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

Running title: One-carbon metabolism and Carnegie stages

F. Parisi[1], M. Rousian[1], A.H.J. Koning[2], S.P. Willemsen[1,3], I. Cetin[4], R.P.M. Steegers-Theunissen[1,5*]

1. Department of Obstetrics and Gynaecology, Erasmus MC, University Medical Centre, PO Box 2040, 3000CA, Rotterdam, The Netherlands
2. Department of Bioinformatics, Erasmus MC, University Medical Centre, PO Box 2040, 3000CA, Rotterdam, The Netherlands
3. Department of Biostatistics, Erasmus MC, University Medical Centre, PO Box 2040, 3000CA, Rotterdam, The Netherlands
4. Centre for Fetal Research Giorgio Pardi, Department of Biomedical and Clinical Sciences, Hospital Luigi Sacco, Università degli Studi di Milano, 20157 Milan, Italy
5. Department of Pediatrics, Division of Neonatology, Erasmus MC, University Medical Centre, PO Box 2040, 3000CA, Rotterdam, The Netherlands

*Correspondence address

Department of Obstetrics and Gynaecology, Erasmus MC, University Medical Centre, PO Box 2040, 3000CA, Rotterdam, The Netherlands. Tel: 0031-10-7038256; Fax: 0031-10-7036815; E-mail: r.steegers@erasmusmc.nl

ABSTRACT

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

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

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

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

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

Main results and the role of chance: We performed a median of three 3D US scans per pregnancy (range 1-5) resulting in 600 good quality datasets for the Carnegie stage annotation (80.5%). Vitamin B12 was positively associated with embryonic development in the total study population (β=0.001 (95% CI: 0.000; 0.002), $p<0.05$) and in the subgroup

of strictly dated spontaneous pregnancies (β=0.002 (95% CI: 0.001; 0.003), p<0.05). Low vitamin B12 concentrations (-2 standard deviation (SD), 73.4 pmol/l) are associated with delayed embryonic development by 1.4 days (95% CI: 1.3-1.4) compared to high concentrations (+2SD, 563.1 pmol/l). RBC folate was positively associated with Carnegie stages only in IVF/ICSI pregnancies (β=0.001 (95% CI: 0.0005; 0.0015), p<0.05). Low RBC folate concentrations (-2SD, 875.4 nmol/l) were associated with a 1.8-day delay (95% CI: 1.7-1.8) in development compared to high concentrations (+2SD, 2119.9 nmol/l). tHcy was negatively associated with embryonic development in the total study population (β = -0.08 (95% CI: -0.14; -0.02), p<0.01), as well as in the IVF/ICSI subgroup (β= -0.08 (95% CI: -0.15; -0.01), p<0.05). High tHcy concentrations (+2SD, 10.4 µmol/l) were associated with a delay of 1.6 days (95% CI: 1.5-1.7) in embryonic development compared to low concentrations (-2 SD, 3.0 µmol/l).

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

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

Study funding/competing interest(s): The work was funded by the Department of Obstetrics and Gynaecology, Erasmus MC, University Medical Centre, Rotterdam, The Netherlands. RPMST is CSO of the startup company Slimmere Zorg and CEO of eHealth Care Solutions. The authors declare no conflicts of interest.

Trial registration number: not applicable.

Key words: Carnegie stage, maternal one-carbon metabolism, homocysteine, folate, vitamin B12.

INTRODUCTION

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

The introduction of high resolution three-dimensional ultrasound (3D US) scans combined with visualization in immersive virtual reality (VR) systems, providing real depth perception and more sensitive embryonic size measurements and morphological evaluations, has markedly improved the opportunity to accurately study the periconceptional period (time window: 14 weeks pre-conception to 10 weeks post-conception) (Rousian et al., 2010; Baken et al., 2014; Steegers-Theunissen et al., 2013). So far these innovative techniques were used to study embryonic crown-rump length

(CRL) and volume (EV) trajectories as non-invasive measures of first trimester embryonic growth (Steegers-Theunissen et al., 2016). On the other hand, the Carnegie stages of human embryonic development were introduced as a century old morphological classification of fixated embryos dividing the embryonic period (58 post-conceptional days) into 23 stages (O'Rahilly et al., 1987). The combination of 3D US and VR visualization will allow us to investigate embryonic morphological development *in vivo,* according to the longitudinal annotation of the Carnegie stages (O'Rahilly and Müller, 2010; Blaas et al., 1998; Verwoerd-Dikkeboom et al., 2008). Despite the fact that the normal sequence of developmental events is constant and predictable in every embryo, different times and velocities can occur, making comparisons possible and worthwhile.

Here, we aim to investigate the associations between periconceptional maternal biomarkers of I-C metabolism and first trimester embryonic development, using serial Carnegie stage annotation obtained by 3D US and VR.

MATERIALS AND METHODS

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

Study population and sample

All women before 8^{+0} weeks of gestation who conceived spontaneously, or after intrauterine insemination (IUI), *in vitro* fertilization (IVF), intracytoplasmatic sperm injection (ICSI) or cryopreserved embryo transfer, were eligible for participation between

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

General data

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

Blood sample analysis

One first trimester fasting venous blood sample for serum vitamin B12, RBC folate and plasma tHcy assessment was collected at enrolment and drawn in a vacutainer ethylenediamine tetraacetate (EDTA) tube and in a dry vacutainer tube (BD diagnostics, Plymouth, UK). The dry vacutainer tubes were centrifuged at 2,000 xg, serum was collected and analyzed for vitamin B12 measurement using an immunoelectro-chemoluminescence assay (E170; Roche Diagnostics GmbH, Mannheim, Germany). Plasma was separated by centrifugation within one hour for determination of tHcy by using a sensitive liquid chromatography tandem mass spectrum method (HPLC-Tandem MS, Waters Micromass Quattro Premier XE Mass Spectrometer with Acquity UPLC system, Milford, Massachusetts, United States). EDTA-blood was kept on ice and 0.1 ml EDTA blood was hemolysed with 0.9 ml freshly prepared 1.0% ascorbic acid. The hematocrit was determined with the ADVIA 120 Hematology Analyzer (Bayer Diagnostics, Leverkusen, Germany). RBC folate was calculated with the following formula: (nM hemolysate folate × 10/hematocrit) - [nM serum folate × (1- hematocrit) /hematocrit] = nM RBC folate.

Ultrasound data

From 6^{+0} up to 10^{+2} weeks of gestation, all included women underwent serial 3D US scans performed by trained researchers using the high frequency (4.5 – 11.9 MHz) vaginal

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

Statistical analysis

In order to evaluate selection bias, we compared maternal baseline characteristics and biomarker concentrations between excluded and included pregnancies using Chi-square or exact tests for ordinal variables and Mann-Whitney U test for continuous variables. Univariable linear regression was performed to evaluate associations between maternal baseline characteristics and biomarker concentrations.

To estimate associations between maternal biomarkers of I-C metabolism and embryonic development, we treated the Carnegie stages as a continuous variable that was censored at its maximum value of 23. This was used as the response variable in separate linear mixed models estimated for the total study population and secondly for the subgroups of strictly dated spontaneous and IVF/ICSI pregnancies. This analysis allows the linear modelling of longitudinal measurements, taking into account the existing correlation between serial measurements within the same pregnancy and potential confounders for adjustment (parity, alcohol use, smoking, folic acid/multivitamin supplement use, fetal gender, maternal age, BMI and comorbidity). Firstly, we performed a crude analysis with adjustment for gestational age only (model 1) and secondly we adjusted for additional confounders (model 2). Finally, the estimates of embryonic developmental change expressed in days were determined comparing women with high (+2 standard deviation (SD)) and low (-2 SD) concentrations of the biomarkers that were significantly associated with the Carnegie stages in model 2. Due to the exclusion of pregnancies with uncertain dating and the possibility of selection bias, we additionally performed a sensitivity analysis including pregnancies with discordant CRL (n=15). P-values ≤0.05 were considered significant. All analyses were performed using IBM SPSS version 21.0 (Armonk, NY: IBM Corp) and R version 3.2.1 (The R Foundation for Statistical Computing).

Ethical approval

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

RESULTS

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

Embryonic development

Embryonic development according to the Carnegie stages was comparable between the subgroups of strictly dated spontaneous and IVF/ICSI pregnancies (model 2, group effect: β= -0.20, (95% CI: -0.46; 0.05), p=0.12). Table II shows the estimates from linear mixed models. In model 2, vitamin B12 concentrations were positively associated with embryonic development in the total study population and in strictly dated spontaneous pregnancies, resulting in small, albeit significant estimates. In the total study population, low vitamin B12 concentrations (-2 SD, corresponding to 73.4 pmol/l) were associated

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

DISCUSSION

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

Our results are in line with previous data showing associations between maternal I-C metabolism and several reproductive, pregnancy and health outcomes (Solé-Navais et

al., 2016; Yajnik and Deshmukh, 2012). Recently, maternal early pregnancy high tHcy (≥8.31 µmol/L) and low folate concentrations (≤9.10 nmol/L) have been negatively associated with fetal growth parameters, finally affecting birth weight (Bergen et al., 2016). We also showed that an optimal periconceptional RBC folate level is associated with increased first trimester longitudinal CRL measurements compared to the lowest (β= 0.24 √mm (95%CI: 0.04; 0.44), p=0.02) and highest quartile of concentrations (β= 0.29 √mm (95%CI: 0.09; 0.49), p<0.01) (van Uitert et al., 2014). This result emphasizes that CRL accuracy in pregnancy dating is impacted by maternal I-C metabolism, as well as by several maternal characteristics and exposures (van Uitert et al., 2013b). Moreover, embryonic volume (EV) has been described as a more sensitive marker of first trimester growth restriction compared to CRL (Baken et al., 2013). We focused on the Carnegie stages as a century old classification that, together with embryonic size measurements, could implement first trimester investigation and better define a proper embryonic development. Since we excluded all pregnancies with congenital anomalies detected both *in utero* and after birth, our results indicate that even the developmental events of normal ongoing pregnancies are impacted by maternal I-C metabolism. This and previous findings indicate that first trimester growth and development are important embryonic outcomes affected by maternal environment. Nevertheless, CRL, EV and Carnegie stages also represent non-invasive reproducible markers with predictable associations with gestational age, leading to their potential use for pregnancy dating and raising the question which biomarker should be the best candidate (Robinson and Fleming, 1975; O'Rahilly and Müller, 2010). Due to the lack of an optimal pregnancy dating strategy and to unavoidable systematic errors related to the recall of the LMP, imprecise

ovulation/implantation dates and parental characteristics impacting embryonic ultrasound measurements, we defined gestational age based on a known LMP, regular cycle and concordant CRL. In this way, all ultrasound measurements could be read as response variables and outcome measurements. In order to reduce selection bias, we compared maternal baseline characteristics, showing that excluded women had a higher BMI, lower age and RBC folate concentrations. This may be mainly explained by the inclusion of a large population of subfertile women and pregnancies achieved after IVF/ICSI treatment (higher age, lower BMI, higher use of folic acid supplements). We also compared the subgroup of included and excluded spontaneous pregnancies showing indeed no significant results (data not shown). Moreover, the sensitivity analysis including pregnancies with discordant CRL confirmed the detected associations, reducing the possibility of selection bias.

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

297 embryo (Steegers-Theunissen et al., 2013). All these events may finally lead to impaired
298 first trimester development, thereby supporting our results.
299 Our findings also reveal that conception mode seems to modify the associations between
300 blood biomarkers and Carnegie stages, despite the fact that no differences in embryonic
301 development have been detected between the two subgroups. As expected, biomarker
302 concentrations differed between spontaneous and IVF/ICSI pregnancies. Besides higher
303 and longer preconceptional folic acid supplement use in the IVF/ICSI subgroup, also the
304 ovarian stimulation treatment may affect I-C blood biomarker concentrations (Boxmeer et
305 al., 2008). Moreover, the IVF/ICSI technique has been associated with different
306 epigenetic patterns, gene expression and preimplantation embryo phenotype compared
307 to spontaneous conception, possibly affecting embryonic responses to maternal I-C
308 biomarkers and explaining different associations detected in our results (Kroener et al.,
309 2016; Zandstra et al., 2015; Giritharan et al., 2007; Song et al., 2015).
310 The major strength of our study is the longitudinal evaluation of embryonic development
311 using a median of three scans per patient, the use of 3D US with VR visualization and the
312 consequent high success rate of the Carnegie stage assessment. This gives an accurate
313 and precise picture of the course of first trimester development. Confounding by
314 gestational age is minimized by including women with strict pregnancy dating only. The
315 high rate of folic acid supplement use, resulting in an extremely low rate of
316 hyperhomocysteinemia and high RBC folate concentrations, strongly underlines the
317 importance of our results, since even clinically normal values of tHcy and a non-deranged
318 I-C metabolism could impact embryonic development of non-malformed ongoing
319 pregnancies. The most relevant limitation of this study is related to the tertiary care

setting, resulting in expected high rates of folic acid supplement use, chronic comorbidity and pregnancy complications. This may reduce the external validity of our findings. Despite it is reassuring that significant associations were confirmed in IVF/ICSI pregnancies where conception date is known by definition, the implantation date is not known and systematic errors in pregnancy dating are expected. Therefore, it is also possible that the small differences detected in embryonic development reflect an impact on the timing of implantation.

Inadequacies in dietary B vitamins and lifestyle (i.e. smoke, alcohol and coffee consumption) have led to increased dangerous plasma tHcy concentrations in the last decades (Steegers-Theunissen et al., 2013). Our results suggest that this may negatively impact first trimester embryonic development resulting in the highest effect estimates in line with previous findings (Blanco et al., 2016; Steegers-Theunissen et al., 2013). Since plasma tHcy is an overall stable marker within the same individual and in uncomplicated pregnancies, a random periconceptional tHcy measurement is reflective of an individual's status and therefore could represent a potential useful predictor of embryonic development in a clinical setting (McKinley et al., 2001; López-Alarcón et al., 2015). Conversely, the small estimates detected for vitamin B12 and RBC folate may not address for their clinical use as embryonic development predictors. Nevertheless, while reduced CRL measurements have been associated with adverse pregnancy and health outcomes in the offspring (Mook-Kanamori et al., 2010; van Uitert et al., 2013c; Jaddoe et al., 2014), nothing is known about the clinical implications of first trimester developmental delay in ongoing pregnancies.

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

AUTHORS' ROLES

FP contributed to data collection, analysis and interpretation, she wrote the first draft and revised all versions of the manuscript; MR performed embryonic measurements; AHJK provided essential materials (V-scope software); SPW analyzed data and contributed to the interpretation of results; IC supervised the writing of the manuscript; RPMST had primary responsibility for final content, initiated the study and research questions and supervised and contributed to all aspects of the study. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We would thank all participating women and the whole Predict study group for the great effort they put into the Rotterdam Periconception cohort from 2009 and onwards.

FUNDING

The work was funded by the Department of Obstetrics and Gynaecology, Erasmus MC, University Medical Centre, Rotterdam, The Netherlands.

CONFLICT OF INTEREST

No conflict of interest has to be declared by any of the authors regarding the material discussed in the manuscript. RPMST is CSO of the startup company Slimmere Zorg and CEO of eHealth Care Solutions.

REFERENCES

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

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

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

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

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

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

385 Boxmeer JC, Steegers-Theunissen RP, Lindemans J, Wildhagen MF, Martini E, Steegers
386 EA, Macklon NS. Homocysteine metabolism in the pre-ovulatory follicle during ovarian
387 stimulation. *Hum Reprod* 2008;**23**:2570-2576.
388 Finkelstein JL, Layden AJ, Stover PJ. Vitamin B-12 and Perinatal Health. *Adv Nutr*
389 2015;**6**:552-563.
390 Giritharan G, Talbi S, Donjacour A, Di Sebastiano F, Dobson AT, Rinaudo PF. Effect of
391 in vitro fertilization on gene expression and development of mouse preimplantation
392 embryos. *Reproduction* 2007;**134**:63-72.
393 Hogeveen M, Blom HJ, den Heijer M. Maternal homocysteine and small-for-gestational-
394 age offspring: systematic review and meta-analysis. *Am J Clin Nutr* 2012;**1**:130-136.
395 Jakubowski H. Pathophysiological consequences of homocysteine excess. *J Nutr*
396 2006;**136**:1741S-1749S.
397 Jaddoe VW, de Jonge LL, Hofman A, Franco OH, Steegers EA, Gaillard R. First trimester
398 growth restriction and cardiovascular risk factors in childhood. *BMJ* 2014;**348**:g14.
399 Jia L, Li J, He B, Jia Y, Niu Y, Wang C, Zhao R. Abnormally activated one-carbon
400 metabolic pathway is associated with mtDNA hypermethylation and mitochondrial
401 malfunction in the oocytes of polycystic gilt ovaries. *Sci Rep* 2016;**6**:19436.
402 Kalhan SC. One carbon metabolism in pregnancy: Impact on maternal, fetal and neonatal
403 health. *Mol Cell Endocrinol* 2016. In press.
404 Koning AH, Rousian M, Verwoerd-Dikkeboom CM, Goedknegt L, Steegers EA, van der
405 Spek PJ. V-scope: design and implementation of an immersive and desktop virtual reality
406 volume visualization system. *Stud Health Technol Inform* 2009;**142**:136-138.

Kroener L, Wang ET, Pisarska MD. Predisposing Factors to Abnormal First Trimester Placentation and the Impact on Fetal Outcomes. *Semin Reprod Med* 2016;**34**:27-35.

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

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

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

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

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

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

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

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

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

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

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

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

Steegers-Theunissen RP, Verheijden-Paulissen JJ, van Uitert EM, Wildhagen MF, Exalto N, Koning AH, et al. Cohort Profile: The Rotterdam Periconceptional Cohort (Predict Study). *Int J Epidemiol* 2016;**45**:374-381.

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

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

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

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

van Uitert EM, Exalto N, Burton GJ, Willemsen SP, Koning AH, Eilers PH, et al. Human embryonic growth trajectories and associations with fetal growth and birthweight. *Hum Reprod* 2013c;**28**:1753-1761.

van Uitert EM, van Ginkel S, Willemsen SP, Lindemans J, Koning AH, Eilers PH, et al. An optimal periconception maternal folate status for embryonic size: the Rotterdam Predict study. *BJOG* 2014;**121**:821-829.

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

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

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

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

Yajnik CS, Chandak GR, Joglekar C, Katre P, Bhat DS, Singh SN, Janipalli CS, Refsum H, Krishnaveni G, Veena S, et al. Maternal homocysteine in pregnancy and offspring

birthweight: epidemiological associations and Mendelian randomization analysis. *Int J Epidemiol* 2014;**43**:1487-1497.

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

FIGURE LEGENDS

Figure 1. Flow chart of the study population. IUFD: intrauterine fetal death, US: ultrasound, LMP: last menstrual period, CRL: crown-rump length, IUI: intrauterine insemination, IVF: in vitro fertilization, ICSI: intracytoplasmatic sperm injection.

Figure 2. Average regression lines for vitamin B12 (A) and total homocysteine (tHcy) (B) concentrations in the total study population. In model 2, a low vitamin B12 (-2 standard deviation (SD), corresponding to 73.4 pmol/l) delays embryonic development by 1.4 days (95% CI: 1.3-1.4) compared to high concentrations (+2SD, 563.1 pmol/l). Conversely, high tHcy concentrations (+2SD, 10.4 µol/L) delay embryonic development by 1.6 days (95% CI: 1.5-1.7) compared to low concentrations (-2SD, 3.0 µmol/l). GA: gestational age.

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

491

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

492

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

493

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

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

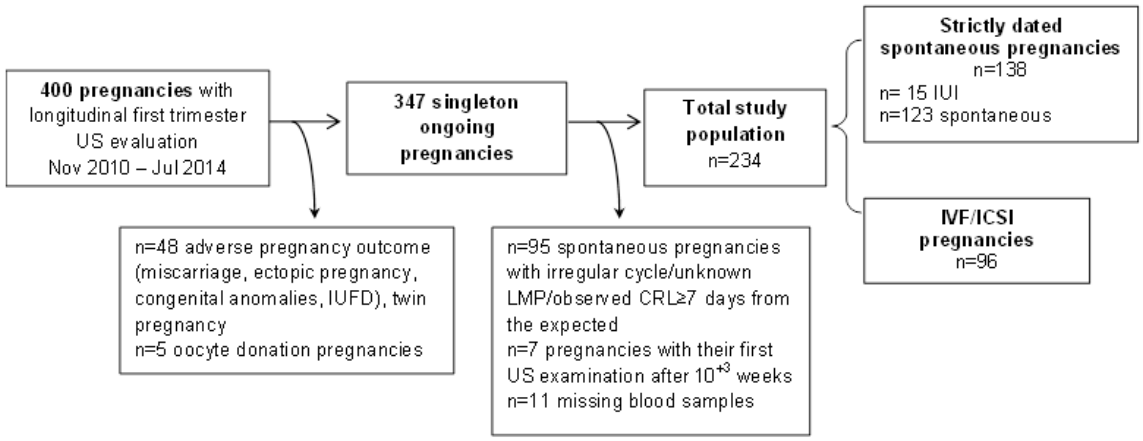
Effect estimates represent the change in Carnegie stage per unit of increase of biomarker concentration. Model 1 shows the crude model with adjustment for gestational age. Model 2 includes adjustment for potential confounders (parity, alcohol use, smoking habit, folic acid use, age, BMI, chronic diseases, fetal gender).

RBC: red blood cell, IVF: *in vitro* fertilization, ICSI: intracytoplasmatic sperm injection, tHcy: total homocysteine; CI: confidence interval.

*p<0.05, **p≤0.01

499 FIGURE 2

500

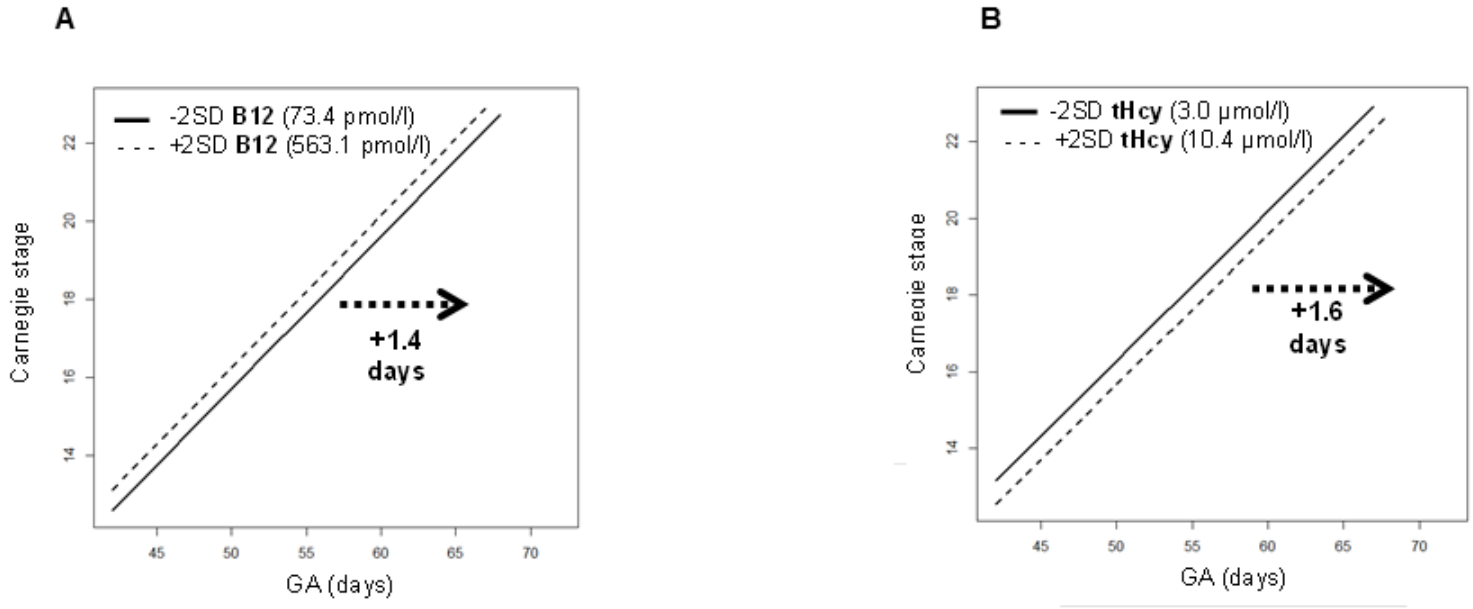


501

502

503 FIGURE 2

504



505