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ARTICLE

Periconceptional maternal folate status and the impact on embryonic head and brain structures: the Rotterdam Periconceptional Cohort

BIOGRAPHY

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KEY MESSAGE

U-shaped associations are shown between the periconceptional maternal red blood cell folate status and embryonic head structures. The clinical implication of these findings on neonatal brain development and functioning needs further investigation.

ABSTRACT

Research question: Does periconceptional maternal folate status influence the size of human embryonic head and brain structures?

Design: The study population was selected from the Rotterdam Periconceptional Cohort conducted at the Erasmus MC. Three-dimensional (3D) ultrasound scans were performed at 9 and 11 weeks of gestational age. Using 3D ultrasound datasets, head volume, head circumference, diencephalon (DTD), mesencephalon (MTD) and left/right telencephalon (TTL/TTR) measurements were performed offline using a virtual reality technique and specialized 3D software. Maternal venous blood samples were taken at study entry to determine red blood cell (RBC) folate. Linear regression models were applied to investigate associations between RBC folate status and embryonic head and brain structures adjusted for gestational age, alcohol use, smoking, maternal age and mode of conception.

Results: RBC folate measurements were available for 144 of the 166 singleton pregnancies eligible for analysis. RBC folate quartiles were defined: 466–1078 nmol/l (Q1), 1079–1342 nmol/l (Q2), 1343–1594 nmol/l (Q3), 1595–2919 nmol/l (Q4), with Q3 being used as reference. At 11 weeks of gestational age, head volume was largest in Q1 ($\beta = 0.866$; $P = 0.004$) and Q4 ($\beta = 0.764$; $P = 0.007$). In addition, head circumference at 11 weeks of gestational age was significantly larger in Q4 ($\beta = 2.745$; $P = 0.03$). There were no statistical significantly associations between the RBC folate quartiles and the sizes of the DTD, MTD, TTL and TTR.

Conclusions: U-shaped associations were shown between the periconceptional maternal RBC folate status and embryonic head volume and head circumference. The clinical implication of these findings needs further investigation.

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KEYWORDS

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Virtual reality

INTRODUCTION

Pregnancy is defined as a period of rapid growth and cell development, during which major changes take place to create a safe environment for the unborn child. Fetal growth and development are susceptible to multiple environmental factors, for example maternal factors such as smoking, alcohol use, mode of conception and natural food folate and synthetic folic acid intake (*Koning et al., 2016*; *Mook-Kanamori et al., 2010*; *Van Dijk et al., 2018*; *van Uitert et al., 2013*).

The development of the growing fetus, especially the brain, is complex. During pregnancy, brain development is one of the earliest processes in embryogenesis, while it is also the last to be completed after birth. The volume of the brain increases dramatically during pregnancy; between the embryonic period and birth there is nearly a 40-fold increase in the weight of the brain (*O'Rahilly and Muller, 2008*). The development starts with the differentiation of neural progenitor cells resulting in the formation of the neural tube, which closes in the third week after conception (*Carlson, 2004*). From that moment on the brain undergoes major structural changes. This complex process is highly dependent on the expression of specific combinations of Hox genes and other transcription factors, and therefore very susceptible to changes in DNA methylation patterns (epigenetics), possibly leading to permanent modifications in gene expression and postnatal phenotypes (*Carlson, 2004*). DNA methylation and synthesis of RNA, lipids and proteins is highly dependent on one-carbon metabolism, an essential metabolism throughout human life for cellular growth and differentiation in general, and in particular highly important for the development of the growing fetus (*Steegers-Theunissen et al., 2013*). Natural folate and synthetic folic acid provide one-carbon moieties for cell multiplication and epigenetic programming. Studies have shown that adequate maternal folate status is associated with multiple beneficial pregnancy outcomes; it is associated with a reduction of neural tube defects and fetal growth restriction (*van Uitert and Steegers-Theunissen, 2013*; *van Uitert et al., 2014*), and neurodevelopmental outcome in later life (*Julvez et al., 2009*; *Roza et al., 2010*; *Veena et al., 2010*).

Measurements of plasma/serum folate reflect the short-term folate status influenced by food consumption, multivitamins and folate pills (*Piyathilake et al., 2007*). However, red blood cell (RBC) folate is a biomarker of the long-term folate status of the previous 2–4 months (*Piyathilake et al., 2007*). Two recent studies showed associations between maternal RBC folate status and embryonic growth depicted by serial crown rump length (CRL) measurements and cerebellum growth in the first trimester (*Koning et al., 2015*; *van Uitert et al., 2014*). These studies divided the RBC folate values into quartiles and showed that RBC folate in the third quartile was significantly associated with increased growth of the embryo (*van Uitert et al., 2014*) and cerebellum (*Koning et al., 2015*). New developments, such as three-dimensional ultrasound (3D-US) techniques, make it possible to perform measurements of the embryonic brain and head with higher precision (*Verwoerd-Dikkeboom et al., 2008*). In addition, it is also possible to determine the embryonic volume using virtual reality techniques, providing a more sensitive way to determine embryonic neurodevelopmental growth (*Verwoerd-Dikkeboom et al., 2008*).

Because major changes in the development of the brain and head take place during the first trimester of pregnancy, this period is of great importance. Therefore, it was postulated that the growth and development of the embryonic head and brain structures in the first trimester are influenced by the maternal folate status. This study investigates whether periconceptional RBC folate status of the mother is associated with first trimester head and brain structure measurements using 3D ultrasound and virtual reality techniques.

MATERIALS AND METHODS

Study population

The study population was selected from the Rotterdam Periconceptional Cohort (Predict Study), an ongoing prospective cohort study investigating the influence of periconceptional gene–environment interactions on parental health, embryonic and fetal development and pregnancy outcomes at the Department of Obstetrics and Gynecology of the Erasmus MC, University Medical Center, Rotterdam, the Netherlands (*Rousian et al., 2021*; *Steegers-Theunissen et al., 2016*). Approval for the study was obtained from the regional Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center, Rotterdam, on 15 October 2004 (MEC 2004-227). Exclusion criteria for the current study were defined as miscarriages, intrauterine fetal death, neonatal death, termination of pregnancy, ectopic pregnancy, congenital anomalies, twin pregnancies, oocyte donation and missing questionnaire and ultrasound data. The study population included naturally conceived pregnancies and pregnancies initiated by assisted reproductive techniques, including IVF with or without intracytoplasmic sperm injection (ICSI) or intrauterine insemination (IUI). Gestational age was calculated according to the last menstrual period (LMP) for natural pregnancies (adjusted for the duration of the menstrual cycle if <25 or >32 days), from the LMP or insemination date plus 14 days for IUI pregnancies or according to the conception date (embryo transfer date for pregnancies conceived after IVF and/or ICSI). It is known that this method might be somewhat unusual for naturally conceived pregnancies; however, the reason for choosing this method is that in investigating the impact of exposures on very small biological differences in embryonic size and growth, the outcome cannot be considered as uniform. When correcting gestational age for CRL, regression to the mean will be established and early onset growth differences cannot be investigated. Information on pregnancy outcomes was collected from the routine second trimester structural anomaly scan and the medical records. Information on maternal characteristics, and dietary and lifestyle habits was collected through self-reported questionnaires. In addition, medical reports were the main source for pregnancy complications, congenital malformations and neonatal outcome data. The first visit in this prospective cohort study was always before 9+6 weeks of gestational age, depending on the time of the first ultrasound.

Ultrasound data

The 3D-US scans were performed on the Voluson E8 system (GE Medical Systems, Zipf, Austria) using a 6–12 MHz transvaginal transducer at 9 and 11 weeks of gestational age. Ultrasound examinations were performed using the 'ALARA principle' (As Low As Reasonably Achievable): the total scanning time was kept below 45 min and the thermal index and mechanical index were kept

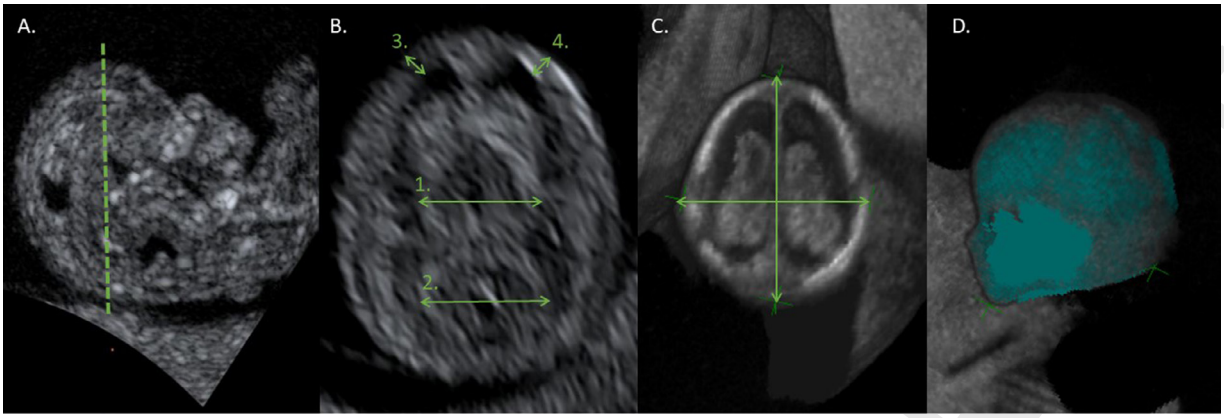

FIGURE 1 Embryonic brain and head measurements. (A) Mid-sagittal plane with diagrammatic reproduction of the position in which the axial plane is made to perform the embryonic brain measurements, 9 weeks of gestational age. (B) Axial plane, 9 weeks of gestational age; 1 = DTD, 2 = MTD, 3 = TTL, 4 = TTR. (C) BPD and OFD measurements in the axial plane. Callipers for measuring the BPD were placed perpendicular to the midline and the OFD callipers were placed along the midline. (D) Head volume after segmentation; hyperechoic structures were segmented selecting an upper (255) and lower (60) grey level threshold and an upper (80–90) threshold for the SD, before placing the seed point. Hypoechoic regions including the brain ventricles were directly supplemented with voxels by using a virtual 'brush'. BPD = biparietal diameter; DTD = diencephalon total diameter; MTD = mesencephalon total diameter; OFD = occipital frontal diameter; TTL = telencephalon thickness left; TTR = telencephalon thickness right.

within advised ranges as described in the safety statements of the International Society of Ultrasound in Obstetrics and Gynecology, to ensure safety (*Salvesen et al., 2021*). When performing Doppler ultrasound, exposure time was kept as short as possible (usually no longer than 5–10 min) (*Salvesen et al., 2021*). The offline embryonic head measurements were performed using the desktop version of the BARCO I-Space virtual reality system. Embryonic brain measurements were performed offline using specialized 3D software (4D View, version 5.0, GE Medical Systems). The head and brain measurements could only be performed when the quality of the ultrasound was high enough; this required 3D-US datasets without motion artefacts and clear demarcations of the embryonic head and brain structures. The techniques and reliability of these embryonic head and brain measurements have been described and published previously by the study group (*Gijtenbeek et al., 2014*; *Koning et al., 2016*).

As mentioned previously, embryonic head volume and head circumference measurements were performed using the virtual reality desktop system. What was actually measured were biparietal diameter (BPD), occipital frontal diameter (OFD) and head volume. The head circumference was calculated using the BPD and OFD with the following formula (*Verwoerd-Dikkeboom et al., 2010*):

$$HC = \pi\left[0.75(BPD + OFD) - \sqrt{\frac{BPD \times OFD}{4}}\right]$$

Both lateral ventricles and the vertical midline were the reference points for the BPD and OFD and had to be visible in an axial plane to perform these measurements. BPD and OFD were both measured by placing callipers on the outer borders of the head. Callipers for measuring the BPD were placed perpendicular to the midline and the OFD callipers were placed along the midline (*Salomon et al., 2013*); these measurements are shown in FIGURE 1. The placement of the callipers was checked from different points of view. BPD measurements were verified in a coronal plane and OFD measurements in a mid-sagittal plane.

Head volume measurements were performed using the virtual reality application in a standardized manner as described in detail previously (*Koning et al., 2016*). Reference points, the lowest point of the chin and lowest point of the fourth ventricle in the mid-sagittal plane, are shown in FIGURE 1 and were determined to create a line between the head and the lower body part of the embryo. This line was formed to separate the head by removing the voxels below these two reference points. In order to measure head volume, hyperechoic structures were segmented using a region growing approach, selecting an upper (255) and lower (60) grey level threshold and an upper (80–90) threshold for the SD, before placing the seed point. In addition, hypoechoic regions including the brain ventricles were directly supplemented with voxels by using a virtual 'brush'. The head volume after segmentation is also shown in FIGURE 1. In addition, the embryonic volume measurements were performed using the virtual reality desktop system (*Rousian et al., 2010*).

The brain structure measurements were performed using specialized 3D software. Measurements of the brain, including diencephalon (DTD), mesencephalon (MTD) and left and right telencephalon (TTL/TTR), were also performed in an axial plane. First, the borders of the DTD and MTD were determined, and then the maximal thickness was measured using outer border callipers. The hemispheres of the telencephalon were measured in the same axial plane in a line of a 45-degree angle from the longitudinal axis of the embryonic brain (*Gijtenbeek et al., 2014*; *Husen et al., 2021*). The brain structures are shown in FIGURE 1.

The methods and reliability of the embryonic brain measurements and virtual reality head measurements have been described in detail previously by the study group, with intraclass correlation coefficient values of the inter- and intra-observer variability analysis above 0.98, representing very good reliability between the measurements (*Gijtenbeek et al., 2014*; *Koning et al., 2016*). To enhance precision of the measurements, one researcher was trained according to protocol and performed all measurements.

RBC folate

At the first visit, venous blood samples were obtained in a 8.5-ml Vacutainer ethylenediaminetetraacetic acid (EDTA)

tube (BD Diagnostics, Plymouth, Cornwall, UK) to determine the RBC folate status. After collecting the venous blood samples, the hemolysate was prepared by diluting 0.1 ml of full blood in 0.9 ml of freshly prepared 1.0% ascorbic acid. The haematocrit of the remaining EDTA full blood was determined on a Sysmex XE-2100 Hematology Analyser (Sysmex Europe GmbH, Norderstedt, Germany) and an electrochemiluminescence immunoassay (Modular E170, Roche GmbH, Mannheim, Germany) was used to measure the folate in serum. Before the folate analysis, the hemolysate was centrifuged at 1000*g* with a temperature condition of 18°C for 5 min. The RBC folate concentration was calculated using the hemolysate folate concentration with the following formula: (nmol/l hemolysate folate × 10/haematocrit) – [nmol/l serum folate × (1 – haematocrit)/ haematocrit] = nmol/l RBC folate.

Statistical analyses

Linear regression models to determine associations between RBC folate quartiles and embryonic head and brain measurements were applied using SPSS. *P*-values <0.05 were valued as statistically significant and a trend was defined by a *P*-value <0.1. Descriptive statistics calculated the general characteristics for the total study population. In addition, general characteristics of the study population were calculated, stratified by RBC folate quartiles after the quartiles were determined. Success rates, medians and ranges for all embryonic head and brain measurements per gestational age were evaluated. Two models were used to investigate the association between the RBC folate quartiles and the embryonic head and brain measurements. The first model was created using the embryonic head and brain measurements as outcome and the RBC folate quartiles as predictor adjusted for gestational age. The second model was adjusted for additional confounders: alcohol use, smoking, maternal age and mode of conception. In addition, the significant outcomes, in both models 1 and 2, were adjusted for embryonic volume, considered as an accurate measurement for embryonic growth (*Rousian et al., 2010*), to be able to differentiate whether differences in embryonic head and brain structures are due to differences in embryonic volume or due to the maternal RBC folate status entered in the model.

TABLE 1 GENERAL CHARACTERISTICS OF THE TOTAL STUDY POPULATION

Maternal characteristic	Total (*n* = 166)	Missing, *n* (%)
Age at enrolment, years	32.1 ± 4.8	3 (1.8)
Nulliparous	71 (42.8)	0 (0)
Geographical origin		
Dutch	124 (74.7)	
Other Western	10 (6.0)	1 (0.6)
Non-Western	31 (18.7)	
Pre-pregnancy BMI, kg/m^2, median (range)	23.0 (15.2–39.7)	10 (6.0)
Educational level		
Low	20 (12.0)	
Middle	64 (38.6)	1 (0.6)
High	81 (48.8)	
Mode of conception		0 (0)
Natural	116 (69.9)	
IVF/ICSI	50 (30.1)	
Folic acid supplement use, moment of initiation	160 (96.4)	0 (0)
Preconceptional	114 (68.7)	8 (4.8)
Postconceptional	44 (26.5)	
Periconceptional:		
Smoking, yes	28 (16.9)	2 (1.2)
Alcohol use, yes	45 (27.1)	3 (1.8)
Neonatal characteristics		
Birthweight, g, median (range)	3285 (665–4380)	2 (1.2)
Gestational age at birth, days, median (range)	273 (182–292)	2 (1.2)
Gender, male	83 (50)	3 (1.8)

Data are presented as mean ± SD or *n* (%) unless otherwise stated. Missing values are presented as numbers with corresponding percentage.

BMI = body mass index; IVF/ICSI = IVF with or without intracytoplasmic sperm injection.

RESULTS

Study population

This study included 175 pregnant women with available first trimester 3D-US scans. In total, nine participants were excluded according to the described exclusion criteria; 166 participants were eligible for analysis (Supplementary Figure 1). RBC folate measurements were available for 144 of the 166 singleton pregnancies eligible for analysis. General characteristics of the total study population are presented in TABLE 1.

RBC folate

A total of 135 women used the standard dosage of folic acid supplementation in the Netherlands, which is 0.4/0.5 mg/ day. Four women in the study population used 5.0 mg/day and five women did not use folic acid during pregnancy. The overall median (range) folate concentration was 1343 (466–2919) nmol/l. The distribution of the RBC folate quartiles in the study population was: quartile 1 (Q1) = 466–1078 nmol/l, quartile 2 (Q2) = 1079–1342 nmol/l, quartile 3 (Q3) = 1343–1594 nmol/l and quartile 4 (Q4) = 1595–2919 nmol/l. General characteristics of the study population stratified by RBC folate quartiles for the included pregnancies are represented in Supplementary Table 1. In the higher quartiles (RBC folate Q3 and Q4), preconceptional folic acid use was more common, namely around 90% (Supplementary Table 1). The time period of onset of supplementation corresponds to RBC folate concentrations: preconceptional initiation of folic acid supplement use = 1472 (846–2919) nmol/l; postconceptional initiation of folic acid supplement use = 1098 (466–1699) nmol/l.

Success rates and means of the measurements

The success rates and medians with their corresponding ranges

TABLE 2 ASSOCIATIONS BETWEEN RED BLOOD CELL FOLATE IN QUARTILES AND FIRST TRIMESTER EMBRYONIC HEAD VOLUME AND HEAD CIRCUMFERENCE

		Model 1			Model 2		
		β	95% CI	*P*-value	β	95% CI	*P*-value
HV	GA 9 weeks						
	RBC Q1	*0.169*	*–0.025, 0.363*	*0.09*	*0.195*	*–0.015, 0.405*	*0.07*
	RBC Q2	0.072	–0.134, 0.278	0.49	0.081	–0.150, 0.312	0.49
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	0.233	0.013, 0.452	0.04*	*0.239*	*–0.004, 0.481*	*0.05*
	GA 11 weeks						
	RBC Q1	0.735	0.169, 1.300	0.01*	0.866	0.286, 1.447	0.004*
	RBC Q2	0.422	–0.124, 0.967	0.13	*0.536*	*–0.052, 1.124*	*0.07*
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	0.661	0.116, 1.205	0.02*	0.764	0.209, 1.319	0.007*
HC	GA 9 weeks						
	RBC Q1	1.359	–0.962, 3.680	0.25	1.380	–1.107, 3.867	0.27
	RBC Q2	1.156	–1.312, 3.625	0.35	0.803	–1.926, 3.532	0.56
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	1.577	–1.049, 4.203	0.24	1.366	–1.500, 4.231	0.35
	GA 11 weeks						
	RBC Q1	0.458	–2.017, 2.933	0.71	0.973	–1.539, 3.486	0.44
	RBC Q2	–0.107	–2.495, 2.282	0.93	0.396	–2.151, 2.943	0.76
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	*2.162*	*–0.223, 4.547*	*0.07*	2.745	0.340, 5.149	0.03*

Depicted are the effect estimates of RBC folate in association with HV and HC using linear regression models. The effect estimates (β), 95% confidence interval (CI) and *P*-values are depicted for two separate models. Significant findings are marked with *. Trends $P < 0.1$ are depicted in italic.

Model 1: adjusted for gestational age.

Model 2: model 1 + adjustment for maternal age, smoking, alcohol use, IVF/ICSI.

CI = confidence interval; GA = gestational age; HC = head circumference; HV = head volume; ICSI = intracytoplasmic sperm injection; RBC Q = red blood cell folate in quartiles.

for the embryonic head and brain measurements per gestational age are shown in Supplementary Table 2. In total, 276 3D-US results were available to perform the embryonic head and brain measurements. Success rates of the head volume and head circumference measurements were at least 86.8%. Success rates of the DTD and MTD measurements were at least 66.7%, while those of the TTR and TTL measurements varied between 51.8% and 57.2%. Supplementary Table 3 shows the quality of the ultrasound measurements used.

Linear regression analyses

The results of the linear regression models of the association between the RBC folate quartiles and embryonic head and brain measurements are shown in TABLE 2 and TABLE 3, respectively. RBC folate Q3 was used as reference to compare with the other quartiles because it resulted in the lowest values in most of the embryonic measurement analyses. Model 1 adjusted for gestational age and model 2 adjusted for gestational age, maternal age, smoking, alcohol use and mode of conception. There is a distinction between the results, represented in different models, depending on the adjustment of confounders.

Head measurement outcomes

The linear regression model showed the smallest head volume and head circumference measurements in RBC folate Q3 (1343–1594 nmol/l) compared with Q1 (466–1078 nmol/l), Q2 (1079–1342 nmol/l) and Q4 (1595–2919 nmol/l) at both 9 and 11 weeks of gestational age in most cases. At 9 weeks of gestational age, a significantly larger head volume measurement in the upper RBC folate quartile (Q4) was shown in model 1 (β = 0.233, 95% CI 0.013, 0.452, $P = 0.04$); in model 2 the values were β = 0.239, 95% CI –0.004, 0.481, $P = 0.05$). At 11 weeks of gestational age, the significantly largest head volume measurements were shown in RBC folate Q1 in model 1 (β = 0.735, 95% CI 0.169, 1.300, $P = 0.01$) and the fully adjusted model 2 (β = 0.886, 95% CI 0.286, 1.447, $P = 0.004$). The second largest head volume measurements were shown in RBC folate Q4 in model 1 (β = 0.661, 95% CI 0.116, 1.205, $P = 0.02$) and in model 2 (β = 0.764, 95% CI 0.209, 1.319, $P = 0.007$). The significantly largest head circumference measurements occurred in RBC folate Q4 at 11 weeks of gestational age in the fully adjusted model 2 (β = 2.745, 95% CI 0.340, 5.149, $P = 0.03$).

Supplementary Table 4 shows the linear regression model outcomes of the head measurements (head volume and head circumference), adjusted for embryonic volume: at 9 weeks of gestational age

TABLE 3 ASSOCIATIONS BETWEEN RED BLOOD CELL FOLATE IN QUARTILES AND FIRST TRIMESTER EMBRYONIC BRAIN STRUCTURES; DIENCEPHALON, MESENCEPHALON, AND LEFT AND RIGHT TELENCEPHALON

		Model 1			Model 2		
		β	95% CI	*P*-value	β	95% CI	*P*-value
DTD	GA 9 weeks						
	RBC Q1	0.118	−0.191, 0.428	0.45	0.188	−0.146, 0.521	0.26
	RBC Q2	0.047	−0.253, 0.348	0.75	0.079	−0.266, 0.424	0.65
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	−0.040	−0.390, 0.310	0.82	−0.015	−0.404, 0.373	0.94
	GA 11 weeks						
	RBC Q1	*0.298*	*0.001, 0.596*	*0.05*	*0.301*	*−0.006, 0.609*	*0.06*
	RBC Q2	0.092	−0.194, 0.378	0.53	0.022	−0.283, 0.326	0.89
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	0.159	−0.127, 0.444	0.27	0.117	−0.178, 0.412	0.43
MTD	GA 9 weeks						
	RBC Q1	0.111	−0.147, 0.368	0.39	0.178	−0.094, 0.449	0.20
	RBC Q2	0.009	−0.242, 0.259	0.95	0.011	−0.270, 0.292	0.94
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	−0.045	−0.336, 0.247	0.76	−0.032	−0.348, 0.284	0.84
	GA 11 weeks						
	RBC Q1	0.061	−0.165, 0.287	0.59	0.031	−0.195, 0.257	0.79
	RBC Q2	0.117	−0.100, 0.334	0.29	0.041	−0.183, 0.264	0.72
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	0.066	−0.151, 0.283	0.55	0.005	−0.211, 0.221	0.96
TTR	GA 9 weeks						
	RBC Q1	−0.035	−0.111, 0.041	0.36	−0.046	−0.131, 0.039	0.28
	RBC Q2	0.009	−0.066, 0.085	0.81	0.000	−0.088, 0.089	0.99
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	−0.023	−0.121, 0.075	0.64	−0.035	−0.161, 0.091	0.58
	GA 11 weeks						
	RBC Q1	0.071	−0.033, 0.174	0.18	0.073	−0.034, 0.180	0.18
	RBC Q2	−0.009	−0.114, 0.096	0.86	−0.012	−0.126, 0.102	0.84
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	0.049	−0.050, 0.148	0.33	0.039	−0.061, 0.139	0.44
TTL	GA 9 weeks						
	RBC Q1	−0.010	−0.087, 0.068	0.80	−0.016	−0.099, 0.067	0.70
	RBC Q2	−0.024	−0.101, 0.052	0.53	−0.013	−0.100, 0.074	0.71
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	0.003	−0.097, 0.103	0.95	0.027	−0.096, 0.151	0.66
	GA 11 weeks						
	RBC Q1	0.073	−0.032, 0.176	0.17	0.067	−0.041, 0.176	0.22
	RBC Q2	0.042	−0.064, 0.145	0.43	0.010	−0.103, 0.122	0.86
	RBC Q3 (REF)	–	–	–	–	–	–
	RBC Q4	0.030	−0.074, 0.132	0.56	0.022	−0.081, 0.126	0.67

Depicted are the effect estimates of RBC folate status on DTD, MTD, TTR and TTL of the linear regression models. The effect estimates (β), 95% confidence interval (CI) and *P*-values are depicted for two separate models. Trends $P < 0.1$ are depicted in italic.

Model 1: adjusted for gestational age.

Model 2: model 1 + adjustment for maternal age, smoking, alcohol use, IVF/ICSI.

CI = confidence interval; DTD = diencephalon total diameter; GA = gestational age; ICSI = intracytoplasmic sperm injection; MTD = mesencephalon total diameter; TTL = telencephalon thickness left; RBC Q = red blood cell folate in quartiles; TTR = telencephalon thickness right.

a significantly largest head volume measurement in the upper quartile (Q4) was shown in model 1 (β = 0.113, 95% CI 0.002, 0.223, *P* = 0.046) and a trend was shown in model 2 (β = 0.110, 95% CI –0.008, 0.227, *P* = 0.07). At 11 weeks of gestational age a significantly largest head volume measurement in the lower RBC folate quartile (Q1) was shown in model 1 (β = 0.444, 95% CI 0.077, 0.811, *P* = 0.02) and model 2 (β = 0.409, 95% CI 0.015, 0.802, *P* = 0.042).

Brain measurement outcomes

As shown in TABLE 3, there was no statistically significant association between the different RBC folate quartiles and brain measurements, at both 9 and 11 weeks of gestational age in both models.

DISCUSSION

This explorative prospective cohort study, conducted at a tertiary hospital setting and including 144 pregnancies, showed that sizes of the human embryonic head (head volume, head circumference) are related to maternal RBC folate status. The different RBC folate quartiles showed a U-shaped association with the head measurements, with RBC folate Q3 as reference and thereby the smallest measurements and the largest measurements in Q1 and Q4. There was no statistically significant association between the different RBC folate quartiles and the sizes of the DTD, MTD, TTL and TTR, at both 9 and 11 weeks of gestational age. However, measurements of DTD and TTL at 11 weeks of gestational age showed approximately the same U-shaped trend between the variables as the previously discussed head measurements, with RBC folate Q3 as the variable showing the smallest measurements and Q1 showing the largest. TTR at 11 weeks of gestational age showed a similar pattern with the difference that the RBC folate Q2 provided the smallest measurements instead of Q3. Measurements of the DTD at 9 weeks of gestational age and MTD measurements at both 9 and 11 weeks of gestational age showed a totally different pattern, with the largest measurements in the two lower RBC folate quartiles. It was concluded that maternal RBC folate status is associated with head sizes. Additional adjustment for embryonic volume also shows a comparable U-shaped trend at 9 and 11 weeks of gestational age of the head volume measurements, although effect sizes were reduced and there were fewer statistically significant differences. No significant difference was shown between the head volume measurement in the upper quartile (Q4) and the reference quartile (Q3) in the adjusted model 2.

Strengths of this study are the prospective study design, the use of precise methods to measure the human embryonic head and brain structures (3D-US and virtual reality techniques), allowing longitudinal measurements of innovative, very small, embryonic brain markers like the volume of the embryonic head and biometric measurements of the embryonic brain. High success rates of head volume and head circumference (around 90%) and acceptable success rates were achieved for MTD and DTD, but TTL and TTR were particularly difficult to measure and high ultrasound quality was required to make measurements of these very small structures possible. The quality of the ultrasound datasets may be influenced by movement, shadowing and the body mass index of the mother. It should be mentioned that the brain structures are small, especially in the first trimester, and this is accompanied by small differences between the measurements. For that reason it is difficult to find differences between the different RBC folate quartiles and brain measurements.

The study population originates from the Rotterdam Periconceptional Cohort, a population that was collected from a tertiary hospital (Erasmus MC). A high number of patients used folic acid supplements before and/or during pregnancy (96.4%). The high percentage of assisted reproductive technique pregnancies in the study population (30.1%) can partially explain the high folate status, because these patients are mostly well prepared (preconceptional folic acid supplement use, healthy lifestyle and nutrition). Therefore it is important to perform more research to elucidate whether these outcomes are also applicable in the general population for external validity. Only 11 patients (6%) in the current study population had a rather low RBC folate status (<906 nmol/l) than believed to be optimal for the prevention of neural tube defects (*Daly et al., 1995*; *World Health Organization, 2015*). Furthermore, excessive concentrations of RBC folate are also considered to have an effect on neurodevelopmental outcome (*Barua, Kuizon, and Junaid, 2014*; *Gao et al., 2016*). Folate and synthetic folic acid, as a cofactor in one-carbon metabolism during gestation, may exert epigenetic effects (*Barua et al., 2014*). By causing alterations of gene expression as a result of aberrant methylation, excessive folic acid supplementation may influence other normal biological processes, such as embryonic and fetal brain development (*Barua et al., 2014*).

Measurement of RBC folate as predictor is a strength of the study, as RBC folate is not affected by food intake, which results in short-term fluctuations in serum/plasma folate concentrations. In addition, in higher quartiles, including RBC folate Q3 and Q4, preconceptional folic acid use was more common, namely around 90% of the participants. This observation can be explained by the fact that the RBC folate reflects the past 2–4 months and it is therefore possible to represent the periconceptional maternal folate status.

Precise pregnancy dating is of great importance because it affects the results and could make them potentially unreliable. To prevent this, a precise method was used for the pregnancy dating described in the Methods section before adjusting the analysis for gestational age.

Additional adjustment for embryonic volume showed a comparable U-shaped trend of the head volume measurements in this study population, which suggests a specific influence of the maternal RBC folate status on the development of the embryonic head. The non-significance of these results in this small sample size suggests that the influence of the maternal RBC folate status on the head volume is not independent from the development of the embryonic volume. However, despite this the influence seems bigger on the embryonic head. Furthermore, this analysis was adjusted for the mode of conception, which was shown to have a significant effect on embryonic brain measurements in the first trimester (*Husen et al., 2021*). However, in particular because of the small sample size, the results do not exclude residual confounding. Therefore, a future study using a larger sample size should address this issue. Methylenetetrahydrofolate reductase (MTHFR) polymorphism will result in alterations in folate metabolism and

these patients can have slightly higher homocysteine concentrations (*Liew and Gupta, 2015*). Personalized folic acid supplementation dosage could be a solution for these patients. However, the MTHFR polymorphism has not been measured in this study population and contributions of folic acid fortification to the RBC folate concentrations were not considered. In the Netherlands folic acid fortification is used in only a limited number of foods. Unfortunately there is limited precision in the measurement of folic acid from fortified foods, which is considered a limitation in this study.

Previous studies investigated the association between RBC folate quartiles and growth trajectories of CRL and cerebellum (*Koning et al., 2015*; *van Uitert et al., 2014*). *Van Uitert et al. (2014)* showed that RBC folate in the third quartile (1513–1812 nmol/l) was significantly associated with an increased CRL compared with the first two lower quartiles (814–1512 nmol/l) and the upper quartile (1813–2936 nmol/l). *Koning et al. (2015)* also showed the highest growth of the cerebellum measurements in the third RBC folate quartile (1538–1813 nmol/l). However, if these data are compared with the study outcomes, RBC folate Q3 does not cause the largest measurements, but the smallest measurements. The different outcomes can partly be explained by the fact that the RBC folate ranges per quartile differed between the current study and the previous two studies, which were performed in the same population and therefore had almost equivalent quartiles. In general it can be concluded that in the current study, Q1 and Q2 together largely correspond to the Q1 terms of both *Koning et al. (2015)* and *van Uitert et al. (2014)*. Q3 in this study largely overlaps also with Q2 and Q4 has overlap with both Q3, as Q4 of the other articles. In conclusion, it would be better to define certain RBC folate ranges where the largest/smallest measurements occur, instead of according to quartiles, because quartiles may differ between studies.

It is complicated to draw a clear and consistent conclusion based on these results. The truth will most likely be somewhere between the overlap of ranges of the current study and the previous studies.

Several studies that have been conducted to investigate the association between maternal serum folate and neurodevelopmental outcomes in offspring, including autism spectrum disorder (ASD), have shown that elevated concentrations are associated with increased risk of ASD (*Egorova et al., 2020*; *Wiens and DeSoto, 2017*). Because ASD is associated with macrocephaly, future research in a large population-based longitudinal birth cohort is very important to investigate the consequences of early prenatal neurodevelopmental growth and developmental differences leading to psychiatric disorders during the life course.

The clinical relevance of these small differences in the embryonic head and brain structure measurements in this explorative, hypothesis-driven and hypothesis-generating study needs to be further elucidated. Therefore, the next step is to carry out additional research to investigate the impact of the size of the early embryonic head and brain structures for neurodevelopment of the child in the early and late life course. Because of the lack of analyses after the first trimester and after birth in the current study, conclusions on fetal and neonatal outcome could not be drawn. For this purpose, further research is necessary with additional analyses at different times during and after pregnancy. Moreover, it would be interesting to investigate RBC folate concentrations within abnormal ranges and their association with growth and development of the fetal head and brain. In addition, a larger sample size is needed to increase power, especially for the very small brain measurements.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2021.11.002.

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