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Moderately elevated first trimester fasting plasma total homocysteine is associated with increased probability of miscarriage. The Reus-Tarragona Birth Cohort Study.

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Highlights

- *The associations between moderately elevated first trimester homocysteine, folate status, SNPs affecting folate metabolism and transport and miscarriage were investigated in a prospective cohort study.*
- *Fasting plasma total homocysteine (tHcy), folate, vitamin B_{12} and red blood cell (RBC) folate as well as the MTHFR 677C>T (rs1801133) and SLC19A1 80G>A (rs1051266) polymorphisms were determined in blood samples collected before 12 gestational weeks.*
- *The RR (95% CI) of miscarriage in women with first trimester tHcy ≥ P_{90} (7.1 µmol/L) compared to those with tHcy lower than this was 2.5 (1.1, 5.7).*
- *First trimester RBC folate status in 50.8 % of the participants was below the recommended threshold by the WHO to prevent neural tube defects (< 906 nmol/L).*
- *First trimester RBC folate concentration < 906 nmol/L was associated with increased risk of miscarriage compared to optimal RBC folate status: RR (95% CI): 2.1 (1.0, 4.5).*
- *First trimester folic acid supplement use ≥ 400 µg/d was associated with greater probability (OR (95% CI) of having optimal RBC folate status (15.1 (3.5, 64.9) while smoking reduced it (0.5 (0.3, 0.9).*

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Abstract

The association between elevated early pregnancy fasting plasma total homocysteine (tHcy) and miscarriage risk was investigated prospectively in participants (n=544) from the Reus-Tarragona Birth Cohort study. Pregnancy was confirmed before 12 gestational weeks (GW) by ultrasound scan and a fasting blood sample collected. Pregnancies with complications other than miscarriages were excluded. Miscarriages were diagnosed by ultrasound scan and gestational age at the time of miscarriage estimated by embryo size, where possible. Cases in which blood samples were collected more than a week after the miscarriage, or the miscarriage was of known cause, were excluded.

Fasting plasma folate, vitamin B_{12}, tHcy, cotinine (biomarker of smoking), red blood cell (RBC) folate, *MTHFR* 677C>T (rs1801133) and *SLC19A1* 80G>A (rs1051266) genotypes were determined.

The exposed group consisted of participants with first trimester tHcy $\geq P_{90}$ (7.1 μmol/L) (n=57) and unexposed of those with tHcy $<P_{90}$ (n=487). Adherence to folic acid supplement recommendations, plasma folate, plasma vitamin B_{12}, RBC folate and prevalence of optimal RBC folate status (≥906 μmol/L) were lower in the exposed compared to unexposed group. The prevalences of the *MTHFR* 677TT genotype and miscarriage were higher in the exposed group. The relative risks (95% CI) of pregnancy ending in miscarriage were 2.5 (1.1, 5.7) and 2.1 (1.0, 4.5) for participants in the high tHcy and suboptimal RBC folate groups (compared to the reference groups) respectively. Adherence to folic acid supplement recommendations was positively associated, while the *MTHFR* 677 TT versus CC genotype and smoking versus non-smoking were negatively associated, with RBC folate status.

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Keywords

Early pregnancy, homocysteine, Red blood cell folate, miscarriage, Reus-Tarragona Birth Cohort.

1. Introduction

1.1 Background

Moderately elevated fasting plasma total homocysteine (tHcy) has been associated with various pregnancy complications or adverse outcomes such as neural tube defects [1], preeclampsia [2] or low birth weight [3, 4], among others [5]. Homocysteine metabolism is regulated by gene-nutrient interactions and depends on dietary B-group vitamins: folate, vitamin B_{12}, pyridoxine, and riboflavin, choline and betaine. The *MTHFR* 677C>T (rs1801133) and *SLC19A1* 180G>A (rs1051266) polymorphisms affect the role of folate in homocysteine metabolism and folate transport, respectively. They have negative effects on folate status and are associated with elevated tHcy [6].

1.2 Evidence to date regarding tHcy and miscarriage/ pregnancy loss

Numerous studies have investigated the association between tHcy and recurrent pregnancy loss or miscarriage. However, early pregnancy tHcy determinations from the index pregnancy in which miscarriage is clinically diagnosed are difficult to obtain. Most studies compared tHcy, measured after the affected pregnancies have ended, between women with a history of recurrent miscarriage versus normal pregnancy. Some of these studies reported higher tHcy in women with a history of miscarriage compared to normal pregnancy [7-10] and that the probability of history of miscarriage was increased with increasing tHcy concentration [7]. However, in this latter study, vitamin B_{12} deficiency prevalence was high among the miscarriage cases but low in the controls. Other studies did not observe any differences in tHcy between women with history of pregnancy loss compared to normal pregnancies [11-14]. The disparity in results between the aforementioned studies may be due to various reasons. None of them measured tHcy before the clinical diagnosis of miscarriage in the affected pregnancy. tHcy levels

decrease during pregnancy [15] so nonpregnant measurements may not accurately reflect even early pregnancy concentrations. Furthermore, following the pregnancy loss women were taking folic acid supplements in many studies in preparation for the next pregnancy, thus affecting their tHcy. Studies with a reliable measurement of the exposure of interest prior to the miscarriage are lacking. In addition to the limitations regarding the exposure measurements, endpoints based on a clinical diagnosis of miscarriage are also scarce. This is relevant because efforts to differentiate between miscarriages likely resulting from other factors unrelated to tHcy, such as foetal chromosomal abnormalities or maternal infection, are warranted. A prospective study from preconception throughout pregnancy in which conception and pregnancy loss were monitored by urinary hCG concentrations, concluded that elevated tHcy at preconception (≥ 12.4 μmol/L) did not increase the relative risk of early pregnancy loss [16]. Miscarriage diagnosis or causes are not described in this study. Another prospective study, from the first prenatal visit, of 100 pregnancies measured tHcy in blood samples collected between 4-16 GW. No difference in tHcy was observed between the women that went on to miscarry and those that had a normal pregnancy outcome [17]. It is not clear whether the statistical power was sufficient (there were only 12 miscarriages), the timing of sample collection covered a range of 12 weeks which affects tHcy and no information was provided regarding the timing, cause or type of miscarriage. Impaired vitamin B_{12} status was associated with a higher probability of miscarriage in that same study. A large French study reported higher tHcy in samples collected following hospitalization for miscarriage in the index pregnancy compared to elective pregnancy termination controls of similar gestational age [18]. The blood samples in cases and controls were collected soon after the events and detailed information regarding miscarriage diagnosis and exclusion of cases due to known causes is provided.

1.3 Hypothesis and aims

We hypothesised that moderately elevated early pregnancy tHcy is a potential biomarker of idiopathic first trimester miscarriage risk.

The aim of this study was to investigate, prospectively, the association between moderately elevated early pregnancy tHcy and the risk of first trimester miscarriage in the Reus-Tarragona Birth Cohort.

2. Materials and Methods

2.1 Study participants

Women attending their first prenatal clinic at the University hospitals Sant Joan Reus and Joan XXIII Tarragona between 2005 and 2016, with confirmed pregnancy of less than 12 GW, were invited to participate in the Reus Tarragona Birth Cohort (RTBC) study. The study was approved by the Hospitals' Research and ethical committees and signed informed consent following the guidelines of the Declaration of Helsinki was obtained from all participants.

2.2 Blood sample collection

Fasting blood samples were collected at < 12 GW, 15 GW, 24-27 GW, 34GW and nonfasting samples at labour. For the purposes of the present report, only the first blood sample will be considered. Participants that developed pregnancy complications other than miscarriage (preeclampsia, intrauterine growth retardation, gestational hypertension, among others) were excluded (n=75) from this report. A total of 544 pregnancies were included. Samples were stored at −80°C in the Pere Virgili Health Research Institute (IISPV) biobank until analysis.

Clinical, nutritional and lifestyle data were recorded and plasma folate and RBC folate (microbiological assay with *Lactobacillus casei*), [19] plasma vitamin B_{12} (microbiological assay with *Lactobacillus leichmannii*) [20] and homocysteine (tHcy) and cotinine concentrations were determined by liquid-tandem mass spectrometry [21]. The *MTHFR* 677C>T (rs1801133) and *SLC19A1* 180G>A (rs1051266) genotypes were determined by matrix-assisted laser desorption/ionization/time-of-flight MS [22]. (Bevital; www.bevital.no). Data regarding smoking habits was collected from three different sources including interrogation by the investigating team (questionnaire), plasma cotinine determinations and from the prenatal history (recorded by the clinicians).

2. 3 Pregnancy confirmation and miscarriage diagnosis

Between 11 to 13+6 GW, pregnancy was confirmed by ultrasound scan. The majority of the miscarriages were first trimester spontaneous "missed" abortions diagnosed on detection of no foetal heartbeat by ultrasound scan at 12 GW. The remaining miscarriages were in course and diagnosed on referral from the emergency room when the clinical symptoms were manifested. Ultrasound scans revealing absence of foetal heartbeat or empty yolk sac were diagnosed as miscarriage. Gestational age at the time of miscarriage was estimated, where possible, from the crown-rump length or biparietal diameter of the embryo. Cases of miscarriages occurring more than 7 days before blood sample collection, were excluded from the study.

2.4 Statistical analysis.

Participants were classified as exposed to moderately elevated first trimester tHcy (≥ P_{90}: 7.1 µmol/L), n=57, or unexposed (<P_{90}), n=487. Smokers were identified based on plasma cotinine concentration ≥ 10 ng/ml and/ or confirmation of smoking habit by questionnaire or on interrogation by the clinicians during the prenatal check-ups. Quantitative variables with non-normal distributions were natural log transformed for the application of parametric statistical tests. Means between groups were compared using ANOVA and proportions using the Chi-square test. We fitted a Cox regression model to calculate the relative risk (RR) of miscarriage associated with moderately elevated tHcy. The model was adjusted for maternal age and smoking habit (active smoking versus non-smoking during pregnancy). Similarly, another Cox regression model was fitted to determine the RR of miscarriage associated with suboptimal RBC folate status during the first trimester of pregnancy. Predictors of tHcy and RBC folate status were assessed using multiple linear regression analysis and multiple logistics regression analysis respectively. IBM-SPSS software was used for all statistical tests. Significance level was set at $p < 0.05$.

3. Results

3.1 Cases included

Of the miscarriage cases, nine were excluded for the following reasons: chorioamnionitis (n = 3), antiphospholipid syndrome (n = 1), myoma (n= 1), trisomy 18 (n =1), late miscarriage, > 18 GW (n= 2), missing information (n = 1). The 32 miscarriages occurring before 18 GW and of unknown cause were included in the analysis.

3.2 Participant characteristics according to first trimester tHcy category

Participant characteristics are described in Table 1. *SLC19A1* 180G>A genotypes, smoking habits, parity, maternal age and body mass index did not differ between the exposed and unexposed groups. Adherence to folic acid supplement recommendations of 400 µg/d was high in both groups but higher in the group with tHcy < P_{90}. Plasma vitamin B_{12} status, plasma and RBC folate status were lower and the *MTHFR* 677TT genotype prevalence higher in the exposed versus unexposed to moderately elevated tHcy group. The WHO recommends a RBC folate status of 906 nmoL/L or more to prevent neural tube defects [23]. A higher proportion of participants in the high tHcy group had RBC folate concentrations below this recommendation and the proportion of early pregnancy miscarriage was higher in the exposed (high tHcy) than the unexposed group.

	First trimester fasting plasma total homocysteine group		
	≥ P_{90}[1]	< P_{90}	Total
MTHFR 677 C>T genotype, *%*			
CC	23.6 [13/55][2]	35.2 [170/483]	34.0 [183/538]
CT	43.6 [24/55]	49.3 [238/483]	48.7 [262/538]
TT	32.7 [18/55]	15.5 [75/483]**	17.3 [93/538]
SLC19A1 80 G>A genotype, %			
GG	38.2 [21/55]	26.7 [128/480]	27.9 [149/535]
GA	38.2 [21/55]	46.5 [223/480]	45.6 [244/535]
AA	23.6 [13/55]	26.9 [129/480]	26.5 [142/535]
First trimester smoking, *%*	31.6 [18/57]	26.9 [131/487]	27.4 [149/544]
First trimester folic acid use, *%*[3]	89.1 [49/55]	94.5 [446/487]***	93.9 [495/527]
Multiparity, %	47.4 [27/57]	54.2 [264/487]	54.4 [296/544]
Age (years), *mean (95% CI)*	32.4 (30.9, 33.9) [57]	32.1 (31.7, 32.5) [486]	32.2 (31.8, 32.5) [543]
BMI (kg/m2), *mean (95% CI)*	24.6 (23.2, 26.0) [55]	23.8 (23.4, 24.3) [464]	23.9 (23.5, 24.2) [516]
Plasma folate (nmol/L), *geometric mean (95% CI)*	14.4 (11.2, 18.4) [57]	26.7 (25.2, 28.4) [487]***	25.1 (23.6, 26.6) [544]
Plasma vitamin B_{12} (pmol/L), *geometric mean (95% CI)*	283 (261, 343) [57]	369 (358, 381) [487]**	363 (352, 373) [544]
tHcy (µmol/L), *geometric mean (95% CI)*	8.4 (8.0, 8.7) [57]	5.1 (5.0, 5.2) [487]***	5.3 (5.2, 5.4) [544]
RBC folate (nmol/L), *geometric mean (95% CI)*	556 (477, 647) [57]	954 (910, 1001) [474]***	901 (859, 945) [531]
RBC folate < 906 nmol/L, *%*	78.9 [45/57]	46.4 [220/474]***	49.9 [265/531]
Miscarriage, *%*	14.0 [8/57]	4.9 [24/487]*	5.9 [32/544]

Gestational week at miscarriage, *mean (95% CI)*	9.3 (7.8, 10.8) [7]	10.4 (9.1, 11.8) [16]	10.1 (9.1, 11.1) [23]

Table 1. Participant characteristics according to exposure to first trimester fasting plasma total homocysteine category.

Abbreviations: tHcy: fasting plasma total homocysteine, RBC: red blood cell. [1]7.1 µmol/L, [2][n], [3]≥ 400 µg/d versus 400 µg/d. Statistical comparison between 2 groups, Chi square for proportions and ANOVA for quantitative variables: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

3.3 Exposure to first trimester tHcy and risk of miscarriage

The association between first trimester tHcy status and risk of miscarriage is reported in table 2. Participants with tHcy at or above the 90th percentile (7.1 µmol/L) had over twice the risk of having a miscarriage. Risk of miscarriage also increased with increasing maternal age. We assessed whether RBC folate status below 906 nmoL/L affects the risk of miscarriage (Table 2). Women with RBC cell folate status < 906 nmol/L were twice as likely to have a miscarriage compared to women with red blood cell folate ≥ 906 nmol/L, after adjusting for maternal age, parity and smoking.

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	RR[1] (95% CI)	Deviance likelihood ratio, chi square	*n, df*
Unadjusted tHcy[2] model		17.2	544, 1**
tHcy ≥ P_{90} versus < P_{90}[3]	2.85 (1.28, 6.34)		
Adjusted tHcy[2] model		17.5	543, 4**
tHcy ≥ P_{90} vs < P_{90}	2.52 (1.12, 5.68)		
Maternal age (y)	1.13 (1.04, 1.22)		
Unadjusted RBC folate[2] model[4]		2.6	531, 1
RBC folate < 906 versus ≥ 906 nmol/L	1.83 (0.87, 3.81)		
Adjusted RBC folate[2] model[4]		15.0	530, 4**
RBC folate < 906 versus ≥ 906 nmol/L	2.11 (1.00, 4.45)		
Maternal age (y)	1.15 (1.06, 1.24)		

213 **Table 2.** Assessment of Relative risks of pregnancy ending in miscarriage according to early
214 pregnancy fasting plasma total homocysteine (tHcy) and RBC folate status using Cox
215 regression analysis. Abbreviations: tHcy: fasting plasma total homocysteine, RBC: red blood
216 cell. [1]Relative risk; [2]measured at < 12 gestational weeks; [3]P_{90}: 7.1 µmol/L; [4]adjusted for parity
217 (multiparous versus nulliparous) and for smoking versus nonsmoking during pregnancy.
218 **p<0.01.

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220 *3.4 Participant characteristics according to pregnancy outcome*

The participant characteristics according to outcome (miscarriage or normal pregnancy) are reported in Table 3. Women with pregnancies that ended in miscarriage were older, adhered less to the recommendation to take 400 µg/d of folic acid in the form of supplements and more of them had suboptimal folate reserves (indicated by RBC folate concentration, showing folate reserves entering pregnancy) compared to women that went on to have normal pregnancy outcomes.

	Miscarriage n=32	Normal pregnancy n=512
MTHFR 677 C>T genotype, *%*		
CC	36.7 [11/30][1]	33.8 [173/508]
CT	40.0 [12/30]	49.4 [253/508]
TT	23.3 [7/30]	16.8 [86/508]
SLC19A1 80 G>A genotype, %		
GG	30.0 [9/30]	27.7 [140/505]
GA	33.3 [10/30]	46.2 [234/505]
AA	36.7 [11/30]	25.9 [131/505]
First trimester smoking, *%*	21.9 [7/32]	28.0 [145/512]
First trimester folic acid supplement use, *%*	68.0 [17/25]	95.2 [478/502]***
Multiparity, *%*	50.0 [16/32]	53.7 [280/512]
Age (years), *mean (95% CI)*	34.6 (32.9, 36.3) [32]	32.0 (31.6, 32.4) [511]**
BMI (kg/m2), *mean (95% CI)*	23.5 (21.7, 25.3) [15]	23.9 (23.5, 24.3) [501]
Plasma folate (nmol/L), *geometric mean (95% CI)*	20.4 (14.7, 28.2) [32]	25.4 (23.9, 27.0) [512]*
Plasma vitamin B_{12} (pmol/L), *geometric mean (95% CI)*	359 (316, 408) [32]	363 (352, 374) [512]
tHcy (µmol/L), *geometric mean (95% CI)*	6.0 (5.4, 6.7) [32]	5.3 (5.2, 5.4) [512]**
RBC folate (nmol/L), *geometric mean (95% CI)*	837 (679, 1030) [31]	905 (861, 950) [500]
RBC folate < 906 nmol/L, *%*	64.5 [20/31]	49.0 [245/500]#

Table 3. Participant characteristics according to pregnancy outcome. [1][n], Abbreviations: tHcy: fasting plasma total homocysteine, RBC: red blood cell. Statistical comparison between 2 groups, Chi square for proportions and ANOVA for quantitative variables: *p<0.05, **p<0.01, ***p<0.001, #p=0.067.

3.5 Factors predicting first trimester tHcy

The predictors of first trimester tHcy were assessed using multiple lineal regression analysis (table 4). The strongest predictor was *MTHFR* 677 TT genotype, followed by plasma creatinine, parity and plasma folate. In a separate model in which first trimester plasma folate was replaced with RBC folate, the strongest predictor was RBC folate, followed by plasma creatinine, *MTHFR* 677 TT genotype and parity.

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	Beta coefficient	Adjusted R square	n, *df*
Model 1		0.090***	504, 10
MTHFR 677 TT versus CC genotype	0.220***		
Plasma creatinine (µmol/L)	0.180***		
Parity (multiparous versus nulliparous)	-0.126**		
Plasma folate (nmol/L)	-0.109*		
Model 2		0.148***	491, 10
RBC folate (nmol/L)	-0.273***		
Plasma creatinine (µmol/L)	0.192***		
MTHFR 677 TT versus CC genotype	0.185***		
Parity (multiparous versus nulliparous)	-0.119*		

242 **Table 4.** Predictors of first trimester tHcy using multiple lineal regression analysis.

243 Abbreviations: RBC: red blood cell. The dependent variable in both models is ln tHcy. Both

244 models were adjusted for maternal age, plasma B_{12}, smoking, *MTHFR* 677 CT versus CC

245 genotype *SLC19A1* 80 AA vs GG and *SLC19A1* 80 GA vs GG genotypes and. $^{*}p<0.05$,

246 $^{**}p<0.01$, $^{***}p<0.001$.

247

248 *3.6 Factors influencing first trimester RBC folate status*

249 The factors influencing the probability of having optimal RBC folate status in the first

250 trimester of pregnancy were assessed using multiple logistic regression analysis (table 5).

Regular use of folic acid supplements at or above the recommended dose of 400 µg/d strongly increased the probability of having optimal RBC folate status. On the other hand, smoking versus nonsmoking and the *MTHFR* TT versus CC genotype were associated with a 44% and 47% reductions, respectively, in the probability of having optimal RBC folate status.

	OR (95% CI)	Nagelkerke R^2	n, *df*
Model		0.123***	505, 9
First trimester folic acid use (≥ 400 µg/d)	15.1 (3.5, 64.9)		
Smoking vs nonsmoking	0.53 (0.31, 0.91)		
MTHFR 677 TT vs CC genotype	0.56 (0.33, 0.94)		

Table 5. Assessment of predictors of probability of optimal red blood cell folate status (≥ 906 µmol/L) using multiple logistic regression analysis. Model adjusted for maternal age, previous pregnancy, *SLC19A1* 80 AA vs GG and *SLC19A1* 80 GA vs GG genotypes.
***p<0.001.

4. Discussion

4.1 Principal findings

Elevated early pregnancy tHcy was associated with more than double the risk of having a miscarriage. First trimester RBC folate concentration < 906 nmol/L, indicative of suboptimal folate reserves entering pregnancy, was also associated with increased risk of miscarriage. Smoking had a negative effect on RBC folate status while folic acid supplement use at or above the recommended 400 µg/d had a protective effect.

4.2 Comparison with previous studies

Most previous studies determined tHcy after the miscarriage had occurred. A study that did take blood samples between 4 and 12 GW reported no difference in tHcy between miscarriage cases and controls [16]. There were two important differences between that study and ours. Firstly, it only had 12 miscarriage cases. It is unclear whether it was sufficiently powered to detect a difference in tHcy between cases and controls, if it existed. Furthermore, RBC folate status in general was higher in that study than in ours. Secondly, no details regarding timing, types or potential causes of miscarriage were provided. To the best of our knowledge, the other study with tHcy measurements nearest to the miscarriage was in patients being treated for the miscarriage [17]. That study by Gris et al, was large and had blood samples and ultrasound confirmation of the miscarriage close to the time of the event. Miscarriages occurring late in pregnancy or due to chromosomal abnormalities or maternal infection were also excluded from that study. Our findings confirm their findings that miscarriage risk was increased with increasing tHcy concentrations. They reported a twofold increase in risk for tHcy $\geq$ 9.9 μmol/L. This effect size is similar to our observation regarding tHcy $\geq$ 7.1 μmol/L.

4.3 Interpretation

The mechanism for the association between tHcy and miscarriage warrants investigation. It is possible that elevated tHcy in our study may be marking impaired folate status. The most important predictors of first trimester tHcy were RBC folate, followed by the *MTHFR* 677TT genotype. We previously reported in a population study from the same region that adults with the *MTHFR* 677TT genotype had lower folate status (both plasma and RBC folate) as well as higher tHcy than their CC or CT genotype counterparts [6]. We also observed in that same study that 18.8% of the participants had folate deficiency. In contrast to widespread folic acid

use in the Reus Tarragona Birth Cohort, the population study did not include folic acid users. Nevertheless, there is no mandatory fortification with folic acid in Spain and most participants in the Reus Tarragona Birth Cohort did not initiate folic acid supplementation until they were pregnant [24]. Use of the recommended dose of folic acid supplements and plasma folate status were lower in cases than in controls and the percentage of cases with RBC folate below the threshold recommended by the WHO to prevent neural tube defect affected pregnancies, was higher in cases than in controls.

Impaired one carbon metabolism due to low folate status, the *MTHFR* 677C>T polymorphism or other polymorphisms affecting the role of folate or other nutrients in the one carbon metabolic network have been associated with adverse outcomes stemming from anomalies in early pregnancy [5]. It is possible that the physio pathological mechanism leading to embryo developmental abnormalities, impaired placentation and foetal growth is shared, at least in part, in pregnancies affected by suboptimal one carbon metabolism. Impaired chorionic vascularisation in spontaneous miscarriage tissue from women with history of recurrent pregnancy loss and with tHcy > 18.3 µmol/L was reported in a study by Nelen et al [25]. It is also possible that anomalies in DNA methylation and other epigenetic processes arising from impaired one carbon metabolism are involved. However, further research in this field is required to explore and establish the associations between early pregnancy folate status and tissue-specific outcomes, their impact and replication between studies.

4.4 Strengths and limitations

Strengths of this study were that pregnancy was confirmed by ultrasound scan and tHcy was determined before the miscarriage occurred. Few previously reported studies have achieved these measurements due to the difficulty in obtaining them. Late miscarriage cases (caused by

infections or foetal developmental abnormalities) as well as miscarriages due to known causes such as chromosomal abnormalities were also excluded. Strictly, fasting blood samples and confirmation of pregnancy by ultrasound scan before 12 GW were required for eligibility to be included in the study. The study was designed to measure first trimester tHcy as a potential biomarker of adverse pregnancy outcome and blood samples were processed in strict adherence to protocol to prevent homocysteine release from blood cells [26].

Furthermore, RBC folate concentration was determined and is indicative of folate reserves during preconception and the start of pregnancy. It is spared the effects of haemodilution and the initial effects of folic acid supplement use (unlike plasma folate concentration and tHcy, which are sensitive to folic acid supplement use at the time of the blood draw).

Estimation of time of miscarriage in "missed" spontaneous abortions based on changes in transvaginal ultrasound measurements of crown-rump length or parietal circumference of the embryo are susceptible to error depending on time elapsed between cessation of foetal heartbeat and the performance of the scan. Gestational age based on reported date of the last menstrual period by the participants is also subject to error. However, we were able to minimise these errors due to the prospective nature of the study and recording of the timing of the blood samples and ultrasound scans. We stipulated that any miscarriage suspected to have occurred more than 7 days before the blood samples would be excluded.

4.5 Implications

This study shows that in the absence of mandatory fortification with folic acid, women not adhering to the recommended intake of 400 µg/d from folic acid supplements, are more likely not to meet the threshold RBC folate status proposed to offer protection against folate sensitive neural tube defects. This study shows that RBC folate status below this threshold also increases the risk of miscarriage. Smoking was negatively associated with RBC folate status. These

results indicate that the message regarding the importance of periconception folic acid in the prevention of adverse pregnancy outcome needs to be reinforced, and especially in smokers.

5.0 Conclusions

Moderately elevated early pregnancy tHcy is associated with 2.5 times more risk of early miscarriage, of unknown cause. The results provide evidence to support the consideration of early pregnancy tHcy as a potential biomarker of adverse pregnancy outcome.

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Author contributions:

MMM, PC-B and JF-B designed the research; PC-B, MI-P, MB were responsible for the clincial aspects of the study: JH-B, AR, CR-R, MMM were responsible for coordinating the field work of the study as well as data and sample collection, processing and biobanking; PMU and KM: were responsible for the biochemical and genetic determinations carried out at

373 Bevital AS; PC-B, MI-P, MMM, JF-B, JH-B, AR-G and CR-R: analysed the data and wrote
374 the manuscript; MMM and PC-B had primary responsibility for the final content.
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