly, all women and their partners of at least 18 years old with an ongoing intrauterine singleton pregnancy of 6 to 8 weeks gestation were eligible for participation and recruited in 2009 and 2010. The majority of couples were recruited from the outpatient clinic of the Department of Obstetrics and Gynaecology at the Erasmus MC.

Women received weekly transvaginal 3D ultrasound scans from enrolment up to the 13th week of pregnancy. Ultrasound scans were performed with a 6-12 MHz transvaginal probe using GE Voluson E8 equipment and 4D View software (General Electrics Medical Systems, Zipf, Austria). Afterwards the obtained 3D-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. This is a four-walled CAVE™-like (Cave Automatic Virtual Environment) virtual reality system, allowing depth perception and interaction with the projected images [25]. CRL measurements were performed offline using the I-Space and V-Scope software [21], and by placing the callipers at the outer side of crown and rump in the mid-sagittal plane. CRL measurements performed in the I-Space show good agreement with 2D measurements (intraclass correlation coefficient (ICC) 0.997 (95%CI 0.994 to 0.999)) and very good inter- and intraobserver agreement (both ICCs 1.000 (95%CI 0.999 to 1.000)) [23]. All CRL measurements were performed three times by the same researcher, and the mean of these three measurements was used in the analyses.

At enrolment both parents completed a self-administered general questionnaire covering details on age, anthropometrics, ethnicity, education and periconception lifestyle, and all women completed additional items on their obstetric history. Self-reported parental birthweight and gestational age at delivery were used to calculate a parental birthweight *Z*-score based on Dutch reference curves [39]. Paternal characteristics and lifestyle factors considered in the analysis were age, weight, height, BMI, ethnicity (Dutch, other western or non-western), education (low, medium or high), birthweight *Z*-score (continuous), and preconception smoking and alcohol use (yes or no). If birthweight was known but gestational age at birth was missing, the average gestational age of the group was substituted to calculate birthweight *Z*-score. If birthweight was missing but gestational age at birth was reported, birthweight *Z*-score was calculated as the average birthweight *Z*-score at that gestational age. Preconception smoking and alcohol use was defined as smoking any number of cigarettes or drinking any alcoholic beverages up to the moment of conception. Body mass index (BMI) was calculated as weight in kilograms divided by the square of height in metres.

Data on women's first day of the last menstrual period (LMP) and regularity and duration of the menstrual cycle were obtained in a personal interview by the researcher performing the ultrasound at the first visit. We calculated the gestational age from the LMP in spontaneously conceived pregnancies and from the LMP or insemination date plus 14 days in pregnancies conceived through intra-uterine insemination (IUI).

For the current study we selected only spontaneously conceived singleton pregnancies, including pregnancies conceived after intrauterine insemination (IUI). If the first day of the LMP was unknown or the observed CRL differed by more than six days from the expected CRL according to the Robinson curve [160], pregnancies were excluded from the analysis. Furthermore, we selected spontaneous pregnancies with a gestational age based

on a strictly regular menstrual cycle of 28±3 days only. Pregnancies conceived using donor semen were excluded. In addition, we selected only pregnancies that ended in a non-malformed live birth.

Data on pregnancy complications, the infant's date of birth, gender, birthweight and presence of congenital anomalies was obtained from medical records. Gestational age at delivery was calculated from the dating procedure used in the first trimester as described above. Embryonic growth data were studied using all available weekly CRL measurements from ultrasound images performed between 6^{+0} and 12^{+6} weeks.

To assess the association between paternal characteristics and embryonic growth trajectories we performed linear mixed model analysis, with stepwise regression to determine the role of paternal characteristics. Square root transformation of CRL data resulted in linearity of growth with gestational age, and a constant variance independent of gestational age and was therefore used in the analysis. As the coefficients resulting from this model are difficult to interpret directly due to the square root transformation, we present model results both in terms of √mm, and as backtransformed embryonic size or differences in millimetres at 6^{+0} and 12^{+0} weeks of gestation.

Firstly, we performed a univariable analysis for all characteristics with and without time interaction, in which we adjusted for gestational age only. In the second, multivariable model, we entered all characteristics for which a *P*-value <.20 was observed in the univariable analysis (forced forward).

Because maternal and paternal characteristics tend to be associated, we assessed whether the characteristics of interest were closely correlated or essentially redundant using PRINCALS analyses prior to further analysis. PRINCALS is a specific form of principal component analysis usually applied to nominal and ordinal data. Principal component analysis investigates whether a set of possibly correlated observations can be converted into a much smaller set of uncorrelated (constructed) variables, called principal components, where the first component is optimized to explain most of the variance. In the usual case such component can be interpreted as some underlying commonality, as in this case the shared characteristics of mother and father; additional components could in our case cover the maternal and paternal specificities. The PRINCALS procedure additionally allows for optimal rescaling of the original variables to achieve better construction and higher explanatory power of the components without loss of interpretation. PRINCALS results showed that maternal and paternal were not closely correlated, i.e. while some commonalities between individual maternal and paternal factors existed, there was no overall common factor.

In our previous study maternal age was positively and maternal periconception smoking and alcohol use were negatively associated with embryonic growth [159]. Therefore, in the next step we additionally adjusted the model obtained from the forward procedure for

these maternal covariates and in addition for fetal gender. As parity was not significantly associated with embryonic growth in our previous study, we did not adjust for parity in the current study [159].

Linear mixed model analyses were performed using PROC MIXED and PRINCALS analysis using PROC PRINQUALS in SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). All other analyses were performed using IBM SPSS Statistics Version 20.0 for Windows software (IBM, Armonk, NY, USA).

RESULTS

Of 259 pregnancies enrolled in the Rotterdam Predict study between 2009 and 2010, 184 fulfilled the criterion of being spontaneously conceived. Sixty-one pregnancies were excluded for using donor semen, unknown LMP, a CRL discrepancy of more than 6 days compared with the Robinson curve, resulting in a miscarriage or fetal/ neonatal demise or congenital anomalies, or a missing paternal questionnaire (**Figure 1**). Of the remaining 123 pregnancies, 81 fulfilled the criterion of being dated on a strictly regular menstrual cycle of 28±3 days (**Figure 1**).

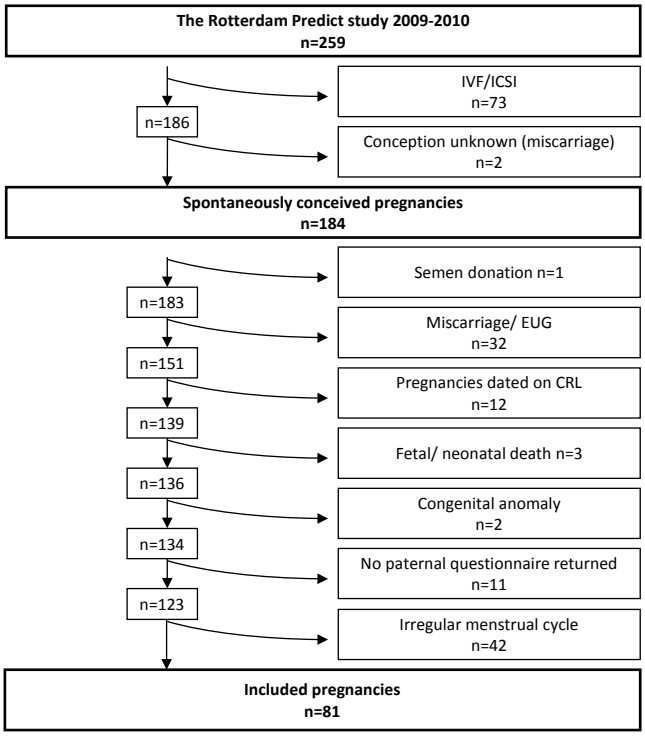


Figure 1 Flowchart of the study population.

The median gestational age at enrolment was 6^{+6} (range 6^{+0}-8^{+4}) weeks, and the median number of visits per pregnancy was 6 (range 4-8). From a total of 502 datasets, 463 (92.2%) were of sufficient quality to perform CRL measurements. We performed a median of 6 (range 3-8) CRL measurements per pregnancy.

Parental and pregnancy characteristics are shown in **Table 1**. Mean paternal age was 34.8 (standard deviation 5.5) years and men predominantly had a high level of education (55.6%) and were of Dutch descent (75.3%). Pregnancy complications occurred in 13 (16.0%) pregnancies. Paternal characteristics were not significantly different between the study population and the pregnancies that were excluded because of an irregular menstrual cycle or IVF/ ICSI treatment. However, mothers more often were non-Western (19.8% vs. 7.3%) and smoked (22.2% vs. 10.2%) and less often initiated folic acid supplement use preconceptionally (73.1% vs. 87.6%; supplementary **Table S1**). Maternal pregnancy complications were less frequent in the study population (3.7% vs. 13.3%). Other characteristics including birth weight and gestational age at delivery did not differ between included and excluded pregnancies.

In the univariable analysis, only paternal height was significantly associated with embryonic growth (P = 0.02, **Table 2**). Every ten centimetre increase in paternal height was associated with a decrease in embryonic growth of 0.14 √mm (95%CI -0.25, -0.02). Retransformation to the original scale showed that an embryo from a 1.90 cm tall father was 1.2 mm (23%) and 4.1 mm (7.0%) smaller compared to an embryo from a 1.70 cm tall father, at 6^{+0} and 12^{+0} weeks gestational age, respectively (**Table 4**).

Next, all variables with a P-value <.20 in the univariable analysis were entered simultaneously into the forward model. In this model, the significance of paternal height attenuated (P = 0.18) and a new positive association between paternal birthweight Z-score and embryonic growth emerged (P = 0.04; **Table 3**). A one point increase in paternal birthweight Z-score was associated with a 0.0016 √mm/day (95%CI 0.0001, 0.0032) increase in embryonic growth (**Table 3**). Retransformed to the original scale, the difference between a paternal birthweight Z-score of +2 compared to -2 was 0.5 mm (12.2%) and 5.9 mm (11.2%) at 6 and 12 weeks of gestational age, respectively (**Table 4**).

Adding maternal age, periconception smoking and alcohol use and fetal gender for the final model provided similar results, where paternal birthweight Z-score remained significantly associated with embryonic growth (P = 0.01; **Tables 3** and **4**). **Figure 2** displays the predicted average regression lines from the final model on the square root scale and after retransformation to the original CRL scale, showing the positive association between paternal birthweight Z-score and embryonic growth.

Table 1 General characteristics of the study population.

	Paternal n=81	missing	**Maternal** n=81	missing
At enrolment				
Age, y (mean±sd)	34.8±5.5	1	32.1±4.7	1
Height, cm (mean±sd)	183±6.4	0	170±6.5	1
Weight, kg	84 (60-125)	0	69 (54-133)	0
BMI, kg/m^2	25.0 (19.7-36.5)	0	23.8 (19.3-48.9)	1
Ethnicity		0		0
Dutch	61 (75.3)		60 (74.1)	
Western other	5 (6.2)		5 (6.2)	
Non Western	15 (18.5)		16 (19.8)	
Education		0		4
Low	11 (13.6)		7 (9.1)	
Intermediate	25 (30.9)		19 (22.5)	
High	45 (55.6)		51 (66.2)	
Preconception alcohol use	60 (74.1)	0	42 (51.9)	0
If yes, # per week	9 (0-32)	4	4 (2-24)	0
Preconception smoking	22 (27.2)	0	18 (22.2)	0
If yes, # per day	6 (0-20)	0	15 (1-20)	0
Periconception folic acid use	-		79 (97.5)	0
If yes, preconception initiation	-		57 (73.1)	1
Primiparous	-		46 (56.8)	0
Parental birth data				
Birth weight (mean±sd), g	3438±557	18	3231±507	7
Gestational age at delivery, wk+[d]	40^{+0} (33^{+0}-44^{+0})	25	40^{+0} (32^{+0}-43^{+4})	17
Birth weight *Z*-score (mean±sd)[†]	-0.22±0.87	17	-0.28±1.11	6
Pregnancy & outcome				
Birth weight (mean±sd), g	3307±594	0		
Gestational age at delivery, wk+[d]	39^{+2} (26^{+5}-42^{+0})	0		
Male infant	39 (48.1)	0		
Complications	13 (16.0)	0		
Maternal	3 (3.7)	0		
Hypertensive disorder	2 (2.5)	0		
Gestational diabetes	1 (1.2)	0		
Fetal	10 (12.3)	0		
Low birth weight (<2500g)	5 (6.2)	0		
Premature delivery (<37 weeks)	5 (6.2)	0		
SGA (<10th customized centile)[‡]	8 (10.0)	1		

Data are presented as median (range) or n (%) unless otherwise specified. sd, standard deviation; BMI, body mass index; SGA, small for gestational age. [†] Birth weight adjusted for gestational age, gender and parity, according to Dutch reference charts [39]. [‡] Defined as weight under the 10th centile for gestational age, gender and parity according to Dutch reference charts [39].

Table 2 Effect estimates from the univariable models for paternal characteristics and lifestyle factors with respect to embryonic crown-rump length (CRL).

Characteristic	Effect estimate (95%CI)	*P*
Linear	**√mm[a]**	
Age, per year increase	0.007 (-0.007, 0.021)	0.35
Height, per cm increase	-0.014 (-0.025, -0.002)	**0.02**
Weight, per kg increase	0.002 (-0.004, 0.009)	0.49
BMI, per point increase	0.021 (-0.001, 0.042)	0.06
Ethnicity		0.10
Dutch	0 [Reference]	
Other western	-0.22 (-0.54, 0.09)	
Non-western	0.14(-0.05, 0.34)	
Education		0.78
Low	0 [Reference]	
Intermediate	0.05 (-0.20, 0.30)	
High	-0.01 (-0.24, 0.23)	
Non-linear	**√mm/ day[a]**	
Birth weight *Z*-score, per one point increase	0.0014 (-0.0002, 0.0029)	0.08
Preconception alcohol use		0.17
No	0 [Reference]	
Yes	-0.0019 (-0.0046, 0.0008)	
Preconception smoking		0.17
No	0 [Reference]	
Yes	-0.0018 (-0.0044, 0.0008)	

CI confidence interval. [a] For continuous variables, effect estimates represent the amount of change in square root CRL (√mm) per unit increase of the variable. For categorical or dichotomous variables, effect estimates represent the difference in square root of CRL compared to the reference group. For birth weight *Z*-score, preconception alcohol use and smoking, effect estimates represent the amount of change in square root CRL per day increase in gestational age, per one point increase in birth weight *Z*-score or compared to the reference group.

Table 3 Effect estimates from the forward and fully adjusted models for paternal characteristics and lifestyle factors with respect to embryonic crown-rump length (CRL).

	Forward[a]		Fully adjusted[b]	
	Effect estimate (95%CI)	***P***	**Effect estimate (95%CI)**	***P***
Characteristic				
Linear	**√mm [c]**		**√mm [c]**	
Height, per cm increase	-0.011 (-0.027, 0.005)	0.18	-0.009 (-0.026, 0.007)	0.26
BMI, per point increase	0.011 (-0.019, 0.099)	0.46	0.013 (-0.189, 0.043)	0.38
Ethnicity		0.39		0.52
Dutch	0 [Reference]		0 [Reference]	
Other western	-0.25 (-0.63, 0.13)		-0.22 (-0.61, 0.17)	
Non-western	-0.15 (-0.63, 0.34)		-0.12 (-0.62, 0.39)	
Non-linear	**√mm/ day [c,d]**		**√mm/ day [c,d]**	
Birth weight *Z*-score, per point increase	0.0016 (0.0001, 0.0032)	**0.04**	0.0019 (0.0004, 0.0034)	**0.01**
Preconception alcohol use		0.12		0.19
No	0 [Reference]		0 [Reference]	
Yes	-0.0028 (-0.0064, 0.0007)		-0.0023 (-0.0057, 0.0012)	
Preconception smoking		0.28		0.51
No	0 [Reference]		0 [Reference]	
Yes	-0.0016 (-0.0044, 0.0013)		-0.0009 (-0.0037, 0.0019)	

CI confidence interval, BMI body-mass index. [a] Adjusted for paternal height, BMI, ethnicity, birth weight *Z*-score, preconception alcohol use and smoking, and gestational age. [b] Forward model additionally adjusted for maternal age, smoking and alcohol use and fetal gender. [c] For continuous variables, effect estimates represent the amount of change in square root CRL (√mm) per unit increase of the variable. For categorical or dichotomous variables, effect estimates represent the difference in square root of CRL compared to the reference group. [d] For birth weight *Z*-score, preconception alcohol use and smoking, effect estimates represent the amount of change in square root CRL per day increase in gestational age, per one point increase in birth weight *Z*-score or compared to the reference group.

Table 4 Estimated embryonic size in millimetres at 6 and 12 weeks of gestation from the univariable, forward and fully adjusted models for paternal height and birth weight *Z*-score.

		Model								
		Univariable[a]			**Forward**[b]			**Fully adjusted**[c]		
		Estimated size (95%CI), mm		*P*	Estimated size (95%CI), mm		*P*	Estimated size (95%CI), mm		*P*
Characteristic	**Categories**	6^{+0} weeks	12^{+0} weeks		6^{+0} weeks	12^{+0} weeks		6^{+0} weeks	12^{+0} weeks	
Height				**0.02**			0.18			0.26
	1.70m	5.2 (4.5, 6.1)	58.5 (55.8, 61.3)		4.9 (3.9, 6.0)	57.5 (54.0, 61.2)		4.9 (3.9, 6.0)	57.4 (53.8, 61.1)	
	1.90m	4.0 (3.6, 4.5)	54.4 (52.7, 56.1)		4.0 (3.4, 4.6)	54.2 (51.9, 56.6)		4.1 (3.5, 4.7)	54.6 (52.2, 57.1)	
Birth weight *Z*-score				0.08			**0.04**			**0.01**
	-2	4.0 (3.2, 4.8)	52.5 (49.4, 55.8)		4.1 (3.2, 5.0)	52.8 (49.4, 56.3)		4.1 (3.3, 5.1)	52.7 (49.2, 56.3)	
	0	4.6 (4.2, 5.0)	55.7 (54.2, 57.2)		4.3 (3.9, 4.8)	55.7 (53.9, 57.5)		4.4 (3.9, 4.8)	55.9 (54.1, 57.7)	
	+2	4.8 (3.8, 5.9)	58.9 (54.9, 63.1)		4.6 (3.5, 5.9)	58.7 (54.1, 63.4)		4.6 (3.5, 5.9)	59.2 (54.5, 64.0)	

CI confidence interval. [a] Adjusted for gestational age only. [b] Univariable model additionally adjusted for paternal height, ethnicity, birth weight *Z*-score, preconception alcohol use and smoking. [c] Forward model additionally adjusted for maternal age, smoking and alcohol use and fetal gender.

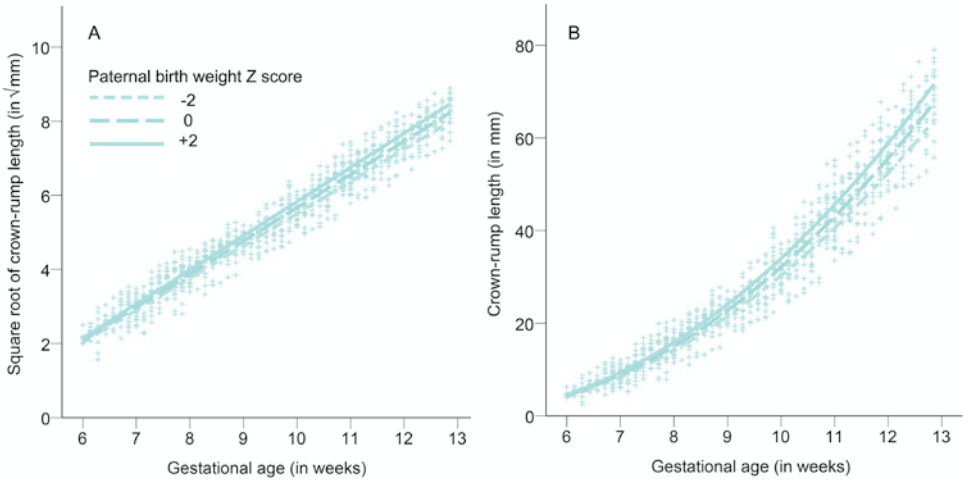


Figure 2 Regression lines for crown-rump length (CRL) conditional on paternal birth weight *Z*-score derived from the final model adjusted for gestational age, paternal height, body-mass index, preconception alcohol use and smoking and ethnicity, and maternal age, periconception smoking and alcohol use and fetal sex, displayed using square root CRL data (A) and after retransformation to the original CRL scale (B).

DISCUSSION

For the first time we demonstrate in a prospective periconceptional cohort study that paternal birthweight is positively associated with embryonic growth trajectories in the offspring independent of maternal characteristics.

One of the main strengths of this study is that we prospectively collected exposure and background data in early pregnancy, and measured with a high precision and reliability the CRL of the embryo in a true three-dimensional hologram from 6 up to 13 weeks gestation. In addition, we used advanced statistical procedures to extract the information of the (internally correlated) paternal and maternal characteristics, to subsequently test correlation between maternal and paternal characteristics, and to adjust for potential confounders.

An important issue is the dependency of embryonic growth on gestational age. We have limited the confounding influence of cycle irregularity by selecting only pregnancies with a known LMP and a strictly regular menstrual cycle. Furthermore, we are aware that anthropometric data and parental birthweight and gestational age at birth obtained from questionnaires are prone to recall bias. Adverse outcomes may be recollected more easy, however, as questionnaires were completed long before delivery recollection is not influenced by outcome, hence recall bias is not selective and will not affect results.

The generalizability of our data is restricted to other high risk populations with a high education and spontaneously conceived pregnancies only. Therefore, cohorts in the general population have to further confirm our findings.

The association between paternal birthweight and embryonic growth has not been studied previously, but is in line with the associations between paternal birth weight and offspring birthweight [161-164], risk of small for gestational age [165] and low birth weight [163]. We have previously shown that embryonic growth is positively associated with birthweight [41], however, in the current study we did not have enough power to show an effect of paternal birthweight on offspring birthweight, which we aim to further investigate in the future.

In other studies associations of paternal height, weight, BMI, education and alcohol use with offspring birthweight have been observed inconsistently [9, 166-168]. In our study the association with paternal height disappeared when the analysis was adjusted for other paternal characteristics and we observed no associations for paternal age, weight, BMI, alcohol use or smoking. With regard to paternal age effects may be U-shaped and therefore more pronounced at the extremes of paternal age [9], however, median paternal age in our study population was around 34 years and the numbers of an extreme low (<20 years) or high (>40 years) paternal age in our study were too small to assess these effects.

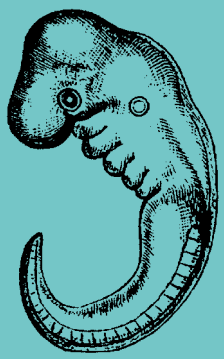
Paternal smoking is difficult to study as a separate risk factor as it may encompass both a direct influence on semen quality and an indirect influence by maternal and therefore embryonic exposure to passive smoking if father continues smoking throughout pregnancy, which is also suggested to have a significant negative effect on birthweight [169]. The absence of an association in our study may be due to the small number of smokers, or may suggest an effect that surfaces later in pregnancy. Effects of some of the studied paternal characteristics on semen quality may suggest that decreased semen quality decreases fertility but not necessarily have consequences for embryonic growth once a viable ongoing pregnancy has been achieved, however, more research on this topic is warranted.

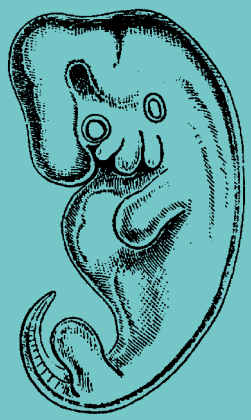
The mechanisms by which paternal birthweight influences embryonic growth remain to be elucidated. Whereas maternal influences continue throughout pregnancy, with the exception of shared lifestyle paternal influences are limited to influences on the semen in the preconception period. The offspring inherits a certain maximum growth potential through the parental chromosomes. As embryonic growth has been positively associated with birthweight [17, 37, 41], offspring from tall parents may exhibit their larger growth potential as early as in the embryonic period. However, if genetics would be the only explanation, we would also expect to have observed an association between paternal height and embryonic growth, which we did not observe. This may be due to a power problem, but could also suggest more complicated or multifactorial underlying mechanisms. The DOHaD paradigm states that intrauterine factors affect birth weight. Here we show a transgenerational effect on embryonic growth using birth weight of the father as proxy for

prenatal programming. We speculate that DNA methylation of embryonic and metabolic genes such as insulin-like growth factor 2 (IGF2) may be responsible for this finding. The IGF2 differentially methylated region (DMR) can be influenced by exposure to folic acid and the Dutch Hunger Winter and has been associated with birthweight and SGA [104, 126]. Soubry et al. observed in human that paternal obesity was associated with hypomethylation of the IGF2 DMR in the offspring [170], and in rodent studies paternal exposure to stress has been associated with changes in DNA methylation in the offspring [165]. These studies substantiate that paternal characteristics can be transmitted to the next generation via epigenetic mechanisms which could potentially alter the extent to which the maximum growth potential inherited through parental chromosomes can be reached. Considering the association between prenatal growth and health and disease in later life this suggests that paternal birthweight can be seen as proxy for prenatal programming, with consequences for the programming of embryonic growth in the next generation. Finally, we cannot exclude that the observed differences may stem not only from a difference in growth velocity but also from differences in the exact moment of conception and endometrium receptivity.

Although paternal birthweight was not significantly associated with education or unhealthy lifestyle behaviours such as smoking and alcohol use, residual confounding due to unmeasured exposures cannot be excluded. Results may to some extent reflect sociodemographic factors that have influenced paternal birthweight and now affect offspring growth as well. The association between paternal birthweight and embryonic growth further underlines the importance of preconception and early prenatal care as effects may not only affect outcome of the current pregnancy but may additionally be passed on transgenerationally. Because in current clinical practice we often date pregnancies based on CRL, differences in embryonic growth according to parental characteristics, albeit small, may suggest that the development of more customized embryonic growth curves could aid in increasing the accuracy in selection of pregnancies at risk for adverse pregnancy outcome.

CHAPTER





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The influence of IVF/ ICSI treatment on human embryonic growth trajectories: the Rotterdam Predict Study

Stefanie C Eindhoven, Evelyne M van Uitert, Joop SE Laven, Sten P Willemsen, Anton HJ Koning, Paul HC Eilers, Niek Exalto, Eric AP Steegers and Régine PM Steegers-Theunissen

Submitted

ABSTRACT

Background IVF/ ICSI treatment has been associated with increased risks of preterm birth, fetal growth restriction and low birth weight. Decreased first trimester crown-rump length (CRL) in the general population has been inversely associated with the same adverse pregnancy outcomes. In the current study we assess whether in vitro fertilization treatment with or without intracytoplasmatic sperm injection (IVF/ ICSI) is associated with first and second trimester embryonic and fetal growth trajectories and birth weight in singleton pregnancies.

Methods In a prospective periconception birth cohort study conducted in a tertiary centre, 146 singleton pregnancies with reliable pregnancy dating and nonmalformed liveborns were investigated, including 88 spontaneous and 58 IVF/ ICSI pregnancies. Serial 3D ultrasound scans were performed from 6 to 12 weeks of gestation. As estimates of embryonic growth, CRL and embryonic volume (EV) were measured using the I-Space virtual reality system. General characteristics were obtained from self-administered questionnaires at enrolment. Fetal growth parameters at 20 weeks and birth weight were obtained from medical records. To assess associations between IVF/ ICSI and embryonic growth trajectories, estimated fetal weight and birth weight, stepwise linear mixed model analyses and linear regression analyses were performed using square root transformed CRL and fourth-root transformed EV.

Results In 146 pregnancies, 934 ultrasound scans were performed of which 849 (90.9%) CRLs and 549 (58.8%) EVs could be measured. Embryonic growth trajectories were comparable between IVF/ ICSI pregnancies and spontaneously conceived pregnancies (CRL: $\beta_{IVF/\ ICSI} = 0.16$ √mm; $P = 0.13$; EV: $\beta_{IVF/\ ICSI} = 0.034$ √cm^3; $P = 0.13$). Estimated fetal weight and birth weight were also comparable between both groups ($\beta_{IVF/\ ICSI} = 6$ grams; $P = 0.36$ and $\beta_{IVF/\ ICSI} = 80$ grams; $P = 0.24$, respectively).

Conclusions The absence of a significant difference in embryonic and fetal growth trajectories suggests safety of IVF/ ICSI treatment with regard to early embryonic growth. However, further research is warranted to ascertain the influence of IVF/ ICSI treatments in a larger study population, and to estimate the impact of the underlying causes of the subfertility and other periconceptional exposures on human embryonic and fetal growth trajectories.

INTRODUCTION

In European countries between 0.5% to 4.9% of live births are conceived using assisted reproductive technologies (ART) and these figures are still rising (de Mouzon, Goossens [171]. Since the introduction of ART, potential side-effects for mother and child are still an issue of concern. Previous research has focused on the association between in vitro fertilization with and without intracytoplasmatic sperm injection (IVF/ ICSI) and fetal outcome in the second and third trimester of pregnancy, and at birth. Increased risks of preterm birth, having a child small for gestational age (SGA) and low birth weight have been reported in association with IVF/ ICSI treatment [7, 172, 173]. Recently, in a population based study an inverse association has been shown between crown-rump length (CRL) measurements at the end of the first trimester of pregnancy and the risk of preterm birth, SGA and low birth weight [16]. Furthermore, associations have been observed between late first trimester CRL and environmental factors, such as maternal age, ethnicity, adherence to an energy-rich dietary pattern, smoking and folic acid supplement use [16, 30]. We consider IVF/ ICSI to be another environmental factor which may influence embryonic growth trajectories. This is supported by studies showing that hormonal stimulation and in vitro culture of the embryo in different media can cause alterations in gene expression patterns in follicular granulosa cells, gametes and early embryos, which could affect subsequent fetal growth and development [174].

The introduction of transvaginal three-dimensional (3D) ultrasound sonography has provided new opportunities for the visualization and very precise measurements of the human embryo in vivo. The Barco I-Space in combination with V-scope visualization software enables actual view and measurement in three dimensions. The perception of depth in the I-Space enables precise and reliable measurements of embryonic biometry in the first trimester of pregnancy [22-24, 175]. The availability of 3D ultrasound and virtual reality has shifted research opportunities from the second and third trimester to the first trimester of pregnancy.

The aim of this study is to investigate the association of IVF/ ICSI treatment with first trimester embryonic growth trajectories, measured by CRL and embryonic volume (EV), and subsequent second trimester estimated fetal weight (EFW) and birth weight in non-malformed singleton livebirths.

METHODS

This study was embedded in the Rotterdam Predict Study, a prospective periconception hospital-based birth cohort study, conducted at the department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Centre in Rotterdam, the Netherlands [41]. Women of at least 18 years of age with vital singleton pregnancies

were enrolled before eight weeks of gestation. Written informed consent was obtained from all participants. Approval of the study was obtained from the Central Committee on Research in The Hague and the local Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Centre in Rotterdam in the Netherlands.

Study population

From January 2009 to November 2010, 259 singleton pregnancies were enrolled. For this study, we included only IVF or IVF/ ICSI conceived pregnancies and spontaneously conceived pregnancies that resulted in the live birth of a child without major congenital malformations. From this point on, we will refer to the group of IVF or IVF/ ICSI pregnancies as IVF/ ICSI. IVF/ ICSI pregnancies were included only when using own oocytes from the participating mother-to-be. Spontaneous pregnancies were included only when gestational age was based on a regular menstrual period of 28±3 days and a known first day of the last menstrual period. All spontaneously conceived pregnancies were dated using the first day of the last menstrual period. Pregnancies in which the observed CRL differed more than six days from the expected CRL according to the Robinson curve were excluded [28]. The gestational age of pregnancies conceived by IVF/ ICSI was calculated by adding 14 days to the number of days between the date of oocyte pick up and the study moment, or in case of a cryopreserved procedure by adding 17 or 18 days to the number of days between the date of embryo transfer and the study moment, depending on the number of days between oocyte pickup and freezing of the embryo.

Data on 146 pregnancies that resulted in a non-malformed live-birth were available for analysis after exclusion of pregnancies conceived after oocyte donation (n=2), ovulation induction treatment (n=9), dated based on an irregular menstrual cycle or missing first day of the last menstrual period (n=38), deviation of the CRL of more than 6 days from the Robinson curve (n=10), or that resulted in a miscarriage (n=42), ectopic pregnancy (n=1), termination of pregnancy due to aneuploidy (n=3), fetal and neonatal death (n=3), congenital anomaly (n=2) and absence of 3D ultrasound images to measure embryonic growth estimates (n=3).

Questionnaires

At enrolment all participants completed a self-administered questionnaire, which was verified by a researcher for completeness and consistency. Questionnaires contained items on age, height, weight, ethnicity, educational level, obstetrical and medical history, mode of conception and periconceptional use of (multi)vitamin and/or folic acid supplements, alcohol and cigarettes.

Ultrasound data

Weekly 3D US were performed from 6^{+0} to 12^{+6} weeks of gestation using the 6-12 MHz transvaginal probe of the GE Voluson E8 Expert System (GE, Zipf, Austria). The 3D data sets were converted by specialized 3D software named 4D View (4D View, version 9.1, GE Medical Systems) and saved as Cartesian (rectangular) volumes. A custom volume

rendering application called V-Scope was used to create an interactive hologram of the ultrasound image in the I-Space [22]. The I-Space is a four-walled CAVE^{TM}-like (Automatic Virtual Environment) virtual reality system at the department of Bioinformatics of the Erasmus MC, allowing depth perception and interaction with the hologram [23]. Images were selected based on the quality of the rendering of the embryo and both CRL and EV were measured in the same image. CRL measurements were performed using an integrated 'tracing tool' allowing 3D length measurement by placing two callipers in the mid-sagittal plane. CRL measurements performed in the I-Space show good agreement with 2D measurements and good inter- and intraobserver agreement [23]. CRL measurements were performed three times in the same scan and the mean value was used for the analysis. EV measurements were performed using a semi-automated segmentation algorithm, using grey values and surrounding variation threshold [175]. We have previously shown that EV measurements can be performed with a good intra- and interobserver agreement [22, 175]. Initially, EV measurements were performed three times and the mean value was used for analysis up to the point where an intraobserver and interobserver agreement of at least 0.90 was achieved, after which EV was measured only once.

Follow-up

Data on the routine second trimester ultrasound examination performed at around 20 weeks of gestation and data on pregnancy complications and birth outcome were obtained from medical records of the hospital registries and community midwives.

Estimated fetal weight was calculated using the Hadlock 2 formula: Log_{10} EFW = 1.326- (0.00326 x AC x FL) + (0.0107 x HC) + (0.0438 x AC) + (0.158 x FL) (FL = femur length, AC = abdominal circumference, HC = head circumference) [29].

Statistical Analysis

To assess the association between IVF/ ICSI treatment and embryonic growth we performed linear mixed model analyses. Models were estimated using mode of conception and gestational age as predictors for CRL and EV. A square root transformation of the CRL and fourth root transformation of the EV was performed to achieve linearity with gestational age and a constant variance independent of gestational age. To model the within subject correlation we used random intercepts and gestational age. First, we performed a univariable analysis in which we adjusted for gestational age only, and tested for time-interaction of conception mode. In the multivariable analyses, linear mixed models were adjusted for potential confounders. Potential confounders were identified by calculating Pearson and Spearman correlation coefficients for conception mode and the maternal characteristics listed in **Table 1**. Linear mixed model analysis with backward stepwise elimination was conducted to estimate the relation between mode of conception and embryonic growth estimates in the multivariable models. Backward selection with $\alpha = 0.2$ was used to determine the final model.

The associations of IVF/ ICSI treatment with estimated fetal weight at 20 weeks of gestation and birth weight were analysed using linear regression. Gestational age was taken into account in all models. Based on the literature multivariable models were adjusted for the following potential confounders; fetal gender, maternal age, ethnicity and parity. Backward selection with $\alpha = 0.2$ was performed to determine the final model.

In all analyses, *P*-values < 0.05 were considered significant. All analyses were performed using SPSS software version 17.0 (SPSS for Windows, SPSS Inc., Chicago, Illinois, USA) and SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

A total of 146 women were included for the analysis. **Table 1** shows the distributions of the maternal characteristics in IVF/ ICSI and spontaneously conceived pregnancies. In the IVF/ ICSI group more women were primigravida ($P < 0.001$), primiparous ($P = 0.002$) and of Dutch ethnicity ($P = 0.02$). All women in the IVF/ ICSI group (100%) initiated folic acid use in the preconception period compared to 61 (71.8%) women with a spontaneously conceived pregnancy ($P < 0.001$). Furthermore, in the IVF/ ICSI group fewer women reported smoking (6.9% compared to 23.9% ($P < 0.01$)) and alcohol use (22.4% compared to 53.4% ($P < 0.001$)) in the periconception period. Comparisons between the general characteristics of the included women with a spontaneously conceived pregnancy and reliable pregnancy dating and the excluded women with a spontaneously conceived pregnancy with a less reliable pregnancy dating revealed no significant differences (**Table 1**).

Conception through IVF/ ICSI was significantly negatively correlated to education ($r_s = -0.178$; $P = 0.034$), being primigravida ($r_s = -0.326$; $P < 0.001$), preconceptional initiation of folic acid use ($r_s = -0.369$; $P < 0.001$), smoking ($r_s = -0.220$; $P = 0.008$) and alcohol use ($r_s = -0.308$; $P < 0.001$).

The median gestational age at the first ultrasound was 6^{+5} (range 6^{+0}-9^{+1}) weeks and the median number of visits was 7 (range 4-7). A total of 934 3D ultrasound scans were performed of which 849 (90.9%) CRLs and 549 (58.8%) EVs could be measured. We performed a median of 6 (range 1-8) CRL and 4 (range 0-7) EV measurements per pregnancy. In three women no EV measurements could be performed.

Testing for time interaction showed no significant interaction of conception mode with gestational age for CRL and EV (all *P*-values > 0.05), and we therefore assumed a linear relation between conception mode and embryonic growth for the remainder of the analyses. **Table 2** depicts the results of the linear mixed model analysis. In the univariable analysis, we observed no significant difference in growth between the two groups for CRL ($P = 0.08$) or EV ($P = 0.16$; **Table 2**). After adjustment for potential confounders results

Table 1 General characteristics of IVF/ ICSI pregnancies and spontaneously conceived pregnancies with reliable pregnancy dating included for the analysis, and the excluded spontaneously conceived pregnancies with a less reliable pregnancy dating.

Characteristics	Included			Excluded	
	IVF/ICSI pregnancies n=58 (39.7%)	Spontaneous pregnancies n=88 (60.3%)	*P* [a]	Spontaneous pregnancies N=36	*P* [b]
Maternal (at enrolment)					
Age, years	33.1 ± 4.2	32.3 ± 4.8	0.32	31.1 ± 4.9	0.10
BMI, kg/m^2 (median (range))	23.9 (18.6-33.0)	23.8 (19.3-48.9)	0.99	23.2 (19.6-35.0)	0.48
Primiparous	47 (81.0)	49 (55.7)	<0.01	19 (52.8)	0.84
Ethnicity			0.02		0.08
Dutch	48 (82.8)	62 (70.5)		28 (80.0)	
Western other	6 (10.3)	5 (5.7)		5 (14.3)	
Non-Western	4 (6.9)	21 (23.9)		2 (5.7)	
Education			0.06		0.57
Low	6 (10.3)	7 (8.4)		3 (9.1)	
Intermediate	24 (41.4)	20 (24.1)		10 (30.3)	
High	28 (48.3)	56 (67.5)		20 (60.6)	
Folic-acid containing supplement					
No	0 (-)	2 (2.3)		0 (-)	
Yes	58 (100.0)	86 (97.7)		36 (100.0)	
Preconception initiation	57 (100.0)	61 (71.8)	<0.001	25 (69.4)	0.83
Postconception initiation	0 (-)	24 (28.2)		11 (30.6)	
Periconception alcohol use	13 (22.4)	47 (53.4)	<0.001	19 (52.8)	1.00
Periconception smoking	4 (6.9)	21 (23.9)	<0.01	6 (16.7)	0.48
Fetal					
Birth weight, grams	3372 (505)	3322 (590)	0.58	3224 (571)	0.47
Male	23 (39.7)	43 (48.9)	0.31	21 (60.0)	0.32

Data are presented as number (%) or mean±standard deviation unless otherwise specified. IVF/ICSI, in vitro fertilization with or without intracytoplasmatic sperm injection.

[a] IVF/ICSI pregnancies vs. spontaneously conceived pregnancies. [b] Spontaneous pregnancies vs. excluded spontaneous pregnancies.

Table 2 Effect estimates for CRL and EV derived from linear mixed models for IVF/ ICSI pregnancies compared to spontaneously conceived pregnancies.

	CRL (√mm)		**EV (^{4}√cm^3)**	
Model	β IVF/ICSI (se)	*P*	β IVF/ICSI (se)	*P*
Unadjusted [a]	0.09 (0.05)	0.08	0.02 (0.02)	0.16
Fully adjusted [b]	0.17 (0.08)	0.08	0.05 (0.03)	0.18
Final [c]	0.16 (0.08)	0.13	0.03 (0.02)	0.13

IVF/ICSI, in vitro fertilization with or without intracytoplasmatic sperm injection; se, standard error; CRL, crown-rump length; EV, embryonic volume

[a] Adjusted for gestational age. [b] Adjusted for gestational age, fetal gender, moment of initiation folic acid use, gravidity, education and periconception smoking and alcohol use. [c] Adjusted after backward elimination; CRL model adjusted for fetal gender, gravidity, moment of initiation folic acid use and periconception smoking and alcohol use; EV model adjusted for moment of initiation of folic acid use and periconception smoking and alcohol use.

Table 3 Effect estimates of IVF/ ICSI treatment for estimated fetal weight and birth weight derived from linear regression models.

	Estimated fetal weight (in grams)		**Birth weight (in grams)**	
Model	β IVF/ICSI (se)	*P*	β IVF/ICSI (se)	*P*
Unadjusted [a]	10 (6.2)	0.13	38 (67.0)	0.57
Fully adjusted [b]	5 (6.8)	0.47	72 (72.8)	0.33
Adjusted after backward selection [c]	6 (6.5)	0.36	80 (67.6)	0.24

IVF/ICSI, in vitro fertilization with or without intracytoplasmatic sperm injection; se, standard error

[a] Model of mode of conception and gestational age. [b] Adjusted for fetal gender, maternal age, parity and ethnicity. [c] Adjusted after backward elimination; Estimated fetal weight adjusted for maternal age and parity; Birth weight adjusted for parity.

from the fully adjusted model and the model after backward selection remained non-significant for CRL (P = 0.08 and P = 0.13, respectively) and EV (P = 0.18 and P = 0.13 respectively; **Table 2**). In **Figure 1** the estimated regression lines for embryonic growth trajectories from the final multivariable linear mixed models are shown.

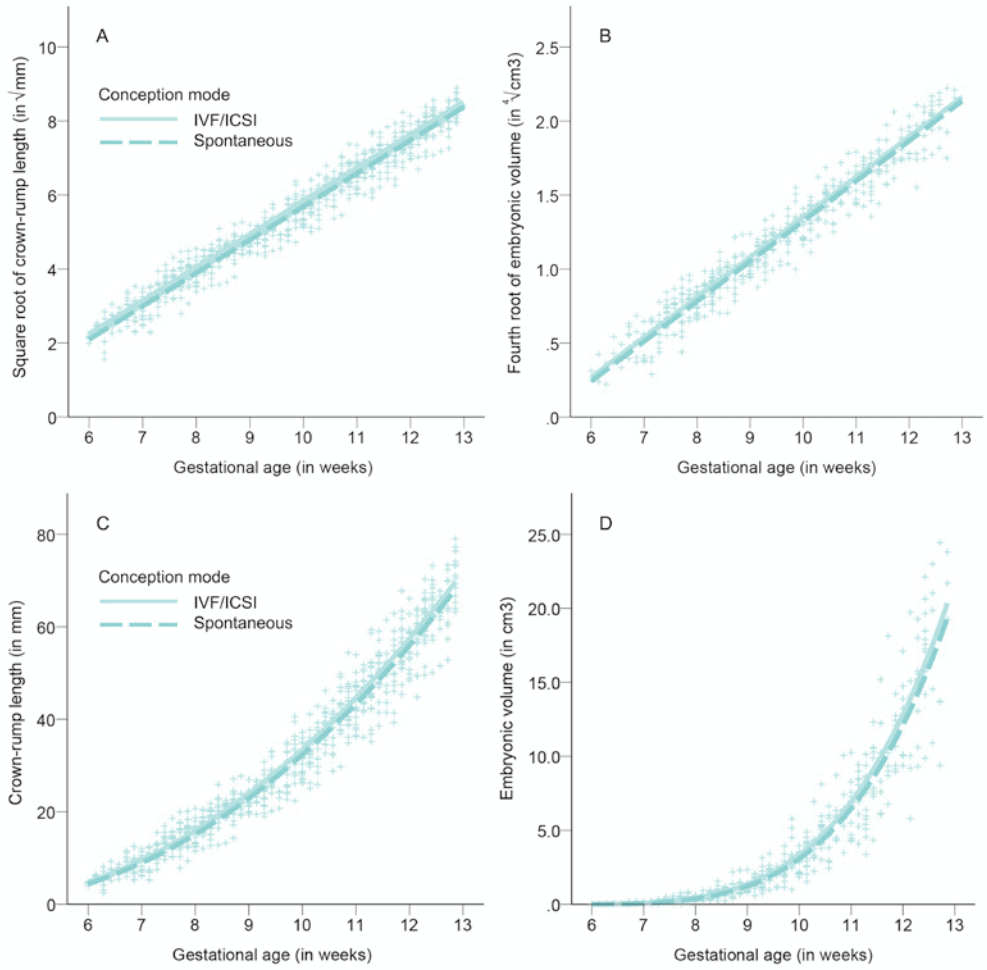

Figure 1 Estimated regression lines from the final multivariable model for in vitro fertilization/ intracytoplasmatic sperm injection (IVF/ ICSI) and spontaneously conceived pregnancies using square root transformed crown-rump length (A) and fourth root transformed embryonic volume (B) and after retransformation to the original scale (C, D).

Table 3 summarises the results of the linear regression analysis of mid-pregnancy estimated fetal weight and birth weight. The univariate regression analysis showed no significant association between mode of conception and estimated fetal weight ($\beta_{IVF/\ ICSI}$ = 10 grams,

P = 0.13) and birth weight ($\beta_{IVF/\ ICSI}$ = 38 grams, P = 0.57). After adjustment for potential confounders the associations with IVF/ ICSI remained non-significant (all P-values > 0.05; **Table 3**). Backward selection did not further alter the results (all P-values > 0.05; **Table 3**). **Figure 2** shows the distribution of mid-pregnancy estimated fetal weight and birth weight in IVF/ ICSI and spontaneously conceived pregnancies.

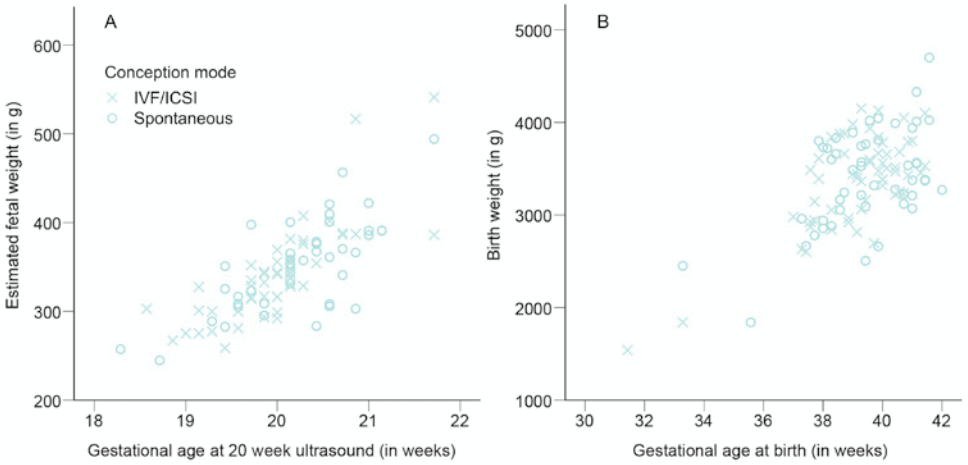

Figure 2 Scatter plot for mid-pregnancy estimated fetal weight (A) and birth weight (B) for in vitro fertilization/intracytoplasmatic sperm injection (IVF/ ICSI) and spontaneously conceived pregnancies.

DISCUSSION

This study demonstrates that first trimester embryonic growth trajectories, mid-pregnancy estimated fetal weight and birth weight are comparable between IVF/ ICSI and spontaneously conceived pregnancies with reliable pregnancy dating.

Our data are new and very precise, because 3D ultrasound measurements have been performed with reliable sophisticated techniques in a prospective study with serial ultrasound measurements from 6^{+0} up to 12^{+6} weeks gestational age. Moreover, follow-up data of the pregnancies was obtained from medical records. Questionnaire and clinical follow-up data enabled adjustment for potential confounders in the analysis.

The success rate of CRL and EV measurements in our study was 90.9% and 58.8%, respectively. The lower success rate of EV compared to CRL measurements can be explained by the increased demands on image quality in order to be able to perform the measurement correctly. For CRL measurements, visibility of crown and rump suffices

and limbs do not have to be fully displayed in the image. To allow EV measurements, the entire embryonic volume has to be captured on the 3D ultrasound image, including all limbs and image quality has to be adequate throughout the embryo. However, success rates of CRL and EV measurements were not different between IVF/ ICSI and spontaneous pregnancies, which renders selection bias unlikely.

In studies on embryonic and fetal growth, pregnancy dating is a recurring issue which has to be addressed carefully. In spontaneous pregnancies dating can be confounded by a delay in implantation, due to differences in endometrial receptivity and an uncertain first day of the last menstrual period due to recall bias. Moreover, there is considerable variation in the duration of the follicular phase between consecutive menstrual cycles [176]. To minimize variation in pregnancy dating we selected women with a regular menstrual interval of 28±3 days and a known first day of their last menstrual period only, thereby increasing the precision of pregnancy dating. The absence of differences between women who conceived spontaneously with and without a regular menstrual cycle of 28±3 days suggest that no selection bias was introduced by excluding these women. In IVF/ ICSI pregnancies on the other hand, the exact timing of fertilization is known, which makes the determination of the duration of pregnancy more accurate and reliable compared to spontaneously conceived pregnancies. However, recent literature suggests differences in endometrial receptivity may play a role in IVF/ ICSI pregnancies as well because of an altered endocrine environment. Ovarian stimulation for IVF/ ICSI alters the luteal phase endometrial development, shown in histological observations and expression of implantation window markers [177]. However, with peri-ovulatory endometrial maturation advancement exceeding three days, no clinical pregnancies were observed [178] suggesting that in ongoing pregnancies potential dating errors are likely to be small.

So far, few studies have focused on the influence of IVF/ ICSI treatment on embryonic and fetal growth. Cooper et al. compared IVF to spontaneously conceived pregnancies with regard to first trimester CRL, second trimester estimated fetal weight and birth weight. They observed no differences in the first and second trimester, but with regard to birth weight, children born after IVF treatment had a 100 g lower birth weight compared to spontaneously conceived pregnancies (3268 g compared to 3368 g, $P = 0.01$) [179]. This difference in birth weight is not in line with our results and may be explained by our strict selection of spontaneously conceived pregnancies with reliable pregnancy dating and inclusion of IVF/ ICSI pregnancies in addition to IVF pregnancies only, or our study sample may be too small to observe small differences. Conway et al. performed a late first trimester cross-sectional study comparing CRL between IVF/ ICSI and spontaneously conceived pregnancies between 9 and 12 weeks of gestational age. Their results also showed no significant differences [180]. Since 3D US emerged, the number of studies investigating first trimester embryonic growth using volume measurements has been rising. In the last few years in several cross-sectional studies EV has been studied and has been associated with CRL, gestational age and other (extra)embryonic structures [181-184]. Rolo et al. and Aviram et al. suggest that when it comes to early diagnosis of embryonic growth

disorders, EV may be superior to CRL because of the exponential correlation between EV and CRL [181, 185]. Therefore, the absence of a significant association between IVF/ ICSI treatment and embryonic growth as measured by CRL is further supported by the absence of a significant association with EV.

It would be of interest to assess the association between embryonic growth and pregnancy outcome, such as having a low birth weight or small for gestational age infant. However, the numbers in our study population were too small to assess those associations.

In conclusion, this is the first study with serial first-trimester measurements to show that IVF/ ICSI treatment appears not to be significantly associated with first trimester embryonic growth trajectories, estimated fetal weight and birth weight. These findings are reassuring, however, further research is warranted to confirm our results and ascertain differences between IVF and IVF/ ICSI treatments in a larger study population, and to estimate the impact of the underlying causes of the subfertility and other periconceptional exposures on human embryonic and fetal growth trajectories.

DISCUSSION

DISCUSSION

In this thesis we investigated longitudinal human embryonic growth in vivo using three-dimensional ultrasound and virtual reality techniques, and associations with parental and environmental influences. Below we discuss the implications of our findings.

HUMAN EMBRYONIC GROWTH

Up to date, clinical practice assumes a more or less uniform embryonic growth throughout the first trimester of pregnancy, as illustrated by the current practice of pregnancy dating using CRL. By studying longitudinal CRL, however, we observed differences in embryonic growth in both spontaneous as well as IVF/ ICSI conceived pregnancies (chapter 1). Differences in embryonic growth were subjectively visible in the longitudinal embryonic growth curves, as well as determined by correlations of the median *Z*-score for embryonic growth with both the calculated estimated fetal weight as well as the direct growth parameters head circumference, biparietal diameter, abdominal circumference and femur length. Because of the repeated measurements performed at different gestational ages in different pregnancies we used *Z*-scores for embryonic growth and fetal growth parameters. To minimize estimation issues we used median *Z*-scores per pregnancy. The origins of the observed differences remain to be elucidated but may include parental and environmental factors.

The use of 3D measurements in a virtual reality environment has enabled the *in vivo* measurement of CRL, EV, and embryonic curvature. The first two measurements have previously shown to be reliable and accurate [23, 175], and here we have shown that this is also true for the measurement of embryonic curvature (chapter 2). Moreover, our data suggest that curvature measurements do not differentiate between vital pregnancies and pregnancies resulting in a miscarriage. However, this could also be a result of the small sample size of the miscarriage cohort in this study.

PERICONCEPTION PARENTAL AND ENVIRONMENTAL FACTORS

In the first chapter of the second part we performed a narrative review of folate determinants and prenatal growth parameters, and observed that although literature suggests an association between folate and second trimester prenatal growth, little is known about the association of folate and embryonic growth (chapter 3). In the next chapters we studied the associations between several parental and environmental factors and embryonic growth and indeed observed differences accordingly (chapters 4, 5, 6). We demonstrated

there appears to be an optimal maternal RBC folate levels with regard to embryonic growth (chapter 4). In addition, maternal age was positively and smoking and alcohol use were negatively associated with embryonic growth in spontaneously conceived pregnancies (chapter 5). Moreover, not only maternal factors but also paternal birth weight was positively associated with embryonic growth, suggesting a more important role for paternal influences than previously perceived (chapter 6).

Finally, we showed that conception though IVF/ ICSI treatment was not associated with differences in embryonic growth (chapter 7). With the still increasing number of pregnancies arising from ART these results seem to be reassuring. However, conflicting literature on differences in birth weight suggests that such differences may arise after the first trimester of pregnancy. Hence, although ART treatment disturbs the periconception endocrine environment, we did not establish a significant association with embryonic growth, which could also be due to very small differences. However, this does not exclude (detrimental) effects of IVF or IVF/ ICSI treatment on postnatal physiology, as recently shown in children and adolescents [186, 187]. It would be interesting to further investigate whether potential differences in embryonic growth may be more pronounced after IVF/ ICSI compared to IVF treatment.

These results suggest that parental and environmental factors affect embryonic growth, the mechanisms of which remain to be elucidated but may include influences on epigenetic, genetic and/or biological mechanisms leading to a direct influence on embryonic growth rate or a more indirect effect through influences on placentation and sex-steroid hormones affecting the menstrual cycle and endometrium receptivity. The timing of the transition from a histiotrophic to a haemotrophic nutrient supply which coincides with a remarkable increase in embryonic growth rate (chapter 1) may play a significant role as well. If the onset of this transition can be influenced by these parental and environmental factors, this might also be an additional explanation for the observed differences in embryonic growth.

METHODOLOGICAL CONSIDERATIONS

The data used for this thesis were collected in the Rotterdam Predict study, a prospective periconception cohort study carried out in a tertiary hospital and therefore its external validity is expected to be limited. Inherent to the main enrolment from a tertiary hospital the proportion of high risk pregnancies and pregnancy complications in our cohort is higher than in the general population. In addition, the selection criterion of enrolment before 8 weeks of gestation automatically leads to a selection of women who know they are pregnant very early in pregnancy. Furthermore, women had to pay weekly visits to our hospital for the ultrasound examinations between 6 and 12 weeks of gestation, leading to an additional selection of women, as they had to have the time and means to do so. These

factors are likely to have contributed to the relatively high proportion of women with a higher education and pregnancies conceived after IVF/ ICSI treatment. Therefore, our results will have to be interpreted with caution and replicated in other cohorts, including pregnancies without a priori risk factors for adverse pregnancy outcome.

A major issue studying human pregnancies is the way of pregnancy dating, i.e. determining the precise duration of the index pregnancy. Pregnancy dating using the LMP is notoriously unreliable due to the anamnestic quality, differences in the duration of the follicular phase of the menstrual cycle as frequently encountered in fertile women and the duration of the diapause. We therefore excluded those pregnancies in which there was a large discrepancy between gestational age based on LMP and CRL.

The Rotterdam Predict study is an ongoing cohort study which we have recently extended to include collection of parental and umbilical cord blood samples and additional prospective data on the course of pregnancy, maternal health and lifestyle during pregnancy. In future studies we will therefore be able to further investigate influences of parental and environmental factors in specific periods before and during pregnancy. Furthermore, the extension of the study population will allow us to study not only periconception influences on embryonic growth but also on adverse outcome, such as preterm birth, fetal growth restriction and congenital anomalies.

IMPLICATIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

In this thesis we have shown that embryonic growth is not uniform but that there are differences in embryonic growth according to parental and environmental factors (chapters 1, 4, 5, 6). These findings raise question marks to our current day clinical practice of pregnancy dating according to first trimester CRL. Although individual differences according to maternal age or paternal birth weight may be small in the order of only one or a few days, future development of customized growth curves may increase the accuracy of pregnancy dating. This in turn could improve the differentiation between (un)necessary interventions where a very exact gestational age is mandatory, such as in the case of decision of treatment in the very preterm infant born at around 24 weeks, or the diagnosis of a fetus small or large for gestational age with all subsequent care.

Although we have shown embryonic growth to be associated with subsequent fetal growth and birth weight (chapter 1), future research should assess whether differences observed in embryonic growth according to parental and environmental factors translate to differences in fetal growth and birth weight. In addition, the (long-term) implications of being large or small in the embryonic period remain to be elucidated.

The observed associations between parental and environmental factors and embryonic growth may bear implications not only for the outcome of the index pregnancy, but also for future pregnancies in the offspring, as indicated by the association between paternal birth weight and offspring embryonic growth. The observed associations underpin the importance of preconception and early pregnancy care. In particular modifiable factors such as periconception folic acid supplement use, smoking and alcohol use provide ways in which we can improve the health of the early embryo with potentially consequences throughout life. However, other cohorts will have to confirm our findings.

Therefore, more research is warranted to unravel underlying mechanisms of the association between maternal, paternal and environmental factors and embryonic growth and to assess the implications for preconception and early pregnancy care, such as the development and implementation of effective lifestyle interventions. Furthermore, more research is needed on the optimal folate level regarding embryonic size and growth, including effects on subsequent fetal growth, pregnancy outcome and postnatal growth and health with potential implications for current folate recommendations.

Ultimately, if an optimal embryonic growth curve can be predicted, customized growth curves could aid in the development of a predictive tool, differentiating between normal and abnormal embryonic growth, further enhancing possibilities for early intervention and prevention strategies.

SUMMARY

SUMMARY

Prenatal growth in the second half of pregnancy and subsequent birth weight have been studied for decades and have been shown to be associated not only with pregnancy outcome but also with health and disease in adult life. Many parental and environmental factors during pregnancy have been shown to influence birth weight. Yet although the embryonic period is perhaps the most important period of prenatal development as this is the period in which organogenesis is completed, first trimester embryonic growth has received far less attention. In this thesis we studied human embryonic growth and associations with periconception parental and environmental exposures.

In the general introduction we provide a background for this thesis. The three-dimensional ultrasound and virtual reality software used in the studies in this thesis are introduced.

In Part One we have shown using longitudinal three-dimensional ultrasound measurements that first trimester embryonic growth trajectories and growth rates show individual variation in both spontaneously as well as in IVF/ ICSI conceived pregnancies (chapter 1). First trimester embryonic growth, particularly from 10 weeks gestation onwards, was associated with fetal growth parameters and birth weight. Furthermore, we showed that embryonic curvature can be measured reliably using three-dimensional ultrasound and virtual reality techniques and that embryonic curvature decreases towards the end of the first trimester (chapter 2). There appears to be no difference in curvature between ongoing pregnancies and pregnancies resulting in a miscarriage.

The non-uniformity of embryonic growth is further assessed in Part Two. Maternal factors are a likely contributor to the observed differences in embryonic growth. We performed a literature search to assess the association between maternal folate status and prenatal growth (chapter 3). Results suggest a positive influence on birth weight of maternal folate status, determined by long-term RBC folate and to a lesser extent by folic acid supplement use and maternal dietary folate intake. However, only a few studies have studied embryonic growth. In our own study population we studied first trimester maternal RBC folate levels with respect to embryonic growth (chapter 4). Maternal first-trimester RBC folate appeared to follow an optimum curve in which both lower and very high levels are associated with reduced embryonic size. In the next chapter we studied several other maternal characteristics with regard to embryonic growth. Maternal age was positively and smoking and alcohol use were negatively associated with embryonic growth in spontaneously conceived pregnancies (chapter 5). Moreover, not only maternal factors but also paternal birth weight was positively associated with embryonic growth (chapter 6). In the final chapter we showed that spontaneously conceived pregnancies and pregnancies conceived through IVF/ ICSI treatment showed comparable embryonic growth (chapter 7).

In the general discussion we address methodological considerations, limitations and implications of the findings reported in this thesis and we provide recommendations for future research.

SAMENVATTING

SAMENVATTING

Prenatale groei in de tweede helft van de zwangerschap en daaropvolgend geboortegewicht worden al jarenlang bestudeerd en blijken niet alleen geassocieerd met zwangerschapsuitkomst maar ook met gezondheid en het optreden van ziekten in het latere leven. Vele ouderlijke en omgevingsfactoren in de zwangerschap zijn van invloed op het geboortegewicht. Echter, hoewel de embryonale periode wellicht de meest belangrijke periode van prenatale groei is aangezien dit de periode is waarin de organogenese plaats vindt, is er veel minder aandacht geweest voor de embryonale groei in het eerste trimester van de zwangerschap. In dit proefschrift bestuderen we de humane embryonale groei en associaties met periconceptionele ouderlijke en omgevingsfactoren.

In de introductie wordt een achtergrond gegeven voor dit proefschrift. Driedimensionale echoscopische en virtual reality technieken die gebruikt zijn in de studies in dit proefschrift worden geïntroduceerd.

In het eerste gedeelte van dit proefschrift hebben we met behulp van driedimensionale echoscopie metingen laten zien dat eerste trimester embryonale groeitrajecten en groeisnelheden individuele variatie vertonen in zowel spontane als IVF/ ICSI zwangerschappen (hoofdstuk 1). Eerste trimester embryonale groei, in het bijzonder vanaf 10 weken, was geassocieerd met daaropvolgende groei van de foetus en het geboortegewicht. Verder lieten we zien dat embryonale kromming betrouwbaar gemeten kan worden met behulp van driedimensionale echoscopie en virtual reality technieken en dat de embryonale kromming afneemt naar het einde van het eerste trimester van de zwangerschap (hoofdstuk 2). Er lijkt geen verschil in kromming te bestaan tussen doorgaande zwangerschappen en zwangerschappen eindigend in een miskraam.

De niet-uniforme embryonale groei wordt verder onderzocht in het tweede gedeelte van dit proefschrift. Zeer waarschijnlijk dragen maternale factoren bij aan de geobserveerde verschillen in embryonale groei. We hebben literatuuronderzoek verricht naar de associatie tussen de maternale foliumzuurstatus en prenatale groei (hoofdstuk 3). Hieruit blijkt een positieve invloed op geboortegewicht van het foliumzuurgehalte in de rode bloedcel van de moeder, dat een indruk geeft van de lange termijn fliumzuurstatus, en in mindere mate door het gebruik van foliumzuur supplementen en het foliumzuurgehalte van het maternale dieet. Er was echter weinig literatuur beschikbaar over de associatie met embryonale groei. In onze eigen studiepopulatie hebben we eerste trimester maternale foliumzuurgehalte in de rode bloedcel onderzocht in relatie tot embryonale groei (hoofdstuk 4). Dit lijkt een optimum curve te volgen waarbij zowel een lage als een hoge spiegel geassocieerd is met een kleiner embryo. In het volgende hoofdstuk hebben we een aantal andere maternale karakteristieken bestudeerd in relatie tot embryonale groei. Maternale leeftijd was positief en periconceptioneel roken en alcoholgebruik negatief geassocieerd met embryonale groei in spontane zwangerschappen (hoofdstuk 5).

Bovendien bleek dat niet alleen maternale factoren maar ook het geboortegewicht van vader positief geassocieerd is met embryonale groei (hoofdstuk 6). In het laatste hoofdstuk hebben we laten zien dat embryonale groei in spontane zwangerschappen en zwangerschappen ontstaan na een IVF/ ICSI behandeling daarentegen vergelijkbaar was (hoofdstuk 7).

In de discussie gaan we in op de methodologische aspecten en limitaties van het onderzoek en de implicaties van de bevindingen en worden aanbevelingen gedaan voor toekomstig onderzoek.

ADDENDUM

SUPPLEMENTARY MATERIAL

Chapter 1 Human embryonic growth trajectories and associations with fetal growth and birth weight

Table S1 Pearson's correlation coefficients for the association between crown-rump length (CRL) and mid-pregnancy fetal growth and birth weight *Z*-scores for all subgroups, for overall first trimester CRL and stratified by early (up to and including 9 weeks gestation) and late (from 10 weeks gestation onwards) CRL.

	Total group			Reliable gestational age*			Conception mode						Uncomplicated pregnancies		
							Spontaneous			IVF/ ICSI					
	n	r	P	n	r	P	n	r	P	n	r	P	n	r	P
Overall CRL															
CRL*EFW	175	0.575	<.001	135	0.565	<.001	121	0.622	<.001	55	0.437	.001	140	0.604	<.001
CRL*HC	177	0.580	<.001	136	0.567	<.001	122	0.645	<.001	56	0.283	.035	141	0.571	<.001
CRL*BPD	173	0.407	<.001	133	0.392	<.001	120	0.482	<.001	54	0.138	.319	138	0.401	<.001
CRL*AC	177	0.507	<.001	136	0.497	<.001	122	0.558	<.001	56	0.402	.002	141	0.523	<.001
CRL*FL	175	0.418	<.001	135	0.405	<.001	121	0.460	<.001	55	0.257	.059	140	0.423	<.001
CRL*BW	184	0.158	.032	145	0.228	.006	127	0.119	.186	58	0.326	.012	156	0.173	.031
Early CRL															
CRL*EFW	172	0.576	<.001	132	0.573	<.001	121	0.629	<.001	52	0.361	.008	137	0.601	<.001
CRL*HC	174	0.587	<.001	133	0.584	<.001	122	0.645	<.001	53	0.318	.020	138	0.577	<.001
CRL*BPD	171	0.426	<.001	131	0.430	<.001	120	0.482	<.001	52	0.255	.068	136	0.419	<.001
CRL*AC	174	0.506	<.001	133	0.505	<.001	122	0.563	<.001	53	0.326	.017	138	0.520	<.001
CRL*FL	172	0.425	<.001	132	0.418	<.001	121	0.466	<.001	52	0.269	.054	137	0.424	<.001
CRL*BW	180	0.109	.146	141	0.179	.034	126	0.079	.380	55	0.231	.090	152	0.123	.130
Late CRL															
CRL*EFW	174	0.546	<.001	134	0.533	<.001	121	0.607	<.001	54	0.286	.036	139	0.584	<.001
CRL*HC	176	0.557	<.001	135	0.541	<.001	122	0.615	<.001	55	0.287	.033	140	0.562	<.001
CRL*BPD	172	0.395	<.001	132	0.389	<.001	120	0.454	<.001	53	0.191	.171	137	0.408	<.001
CRL*AC	176	0.479	<.001	135	0.467	<.001	122	0.550	<.001	55	0.214	.117	140	0.499	<.001
CRL*FL	174	0.399	<.001	134	0.386	<.001	121	0.440	<.001	54	0.237	.084	139	0.416	<.001
CRL*BW	183	0.178	.016	144	0.251	.002	127	0.148	.099	57	0.315	.017	155	0.200	.013

IVF/ICSI In vitro fertilization with or without intracytoplasmic sperm injection; r Pearson's correlation coefficient; EFW Estimated fetal weight; HC Head circumference; BPD Biparietal diameter; AC Abdominal circumference; FL Femur length; BW Birth weight.

* Gestational age based on a menstrual cycle of 28±3 days or conception date.

Appendix: *Z*-scores.

We used the GAMLSS method as implemented in R (version 2.15.1, GAMLSS package version 4.5.1) to estimate centile curves [46]. In this method we assume that the anthropometric measurements follow some parametric distribution of which the parameters depend on (gestational) age. This dependency is modelled by a spline. The distributions we tried came from the Box-Cox Cole and Green (BCCG) and Box-Cox power exponential (BCPE) families with splines of various degrees of freedom. We chose the best fitting models based on information criteria.

The best fitting model of CRL in early pregnancy was a BCPE model with 12 degrees of freedom for the median, 4 degrees of freedom for the coefficient of variation and constant parameters for skewness and kurtosis. For mid-pregnancy parameters we had fewer measurements taken over a more restricted interval of gestational age. Here it was sufficient to use a model in which the median was modelled as a linear function of gestational age while holding the other parameters constant. For estimated fetal weight and abdominal circumference the BCPE provided the better fit, while for the other parameters (biparietal diameter, head circumference and femur length) BCCG was selected. For birth weight we used a BCPE model with 2 degrees of freedom for the median. The other parameters of the distribution were again held constant.

Chapter 2 First trimester human embryonic curvature measurements using 3D ultrasound

Table S1 Reproducibility of crown-rump length (CRL) and total arc length (TAL) measurements.

	Mean difference (%)	SD	ICC
Intra observer variability			
CRL	0.34	1.47	0.999
TAL	2.66	3.11	0.998
Inter observer variability			
CRL	-0.24	2.23	0.999
TAL	-0.05	4.31	0.997

ICC, intraclass correlation coefficient; SD, standard deviation

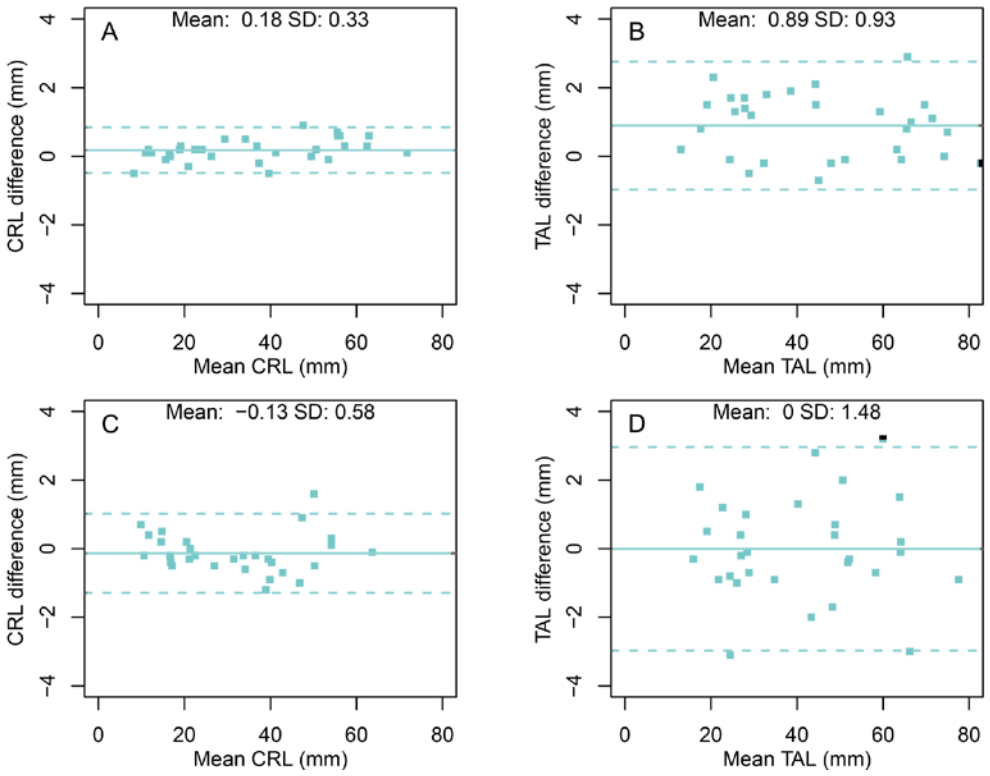

Figure S1 Bland Altman plots for crown-rump length (CRL) and total arc length (TAL) for intraobserver (A and B) and interobserver variability (C and D).

Table S1 Detailed description of study design and results of all included studies. Studies are arranged chronologically by determinant of maternal folate status.

Author Year	Study design: Country; Study population Confounder handling (if applicable)	Folate assay	Results[a)]
RBC folate			
Chanarin [86] 1968	United Kingdom; Cohort evaluation within randomized controlled study of 206 women	M	Mean 3rd trimester RBC folate in mothers with LBW and non-LBW infants were not significantly different (290 nmol/L vs. >340 nmol/L).
Hibbard [61] 1975	United Kingdom; Prospective cohort study of 723 women	n/a	Under 16 weeks gestation, RBC folate <130 µg/ml[b)] were found in 13% normal pregnancies compared with 49% SGA pregnancies (highly significant, p-values n/a).
Rolschau [62] 1979	Denmark; Cohort evaluation within randomized controlled study of multivitamin only or in combination with FA (5000µg) of 36 women with normal pregnancies	PB	RBC folate at birth was significantly correlated with BW when groups were pooled (r=0.53, p<0.001).
Ek [82] 1982	Norway; Cross-sectional study of 139 women with normal pregnancies; women who smoked >1 cigarette/ day and diabetics were excluded	M	RBC folate at birth was significantly correlated with BW (r=0.18, p=0.05). No differences were demonstrated in RBC folate in mothers who gave birth to <3,000g (n=16), 3,000-4,000g (n=80) and >4,000g (n=40) infants. RBC folate mean (SEM): 320.0 (26.8), 330.7 (16.7) and 354.1 (20.7), respectively.
Tamura [109] 1994	United States; Prospective cohort study of 76 women undergoing amniocentesis on indication, e.g., advanced age, or genetic consultation	M	2nd trimester RBC folate was not significantly correlated with BW (r and p values n/a).
Frelut [71] 1995	France; Case-control study of SGA (n=8) vs. AGA (n=13) mothers; women with HIV, low hemoglobin level, hemoglobinopathy, underlying disease which may affect pregnancy outcome, recent blood transfusion, use of antibiotics or antiepileptic drugs were excluded	M	RBC folate in 3rd trimester, but not at birth, was significantly correlated with BW (p<0.02, r n/a). Mean RBC folate in 3rd trimester and at birth was lower in mothers with SGA offspring, albeit not significantly (percentage of SGA compared to AGA mothers with RBC folate levels <200 ng/ml: 3rd trimester 33% vs 56%, at birth 33% vs 78%).
Rondo [76] 1995	Brazil; Case-control study of mother with SGA (n=356) and AGA (n=356) offspring	PB	Mean maternal RBC folate was similar between mothers with SGA and AGA offspring (mean (sd): 300 (130) vs. 310 (140) nmol/L (p=0.20)). Frequency of RBC folate levels below 230 nmol/L at birth was comparable, 36.8 vs. 32.1%, OR (95% CI): 1.17 (0.79-1.72), p=0.41.

Rondo [77] 2000	Brazil; Case-control study of mothers with term SGA (n=315) and AGA (n=321) offspring Adjustment in statistical analysis: Alcohol use (for OR only)	PB	Mean RBC folate was similar in mothers with SGA and AGA offspring, mean (sd): 298 (134) vs. 309 (138) nmol/L, respectively, p>0.10). RBC folate ≤226.5 was not associated with risk of SGA (aOR (95% CI) for SGA 1.08 (0.76-1.51, p >0.50).
Weerd [110] 2003	Netherlands; Prospective cohort study of 194 epileptic women, women who previously delivered a child with a neural tube defect, and controls Adjustment in statistical analysis: Biochemical determinants, age, pre-pregnancy weight, BMI, weight gain until 32 weeks amenorrhea, parity, obstetric and gynecologic history, epilepsy, medication, education, vitamin supplement use and smoking	PB	Regression analysis showed that preconceptional levels and 1st trimester changes in RBC folate were not associated with BW (β and p-values n/a).
Relton [8] 2005	United Kingdom; Prospective cohort study of 683 women with term singleton births without overt congenital anomalies Adjustment in statistical analysis: Neonatal RBC folate, maternal and neonatal vitamin B12, maternal age, parity, smoking and GA at birth	PB	Univariate and multivariate linear regression analysis showed late 1st trimester maternal RBC folate to be a significant predictor of BW (multivariate β BW z-score (95% CI): 0.25 (0.08, 0.42), p=0.005).
Relton [60] 2005	United Kingdom; Prospective cohort study of 998 women with term singleton births without overt congenital anomalies Adjustment in statistical analysis: GA at birth and infant gender (BW z-score)	PB	Late 1st trimester RBC folate in the lowest quintile was associated with a significantly lower BW, compared to the highest quintile (mean difference in BW z-score (95% CI): -0.31 (-0.55, -0.06), p=0.01).
Yajnik [112] 2005	India; Nested case-control study of mothers with SGA (n=30) and AGA (n=50) offspring with live singleton births	PB	2nd and 3rd trimester RBC folate was not significantly different between mothers with SGA and AGA offspring. geometric means (IQR): 912 (744-1,194) vs. 1,053 (784-1,625) nmol/L, p=0.14, and 840 (744-965) vs. 951 (693-1,359) nmol/L, p=0.18, respectively.
Martin [72] 2007	Sweden; Case-control study of healthy non-smoking mothers with SGA (n=30) and AGA (n=52) offspring who did not use medication or a special diet	PB	RBC folate at birth was lower in mothers with SGA than with AGA offspring, mean (SEM): 805 (63) vs. 1,109 (86) nmol/L, p=0.02.
Takimoto [83] 2007	Japan; Prospective cohort study of 94 women with no major pregnancy complications and term singleton births Adjustment in statistical analysis: Maternal age, BMI and parity	M	Multiple regression analyses showed that 1st trimester RBC folate was not related to BW or HC, 2nd trimester RBC folate was significantly related to HC (effect size 0.05mm, p=0.01), but not BW (effect size 0.38g, p=0.27), and 3rd trimester RBC folate was significantly related to BW (effect size 0.36g, p=0.04) but not HC (effect size -0.0003mm, p=0.90).
Baker [81] 2009	United States; Prospective cohort study of 263 adolescent women aged 14-18 years Adjustment in statistical analysis: Smoking, cotinine, SES, ethnicity, maternal age, underweight and obesity	PB	3rd trimester RBC folate was lower in mothers with SGA vs. AGA offspring (geometric mean (95% CI): 545 (477-621) vs. 670 (636-707) nmol/L), adjusted relative risk ratio 0.85 (95% CI 0.73-0.98, p=0.028.

Brough [84] 2010	United Kingdom; Cohort evaluation in randomized study of 353 women with singleton pregnancies and no chronic disease	PB	Late 1st trimester but not 2nd or 3rd trimester RBC folate was weakly associated with HC (r=0.11, p=0.046). In none of the trimesters RBC folate was associated with BW (r and p-values n/a).
Schlotz [85] 2010	United Kingdom; Retrospective cohort study of 100 women without perinatal mortality or major congenital anomalies Adjustment in statistical analysis: Smoking, alcohol use, total energy intake, GA at birth and infant gender	PB	Regression analyses showed a significant association of late 1st trimester RBC folate with HC at birth (β 1.7mm, p=0.031). In adjusted models, RBC folate explained 2.8% of the variance in HC (delta R^2=0.028), but was not associated with BW (p >0.15).
Parazzini [111] 2011	Italy; Prospective cohort study of 244 women	PB	Mean BW did not change significantly across 1st or 2nd trimester RBC folate tertiles (3,312/ 3,345/ 3,288 and wk16 3,318/ 3,365/ 3,284g; all p-values >0.05).
Serum or plasma folate			
Whiteside [66] 1968	Australia; Prospective cohort study of 60 women without low hemoglobin or evidence of any illness	M	1st, 2nd and 3rd trimester serum folate <11.3 vs. >11.3 nmol/L were not associated with BW (3,500 vs 3,500, 3,430 vs 3,710 and 3,370 vs 3,640g; all p-values>0.05).
Baker [114] 1977	United States; Case-control study of mothers with term LBW (n=50) and non-LBW (n=50) offspring, with no overt signs of malnutrition Matching: Matched for maternal age, parity, ethnicity and infant gender	M	No statistically significant differences in folate levels at birth (unclear whether serum or plasma folate was determined) between non-LBW and LBW mothers (mean (sd): 16.5 (6.48) vs. 12.7 (5.80) nmol/L).
Rolschau [62] 1979	Denmark; Cohort evaluation within randomized controlled study of multivitamin only or in combination with FA (5000µg) of 36 women with normal pregnancies	PB	A positive non-significant correlation between plasma folate at birth and BW was established when groups were pooled (r/p values n/a).
Ek [82] 1982	Norway; Cross-sectional study of 139 women with normal pregnancies; smokers who smoked >1 cigarette/ day and diabetics were excluded	M	Plasma folate at birth was non-significantly positively correlated with BW (r/p values n/a). Delivery of a ≤3,000g infant was associated with lower plasma folate at birth compared to 3,001-4,000g, but not >4,000g infants mean (SEM): 5.2 (0.64) vs. 7.6 (0.55) nmol/L; p<0.05.
Mukherjee [89] 1984	United States; Prospective cohort study of 264 women Adjustment in statistical analysis: Nutrients only (vitamin A, carotene, retinol binding protein, transferrin, ceruloplasmin, FA, albumin, total protein, zinc, copper, iron and total iron binding capacity)	PB	In polynomial stepwise regression analysis with nutrient indices, plasma folate was a predictor for BW (β -0.0197 pounds; se/p values n/a; r^2 0.099) but not for HC (β and p-values n/a). Plasma folate was measured in all three trimesters, it is however unclear which measurement was used for this analysis.

Tamura [79] 1992	United States; Nested case-control study within a prospective cohort, of 1) 80 women with SGA risk factors (SGA group), 2) 80 controls with a normal sized infant matched by race and gender to the SGA group, 3) 40 controls with a normal-sized infant matched to 40 women of the SGA group not only by race and infant gender, but also by smoking status at initial prenatal visit, BMI, and a history of a previous LBW infant, 4) 45 women with a normal-sized infant and no known risk factors of having SGA, and 5) 40 women with a large for GA infant Adjustment in statistical analysis: Maternal characteristics known to influence BW, not further described	M	No significant correlation between 2nd trimester plasma folate and BW. In multiple regression analyses, 3rd trimester plasma folate was significantly associated with BW (β and p values n/a). 2nd and 3rd trimester serum folate was lower in SGA mothers than those of each of the other groups (45 vs 50-61 and 42 vs 51-61 nmol/L, respectively), but differences were not consistently significant when multiple-comparison procedures were used. The percentage of SGA (shown in figure, percentages n/a) tended to decrease as serum folate quartiles increased, significant for 3rd but not 2nd trimester, p=0.014 and p=0.134, respectively.
Tamura [109] 1994	United States; Prospective cohort study of 76 women undergoing amniocentesis on indication, e.g. advanced age or genetic consultation	M	No significant correlation between 2nd trimester serum folate and birth weight (r and p-values n/a).
Frelut [71] 1995	France; Case-control study of mother with SGA (n=8) and AGA (n=13) offspring; women with HIV, low hemoglobin or hemoglobinopathy, underlying disease which may affect pregnancy outcome, recent blood transfusion, use of antibiotics or antiepileptic drugs were excluded	M	Plasma folate in the 3rd trimester and at birth was not significantly correlated with BW (r and p-values n/a). Mean plasma folate was lower in SGA mothers but the difference was not significant (levels and p-values n/a).
Scholl [64] 1996	United States; Prospective cohort study of 832 women without serious non-obstetric disease Adjustment in statistical analysis: Time of blood draw, gestation at entry, ethnicity, prior history of preterm delivery or LBW, maternal age and parity	PB	Multiple regression analysis showed that for each 1 nmol/L increase in early 3rd trimester serum folate the odds of LBW declined by 1.5% (aOR (95% CI): 0.985 (0.970, 0.999)).
Stefanidis [113] 1999	Greece; Cross-sectional study of 101 women without risk factors	PB	No significant correlation between serum folate at birth and BW (r value n/a, p>0.05).
Ronnenberg [90] 2002	China; Case-control study of 434 non-smoking women, including LBW (n=33) and/ or SGA (n=65) mothers and controls (n=390 and n=358, respectively); women with subfertility for ≥ 1 year, history of a clinically recognized spontaneous abortion were excluded. Adjustment in statistical analysis: Analytic batch, maternal age, BMI, hemoglobin concentration, vitamin B6, vitamin B12; for LBW also infant gender and GA at birth	PB	Mean preconception plasma folate and the proportions of women with folate levels under <6.8 nmol/L were not significantly different between LBW or SGA case and controls (mean plasma folate (95% CI): LBW: 9.3 (8.1-10.8) vs. 9.1 (8.7-9.4) nmol/L, 24.2% vs. 20.3 % deficient; SGA: 8.8 (7.9-9.7) vs. 9.1 (8.8-9.5) nmol/L, 27.7% vs. 19.3% deficient). No significant associations were observed between birth of LBW or SGA infants and deficiencies of folate in either unadjusted or adjusted logistic regression models (aOR (95% CI) for folate >6.8 nmol/L: LBW: 0.7 (0.3, 1.7); SGA 0.7 (0.4, 1.2)).

Weerd [110] 2003	Netherlands; Prospective cohort study of 194 epileptic women, women who previously delivered a child with a neural tube defect and controls Adjustment in statistical analysis: Biochemical determinants, maternal age, pre-pregnancy weight, BMI, weight gain until 32 weeks amenorrhea, parity, obstetric and gynaecologic history, epilepsy, medication, education, vitamin supplement use and smoking	PB	Regression analysis showed that preconceptional and 1st trimester serum folate were unrelated to BW (β and p-values n/a).
Sram [78] 2005	Czech Republic; Nested case-control study of Europeans and Gypsies from rural area of Teplice (n=444), and Europeans from Prague (n=322) Adjustment in statistical analysis: BMI, environmental tobacco smoke, smoking, parity, infant gender, GA at birth, and education; Stratified for ethnicity and smoking	PB	Plasma folate in the highest tertile (>28.8 nmol/L) at birth significantly decrease SGA risk in Europeans (GA>32 wks: OR 0.44, p=0.037; GA>36 wks OR 0.38, p=0.026). In all Europeans, independent of smoking, and in Gypsies, maternal folate was not associated with BW. However, in smoking mothers from Prague with GA >36 weeks this association was significant (estimate 167 g, p=0.0004).
Takimoto [83] 2007	Japan; Prospective cohort study of 94 women with no major pregnancy complications and term singleton births Adjustment in statistical analysis: Maternal age, BMI and parity	PB	Multiple regression analyses showed that 1st, 2nd and 3rd trimester serum folate was not related to BW or HC (effect sizes: BW -2.60, -3.51 and-0.12g; HC 0.08, -0.03 and 0.004mm; all p-values >0.05).
Baker [81] 2009	United States; Prospective cohort study of 288 adolescent women aged 14-18 years Adjustment in statistical analysis: Smoking, cotinine, SES , ethnicity, maternal age, underweight and obesity	PB	3rd trimester serum folate in SGA births was lower than in non-SGA births (geometric mean (95% CI): 10.9 (9.4, 12.5) vs. 13.1 (12.3, 14.1) nmol/L; adjusted ratio of geometric mean values by multiple regression analysis 0.84 (95% CI 0.71, 0.99), p=0.034).
Faintuch [88] 2009	Brazil; Retrospective cohort study of 13 women who had undergone Roux-and-Y gastric bypass surgery for morbid obesity and subsequently became pregnant within 5 years	n/a	2nd trimester serum folate was correlated with BW (r 0.781, p=0.001).
Dijk [87] 2010	Netherlands; Prospective cohort study of 4,044 women with singleton live births. Adjustment in statistical analysis: GA at birth, depressive symptoms, maternal age, pre-pregnancy BMI, education, primiparity, ethnicity, smoking, alcohol use, hypertension and onset of delivery	PB	2nd trimester serum folate in the lowest quintile (-5 to 13 nmol/L, negative due to standardization) were significantly associated with lower BW in the univariate but not in the adjusted model (adjusted β (95% CI): -3 g (-51, 44), p=0.89), compared to the highest quintile (38-115 nmol/L).
Hogeveen [68] 2010	Netherlands; Prospective cohort study of 366 women with singleton births Adjustment in statistical analysis: GA at birth, maternal age, parity, smoking, FA use, infant gender, homocysteine, cobalamin, methylmalonic acid and creatinine	M	3rd trimester serum folate was not correlated with BW (r 0.04, p>0.15), and not a predictor of BW (β BW standardized for GA (95% CI): 28 (-78, 133). 3rd trimester folate was not significantly associated with LBW. 3rd trimester plasma folate in lowest quartile (<6.11 nmol/L) vs. other quartiles was not associated with LBW (OR (95% CI): 0.93 (0.93, 1.96).

Nilsen [74] 2010	Norway; Prospective cohort study of 2,934 women with singleton births Adjustment in statistical analysis: Maternal age, marital status, education, parity, pre-pregnancy BMI, smoking and GA at blood collection	M	No significant linear trends in mean BW or HC over 2nd trimester plasma folate percentiles (<p25, p25-75, p75; BW: 3,618/ 3,643/ 3,577g; HC: 35.3/ 35.4/ 35.3mm; adjusted p-trend 0.54 and 0.53 respectively). The aOR for SGA increased with lower 2nd trimester plasma folate, but results were not significant due to wide CI (shown in figure, p_{trend}=0.49).
Parazzini [111] 2011	Italy; Prospective cohort study of 244 women	PB	Mean BW did not change significantly across 1st or 2nd trimester plasma folate tertiles (3,353/ 3,303/ 3,269 and 16wk 3,266/ 3,281/ 3,281g; all p-values >0.05).
Folic acid supplement use		**Dosage (µg/ day)**	
Baumslag [67] 1970	South-Africa; Randomized controlled trial of iron (200mg) only or in combination with FA (5000µg) in Bantu (n=128) and white (n=114) women Randomization: No data on differences between groups	5000	In Bantu women who received iron only from 28 weeks onwards, 19/63 (30.2%) had offspring with a BW of <2,270 g (5 pounds) compared to 4/65 (6.2%) in Bantu who received IFA (p=0.0005). No such difference was found in white women.
Giles [92] 1971	Australia; Randomized controlled trial of iron (200mg) with either FA (5000µg) or placebo in 620 women with certain last menstrual periods and a regular cycle ≤35 days Randomization/ Stratification/ Adjustment in statistical analysis: No differences between groups in age or ethnicity, but more primigravidae in FA group; Adjustment for GA at birth, maternal age; Stratified by parity and GA at entry	5000	There was no significant difference in the average BW between the FA and placebo groups initiated at any time in pregnancy (<10wks, 10-20, 20-30, >30 wks).
Iyengar [96] 1971	India; Randomized controlled trial of iron only (60mg) or in combination with different doses of FA (100, 200 or 300µg) in 95 women without low haemoglobin levels Randomization: No data on differences between groups	100/ 200/ 300	Among women who received supplementation from 20-24 weeks onwards, infants of women using iron and 200 or 300µg of FA were about 200g heavier than women using iron only or in combination with 100µg FA (mean BW (SEM): iron only 2,620 (98), +100µg FA 2,680 (92), +200µg FA 2,890 (75), +300µg FA 2,920 (97)g). This was significant in 300µg users only (p<0.05).
Hamilton [93] 1972	Uganda; Randomized controlled trial of iron only (300mg) or in combination with FA (5000µg) in 685 women Randomization: No differences between groups in maternal age, height, parity, abortions, living children, attendances, defaults or hospital admissions	5000	There was no significant difference in the average birth weights between the IFA and iron only groups, in the entire group (mean BW (sd): 3,021 (576) vs. 2,997 (634) g) and in a subgroup of women regularly attending clinic from <20 weeks, (mean BW (sd): 2,948 (556) vs. 2,767 (764) g). Moment of initiation of regimen n/a.

Fleming [115] 1974	Australia; Randomized controlled trial of FA (500µg) and placebo, or iron only (60mg) or combined with FA in 89 women Randomization: No differences between groups in GA at birth, ethnicity, previous abortion, maternal age/ height/ weight	500	Mean BW did not differ significantly between groups that used no supplements, iron only, FA only or IFA from 20 weeks onwards, mean BW 3,476g, 3,310g, 3,278g and 3,395g, respectively.
Iyengar [94] 1975	India; Randomized controlled trial of iron only (60mg) or in combination with FA (500µg) in 189 women with term births Randomization/ Matching/ Stratification: Matched for height and parity; No data on differences between groups; Stratified for height and parity	500	In mothers using supplements from 20-28 weeks onwards, mean BW of infants born to mothers in the iron only group was smaller than of those in the IFA group, mean BW (se): 2,613 (39) vs. 2,813 (39) g, p<0.001. When stratified for parity, significant in primiparas (n=54) only, mean BW (se): 2,441 (40) vs. 2,838 (80), p <0.001.
Rolschau [62] 1979	Denmark; Randomized controlled trial of multivitamins only or with FA (500µg) in 36 women with normal pregnancies Randomization/ Matching: Matched two and two according to parity, smoking, housing conditions, age, and then randomized	500	The average BW in the group of women using FA containing multivitamins from 21-25 weeks onwards was 407g or 12.7% higher than in the multivitamins only group, mean BW (sd): 3,610 (374) vs. 3,203 (444) g, p<0.01.
Tchernia [80] 1982	France; Randomized controlled trial of iron only (105mg) or with FA (350µg) in 1,982 women Randomization: Difference in GA at birth between groups, no adjustment for GA in statistical analysis	350	3rd trimester IFA use was associated with an increased BW compared to iron use only, mean BW (sd): 3,460 (430) vs. 3,303 (375) g, p<0.05.
Fleming [91] 1986	Nigeria; Randomized controlled trial of antimalaria medication (chloroquine and proguanil) and iron (60mg) only or with FA (1000µg) in 200 primigravidae Randomization: No differences between groups in GA at birth, maternal age, height or weight	1000	There was no significant difference in BW between groups using antimalaria medication only vs. antimalaria medication plus FA, or antimalaria medication plus iron vs. antimalaria medication plus IFA from mean 18 weeks onwards (BW and p-values n/a).
Shaw [116] 1997	United States; Cohort study of 734 women Adjustment in statistical analysis: Ethnicity, maternal age and smoking	Any	Use of FA containing vitamins at any time in the periconception period (-1 to +3 months) was not associated with LBW in offspring, aOR (95% CI): 1.2 (0.50 to 3.0), compared to no use of FA containing vitamins.

Rolschau [63] 1999	Denmark; Randomized controlled trial of FA (1000 or 2500µg), compared to non-randomized no FA users in 13,860 women. However, only 8,184 were randomized, and 2,310 received 100 or 200µg FA via vitamins or had been prescribed a rather massive dose (5 mg daily) by some practitioners; these women did not participate in the randomization. Therefore, patients were stratified into groups by dose and initiation (preconception, <20 weeks, after 20 weeks). Ninety-two percent of <20 weeks receiving 1000-2500µg FA were randomized. The remainder, a 'rest group' consisted of 2,721 pregnancies in which no FA was given and 806 where no information was available. Randomization: No data on differences between groups	1000 or 2500	BW did not differ between 2.5 vs. 1mg vs. no FA in the preconception period, except for a higher BW in the 2.5mg group in gestational week 43 compared to no FA (numbers n/a, $p<0.05$). In all following analyses, FA dose groups are combined. LBW was associated with the moment of initiation of FA use (LBW incidence after preconception initiation, <20 weeks gestation initiation and no FA use 40.9, 51.5 and 59.8‰ respectively, $p_{trend}=0.012$). A non-significant lower SGA incidence was seen when FA was given preconceptionally compared to initiation in the first 19 weeks (14.7‰ vs. 17.4‰). However, a significant difference was observed, when all groups (randomized and non-randomized, initiation preconception/ <20wks/ ≥20 wks) were compared (17.3/ 19.5/ 37.0‰, $p_{trend}=0.02$).
Christian [70] 2003	Nepal; Randomized controlled trial of vitamin A (1000µg) only or in combination with FA (400µg) in 1,313 women with live births Randomization: No differences between groups in baseline characteristics	400	Addition of FA to vitamin A supplements from the pre/periconception period onwards had no effect on BW and was not associated with a reduction of LBW risk compared to vitamin A use only, RR (95% CI): 1.00, (0.88, 1.15).
Mitchell [73] 2004	New Zealand; Case-control study of mothers with term SGA and AGA infants without congenital anomalies, and who had returned the FFQ in the 1st (n=503 and n= 573) and 3rd trimester (n=473 and n=546) Adjustment in statistical analysis: Ethnicity, smoking, maternal height and weight, hypertension, SES	Use ≥1/wk	FA use in the periconception period, but not in the 3rd trimester, was associated with a reduced risk of SGA, aOR (95% CI): 0.70 (0.52, 0.94), p=0.02, and 0.70 (0.41, 1.20), p=0.20, respectively.
Charles [56] 2005	Scotland; Randomized controlled trial of placebo or FA (200 or 5000µg) in 2,928 women with live singleton births Randomization/ Adjustment in statistical analysis: Other than an increased percentage of para ≥4 in the placebo group groups were comparable; Adjusted for smoking, social class, parity, maternal height and weight at booking and GA at birth	200 or 5000[a]	FA use from the 2nd trimester onwards was not associated with BW (β (95% CI): FA 200µg -4.6 (-50.6, 41.1), FA 5000µg 17.4 (-28.5, 63.2); $p_{trend}=0.71$) or LBW risk, OR (95% CI): 200µg FA 0.84 (0.49, 1.43), 5mg FA 0.80 (0.45, 1.50), $p_{trend}=0.63$. LBW risk may be reduced when FA use was initiated <17 weeks gestation (OR (95% CI): 200 µg FA 0.47 (0.19, 1.10), 5mg FA 0.46 (0.17, 1.21), $p_{trend}=0.09$).
Katz [117] 2006	Nepal; Prospective cohort study of 4,696 women with live births Adjustment in statistical analysis: Time of measurement, GA at birth, maternal age/ weight/ height, smoking and infant death	400	Addition of FA to vitamin A use from 1st trimester throughout pregnancy did not influence BW, compared to vitamin A use only, regression coefficient estimate (se): -0.96 g (21.78), not significant.

Palma [118] 2008	Spain; Case-control study of LBW (n=41) and non-LBW (n=71) mothers with live singleton births Adjustment in statistical analysis: Smoking, education, maternal age, Kessner index, BMI, obstetric diseases during pregnancy, GA at birth and weight gain during pregnancy	Not specified	FA use at any time during pregnancy for at least one week was not significantly associated with LBW risk, aOR (95% CI): via prenatal care records 1.31 (0.50-3.42) and interview aOR 1.43 (0.59-3.47).
Baker [81] 2009	United States; Prospective cohort study of 498 adolescent women aged 14-18 years Adjustment in statistical analysis: Smoking, cotinine, SES, ethnicity, maternal age, underweight/obesity and energy intake	Not specified	There was no significant association between FA use from the 2nd trimester onwards and SGA risk, OR (95% CI): 0.62 (0.37, 1.04), p=0.069.
Timmermans [10] 2009	Netherlands; Prospective cohort study of 6,353 women with singleton live births Adjustment in statistical analysis: GA at time of ultrasound or birth, maternal age/ height/ weight, parity, ethnicity, infant gender, education and smoking	400-500	Preconception FA use was associated with trends towards significantly larger HC and AC in the 2nd and 3rd trimester compared to non-users, regression coefficients (95% CI): 20 wks HC 0.61 (0.09, 1.12), AC 0.41 (-0.27, 1.08); 30 wks HC 1.34 (0.57, 2.11), AC 1.71 (0.61, 2.80). Similar non-significant trends towards larger femur length were observed. Preconception FA use was associated with 68g higher BW (95% CI 37.2, 99.0) and significantly reduced risks for LBW and SGA compared to non-users, OR (95% CI): 0.43 (0.28, 0.69) and 0.40 (0.22, 0.72). FA use initiated after pregnancy confirmation was associated with non-significant trends towards larger HC and AC, in mid- and late pregnancy, compared to non-users (regression coefficients (95% CI): 20 wks HC 0.10 (-0.41, 0.60), AC 0.05 (-0.61, 0.71); 30 wks: HC 0.63 (-0.12, 1.38), AC 0.32 (-0.75, 1.39)). Similar non-significant trends towards larger femur length were observed. Initiation of FA use after pregnancy confirmation was associated with a 53g higher BW (95% CI 23.60, 83.18), a significantly reduced risk of LBW and a non-significantly reduced SGA risk, aOR (95% CI): 0.61 (0.40, 0.94) and 0.69 (0.42, 1.14), respectively, compared to non-users. Fetal growth was not different between FA use initiating >8 weeks gestation compared to non-users or periconception initiation.
Czeizel [95] 2010	Hungary; Cohort study of 13,612 primiparous women with singleton births, using medically recorded prospective data Adjustment in statistical analysis: Maternal age, SES and GA at birth	5600	BW and risk of LBW did not differ significantly between users of FA only in 1st, 2nd or 3rd trimester, in 1st and 2nd, or in all 3 trimesters, compared to non-users. BW, but not LBW risk, differed significantly between FA users in 2nd and 3rd trimester and non-users, mean BW (sd): 3,257 (455) vs. 3,216 (486) g, p=0.004; LBW 4.2% vs. 5.7%, OR (95% CI): 0.74 (0.34, 1.27).

Hogeveen [68] 2010	Netherlands; Prospective cohort study of 366 women with singleton births Adjustment in statistical analysis: GA at birth, maternal age, parity, smoking, plasma folate, infant gender, homocysteine, cobalamin, methylmalonic acid and creatinine	Not specified	Maternal use of FA was not a significant predictor of standardized BW in a multiple linear regression model.
Mook-Kanamori [16] 2010	Netherlands; Prospective cohort study of 1,631 women with a known first day of last menstrual period or a regular cycle of 24-32 days and singleton births Adjustment in statistical analysis: Duration of last menstrual cycle, GA, infant gender, maternal age, blood pressure, haematocrit level, education, race/ethnicity, smoking and parity	400-500	In multivariate analysis, the associations of 1st trimester FA supplement use with CRL remained significant, (standard deviation score (95% CI): 0.17 (0.33, 0.01), p=0.03). However, after adjustment for multiple testing, the associations were no longer significant.
Nilsen [74] 2010	Norway; Prospective cohort study of 2,934 women with singleton births Adjustment in statistical analysis: Maternal age, marital status, education, parity, pre-pregnancy BMI, smoking and GA at blood collection	None vs. <400 vs. ≥ 400	Regression analysis showed no significant linear trends in mean values of BW or HC across categories of 2nd trimester FA use (none, <400, >400 µg; adjusted p_{trend}=0.44 and 0.44 respectively).
Hossein-nezhad [103] 2011	Iran; Prospective cohort study of 113 non-smoking and non-alcohol using healthy women with singleton births and Apgar score at 5 minutes of ≥7 Stratification: By duration of FA use	1000	Mean BW and HC did not differ significantly between mothers who used FA in the first two trimesters only and those who continued FA use throughout pregnancy (mean BW (sd): 3,153 (390) vs. 3,126 (480)g, p=0.7; HC: 34.70 (1.30) vs. 34.50 (1.74)cm, p=0.5).
Pastor-Valero [75] 2011	Spain; Prospective cohort study of 786 women with spontaneous pregnancies and live singleton births Adjustment in statistical analysis: GA, infant gender, maternal age, country of origin, education, energy intake, smoking, alcohol use, television viewing, gestational diabetes, transient hypertension, gestational weight gain, parity, planned pregnancy, history of medical problems in previous pregnancies, private gynaecologist and maternal and paternal height	No use vs. moderate (≤1000) vs. high use (>1000)	Multiple linear regression analysis showed that mothers using moderate and high doses of FA had non-significant lower-birth-weight babies for GA than non-users, β (95% CI): -22.96 (-101.14, 55.23) and -89.72 (-188.64, 9.21) respectively, p_{trend}=0.087. There was a significant trend across no (9.5%) vs. moderate (15.8%) vs. high (16.7%) FA dose users (p_{trend}= 0.047), but use of high or moderate doses of FA dose was not significantly associated with SGA risk compared to no use, aOR (95% CI): 2.05 (0.98, 4.28) and 1.38 (0.76, 2.51).
Dietary folate intake		Dietary folate assess-ment	
Whiteside [66] 1968	Australia; Prospective cohort study of 60 women without low haemoglobin or evidence of any illness	FFQ	Estimated dietary folate intake was not correlated with BW in any trimester (r/p values n/a).

Johnson [98] 1994	United States; Prospective cohort study of 332 healthy nulliparous African-American women with term singleton births	24h recall, monthly	Mean folate intake throughout pregnancy was not significantly correlated with BW (r/p values n/a). No significant differences in mean folate intake between mothers with LBW vs. normal BW offspring or BW below the median vs. BW above the median (numbers and p-values n/a).
Frelut [71] 1995	France; Case-control study of mothers with SGA (n=8) and AGA (n=13) offspring; women with HIV, low haemoglobin or haemoglobinopathy, underlying disease which may affect pregnancy outcome, recent blood transfusion, use of antibiotics or antiepileptic drugs were excluded	FFQ	3rd trimester dietary folate intake was not different between SGA and AGA mothers (no statistical testing shown).
Scholl [64] 1996	United States; Prospective cohort study of 832 women with no serious non-obstetric diseases Adjustment in statistical analysis: Time of blood draw, gestation at entry, ethnicity, prior history of preterm delivery or LBW, maternal age, and parity	24h recall, three times	Low 3rd trimester mean daily folate intake, including FA from supplements, <240µg/d was associated with increased risk of LBW, compared to adequate intake >400µg/d, aOR (95% CI): 3.33 (1.82, 6.09).
Neggers [69] 1997	United States; Prospective cohort study of 289 predominantly African-American women (70%) at high risk for intra-uterine growth restriction Adjustment in statistical analysis: GA at birth, smoking, race, infant gender, maternal weight, weight gain and dietary zinc	24h recall, at 18 and 30 weeks GA	Average dietary folate intake, including FA, in 2nd and 3rd trimester was a significant predictor for BW, β 0.05, p=0.03. A maternal folate intake increase from 10th to 90th percentile increased BW by 47.6 g (p=0.03). No significant association between folate intake and LBW risk, mean folate intake (sd) 1,131 (509) µg/d); OR (95% CI): 1.1 (0.7, 1.7).
Mathews [97] 1999	United States; Prospective cohort study of 624 healthy white primiparous women Adjustment in statistical analysis: Infant gender, GA, maternal height and smoking	FFQ	Dietary folate intake, including FA, at 9-20 weeks was positively associated with BW, but no longer significant after adjustment for confounders, expected change per tertile change (95% CI): 47g (-9.1, 103.2), p=0.11. There was no significant association between 3rd trimester dietary folate intake and BW (numbers and p-values n/a).
Takimoto [83] 2007	Japan; Prospective cohort study of 94 women with no major pregnancy complications and term singleton births Adjustment in statistical analysis: Maternal age, BMI and parity	Weighing and recording, 3/7 days before visit	Multiple regression analyses showed that 1st, 2nd and 3rd trimester dietary folate intake was not associated with BW or HC (effect sizes: BW 1.07, 0.48 and 0.008g; HC 0.0001, 0.009 and -0.006mm; all p-values >0.05).

Watanabe [119] 2008	Japan; Prospective cohort study of 197 healthy women with singleton births without congenital anomalies Adjustment in statistical analysis: Maternal age, parity and infant sex; Unclear whether additionally adjusted for GA at birth and smoking	FFQ	Multiple regression analysis showed that 1st, 2nd and 3rd trimester dietary folate intake was not associated with BW, effect size 0.6 (p=0.21), -0.2 (p=0.63) and -0.8 (p=0.06)) g, respectively.
Baker [81] 2009	United States; Prospective cohort study of 290 adolescent women aged 14-18 years Adjustment in statistical analysis: Smoking, cotinine, SES, ethnicity, maternal age, underweight, obesity and energy intake	24h recall, 3 non consecutive days	Women with 3rd trimester dietary folate intake, including FA, in the lowest quartile (<187 µg/d) were more likely to deliver SGA infants than those with higher intakes. This difference persisted after adjustment for energy intake and confounders, aOR (95% CI): 3.13 (1.23, 7.91), p=0.016. Results were similar when folate intake was calculated as dietary folate equivalents or when intake from supplements was excluded.
Kordas [65] 2009	Mexico; Retrospective cohort study of 474 healthy women with term singleton births Adjustment in statistical analysis: Maternal age and height, pre-pregnancy BMI, education, parity, marital status, ever smoking, postpartum calf circumference, GA at birth and infant gender	FFQ	With every 100µg/d increase in dietary folate intake, including FA, BW increased by 4g (se 0.02, p<0.05). Women with daily folate intake in the highest quartile (1,080–4,898mg/d) had newborns who weighed 54.8 g more than women in the lowest quartile (279µg/d) (p_{trend}=0.08). BW was not different between mothers who consumed dietary folate at levels below 400µg/d and higher. Maternal folate intake was not associated with HC.
Bawadi [120] 2010	Jordan; Retrospective cohort study of 700 healthy women Adjustment in statistical analysis: Parity, pre-gestational BMI and weight gain during pregnancy	FFQ	Dietary folate intake, including FA, at birth was not associated with BW (β -110 g, p=0.78).
Nilsen [74] 2010	Norway; Prospective cohort study of 2,934 women with singleton births Adjustment in statistical analysis: Maternal age, marital status, education, parity, pre-pregnancy BMI, smoking and GA at blood collection	FFQ	Regression analysis showed no significant linear trends in mean values of BW or HC over the percentiles (<p25, p25-75, p75) of 2nd trimester dietary folate intake (adjusted p_{trend}=1.00 and 0.42 including, and 0.99 and 0.27 excluding FA). SGA prevalence according to 1st/2nd trimester food quartiles and categories of FA use suggested a potential dose response relation ranging from 11.2 to 5.3% of SGA with higher percentages at lower levels of food intake. However, no significant trends were observed in the risk of SGA over food folate quartiles or supplemental categories in separate analyses after adjustment for each other, total energy intake, and other covariates, p_{trend}=0.42 including and 0.16 excluding FA .

Schlotz [85] 2010	United Kingdom; Retrospective cohort study of 100 women without a history of perinatal mortality or infant with major congenital anomalies Adjustment in statistical analysis: Smoking, alcohol use, total energy intake, GA at birth and infant gender	FFQ	A non-significant trend towards a positive association of dietary folate, including FA, in early pregnancy with HC, adjusted for daily energy intake was shown, β 1.5 mm, p=0.083. In adjusted models, total folate intake in early pregnancy explained 1.8% of the variance in HC (delta R^2=0.018). No association was found for 3rd trimester total folate intake and HC (β -0.5 mm, p=0.64). Total folate intake in early pregnancy and in the 3rd trimester were unrelated to BW adjusted for GA (p >0.15).
Pastor-Valero [75] 2011	Spain; Prospective cohort study of 786 women with spontaneous pregnancies and live singleton births Adjustment in statistical analysis: GA, infant gender, maternal age, country of origin, education, energy intake, smoking, alcohol use, television viewing, gestational diabetes, transient hypertension, gestational weight gain, parity, planned pregnancy, history of medical problems in previous pregnancies, private gynaecologist and maternal and paternal height	FFQ	Increasing quintiles of 1st trimester dietary folate intake were significantly associated with higher BW, p_{trend}=0.001, although mean weight for the 4th quintile was somewhat lower than those for the 3rd quintile. Increasing quintiles of dietary folate were associated with decreasing risk for SGA, with maximum protection from intakes above 245.4 µg/d, 3rd quintile; p_{trend}=0.002.

a) All anthropometric measurements refer to infant measurements at birth unless specified otherwise; b) Recalculation to nmol/L revealed an improbable RBC folate level. n/a not available from the original study; IFA supplement containing iron and folic acid; M Microbiological; PB protein binding; RBC Red blood cell; (L)BW (low) birth weight; SGA small for gestational age; AGA appropriate birth weight for gestational age; HC head circumference; AC abdominal circumference; (a)OR (adjusted) odds ratio; sd standard deviation, CI confidence interval, SE(M) standard error (of the mean); FFQ Food frequency questionnaire; GA gestational age; FFQ Food frequency questionnaire.

Table S1 Mean crown-rump length in millimetres with 95% reference intervals at 6, 8, 10 and 12 weeks gestation across RBC folate quartiles derived from the univariate model and data by Pexsters et al. and Robinson et al. [28, 135]

	Gestational age (weeks^{+d})			
	6^{+0}	8^{+0}	10^{+0}	12^{+0}
Our data				
Q1	4.3 (2.1, 7.4)	15.0 (10.5, 20.4)	32.2 (25.2, 40.1)	55.8 (46.1, 66.5)
Q2	4.5 (2.2, 7.6)	15.3 (10.7, 20.7)	32.6 (25.5, 40.5)	56.3 (46.6, 67.0)
Q3	5.5 (2.9, 8.9)	17.1 (12.2, 22.8)	35.2 (27.8, 43.4)	59.7 (49.7, 70.7)
Q4	4.2 (2.0, 7.2)	14.7 (10.2, 20.1)	31.8 (24.8, 39.6)	55.3 (45.6, 65.8)
Total	4.6 (2.2, 7.9)	15.5 (10.7, 21.2)	32.9 (25.6, 41.2)	56.8 (46.6, 67.9)
Other studies				
Pexsters et al.[135]	1.9 (0.4, 4.5)	15.3 (10.5, 19.6)	33.5 (25.9, 39.2)	56.6 (46.5, 63.3)
Robinson et al.[28]	n/a	17.0 (12.1, 21.9)	33.0 (25.8, 40.2)	58.3 (48.9, 67.7)

Table S1 General characteristics of the study population compared to the women who were excluded because of a less strictly regular menstrual cycle of $28^{\pm 3}$ days or conception through in vitro fertilization (IVF/ ICSI).

	Included		**Excluded**					
Characteristics	(n = 87)	Missing	Spontaneous irregular (n = 43)	Missing	*P*[a]	IVF/ICSI (n=58)	Missing	*P*[a]
Maternal (at enrolment)								
Age, y (mean±sd)	32.3±4.8	0	30.2±5.2	1	.025	33.1±4.2	2	.276
Ethnicity		0		1	.046		1	.055
Dutch	63 (72.4)		33 (78.6)			48 (84.2)		
Other western	5 (5.7)		6 (14.3)			5 (8.8)		
Non western	19 (21.8)		3 (7.0)			4 (7.0)		
Education		5		4	.481		0	.040
Low	5 (6.1)		4 (10.3)			6 (10.3)		
Middle	20 (24.4)		12 (30.8)			24 (41.4)		
High	57 (69.5)		23 (59.0)			28 (48.3)		
BMI, kg/m^2	23.7 (22.3-25.9)	1	23.7 (21.5-25.7)	0	.550	23.9(21.6-27.3)	0	.826
Primiparous	50 (57.5)	0	22 (51.2)	0	.496	47 (81.0)	0	.003
Periconception alcohol use	47 (54.0)	0	25 (58.1)	0	.657	13 (22.4)	0	.000
Periconception smoking	21 (24.1)	0	6 (14.0)	0	.178[c]	4 (6.9)	0	.007[c]
None	66 (75.9)		37 (86.0)			54 (93.1)		
1-9/ day	8 (9.2)		5 (11.6)			3 (5.2)		
≥10/ day	13 (14.9)		1 (2.3)			1 (1.7)		
Preconception initiation of folic acid supplement use	62 (72.1)	1	31 (72.1)	0	1.00	57(100.0)	1	.000

Table S1 (continued)

Characteristics	Included (n = 87)	Missing	Excluded Spontaneous irregular (n = 43)	Missing	*P* [a]	IVF/ICSI (n=58)	Missing	*P* [a]
Pregnancy and outcome								
Infant gender, male	42 (48.3)	0	25 (58.1)	0	.290	23 (39.7)	0	.307
Birth weight, g (mean±sd)	3309±585	0	3202±574	0	.325[d]	3372±505	0	.504
Gestational age at delivery, wk[+d]	39^{+2} (38^{+2}-40^{+5})	0	39^{+5} (38^{+2}-40^{+3})	0	.804	39^{+4} (38^{+2}-40^{+1})	0	.725
Total pregnancy complications	15 (17.2)	0	13 (30.2)	0	.090	7 (12.1)	0	.395
Maternal pregnancy complication	4 (4.6)	0	9 (20.9)	0	.003	4 (6.9)	0	.714
Hypertensive complication	3 (3.4)	0	9 (20.9)	0	.001	2 (3.4)	0	1.00
Gestational diabetes	1 (1.1)	0	0 (0.0)	0	.480	3 (5.2)	0	.302
Fetal complication	11 (12.6)	0	6 (14.0)	0	.835	3 (5.2)	0	.136
Low birth weight (<2500g)	6 (6.9)	0	5 (11.6)	0	.362	2 (3.4)	0	.476
Premature delivery (before 37 wk)	6 (6.9)	0	5 (11.6)	0	.362	2 (3.4)	0	.373
SGA (< 10^{th} customized centile)[b]	8 (9.4)	2	3 (7.0)	0	.642	1 (1.7)	0	.063.

Data are presented as median (interquartile range) or n(%) unless otherwise specified. Sd, standard deviation; BMI, body-mass index; SGA, small for gestational age.
[a] *P*-value for included versus excluded group. [b] Defined as weight under the tenth centile for gestational age, gender and parity according to Dutch reference charts [39]. [c] Comparison of distribution of periconception smoking in categories $P = 0.08$ and $P = 0.02$ for the included compared to the spontaneous irregular and IVF/ICSI pregnancies, respectively. [d] Comparison of birth weight for gestational age $P = 0.174$.

Table S2 Effect estimates from the univariate models for body mass index, parity and moment of initiation of folic acid use with respect to embryonic crown-rump length (CRL).

Characteristic	Effect estimate (95%CI), √mm[a]	*P*
Body mass index, kg/m^2	0.009 (-0.012, 0.030)	0.39
Parity		
Primiparous	0 [Reference]	
Multiparous	0.040 (-0.112, 0.192)	0.60
Moment of initiation of folic acid use		
Preconception	0 [Reference]	
Postconception	0.071 (-0.097, 0.240)	0.40

CI confidence interval. [a] For continuous variables, effect estimates represent the amount of change in CRL (√mm) per unit increase of the variable. For categorical or dichotomous variables, effect estimates represent the difference in square root of CRL compared to the reference group.

Table S1 General characteristics of the study population compared to those of pregnancies that were excluded because of a less strictly regular menstrual cycle of $28^{\pm 3}$ days or conception through IVF/ ICSI.

	Included				**Excluded**				***P***	
	Spontaneous and strictly regular cycle				Irregular cycle or IVF/ ICSI					
	Paternal n=81	missing	Maternal n=81	missing	Paternal n=98	missing	Maternal n=98	missing	Paternal	Maternal
At enrolment										
Age, y (mean±sd)	34.8±5.5	1	32.1±4.7	1	34.8±5.5	3	31.9±4.8	3	0.99	0.85
Height, cm (mean±sd)	183±6.4	0	170±6.5	1	185±7.0	0	169±6.5	0	0.06	0.51
Weight, kg	84 (60-125)	0	69 (54-133)	0	85 (62-131)	0	68 (53-108)	0	0.92	0.60
BMI, kg/m^2	25.0 (19.7-36.5)	0	23.8 (19.3-48.9)	1	24.8 (17.6-40.9)	0	23.8 (18.6-35.0)	0	0.36	0.71
Ethnicity		0		0		0		2	0.19	0.03
Dutch	61 (75.3)		60 (74.1)		82 (83.7)		78 (81.3)			
Western other	5 (6.2)		5 (6.2)		7 (7.1)		11 (11.5)			
Non Western	15 (18.5)		16 (19.8)		9 (9.2)		7 (7.3)			
Education		0		4		1		4	0.47	0.21
Low	11 (13.6)		7 (9.1)		15 (15.5)		10 (10.6)			
Intermediate	25 (30.9)		19 (22.5)		37 (38.1)		34 (36.2)			
High	45 (55.6)		51 (66.2)		45 (46.4)		50 (53.2)			
Preconception alcohol use	60 (74.1)	0	42 (51.9)	0	70 (71.4))	0	37 (37.8)	0	0.69	0.06
If yes, # per week	9 (0-32)	4	4 (2-24)	0	7 (0-31)	5	4 (2-18)	0	0.19	0.82
Preconception smoking	22 (27.2)	0	18 (22.2)	0	31 (31.6)	0	10 (10.2)	0	0.51	0.03
If yes, # per day	6 (0-20)	0	15 (1-20)	0	13 (0-30)	3	5 (1-15)	0	0.10	0.04
Periconception folic acid use	-		79 (97.5)	0	-		98 (100.0)	0	-	0.12
If yes, preconception initiation	-		57 (73.1)	1	-		85 (87.6)	1	-	0.01
Primiparous	-		46 (56.8)	0	-		67 (68.4)	0	-	0.11

Parental birth data										
Birthweight (mean±sd), g	3438±557	18	3231±507	7	3491±564	17	3201±467	6	0.65	0.69
Gestational age at delivery, wk+[d]	40^{+0} (33^{+0}-44^{+0})	25	40^{+0} (32^{+0}-43^{+4})	17	40^{+0} (32^{+0}-44^{+0})	27	40^{+0} (32^{+0}-43^{+0})	13	0.61	0.43
Birthweight *Z*-score (mean±sd)[†]	-0.22±0.87	17	-0.28±1.11	6	-0.17±1.15	14	-0.40±1.04	4	0.77	0.46
Pregnancy & outcome										
Conception through IVF/ ICSI	0 (0.0)	0			56 (57.1)	0				
Birthweight (mean±sd), g	3307±594	0			3293±546	0			0.86	
Gestational age at delivery, wk+[d]	39^{+2} (26^{+5}-42^{+0})	0			39^{+4} (31^{+3}-41^{+3})	0			0.68	
Male infant	39 (48.1)	0			46 (46.9)	0			0.87	
Complications	13 (16.0)	0			20 (20.4)	0			0.45	
Maternal	3 (3.7)	0			13 (13.3)	0			0.03	
Hypertensive disorder	2 (2.5)	0			11 (11.2)	0			0.03	
Gestational diabetes	1 (1.2)	0			3 (3.1)	0			0.39	
Fetal	10 (12.3)	0			9 (9.2)	0			0.49	
LBW (<2500g)	5 (6.2)	0			7 (7.1)	0			0.80	
Premature delivery (<37 weeks)	5 (6.2)	0			7 (7.1)	0			0.80	
SGA (<10^{th} customized centile)[‡]	8 (10.0)	1			4 (4.1)	0			0.12	

Data are presented as median (range) or n (%) unless otherwise specified. sd, standard deviation; BMI, body mass index; IVF/ICSI, in vitro fertilization with or without intra-cytoplasmic sperm injection; SGA, small for gestational age. * Gestational age based on a menstrual cycle of 28±3 days or conception date. [†] Birthweight adjusted for gestational age, gender and parity, according to Dutch reference charts [39]. [‡] Defined as weight under the 10^{th} centile for gestational age, gender and parity according to Dutch reference charts [39].

REFERENCES

1. Larsen, W.J., *Human Embryology*. 3rd ed, ed. L.S. Sherman, S.S. Potter, and W.J. Scott2001, Philadelphia (PA): Churchill Livingstone.
2. Barker, D.J., *The origins of the developmental origins theory*. J Intern Med, 2007. 261(5): p. 412-7.
3. Steegers-Theunissen, R.P., J. Twigt, V. Pestinger, and K.D. Sinclair, *The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism*. Hum Reprod Update, 2013. 19(6): p. 640-55.
4. Gluckman, P.D., M.A. Hanson, C. Cooper, and K.L. Thornburg, *Effect of in utero and early-life conditions on adult health and disease*. N Engl J Med, 2008. 359(1): p. 61-73.
5. Nafee, T.M., W.E. Farrell, W.D. Carroll, A.A. Fryer, and K.M. Ismail, *Epigenetic control of fetal gene expression*. BJOG, 2008. 115(2): p. 158-68.
6. Gardosi, J., A. Chang, B. Kalyan, D. Sahota, and E.M. Symonds, *Customised antenatal growth charts*. Lancet, 1992. 339(8788): p. 283-7.
7. Helmerhorst, F.M., D.A. Perquin, D. Donker, and M.J. Keirse, *Perinatal outcome of singletons and twins after assisted conception: a systematic review of controlled studies*. BMJ, 2004. 328(7434): p. 261.
8. Relton, C.L., M.S. Pearce, and L. Parker, *The influence of erythrocyte folate and serum vitamin B12 status on birth weight*. Br J Nutr, 2005. 93(5): p. 593-599.
9. Shah, P.S., *Paternal factors and low birthweight, preterm, and small for gestational age births: a systematic review*. Am J Obstet Gynecol, 2010. 202(2): p. 103-23.
10. Timmermans, S., V.W. Jaddoe, A. Hofman, R.P. Steegers-Theunissen, and E.A. Steegers, *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(5): p. 777-785.
11. Wang, X., B. Zuckerman, C. Pearson, et al., *Maternal cigarette smoking, metabolic gene polymorphism, and infant birth weight*. JAMA, 2002. 287(2): p. 195-202.
12. Hammiche, F., J.S. Laven, J.C. Boxmeer, G.R. Dohle, E.A. Steegers, and R.P. Steegers-Theunissen, *Sperm quality decline among men below 60 years of age undergoing IVF or ICSI treatment*. J Androl, 2011. 32(1): p. 70-6.
13. Sadeu, J.C., C.L. Hughes, S. Agarwal, and W.G. Foster, *Alcohol, drugs, caffeine, tobacco, and environmental contaminant exposure: reproductive health consequences and clinical implications*. Crit Rev Toxicol, 2010. 40(7): p. 633-52.
14. Bottomley, C. and T. Bourne, *Dating and growth in the first trimester*. Best Pract Res Clin Obstet Gynaecol, 2009. 23(4): p. 439-52.
15. Bukowski, R., G.C. Smith, F.D. Malone, et al., *Fetal growth in early pregnancy and risk of delivering low birth weight infant: prospective cohort study*. BMJ, 2007. 334(7598): p. 836.
16. Mook-Kanamori, D.O., E.A. Steegers, P.H. Eilers, H. Raat, A. Hofman, and V.W. Jaddoe, *Risk factors and outcomes associated with first-trimester fetal growth restriction*. JAMA, 2010. 303(6): p. 527-534.
17. Smith, G.C., M.F. Smith, M.B. McNay, and J.E. Fleming, *First-trimester growth and the risk of low birth weight*. N Engl J Med, 1998. 339(25): p. 1817-22.

18. Lumley, J., L. Watson, M. Watson, and C. Bower, *Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects*. Cochrane Database Syst Rev, 2000(2): p. CD001056.
19. World Health Organization (WHO), *Prevention of neural tube defects. Standards for maternal and neonatal care*, 2006: http://www.who.int/reproductivehealth/publications/maternal_perinatal_health/neural_tube_defects.pdf
20. Finkelstein, J.D., *Methionine metabolism in mammals*. J Nutr Biochem, 1990. 1(5): p. 228-37.
21. Koning, A.H., M. Rousian, C.M. Verwoerd-Dikkeboom, L. Goedknegt, E.A. Steegers, and P.J. van der Spek, *V-scope: design and implementation of an immersive and desktop virtual reality volume visualization system*. Stud Health Technol Inform, 2009. 142: p. 136-8.
22. Rousian, M., A.H. Koning, R.H. van Oppenraaij, et al., *An innovative virtual reality technique for automated human embryonic volume measurements*. Hum Reprod, 2010. 25(9): p. 2210-6.
23. Verwoerd-Dikkeboom, C.M., A.H. Koning, W.C. Hop, et al., *Reliability of three-dimensional sonographic measurements in early pregnancy using virtual reality*. Ultrasound Obstet Gynecol, 2008. 32(7): p. 910-6.
24. Verwoerd-Dikkeboom, C.M., A.H. Koning, W.C. Hop, P.J. van der Spek, N. Exalto, and E.A. Steegers, *Innovative virtual reality measurements for embryonic growth and development*. Hum Reprod, 2010. 25(6): p. 1404-10.
25. Cruz-Neira C, S.D., DeFanti T, *Surround-screen projection-based virtual reality: the design and implementation of the CAVE (tm)*. In: Proceedings of the 20th Annual Conference on Computer Graphics and Interactive Techniques 1993, S. Cunningham, editor. New York: ACM Press. p. 135-142.
26. Bonsel, G.J., E. Birnie, S. Denktas, J. Poeran, and E.A.P. Steegers, *Dutch report: Lijnen in de Perinatale Sterfte, Signalementstudie Zwangerschap en Geboorte 2010*. 2010, Available at: www.nvk.nl/Nieuws/Dossiers/DossierPerinatalezorg.aspx. Erasmus MC: Rotterdam.
27. Center for Disease Control and Prevention, *Prevalence of Selected Maternal and Infant Characteristics, Pregnancy Risk Assessment Monitoring System (PRAMS), 1997*. In: CDC Surveillance Summaries 1999, MMWR.
28. Robinson, H.P. and J.E. Fleming, *A critical evaluation of sonar "crown-rump length" measurements*. Br J Obstet Gynaecol, 1975. 82(9): p. 702-10.
29. Hadlock, F.P., R.B. Harrist, R.S. Sharman, R.L. Deter, and S.K. Park, *Estimation of fetal weight with the use of head, body, and femur measurements--a prospective study*. Am J Obstet Gynecol, 1985. 151(3): p. 333-7.
30. Bottomley, C., A. Daemen, F. Mukri, et al., *Assessing first trimester growth: the influence of ethnic background and maternal age*. Hum Reprod, 2009. 24(2): p. 284-90.
31. Deter, R.L., J.E. Buster, P.R. Casson, and S.A. Carson, *Individual growth patterns in the first trimester: evidence for difference in embryonic and fetal growth rates*. Ultrasound Obstet Gynecol, 1999. 13(2): p. 90-8.
32. Burton, G.J., J. Hempstock, and E. Jauniaux, *Nutrition of the human fetus during the first trimester--a review*. Placenta, 2001. 22 Suppl A: p. S70-7.
33. Merce, L.T., M.J. Barco, J.L. Alcazar, R. Sabatel, and J. Troyano, *Intervillous and uteroplacental circulation in normal early pregnancy and early pregnancy loss assessed by 3-dimensional power Doppler angiography*. Am J Obstet Gynecol, 2009. 200(3): p. 315 e1-8.

34. Burton, G.J., E. Jauniaux, and A.L. Watson, *Maternal arterial connections to the placental intervillous space during the first trimester of human pregnancy: the Boyd collection revisited.* Am J Obstet Gynecol, 1999. 181(3): p. 718-24.
35. Burton, G.J. and E. Jauniaux, *Oxidative stress.* Best Pract Res Clin Obstet Gynaecol, 2011. 25(3): p. 287-99.
36. Dickey, R.P. and R.F. Gasser, *Ultrasound evidence for variability in the size and development of normal human embryos before the tenth post-insemination week after assisted reproductive technologies.* Hum Reprod, 1993. 8(2): p. 331-7.
37. Salomon, L.J., S. Hourrier, R. Fanchin, Y. Ville, and P. Rozenberg, *Is first-trimester crown-rump length associated with birthweight?* BJOG, 2011. 118(10): p. 1223-8.
38. Pedersen, N.G., F. Figueras, K.R. Wojdemann, A. Tabor, and J. Gardosi, *Early fetal size and growth as predictors of adverse outcome.* Obstet Gynecol, 2008. 112(4): p. 765-71.
39. The Netherlands Perinatal Registry, *Perinatal Care in The Netherlands 2008 (in Dutch: Perinatale zorg in Nederland 2008)*, 2011, The Netherlands Perinatal Registry: Utrecht.
40. Rousian, M., W.C. Hop, A.H. Koning, P.J. van der Spek, N. Exalto, and E.A. Steegers, *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(5): p. 1181-1189.
41. van Uitert, E.M., N. Exalto, G.J. Burton, et al., *Human embryonic growth trajectories and associations with fetal growth and birthweight.* Hum Reprod, 2013. 28(7): p. 1753-61.
42. van Uitert, E.M. and R.P. Steegers-Theunissen, *Influence of maternal folate status on human fetal growth parameters.* Mol Nutr Food Res, 2013. 57(4): p. 582-95.
43. Bottomley, C., A. Daemen, F. Mukri, et al., *Functional linear discriminant analysis: a new longitudinal approach to the assessment of embryonic growth.* Hum Reprod, 2009. 24(2): p. 278-83.
44. Philipp, T. and D.K. Kalousek, *Neural tube defects in missed abortions: embryoscopic and cytogenetic findings.* Am J Med Genet, 2002. 107(1): p. 52-7.
45. O'Rahilly, R. and F. Muller, *Developmental stages in human embryos: revised and new measurements.* Cells Tissues Organs, 2010. 192(2): p. 73-84.
46. Rigby, R.A. and D.M. Stasinopoulos, *Generalized additive models for location, scale and shape.* Journal of the Royal Statistical Society Series C-Applied Statistics, 2005. 54: p. 507-544.
47. Khoshnood, B., R. Greenlees, M. Loane, H. Dolk, E.P.M. Committee, and E.W. Group, *Paper 2: EUROCAT public health indicators for congenital anomalies in Europe.* Birth Defects Res A Clin Mol Teratol, 2011. 91 Suppl 1: p. S16-22.
48. Cameron, M. and P. Moran, *Prenatal screening and diagnosis of neural tube defects.* Prenat Diagn, 2009. 29(4): p. 402-11.
49. van Straaten, H.W. and A.J. Copp, *Curly tail: a 50-year history of the mouse spina bifida model.* Anat Embryol (Berl), 2001. 203(4): p. 225-37.
50. Peeters, M.C., J.W. Hekking, K. Shiota, J. Drukker, and H.W. Van Straaten, *Differences in axial curvature correlate with species-specific rate of neural tube closure in embryos of chick, rabbit, mouse, rat and human.* Anat Embryol (Berl), 1998. 198(3): p. 185-94.
51. McLean, E., B. de Benoist, and L.H. Allen, *Review of the magnitude of folate and vitamin B12 deficiencies worldwide.* Food Nutr Bull, 2008. 29(2 Suppl): p. S38-51.

52. MRC, *Prevention of neural tube defects: results of the Medical Research Council Vitamin Study*. MRC Vitamin Study Research Group. Lancet, 1991. 338(8760): p. 131-7.
53. Czeizel, A.E. and I. Dudas, *Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation*. N Engl J Med, 1992. 327(26): p. 1832-5.
54. Lumley, J., L. Watson, M. Watson, and C. Bower, *Periconceptional supplementation with folate and/or multivitamins for preventing neural tube defects*. Cochrane Database Syst Rev, 2001(3): p. CD001056.
55. Mahomed, K., *Folate supplementation in pregnancy*. Cochrane Database Syst Rev, 2000(2): p. CD000183.
56. Charles, D.H., A.R. Ness, D. Campbell, G.D. Smith, E. Whitley, and M.H. Hall, *Folic acid supplements in pregnancy and birth outcome: re-analysis of a large randomised controlled trial and update of Cochrane review*. Paediatr Perinat Epidemiol, 2005. 19(2): p. 112-124.
57. Carmel, R., *Folate deficiency*. In: Homocysteine in health and disease., R.a.J. Carmel, D.W., Editor 2001, Cambridge University Press: Cambridge. p. 271-288.
58. van der Put, N.M., R.P. Steegers-Theunissen, P. Frosst, et al., *Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida*. Lancet, 1995. 346(8982): p. 1070-1.
59. Shane, B., *Folate status assessment history: implications for measurement of biomarkers in NHANES*. Am J Clin Nutr, 2011. 94(1): p. 337S-342S.
60. Relton, C.L., M.S. Pearce, J. Burn, and L. Parker, *An investigation of folate-related genetic factors in the determination of birthweight*. Paediatr Perinat Epidemiol, 2005. 19(5): p. 360-367.
61. Hibbard, B.M., *Folates and the fetus*. S Afr Med J, 1975. 49(30): p. 1223-1226.
62. Rolschau, J., J. Date, and K. Kristoffersen, *Folic acid supplement and intrauterine growth*. Acta Obstet Gynecol Scand, 1979. 58(4): p. 343-346.
63. Rolschau, J., K. Kristoffersen, M. Ulrich, P. Grinsted, E. Schaumburg, and N. Foged, *The influence of folic acid supplement on the outcome of pregnancies in the county of Funen in Denmark. Part I*. Eur J Obstet Gynecol Reprod Biol, 1999. 87(2): p. 105-110; discussion 103-104.
64. Scholl, T.O., M.L. Hediger, J.I. Schall, C.S. Khoo, and R.L. Fischer, *Dietary and serum folate: their influence on the outcome of pregnancy*. Am J Clin Nutr, 1996. 63(4): p. 520-525.
65. Kordas, K., A.S. Ettinger, H. Lamadrid-Figueroa, et al., *Methylenetetrahydrofolate reductase (MTHFR) C677T, A1298C and G1793A genotypes, and the relationship between maternal folate intake, tibia lead and infant size at birth*. Br J Nutr, 2009. 102(6): p. 907-914.
66. Whiteside, M.G., B. Ungar, and D.C. Cowling, *Iron, folic acid and vitamin B12 levels in normal pregnancy, and their influence on birth-weight and the duration of pregnancy*. Med J Aust, 1968. 1(9): p. 338-342.
67. Baumslag, N., T. Edelstein, and J. Metz, *Reduction of incidence of prematurity by folic acid supplementation in pregnancy*. Br Med J, 1970. 1(5687): p. 16-17.
68. Hogeveen, M., H.J. Blom, E.H. van der Heijden, et al., *Maternal homocysteine and related B vitamins as risk factors for low birthweight*. Am J Obstet Gynecol, 2010. 202(6): p. 572 e571-576.
69. Neggers, Y.H., R.L. Goldenberg, T. Tamura, S.P. Cliver, and H.J. Hoffman, *The relationship between maternal dietary intake and infant birthweight*. Acta Obstet Gynecol Scand Suppl, 1997. 165: p. 71-75.

70. Christian, P., S.K. Khatry, J. Katz, et al., *Effects of alternative maternal micronutrient supplements on low birth weight in rural Nepal: double blind randomised community trial.* BMJ, 2003. 326(7389): p. 571.
71. Frelut, M.L., G.P. de Courcy, J.P. Christides, P. Blot, and J. Navarro, *Relationship between maternal folate status and foetal hypotrophy in a population with a good socio-economical level.* Int J Vitam Nutr Res, 1995. 65(4): p. 267-271.
72. Martin, H., B. Lindblad, and M. Norman, *Endothelial function in newborn infants is related to folate levels and birth weight.* Pediatrics, 2007. 119(6): p. 1152-1158.
73. Mitchell, E.A., E. Robinson, P.M. Clark, et al., *Maternal nutritional risk factors for small for gestational age babies in a developed country: a case-control study.* Arch Dis Child Fetal Neonatal Ed, 2004. 89(5): p. F431-435.
74. Nilsen, R.M., S.E. Vollset, A.L. Monsen, et al., *Infant birth size is not associated with maternal intake and status of folate during the second trimester in Norwegian pregnant women.* J Nutr, 2010. 140(3): p. 572-579.
75. Pastor-Valero, M., E.M. Navarrete-Munoz, M. Rebagliato, et al., *Periconceptional folic acid supplementation and anthropometric measures at birth in a cohort of pregnant women in Valencia, Spain.* Br J Nutr, 2011. 105(9): p. 1352-1360.
76. Rondo, P.H., R. Abbott, L.C. Rodrigues, and A.M. Tomkins, *Vitamin A, folate, and iron concentrations in cord and maternal blood of intra-uterine growth retarded and appropriate birth weight babies.* Eur J Clin Nutr, 1995. 49(6): p. 391-399.
77. Rondo, P.H. and A.M. Tomkins, *Folate and intrauterine growth retardation.* Ann Trop Paediatr, 2000. 20(4): p. 253-258.
78. Sram, R.J., B. Binkova, Z. Lnenickova, I. Solansky, and J. Dejmek, *The impact of plasma folate levels of mothers and newborns on intrauterine growth retardation and birth weight.* Mutat Res, 2005. 591(1-2): p. 302-310.
79. Tamura, T., R.L. Goldenberg, L.E. Freeberg, S.P. Cliver, G.R. Cutter, and H.J. Hoffman, *Maternal serum folate and zinc concentrations and their relationships to pregnancy outcome.* Am J Clin Nutr, 1992. 56(2): p. 365-370.
80. Tchernia, G., I. Blot, A. Rey, J.P. Kaltwasser, J. Zittoun, and E. Papiernik, *Maternal folate status, birthweight and gestational age.* Dev Pharmacol Ther, 1982. 4 Suppl: p. 58-65.
81. Baker, P.N., S.J. Wheeler, T.A. Sanders, et al., *A prospective study of micronutrient status in adolescent pregnancy.* Am J Clin Nutr, 2009. 89(4): p. 1114-24.
82. Ek, J., *Plasma and red cell folate in mothers and infants in normal pregnancies. Relation to birth weight.* Acta Obstet Gynecol Scand, 1982. 61(1): p. 17-20.
83. Takimoto, H., N. Mito, K. Umegaki, et al., *Relationship between dietary folate intakes, maternal plasma total homocysteine and B-vitamins during pregnancy and fetal growth in Japan.* Eur J Nutr, 2007. 46(5): p. 300-306.
84. Brough, L., G.A. Rees, M.A. Crawford, R.H. Morton, and E.K. Dorman, *Effect of multiple-micronutrient supplementation on maternal nutrient status, infant birth weight and gestational age at birth in a low-income, multi-ethnic population.* Br J Nutr, 2010. 104(3): p. 437-445.
85. Schlotz, W., A. Jones, D.I. Phillips, C.R. Gale, S.M. Robinson, and K.M. Godfrey, *Lower maternal folate status in early pregnancy is associated with childhood hyperactivity and peer problems in offspring.* J Child Psychol Psychiatry, 2010. 51(5): p. 594-602.

86. Chanarin, I., D. Rothman, A. Ward, and J. Perry, *Folate status and requirement in pregnancy.* Br Med J, 1968. 2(5602): p. 390-394.
87. Van Dijk, A.E., M. Van Eijsden, K. Stronks, R.J. Gemke, and T.G. Vrijkotte, *Maternal depressive symptoms, serum folate status, and pregnancy outcome: results of the Amsterdam Born Children and their Development study.* Am J Obstet Gynecol, 2010. 203(6): p. 563 e561-567.
88. Faintuch, J., M.C. Dias, E. de Souza Fazio, et al., *Pregnancy nutritional indices and birth weight after Roux-en-Y gastric bypass.* Obes Surg, 2009. 19(5): p. 583-589.
89. Mukherjee, M.D., H.H. Sandstead, M.V. Ratnaparkhi, L.K. Johnson, D.B. Milne, and H.P. Stelling, *Maternal zinc, iron, folic acid, and protein nutriture and outcome of human pregnancy.* Am J Clin Nutr, 1984. 40(3): p. 496-507.
90. Ronnenberg, A.G., M.B. Goldman, D. Chen, et al., *Preconception homocysteine and B vitamin status and birth outcomes in Chinese women.* Am J Clin Nutr, 2002. 76(6): p. 1385-1391.
91. Fleming, A.F., G.B. Ghatoura, K.A. Harrison, N.D. Briggs, and D.T. Dunn, *The prevention of anaemia in pregnancy in primigravidae in the guinea savanna of Nigeria.* Ann Trop Med Parasitol, 1986. 80(2): p. 211-233.
92. Giles, P.F., A.G. Harcourt, and M.G. Whiteside, *The effect of prescribing folic acid during pregnancy on birth-weight and duration of pregnancy. A double-blind trial.* Med J Aust, 1971. 2(1): p. 17-21.
93. Hamilton, P.J., D.A. Gebbie, N.E. Wilks, and F. Lothe, *The role of malaria, folic acid deficiency and haemoglobin AS in pregnancy at Mulago hospital.* Trans R Soc Trop Med Hyg, 1972. 66(4): p. 594-602.
94. Iyengar, L. and K. Rajalakshmi, *Effect of folic acid supplement on birth weights of infants.* Am J Obstet Gynecol, 1975. 122(3): p. 332-336.
95. Czeizel, A.E., E.H. Puho, Z. Langmar, N. Acs, and F. Banhidy, *Possible association of folic acid supplementation during pregnancy with reduction of preterm birth: a population-based study.* Eur J Obstet Gynecol Reprod Biol, 2010. 148(2): p. 135-140.
96. Iyengar, L., *Folic acid requirements of Indian pregnant women.* Am J Obstet Gynecol, 1971. 111(1): p. 13-16.
97. Mathews, F., P. Yudkin, and A. Neil, *Influence of maternal nutrition on outcome of pregnancy: prospective cohort study.* BMJ, 1999. 319(7206): p. 339-343.
98. Johnson, A.A., E.M. Knight, C.H. Edwards, et al., *Dietary intakes, anthropometric measurements and pregnancy outcomes.* J Nutr, 1994. 124(6 Suppl): p. 936S-942S.
99. Timmermans, S., R.P. Steegers-Theunissen, M. Vujkovic, et al., *The Mediterranean diet and fetal size parameters: the Generation R Study.* Br J Nutr, 2012: p. 1-11.
100. McNulty, H. and K. Pentieva, *Folate bioavailability.* Proc Nutr Soc, 2004. 63(4): p. 529-36.
101. Lu, N., M.E. Samuels, and K.C. Huang, *Dietary behavior in relation to socioeconomic characteristics and self-perceived health status.* J Health Care Poor Underserved, 2002. 13(2): p. 241-57.
102. Timmermans, S., V.W. Jaddoe, J.P. Mackenbach, A. Hofman, R.P. Steegers-Theunissen, and E.A. Steegers, *Determinants of folic acid use in early pregnancy in a multi-ethnic urban population in The Netherlands: the Generation R study.* Prev Med, 2008. 47(4): p. 427-32.

103. Hossein-nezhad, A., K. Mirzaei, Z. Maghbooli, A. Najmafshar, and B. Larijani, *The influence of folic acid supplementation on maternal and fetal bone turnover.* J Bone Miner Metab, 2011. 29(2): p. 186-192.
104. Steegers-Theunissen, R.P., S.A. Obermann-Borst, D. Kremer, et al., *Periconceptional maternal folic acid use of 400 microg per day is related to increased methylation of the IGF2 gene in the very young child.* PLoS One, 2009. 4(11): p. e7845.
105. Waterland, R.A., D.C. Dolinoy, J.R. Lin, C.A. Smith, X. Shi, and K.G. Tahiliani, *Maternal methyl supplements increase offspring DNA methylation at Axin Fused.* Genesis, 2006. 44(9): p. 401-6.
106. Lillycrop, K.A., E.S. Phillips, C. Torrens, M.A. Hanson, A.A. Jackson, and G.C. Burdge, *Feeding pregnant rats a protein-restricted diet persistently alters the methylation of specific cytosines in the hepatic PPAR alpha promoter of the offspring.* Br J Nutr, 2008. 100(2): p. 278-82.
107. Steegers-Theunissen, R.P., *Inaugural lecture: 'New life in a changing environment'* 2010, Alphen aan den Rijn: Demmenie Grafimedia.
108. Hanson, M.A. and P.D. Gluckman, *Developmental origins of health and disease: new insights.* Basic Clin Pharmacol Toxicol, 2008. 102(2): p. 90-3.
109. Tamura, T., E.W. Weekes, R. Birch, et al., *Relationship between amniotic fluid and maternal blood nutrient levels.* J Perinat Med, 1994. 22(3): p. 227-234.
110. de Weerd, S., R.P. Steegers-Theunissen, T.M. de Boo, C.M. Thomas, and E.A. Steegers, *Maternal periconceptional biochemical and hematological parameters, vitamin profiles and pregnancy outcome.* Eur J Clin Nutr, 2003. 57(9): p. 1128-1134.
111. Parazzini, F., F. Chiaffarino, E. Ricci, L. Improta, and G. Monni, *Homocysteine, red cell, and plasma folate concentrations and birth weight in Italian women: results from a prospective study.* J Matern Fetal Neonatal Med, 2011. 24(3): p. 427-431.
112. Yajnik, C.S., S.S. Deshpande, A.V. Panchanadikar, et al., *Maternal total homocysteine concentration and neonatal size in India.* Asia Pac J Clin Nutr, 2005. 14(2): p. 179-181.
113. Stefanidis, K., T. Stefos, A. Vekris, A. Sotiriadis, N. Dalkalitsis, and D. Lolis, *Folate status during labor: relationship with pregnancy outcome.* J Matern Fetal Med, 1999. 8(2): p. 61-63.
114. Baker, H., I.S. Thind, O. Frank, B. DeAngelis, H. Caterini, and D.B. Louria, *Vitamin levels in low-birth-weight newborn infants and their mothers.* Am J Obstet Gynecol, 1977. 129(5): p. 521-524.
115. Fleming, A.F., J.D. Martin, R. Hahnel, and A.J. Westlake, *Effects of iron and folic acid antenatal supplements on maternal haematology and fetal wellbeing.* Med J Aust, 1974. 2(12): p. 429-436.
116. Shaw, G.M., R.F. Liberman, K. Todoroff, and C.R. Wasserman, *Low birth weight, preterm delivery, and periconceptional vitamin use.* J Pediatr, 1997. 130(6): p. 1013-1014.
117. Katz, J., P. Christian, F. Dominici, and S.L. Zeger, *Treatment effects of maternal micronutrient supplementation vary by percentiles of the birth weight distribution in rural Nepal.* J Nutr, 2006. 136(5): p. 1389-1394.
118. Palma, S., R. Perez-Iglesias, D. Prieto, R. Pardo, J. Llorca, and M. Delgado-Rodriguez, *Iron but not folic acid supplementation reduces the risk of low birthweight in pregnant women without anaemia: a case-control study.* J Epidemiol Community Health, 2008. 62(2): p. 120-124.
119. Watanabe, H., H. Fukuoka, T. Sugiyama, Y. Nagai, K. Ogasawara, and N. Yoshiike, *Dietary folate intake during pregnancy and birth weight in Japan.* Eur J Nutr, 2008. 47(6): p. 341-347.

120. Bawadi, H.A., O. Al-Kuran, L.A. Al-Bastoni, et al., *Gestational nutrition improves outcomes of vaginal deliveries in Jordan: an epidemiologic screening.* Nutr Res, 2010. 30(2): p. 110-117.
121. Abramowicz, J.S., *Fetal Doppler: how to keep it safe?* Clin Obstet Gynecol, 2010. 53(4): p. 842-50.
122. Houston, L.E., A.O. Odibo, and G.A. Macones, *The safety of obstetrical ultrasound: a review.* Prenat Diagn, 2009. 29(13): p. 1204-12.
123. The British Medical Ultrasound Society, *Guidelines for the safe use of diagnostic ultrasound equipment*, 2009, BMUS: London.
124. Achon, M., L. Reyes, E. Alonso-Aperte, N. Ubeda, and G. Varela-Moreiras, *High dietary folate supplementation affects gestational development and dietary protein utilization in rats.* J Nutr, 1999. 129(6): p. 1204-8.
125. Pickell, L., K. Brown, D. Li, et al., *High intake of folic acid disrupts embryonic development in mice.* Birth Defects Res A Clin Mol Teratol, 2011. 91(1): p. 8-19.
126. Heijmans, B.T., E.W. Tobi, A.D. Stein, et al., *Persistent epigenetic differences associated with prenatal exposure to famine in humans.* Proc Natl Acad Sci U S A, 2008. 105(44): p. 17046-9.
127. Sinclair, K.D., C. Allegrucci, R. Singh, et al., *DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status.* Proc Natl Acad Sci U S A, 2007. 104(49): p. 19351-6.
128. Matthews, R.G. and S.C. Daubner, *Modulation of methylenetetrahydrofolate reductase activity by S-adenosylmethionine and by dihydrofolate and its polyglutamate analogues.* Adv Enzyme Regul, 1982. 20: p. 123-31.
129. Nijhout, H.F., M.C. Reed, P. Budu, and C.M. Ulrich, *A mathematical model of the folate cycle: new insights into folate homeostasis.* J Biol Chem, 2004. 279(53): p. 55008-16.
130. Ashokkumar, B., Z.M. Mohammed, N.D. Vaziri, and H.M. Said, *Effect of folate oversupplementation on folate uptake by human intestinal and renal epithelial cells.* Am J Clin Nutr, 2007. 86(1): p. 159-66.
131. Steegers-Theunissen, R.P., E.A. Steegers, R. de Boer, C.M. Thomas, M.D. Kloosterman, and T.K. Eskes, *Elevated folate levels in amniotic fluid after oral supplementation.* Eur J Obstet Gynecol Reprod Biol, 1990. 36(3): p. 283-91.
132. Molloy, A.M., J.L. Mills, J. McPartlin, P.N. Kirke, J.M. Scott, and S. Daly, *Maternal and fetal plasma homocysteine concentrations at birth: the influence of folate, vitamin B12, and the 5,10-methylenetetrahydrofolate reductase 677C-->T variant.* Am J Obstet Gynecol, 2002. 186(3): p. 499-503.
133. Kelly, P., J. McPartlin, M. Goggins, D.G. Weir, and J.M. Scott, *Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements.* Am J Clin Nutr, 1997. 65(6): p. 1790-5.
134. Obeid, R., M. Kasoha, S.H. Kirsch, W. Munz, and W. Herrmann, *Concentrations of unmetabolized folic acid and primary folate forms in pregnant women at delivery and in umbilical cord blood.* Am J Clin Nutr, 2010. 92(6): p. 1416-22.
135. Pexsters, A., A. Daemen, C. Bottomley, et al., *New crown-rump length curve based on over 3500 pregnancies.* Ultrasound Obstet Gynecol, 2010. 35(6): p. 650-5.
136. American Academy of Pediatrics, Committee on Genetics, *Folic acid for the prevention of neural tube defects.* Pediatrics, 1999. 104(2 Pt 1): p. 325-7.

137. Daly, L.E., P.N. Kirke, A. Molloy, D.G. Weir, and J.M. Scott, *Folate levels and neural tube defects. Implications for prevention.* JAMA, 1995. 274(21): p. 1698-702.
138. Smith, A.D., Y.I. Kim, and H. Refsum, *Is folic acid good for everyone?* Am J Clin Nutr, 2008. 87(3): p. 517-33.
139. Steegers-Theunissen, R.P. and E.A. Steegers, *Nutrient-gene interactions in early pregnancy: a vascular hypothesis.* Eur J Obstet Gynecol Reprod Biol, 2003. 106(2): p. 115-7.
140. Bakker, R., E.A. Steegers, A. Obradov, H. Raat, A. Hofman, and V.W. Jaddoe, *Maternal caffeine intake from coffee and tea, fetal growth, and the risks of adverse birth outcomes: the Generation R Study.* Am J Clin Nutr, 2010. 91(6): p. 1691-8.
141. Prabhu, N., N. Smith, D. Campbell, et al., *First trimester maternal tobacco smoking habits and fetal growth.* Thorax, 2010. 65(3): p. 235-40.
142. Sarris, I., C. Bottomley, A. Daemen, et al., *No influence of body mass index on first trimester fetal growth.* Hum Reprod, 2010. 25(8): p. 1895-9.
143. Mahendru, A., A. Daemen, T. Everett, et al., *Impact of ovulation and implantation timing on first trimester crown-rump length and gestational age.* Ultrasound Obstet Gynecol, 2012.
144. Jukic, A.M. and A.J. Wilcox, *The events of early pregnancy: prying open the black box.* Ultrasound Obstet Gynecol, 2012. 40(6): p. 617-8.
145. Chiazze, L., Jr., F.T. Brayer, J.J. Macisco, Jr., M.P. Parker, and B.J. Duffy, *The length and variability of the human menstrual cycle.* JAMA, 1968. 203(6): p. 377-80.
146. Kolstad, H.A., J.P. Bonde, N.H. Hjollund, et al., *Menstrual cycle pattern and fertility: a prospective follow-up study of pregnancy and early embryonal loss in 295 couples who were planning their first pregnancy.* Fertil Steril, 1999. 71(3): p. 490-6.
147. Munster, K., L. Schmidt, and P. Helm, *Length and variation in the menstrual cycle--a cross-sectional study from a Danish county.* Br J Obstet Gynaecol, 1992. 99(5): p. 422-9.
148. Henderson, J., R. Gray, and P. Brocklehurst, *Systematic review of effects of low-moderate prenatal alcohol exposure on pregnancy outcome.* BJOG, 2007. 114(3): p. 243-52.
149. Jaddoe, V.W., R. Bakker, A. Hofman, et al., *Moderate alcohol consumption during pregnancy and the risk of low birth weight and preterm birth. The generation R study.* Ann Epidemiol, 2007. 17(10): p. 834-40.
150. O'Leary, C.M., N. Nassar, J.J. Kurinczuk, and C. Bower, *The effect of maternal alcohol consumption on fetal growth and preterm birth.* BJOG, 2009. 116(3): p. 390-400.
151. Abbott, L.C. and U.H. Winzer-Serhan, *Smoking during pregnancy: lessons learned from epidemiological studies and experimental studies using animal models.* Crit Rev Toxicol, 2012. 42(4): p. 279-303.
152. Kay, H.H., K.M. Grindle, and R.R. Magness, *Ethanol exposure induces oxidative stress and impairs nitric oxide availability in the human placental villi: a possible mechanism of toxicity.* Am J Obstet Gynecol, 2000. 182(3): p. 682-8.
153. Guerrero-Preston, R., L.R. Goldman, P. Brebi-Mieville, et al., *Global DNA hypomethylation is associated with in utero exposure to cotinine and perfluorinated alkyl compounds.* Epigenetics, 2010. 5(6): p. 539-46.
154. Suter, M., J. Ma, A. Harris, et al., *Maternal tobacco use modestly alters correlated epigenome-wide placental DNA methylation and gene expression.* Epigenetics, 2011. 6(11): p. 1284-94.

155. Kaminen-Ahola, N., A. Ahola, M. Maga, et al., *Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model.* PLoS Genet, 2010. 6(1): p. e1000811.
156. Heyn, H., N. Li, H.J. Ferreira, et al., *Distinct DNA methylomes of newborns and centenarians.* Proc Natl Acad Sci U S A, 2012. 109(26): p. 10522-7.
157. Joseph, K.S., A.C. Allen, L. Dodds, L.A. Turner, H. Scott, and R. Liston, *The perinatal effects of delayed childbearing.* Obstet Gynecol, 2005. 105(6): p. 1410-8.
158. Odibo, A.O., D. Nelson, D.M. Stamilio, H.M. Sehdev, and G.A. Macones, *Advanced maternal age is an independent risk factor for intrauterine growth restriction.* Am J Perinatol, 2006. 23(5): p. 325-8.
159. van Uitert, E.M., N. van der Elst-Otte, J.J. Wilbers, et al., *Periconception maternal characteristics and embryonic growth trajectories: the Rotterdam Predict study.* Hum Reprod, 2013. 28(12): p. 3188-96.
160. Robinson, H.P., *Sonar measurement of fetal crown-rump length as means of assessing maturity in first trimester of pregnancy.* Br Med J, 1973. 4(5883): p. 28-31.
161. Coutinho, R., R.J. David, and J.W. Collins, Jr., *Relation of parental birth weights to infant birth weight among African Americans and whites in Illinois: a transgenerational study.* Am J Epidemiol, 1997. 146(10): p. 804-9.
162. Little, R.E., *Mother's and father's birthweight as predictors of infant birthweight.* Paediatr Perinat Epidemiol, 1987. 1(1): p. 19-31.
163. Magnus, P., H.K. Gjessing, A. Skrondal, and R. Skjaerven, *Paternal contribution to birth weight.* J Epidemiol Community Health, 2001. 55(12): p. 873-7.
164. Mattsson, K. and L. Rylander, *Influence of maternal and paternal birthweight on offspring birthweight - a population-based intergenerational study.* Paediatr Perinat Epidemiol, 2013. 27(2): p. 138-44.
165. Franklin, T.B., H. Russig, I.C. Weiss, et al., *Epigenetic transmission of the impact of early stress across generations.* Biol Psychiatry, 2010. 68(5): p. 408-15.
166. Klebanoff, M.A., *Paternal and maternal birthweights and the risk of infant preterm birth.* Am J Obstet Gynecol, 2008. 198(1): p. 58 e1-3.
167. Winikoff, B. and C.H. Debrovner, *Anthropometric determinants of birth weight.* Obstet Gynecol, 1981. 58(6): p. 678-84.
168. Rice, F. and A. Thapar, *Estimating the relative contributions of maternal genetic, paternal genetic and intrauterine factors to offspring birth weight and head circumference.* Early Hum Dev, 2010. 86(7): p. 425-32.
169. Zhang, J. and J.M. Ratcliffe, *Paternal smoking and birthweight in Shanghai.* Am J Public Health, 1993. 83(2): p. 207-10.
170. Soubry, A., J.M. Schildkraut, A. Murtha, et al., *Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort.* BMC Med, 2013. 11: p. 29.
171. de Mouzon, J., V. Goossens, S. Bhattacharya, et al., *Assisted reproductive technology in Europe, 2007: results generated from European registers by ESHRE.* Hum Reprod, 2012. 27(4): p. 954-966.
172. Jackson, R.A., K.A. Gibson, Y.W. Wu, and M.S. Croughan, *Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis.* Obstet Gynecol, 2004. 103(3): p. 551-63.

173. Wisborg, K., H.J. Ingerslev, and T.B. Henriksen, *In vitro fertilization and preterm delivery, low birth weight, and admission to the neonatal intensive care unit: a prospective follow-up study.* Fertil Steril, 2010. 94(6): p. 2102-6.
174. Mantikou, E., M.A. Youssef, M. van Wely, et al., *Embryo culture media and IVF/ ICSI success rates: a systematic review.* Hum Reprod Update, 2013. 19(3): p. 210-20.
175. Rousian, M., C.M. Verwoerd-Dikkeboom, A.H. Koning, et al., *Early pregnancy volume measurements: validation of ultrasound techniques and new perspectives.* BJOG, 2009. 116(2): p. 278-85.
176. Macklon, N.S. and B.C. Fauser, *Impact of ovarian hyperstimulation on the luteal phase.* J Reprod Fertil Suppl, 2000. 55: p. 101-8.
177. Tavaniotou, A., J. Smitz, C. Bourgain, and P. Devroey, *Ovulation induction disrupts luteal phase function.* Ann N Y Acad Sci, 2001. 943: p. 55-63.
178. Bourgain, C. and P. Devroey, *The endometrium in stimulated cycles for IVF.* Hum Reprod Update, 2003. 9(6): p. 515-22.
179. Cooper, A.R., K.E. O'Neill, J.E. Allsworth, et al., *Smaller fetal size in singletons after infertility therapies: the influence of technology and the underlying infertility.* Fertil Steril, 2011. 96(5): p. 1100-6.
180. Conway, D.A., J. Liem, S. Patel, K.J. Fan, J. Williams, 3rd, and M.D. Pisarska, *The effect of infertility and assisted reproduction on first-trimester placental and fetal development.* Fertil Steril, 2011. 95(5): p. 1801-4.
181. Rolo, L.C., L.M. Nardozza, E. Araujo Junior, P.M. Nowak, J. Bortoletti Filho, and A.F. Moron, *Measurement of embryo volume at 7-10 weeks' gestation by 3D-sonography.* J Obstet Gynaecol, 2009. 29(3): p. 188-91.
182. Bagratee, J.S., L. Regan, V. Khullar, C. Connolly, and J. Moodley, *Reference intervals of gestational sac, yolk sac and embryo volumes using three-dimensional ultrasound.* Ultrasound Obstet Gynecol, 2009. 34(5): p. 503-9.
183. Blaas, H.G., S.H. Eik-Nes, and J.B. Bremnes, *The growth of the human embryo. A longitudinal biometric assessment from 7 to 12 weeks of gestation.* Ultrasound Obstet Gynecol, 1998. 12(5): p. 346-54.
184. Blaas, H.G., S.H. Eik-Nes, S. Berg, and H. Torp, *In-vivo three-dimensional ultrasound reconstructions of embryos and early fetuses.* Lancet, 1998. 352(9135): p. 1182-6.
185. Aviram, R., D.K. Shpan, O. Markovitch, A. Fishman, and R. Tepper, *Three-dimensional first trimester fetal volumetry: comparison with crown rump length.* Early Hum Dev, 2004. 80(1): p. 1-5.
186. Ceelen, M., M.M. van Weissenbruch, J.C. Roos, J.P. Vermeiden, F.E. van Leeuwen, and H.A. Delemarre-van de Waal, *Body composition in children and adolescents born after in vitro fertilization or spontaneous conception.* J Clin Endocrinol Metab, 2007. 92(9): p. 3417-23.
187. Ceelen, M., M.M. van Weissenbruch, J.P. Vermeiden, F.E. van Leeuwen, and H.A. Delemarre-van de Waal, *Cardiometabolic differences in children born after in vitro fertilization: follow-up study.* J Clin Endocrinol Metab, 2008. 93(5): p. 1682-8.

AUTHORS AND AFFILIATIONS

Erasmus MC, University Medical Centre, Rotterdam, The Netherlands

Department of Obstetrics and Gynaecology
Prof.dr. R.P.M. Steegers-Theunissen
Prof.dr. J.S.E. Laven
Prof.dr. E.A.P. Steegers
Prof.dr. G.J. Bonsel
Dr. N. Exalto
Dr. A.H.J. Koning
Drs. H. Bogers
Drs. I.A.L. Groenenberg
Drs. J.J. Wilbers
Drs. N. van der Elst-Otte
Drs. S. van Ginkel
Drs. E.D.H. van der Mooren

Department of Biostatistics
Prof.dr. P.H.C. Eilers
Drs. S.P. Willemsen

Department of Bioinformatics
Dr. A.H.J. Koning

Department of Public Health
Drs. G.J.J.M. Borsboom

Department of Clinical Chemistry
Prof.dr. J. Lindemans

Centre for Trophoblast Research, University of Cambridge, Cambridge, UK

Prof.dr. G.J. Burton

ABBREVIATIONS

3D	Three-dimensional
AC	Abdominal circumference
BPD	Biparietal diameter
BW	Birth weight
CI	Confidence interval
CRL	Crown-rump length
EV	Embryonic volume
FA	Folic acid
FL	Femur length
GA	Gestational age
HC	Head circumference
ICC	Intraclass correlation coefficient
ICSI	Intracytoplasmic sperm injection
IGF	Insulin-like growth factor
IQR	Interquartile range
IVF	In vitro fertilization
LBW	Low birth weight
LMP	Last menstrual period
NTD	Neural tube defect
OR	Odds ratio
RBC	Red blood cell
SD	Standard deviation
SGA	Small for gestational age
TAL	Total arc length

BIBLIOGRAPHY

This thesis

Chapter 1

van Uitert, E. M., Exalto, N., Burton, G. J., Willemsen, S. P., Koning, A. H. J., Eilers, P. H. C., Laven, J. S. E., Steegers, E. A. P., Steegers-Theunissen, R. P. M., Human embryonic growth trajectories and associations with fetal growth and birthweight. Hum Reprod, 2013. 28(7): p. 1753-61.

Chapter 2

Bogers, H., van Uitert, E. M., van Ginkel, S., van der Mooren, E., Groenenberg, I. A. L., Eilers, P. H. C., Exalto, N., Steegers, E. A. P., Steegers-Theunissen, R. P. M., First trimester human embryonic curvature measurements using 3D ultrasound. Submitted for publication.

Chapter 3

van Uitert, E. M. and R. P. M. Steegers-Theunissen, Influence of maternal folate status on human fetal growth parameters. Mol Nutr Food Res, 2013. 57(4): p. 582-95.

Chapter 4

van Uitert, E. M., van Ginkel, S., Willemsen, S. P., Lindemans, J., Koning, A. H. J., Eilers, P. H. C., Exalto, N., Laven, J. S. E., Steegers, E. A. P., Steegers-Theunissen, R. P. M., An optimal periconception maternal folate status for embryonic size: The Rotterdam Predict study. BJOG 2014; Feb 12. Epub ahead of print.

Chapter 5

van Uitert, E. M., van der Elst-Otte, N., Wilbers, J. J., Exalto, N., Willemsen, S. P., Eilers, P. H., Koning, A. H., Steegers, E. A., Steegers-Theunissen, R. P., Periconception maternal characteristics and embryonic growth trajectories: the Rotterdam Predict study. Human Reprod, 2013. 28(12): p. 3188-96.

Chapter 6

van Uitert, E. M., Steegers, E. A. P., Bonsel, G. J., Borsboom, G. J. J. M., Koning, A. H. J., Laven, J. S. E., Exalto, N., Steegers-Theunissen, R. P. M., Human embryonic growth trajectories: does the father matter? The Rotterdam Predict study. Submitted for publication.

Chapter 7

Eindhoven, S. C., van Uitert, E. M., Laven, J. S. E., Willemsen, S. P., Koning, A. H. J., Eilers, P. H. C., Exalto, N., Steegers E. A. P., Steegers-Theunissen, R. P. M., The influence of IVF/ ICSI treatment on human embryonic growth trajectories: the Rotterdam Predict Study. Submitted for publication.

Other publications

Smedts, H. P. M., van Uitert, E. M., Valkenburg, O., Laven, J. S. E., Eijkemans, M. J. C., Lindemans, J., Steegers, E. A. P., Steegers-Theunissen, R. P. M., A derangement of the maternal lipid profile is associated with an elevated risk of congenital heart disease in the offspring. Nutr Metab Cardiovasc Dis, 2012. 22(6): p. 477-85.

PHD PORTFOLIO

General courses

Biomedical English Writing and Communication	2010
Introduction to data-analysis	2010
Classical data-analysis	2010
Courses for the quantitative researcher	2010
Repeated measurements in clinical studies	2011

Seminars and workshops

Wladimiroff Wetenschapsdag, Rotterdam, The Netherlands	2009-2012
Weekly research meetings, department of Obstetrics and Prenatal Medicine, Rotterdam, The Netherlands	2009-2013
Weekly research meetings, department of Fertility, Rotterdam, The Netherlands	2009-2013
Wetenschapslunch cluster 12, Rotterdam, The Netherlands	2009-2013
PhD day	2010

Presentations

Reprotox meeting, Soest, The Netherlands. Oral presentation.	2009
Research meeting department of Obstetrics and Prenatal Medicine, Rotterdam, The Netherlands. Oral presentations.	2009-2013
Research meeting department of Fertility, Rotterdam, The Netherlands. Oral presentations.	2009-2013
Wladimiroff Award, Rotterdam, The Netherlands. Oral presentation.	2010
Reprotox meeting, Soest, The Netherlands. Oral presentation.	2011
Symposium Jonge Zwangerschap, Hoofddorp, The Netherlands. Oral presentation.	2011
Society of Gynecological Investigations, Miami, USA. Poster presentation.	2011
Society of Gynecological Investigations, San Diego, USA. Poster presentation.	2012
European Periconception Congress, Rotterdam, The Netherlands. Oral presentation.	2012
Symposium Jonge Zwangerschap, Rotterdam, The Netherlands. Oral presentation.	2012

Supervising students

Jet Wilbers, 4th year medical student, Master Thesis	2009-2010
Evelien Broekman, 6th year medical student, extracurricular	2010
Jairo Avella, ERACOLE student	2010
Adriënne Eggens, 6th year medical student, Master Thesis	2011
Sharon van Ginkel, 4th year medical student, Master Thesis	2011
Elisabeth van der Mooren, 6th year medical student, Master Thesis	2012
Stephanie Eindhoven, master student in Clinical Research, Master Thesis	2010-2012

ABOUT THE AUTHOR

Evelyne van Uitert was born in 1983 in Stanford, USA. After her first years in California, USA and Hamburg, Germany she grew up in Delden, the Netherlands. After attending middle school in Delden and high school in Hengelo, she returned to the USA in 2001 for a college year at Alma College, Michigan. Back in the Netherlands she was not selected in the lottery to enrol in medicine the first time and began the study of psychology at the University of Utrecht. The following year she was accepted as a medical student at the Erasmus University Medical Centre in Rotterdam. After graduation in 2009 she began studying towards PhD degree at the department of Obstetrics & Gynaecology at the Erasmus MC under the supervision of prof.dr. R.PM. Steegers-Theunissen and prof.dr. J.S.E. Laven. In 2013 she began working as a resident in Obstetrics & Gynaecology at the Albert Schweitzer Hospital in Dordrecht (mw. L.N. Hofman).

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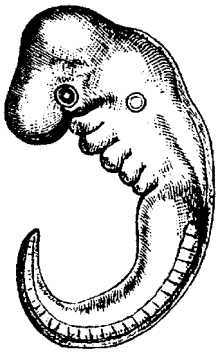

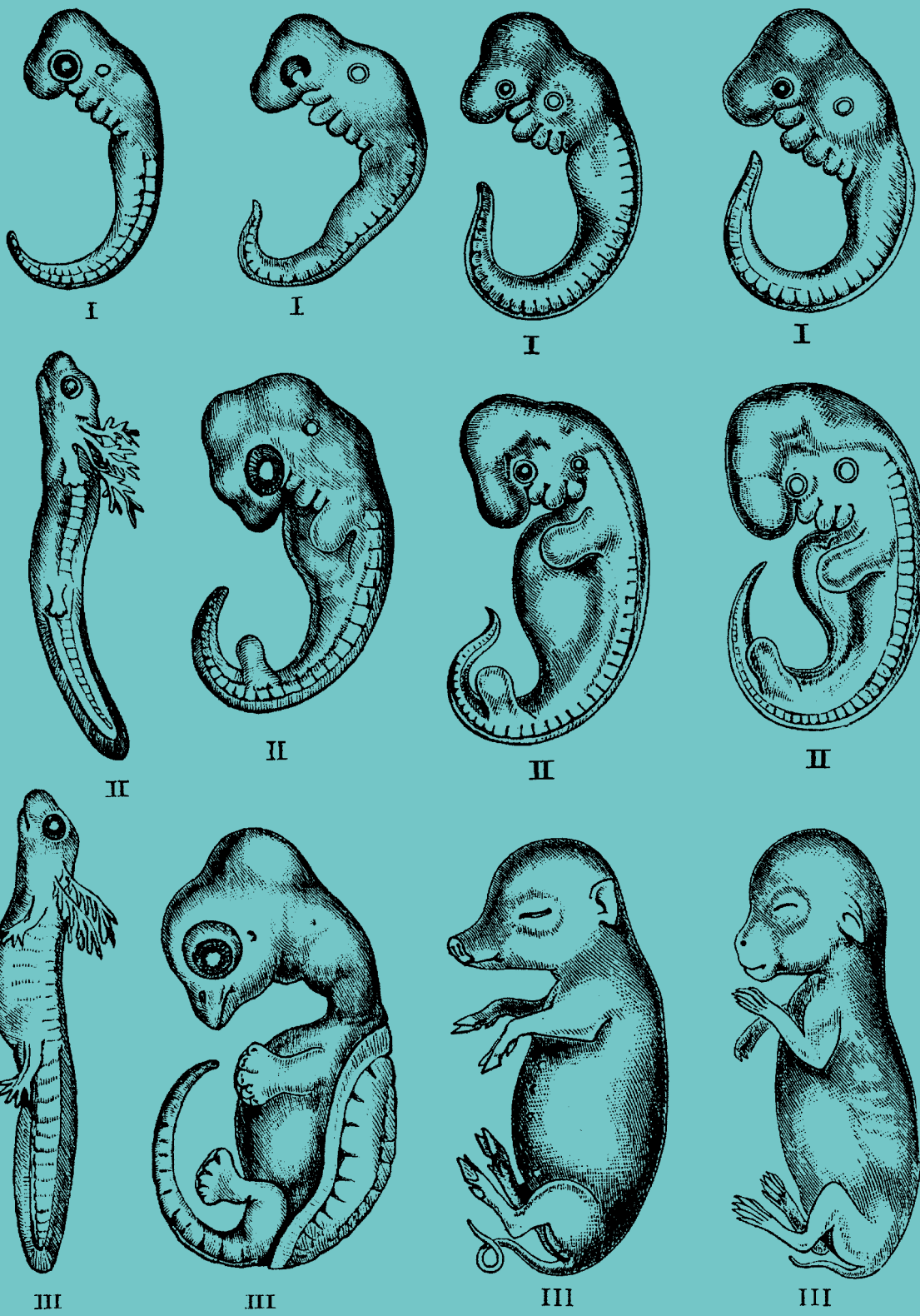
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