Perinatal Outcomes

Investigating the causal effect of maternal vitamin B12 and folate levels on offspring birthweight

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Abstract

Background: Lower maternal serum vitamin B12 (B12) and folate levels have been associated with lower offspring birthweight, in observational studies. The aim of this study was to investigate whether this relationship is causal.

Methods: We performed two-sample Mendelian randomization (MR) using summary data on associations between genotype-B12 (10 genetic variants) or genotype-folate (four genetic variants) levels from: a genome-wide association study of 45 576 individuals (sample 1); and both maternal- and fetal-specific genetic effects on offspring birthweight from the latest Early Growth Genetics consortium meta-analysis with 297 356 individuals reporting their own birthweight and 210 248 women reporting their offspring's birthweight (sample 2). We used the inverse variance weighted method, and sensitivity analyses to account for pleiotropy, in addition to excluding a potentially pleiotropic variant in the *FUT2* gene for B12 levels.

Results: We did not find evidence for a causal effect of maternal or fetal B12 levels on offspring birthweight. The results were consistent across the different methods. We found a positive causal effect of maternal folate levels on offspring birthweight [0.146 (0.065, 0.227), which corresponds to an increase in birthweight of 71 g per 1 standard deviation

higher folate]. We found some evidence for a small inverse effect of fetal folate levels on their own birthweight [−0.051 (−0.100, −0.003)].

Conclusions: Our results are consistent with evidence from randomized controlled trials that higher maternal folate levels increase offspring birthweight. We did not find evidence for a causal effect of B12 levels on offspring birthweight, suggesting previous observational studies may have been confounded.

Key words: Maternal genetic effect, fetal genetic effect, vitamin B12, folate, birthweight, Mendelian randomization

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Key Messages

- We show that maternal two-sample Mendelian randomization can be used to identify potential causal effects of maternal exposures on offspring outcomes.
- Our study finds that higher maternal folate levels across the distribution increase offspring mean birthweight, in accordance with previous randomized controlled trials.
- We did not find evidence for a causal effect of maternal B12 levels on offspring birthweight, suggesting previous observational studies may have been confounded.

Background

Low birthweight is not only associated with lower infant survival, but also increased future risk of chronic diseases in offspring such as type two diabetes mellitus and cardiovascular disease. The Developmental Origins of Health and Disease (DOHaD) hypothesis provides an explanation for these robust observational associations,[1–6] stipulating that impaired fetal growth and development *in utero* lead to developmental compensations that programme the offspring to increased risk of disease in later life.[4] In other words, maternal under- or overnutrition during pregnancy not only exerts detrimental effects on offspring birthweight, but is hypothesized to cause long-term effects on the cardiometabolic health of the offspring. Therefore, identifying causal relationships between maternal nutrition and offspring birthweight could provide information on modifiable maternal exposures to ensure that more babies are born within a healthy weight range.

Low maternal folate (vitamin B9) and vitamin B12 levels have previously been linked with low offspring birthweight and increased risk of preterm birth.[7–11] The combination of high folate and low vitamin B12 levels has also been associated with low birthweight.[12] However, the association between B12 levels and offspring birthweight has been questioned, with a systematic review and meta-analysis showing no evidence for an association [5.1 g change in birthweight per 1 standard deviation (SD) increase in B12 levels (95% confidence interval: −10.9 g,

21.0 g)].[13] For folate, data from a meta-analysis suggested a 2% (95% confidence interval: 0.7%, 3.5%) higher birthweight for every 2-fold higher folate intake,[8] and a Cochrane review suggested a 135 g (95% confidence interval: 47.85 g, 223.68 g) increase in birthweight with folic acid supplement use.[14] However, the observational association between maternal folate and B12 levels and offspring birthweight could be confounded by other maternal characteristics, such as high body mass index (BMI) and gestational diabetes.

To ensure pregnant women have adequate levels of folate during their pregnancy to prevent neural tube defects, folic acid supplements are recommended to all women planning pregnancy and during the antenatal period. In addition, many countries fortify certain foods with folic acid. For vitamin B12 recommendations vary, with most high-income countries not recommending supplements unless there is clear deficiency. Folate and B12, along with vitamin B6 and B2, are coenzymes or integral components of coenzymes that are involved in one-carbon metabolism, also known as the folate metabolism pathway.[15] One-carbon metabolism is essential for DNA synthesis and DNA methylation,[15,16] which is particularly important during pregnancy because of the cell division and differentiation occurring *in utero*. The interplay between folate and B12 is important, both because a folate deficiency can be induced by a B12 deficiency[17] and because high folate levels can mask a B12 deficiency.[18] The interrelationship

between these two vitamins is best explained by the methyl trap hypothesis. This hypothesis states that a vitamin B12 deficiency can lead to lowered levels of methionine synthetase, which results in a functional folate deficiency by trapping an increased proportion of folate as the 5-methyl derivative.[17] Low folate and B12 levels may be caused by low dietary intake, lack of intrinsic factor needed to absorb vitamin B12, poor absorption of ingested folate/B12 or an alteration of folate metabolism due to genetic defects or drug interactions.

Conclusions regarding causality cannot easily be drawn from observational multivariable regression association studies. Mendelian randomization (MR) is a method that uses genetic variants as instrumental variables to provide information on causality in observational studies.[19] Offspring birthweight is thought to be influenced by offspring genetics, maternal genetics operating on the intrauterine environment, and environmental factors. MR investigating the effect of maternal environmental exposures on offspring birthweight is therefore more complicated, because we have both maternal and offspring genetics (which are correlated, $r \approx 0.5$) influencing the outcome of interest. A recently described statistical method, based on structural equation modelling, can be used to estimate the independent effect of maternal genotypes on offspring outcomes by conditioning on offspring genotype.[20] These maternal-specific genetic effects can then be used in a two-sample MR framework to estimate the causal effect of maternal environmental exposures, such as B12 or folate levels, on offspring birthweight.[21] The aim of this study was to investigate whether there is a causal relationship between serum levels of maternal B12 or folate and offspring birthweight.

Methods

Publicly available summary results statistics (effect estimates and their standard errors for each SNP's effect on birthweight, folate levels and B12 levels) were used for the main Mendelian randomization analysis and therefore no additional ethics approval was required. Ethical approval for the Exeter Family of Childhood Health was given by the North and East Devon (UK) Local Research Ethics Committee (approval number 1104), and informed consent was obtained from the parents of the newborns. 23andMe data (used in the maternal gestational age analysis) were collected in compliance with all applicable laws, from human subjects under an Institutional Review Board-approved protocol in accordance with US federal guidelines for 'Protection of Human Subjects', and the 23andMe activities described herein fall within the scope of the

approved protocol. Only aggregate, anonymized data were provided from 23andMe for analysis in this study.

Exposure measurements

We extracted summary results data on 10 single nucleotide polymorphisms (SNPs) that were robustly associated with serum B12 levels and four SNPs that were robustly associated with folate levels, in the largest genome-wide association study (GWAS) of these phenotypes to date[22] ($N = 45\,576$ individuals of Danish and Icelandic ancestry; Supplementary Table S1, available as Supplementary data at *IJE* online). The *FUT6* variant, rs7788053, was identified in the GWAS as associated with B12 levels but was not included in our analysis, as it is monoallelic in the CEU (Utah Residents from North and West Europe) [https:// ldlink.nci.nih.gov/] population. The SNP was not available in the GWAS of birthweight and therefore was left out of the analysis.

We obtained summary results data from the two Danish cohorts in the GWAS ($N = 8424$) for the 10 SNPs associated with B12 levels and four SNPs associated with folate levels. Due to the interplay between folate and B12 levels, we used these data to investigate the causal effect of B12 levels on folate levels and of folate levels on B12 levels.

Outcome measurements

We obtained summary results statistics from the latest GWAS of birthweight published by the Early Growth Genetics (EGG) consortium,[23] which included full-term births (defined as 37 or more weeks of gestation where gestational age was available, or a birthweight of 2.5–4.5 kg for UK Biobank). The GWAS of birthweight included 297 356 individuals reporting their own birthweight and 210 248 women reporting their offspring's birthweight. Due to the correlation between maternal and fetal genotypes, and the influence of both genotypes on offspring birthweight, the authors used a structural equation model[20] to estimate the independent maternal and fetal genetic effects on birthweight (this model is similar to a conditional linear model, accounting for both maternal and fetal genotypes). We extracted publicly available summary results data for the maternal- and fetal-specific effects on birthweight, generated using the structural equation models as described by Warrington and colleagues,[23] published online by the EGG consortium [https://egg-consortium. org/birth-weight-2019.html] (Supplementary Table S2, available as Supplementary data at *IJE* online). The *MMACHC* SNP, rs12272669, which is associated with B12 levels, was not available in the GWAS summary results statistics for birthweight, so we extracted an SNP in

high linkage disequilibrium (LD), rs11234541 ($R^2 = 0.942$, D' = 1) [https://ldlink.nci.nih.gov/].

In the folate GWAS, the *MTHFR* SNP, rs17421511, was identified to be associated with folate in an analysis that conditioned on another *MTHFR* SNP, rs1801133. Therefore, we performed an approximate conditional and joint (COJO) analysis[24] in genome-wide complex trait analysis (GCTA)[25] using European ancestry meta-analysis summary statistics for the maternal- and fetal-specific effects on birthweight, conditioning on rs1801133. The reference panel for determining LD was made up of 47 674 unrelated UK Biobank participants defined by the UK Biobank as having British ancestry.[26] Unrelated individuals with British ancestry were identified on the basis of identity by descent (IBD) sharing of <0.1, and from there a random sample of individuals with identity by state (IBS) <0.025 were selected as the reference panel. We extracted the maternal- and fetal-specific effect estimates for rs17421511 from the COJO analysis (Supplementary Table S2).

Statistical analysis

To assess instrument strength for the standard inverse variance weighted (IVW) MR analysis, we calculated the approximate F statistics for each of the folate and B12 SNPs that will be used in our two-sample MR study [i.e. $F \approx (\beta/SE)^2$] (Supplementary Table S1). In the case of MR Egger regression analysis,[27] the F statistics from the individual markers are not a sufficient indicator of instrument strength. Rather, Bowden and colleagues[28] show that an I^2_{GX} statistic can be used to quantify instrument strength in MR Egger analyses. The authors show that a high value of I^2_{GX} (i.e. close to one) suggests that the instrument effect sizes are estimated well and that measurement error/weak instrument bias is unlikely to affect the results of standard MR Egger analyses. On the other hand, if I^2_{GX} is less than 0.9, inference from MR Egger should be interpreted with caution and some alternative sensitivity analyses should be considered. As such, we calculated I^2_{GX} values for our SNPs to assess the instrument strength in the MR Egger analysis.

We performed two-sample, IVW MR analysis to estimate the causal effect of maternal B12 or folate levels on offspring birthweight. We performed a secondary analysis investigating the causal relationship between an individual's own B12 or folate levels and their own birthweight, using the results of the fetal-specific genetic effects on birthweight. Analyses were performed with the TwoSampleMR package[29] [https://github.com/MRCIEU/TwoSampleMR] in R version 3.5.2 [https://cran.r-project.org/]. We performed a test of heterogeneity of causal effect estimates across each of the SNPs using Cochran's Q. If

heterogeneity was detected, leave-one-out IVW analysis was performed to assess the effect of the individual SNPs on the overall causal estimate. We tested for directional pleiotropy using the MR Egger intercept.[27] Additionally, we conducted sensitivity analysis to adjust for any directional pleiotropy, using MR Egger regression,[27] weighted median,[30] simple and weighted mode estimation.[31] We acknowledge that these sensitivity analyses perform well with large numbers of SNPs and may not perform well in our analyses for effects of folate where we only have four SNPs; however, we include the results of the MR Egger for completeness. As the weighted and simple mode estimator select the group of SNPs that produce the most common causal effect on the outcome, we chose not to perform these analysis for folate where only four SNPs were available.

Further sensitivity analyses were conducted for the B12 analysis excluding the *FUT2* variant, rs602662. The FUT2 protein, which is expressed in the small intestine, is a part of the protein glycosylation pathway and is involved in the absorption and modification of several nutrients.[32–34] Therefore it may reflect nutritional status, which in turn could affect a multitude of lifestyle-associated traits and diseases. In a previous MR study investigating the causal relationship between B12 and BMI,[35] the causal relationship identified between low B12 levels and higher BMI attenuated to the null when the variant in the *FUT2* gene was excluded from the analysis.[35] We therefore performed the MR analyses both with and without the *FUT2* variant, to assess the effect of this potentially pleiotropic variant on the causal estimate, as has been done in previous papers.[35,36]

Three of the variants associated with folate levels were located within the *MTHFR* gene. We looked up the LD between the variants (Supplementary Table S3, available as Supplementary data at *IJE* online) and conducted a leave-one-out MR analysis as a sensitivity analysis to assess the influence of the individual SNPs.

Finally, we performed IVW MR analysis and sensitivity analyses to adjust for directional pleiotropy, to assess the causal effect of B12 levels on folate and of folate levels on B12. If evidence for a casual effect is identified, we planed to use two-step MR[37] to investigate whether there was evidence that one maternal exposure mediates the relationship between the other and offspring birthweight.

Follow-up analysis of gestational duration

Gestational duration is a major determinant of birthweight. Since gestational duration was unavailable for >85% of individuals in the GWAS of birthweight,[23] it is possible that a causal relationship between maternal B12

or folate and birthweight could be driven by a relationship with gestational duration. Therefore, we performed a two-sample IVW MR analysis to estimate the causal effect of maternal B12 and folate levels on gestational duration. We obtained summary results data from the latest maternal GWAS study of gestational duration of 43 568 women of European ancestry from 23andMe, presented in Zhang *et al.*[38] For the fetal GWAS of gestational duration, we obtained summary results data from the latest EGG Consortium study of 84 689 individuals.[39] For the B12 analysis, the *MMACHC* SNP rs12272669 was not available in the maternal or fetal gestational duration GWAS (and no SNPs in high LD ($R^2 > 0.8$) were available), leaving a total of nine SNPs for analysis. The effect allele frequencies were not available in the publicly available summary results data from the fetal GWAS of gestational duration, so we removed the *MMAA* SNP, rs2270665, from the B12 analysis as it is a palindromic SNP and we could not confirm that the direction of effect was the same as in the B12 summary statistics. We conducted sensitivity analysis using MR Egger regression,[27] weighted median,[30] simple and weighted mode estimation[31] to adjust for any potentially pleiotropic SNPs. Similar to the birthweight analysis, we caution the interpretation of the folate sensitivity analyses, due to the small number of SNPs available.

Checking SNP associations with B12 and folate levels in pregnant women from the Exeter Family Study of Childhood Health study

We performed an association analysis of the SNPs associated with B12/folate and the serum level of the two vitamins, measured at 28 weeks of gestation[40] in a total of 871 pregnant women from the Exeter Family Study of Childhood Health (EFSOCH).[41] Genome-wide genotyping and imputation in this study have been described previously.[42] The outcome variables were inverse-normal transformed before analysis, and the linear regression analysis was adjusted for maternal age and five ancestry principal components. The rs12272669 variant in the *MMACHC* gene was not available, so rs11234541 in high LD ($r^2 = 0.9421$ in CEU) was used as a proxy. This SNP had low imputation quality in the EFSOCH study (INFO = 0.68), and the results should be interpreted with care.

Informed consent

Only publicly available summary results data were used in the birthweight MR analyses. Participants in EFSOCH gave informed consent, and ethical approval was obtained from local review committee. 23andMe data (used in the maternal gestational age analysis) were collected in compliance with all applicable laws, from human subjects under an Institutional Review Board-approved protocol in accordance with US federal guidelines for 'Protection of Human Subjects', and the 23andMe activities described herein fall within the scope of the approved protocol. Only aggregate, anonymized data were provided from 23andMe for analysis in this study.

Results

The 10 variants included in the MR analysis of maternal serum B12 levels explained 5.24% of the variance in serum B12 concentrations in the original GWAS (3.82% without the *FUT2* variant, rs602662). The four variants included in the MR analysis of maternal folate levels explained 1.3% of the total variation in serum folate concentration in the original GWAS. F statistics for individual SNPs ranged from 35 to 623 for B12 and from 48 to 203 for folate levels. The magnitude of the SNP effects on serum B12 and folate concentrations measured in pregnant women from the EFSOCH study were consistent with those from the original GWAS (Supplementary Figures S1 and S2, available as Supplementary data at *IJE* online), apart from the *MMACHC* variant, rs12272669, which appeared to have little effect in pregnant women at 28 weeks of gestation (heterogeneity $P = 0.0002$). However, we used a proxy for this variant in the EFSOCH study, rs11234541, which was in high LD with rs12272669 but had low imputation quality.

The causal effect estimates of maternal and fetal B12 and folate levels on birthweight from the IVW analysis and sensitivity analyses are shown in Table 1. We found no evidence for a causal effect of maternal B12 levels on offspring birthweight [0.009 SD change in birthweight per 1 SD higher B12 ($SE_{IVW} = 0.012$), $P_{IVW} = 0.469$; Figure 1], or of fetal B12 levels on their own birthweight [−0.012 SD change in birthweight per 1 SD higher B12 ($SE_{IVW} = 0.017$), $P_{IVW} = 0.478$; Supplementary Figure S3, available as Supplementary data at *IJE* online]. The effect estimates were similar when excluding the *FUT2* variant, rs602662 (Supplementary Table S4, available as Supplementary data at *IJE* online).

We found evidence of a positive causal effect of maternal folate levels on offspring birthweight [0.146 SD change in birthweight per 1 SD higher folate ($SE_{IVW} = 0.041$), $P_{IVW} = 4 \times 10^{-4}$; Figure 2]. Furthermore, we identified a negative effect of fetal folate levels on their own birthweight, although the effect size was nearly a third of the maternal folate causal effect estimate [−0.051 SD change in birthweight per 1 SD higher folate ($SE_{IVW} = 0.025$), $P_{IVW} = 0.037$; Supplementary Figure S4].

Table 1 Mendelian randomization estimates of the causal effect of folate and vitamin B12 levels on birthweight. We estimated causal effects for maternal folate and B12 levels on offspring birthweight (Maternal) and offspring's folate and B12 levels on their own birthweight (Fetal). Causal effects are estimated using five MR models: inverse variance weighted (IVW), weighted median, simple mode, weighted mode and MR Egger regression. Causal effect estimates are the difference in mean birthweight (in standard deviation; SD) per 1 SD higher folate/B12

Method	Folate				B12			
	Number of SNPs	Causal effect estimate	SE	*P*-value	Number of SNPs	Causal effect estimate	SE	*P*-value
Maternal:								
Inverse variance weighted	4	0.1459	0.0413	0.0004	10	0.0088	0.0121	0.4694
Weighted median	4	0.1443	0.0339	2.04×10^{-05}	10	0.0114	0.0138	0.4078
Simple mode	-	–	–	–	10	0.0041	0.0189	0.8348
Weighted mode	-	–	–	–	10	0.0058	0.0135	0.6782
MR Egger	4	0.4378	0.1507	0.1009	10	−0.0031	0.0210	0.8852
Fetal:								
Inverse variance weighted	4	−0.0514	0.0247	0.0372	10	−0.0118	0.0166	0.4776
Weighted median	4	−0.0516	0.0269	0.0548	10	0.0031	0.0127	0.8068
Simple mode	-	–	–	–	10	−0.0069	0.0220	0.7612
Weighted mode	-	–	–	–	10	0.0027	0.0138	0.8504
MR Egger	4	−0.0536	0.1266	0.7135	10	−0.0012	0.0291	0.9686

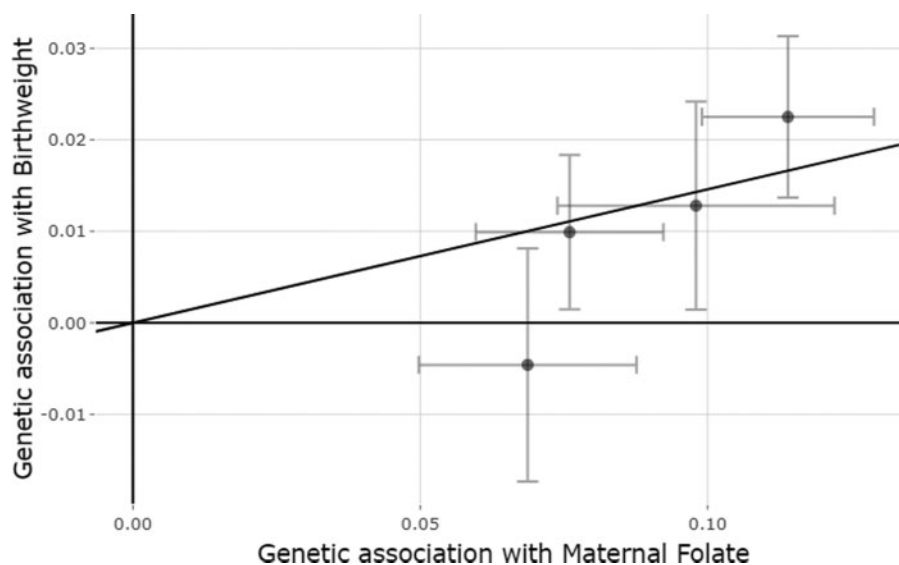1 SD in birthweight correspond to 484 g.[23]

B12: vitamin B12; SNP: single nucleotide polymorphism; SE: standard error.
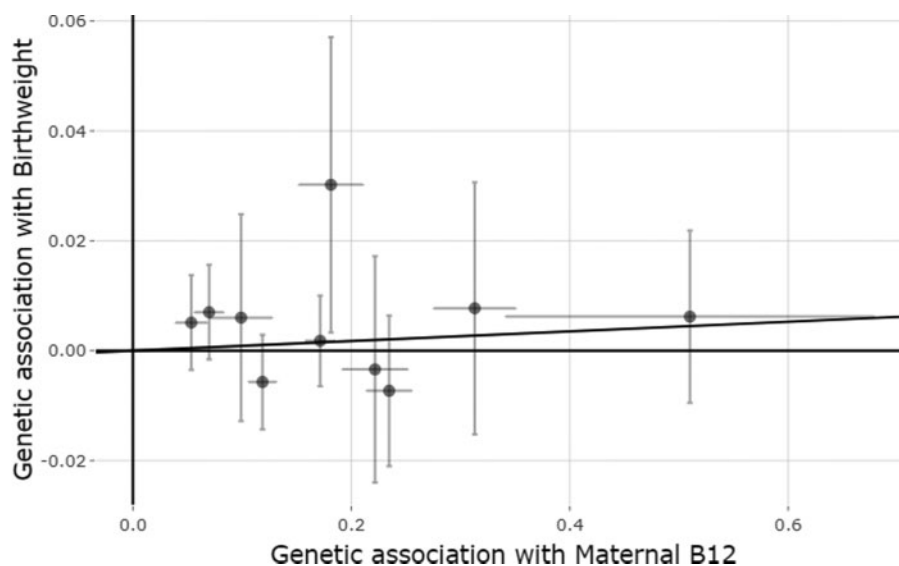
Figure 1 Mendelian randomization estimates of the causal effect of maternal B12 levels on offspring birthweight. The x-axis shows the single nucleotide polymorphism (SNP) effect, and standard error, on vitamin B12 levels for each of the 10 SNPs, and the y-axis shows the SNP effect, and standard error, on offspring birthweight. The regression line for the inverse variance weighted Mendelian randomization method is shown

There was evidence of heterogeneity in the causal effect estimates of fetal B12 levels on their own birthweight across the individual SNPs ($Q = 27.790$, $P = 0.001$), but no strong evidence of heterogeneity in the causal effect estimates of maternal B12 levels on offspring birthweight ($Q = 12.486$, $P = 0.162$). To investigate the heterogeneity further, we performed leave-one-out analysis of fetal B12 levels on their own birthweight (Supplementary Table S5,

available as Supplementary data at *IJE* online). As indicated by the heterogeneity test, there was some variability in the causal estimates, particularly when leaving the *MMACHC* SNP, rs12272669, or the *FUT2* SNP, rs602662, out of the analysis. Even though there are no known pleiotropic pathways for the *MMACHC* gene, we a priori expected the *FUT2* gene to have pleiotropic effects.[35,36] The causal effect estimates of maternal folate

Figure 2 Mendelian randomization estimates of the causal effect of maternal folate levels on offspring birthweight. The x-axis shows the single nucleotide polymorphism (SNP) effect, and standard error, on folate levels for each of the four SNPs, and the y-axis shows the SNP effect, and standard error, on offspring birthweight. The regression line for the inverse variance weighted Mendelian randomization method is shown

levels on offspring birthweight across the individual SNPs also showed some heterogeneity (Q = 6.904, P = 0.075), but no evidence for the fetal folate levels (Q = 0.018, P = 0.999). To investigate the potential influence of the SNPs in LD in the *MTHFR* gene and potential heterogeneity identified for the maternal folate levels, we performed a leave-one-out analysis on both maternal and fetal folate levels on birthweight (Supplementary Table S6, available as Supplementary data at *IJE* online). When the *MTHFR* SNP rs1801133 was excluded from the analysis, the causal effect was attenuated for the effect of maternal folate levels (0.099, SE = 0.051, P = 0.051), but stayed the same and for fetal folate levels (−0.051, SE = 0.034, P = 0.132).

There was no evidence of directional pleiotropy from the MR Egger regression analysis for either B12 or folate levels on birthweight (Egger intercept for maternal B12 levels = 0.003, P = 0.500; Egger intercept for fetal B12 levels = −0.002, P = 0.662; Egger intercept for maternal folate levels = −0.028, P = 0.187; Egger intercept for fetal folate levels = 0.000, P = 0.988), although again highlighting that the MR Egger result from the folate analysis would be unreliable due to the small number of SNPs. This is further supported by the low I^2_{GX} of 0.83 for the folate analysis, which is below the suggested cut-off of 0.9, suggesting that the MR Egger results could be influenced by measurement error or weak instrument bias. Bowden's I^2_{GX} was 0.98 for B12, suggesting little influence in the MR Egger analyses from measurement error or weak instrument bias.

We were unable to detect evidence for a causal effect of B12 levels on folate levels in the two Danish cohorts (Supplementary Table S7, available as Supplementary data

at *IJE* online), indicating that maternal folate levels are unlikely to mediate a relationship between maternal B12 levels and offspring birthweight. In the IVW analysis, we detected a small causal effect of folate levels on B12 levels in the two Danish cohorts (0.155 SD change in B12 per 1 SD higher folate (SE$_{IVW}$ = 0.077), P_{IVW} = 0.043; Supplementary Table S8, available as Supplementary data at *IJE* online). However, given there is no evidence of a causal effect of maternal B12 levels on offspring birthweight, it is unlikely that B12 levels mediate the relationship between maternal folate levels and offspring birthweight.

Last, we did not find any evidence of a causal effect of maternal or fetal B12 or folate levels on gestational duration (Table 2). There was no evidence of directional pleiotropy from the MR Egger regression analysis or of heterogeneity between the causal estimates at each SNP (Maternal: Directional Pleiotropy: P_{B12} = 0.28, P_{Folate} = 0.87; Heterogeneity: P_{B12} = 0.08, P_{Folate} = 0.63; Fetal: Directional pleiotropy: P_{B12} = 0.59, P_{Folate} = 0.38; Heterogeneity: P_{B12} = 0.12, P_{Folate} = 0.21).

Discussion

We did not find any evidence for a causal effect of maternal B12 levels on offspring birthweight [0.009 (−0.015, 0.033) SD change in birthweight per 1 SD higher B12, corresponding to a 4 g increase (−7 g, 16 g) in birthweight per 1 SD higher B12], nor was there evidence that fetal B12 levels influenced their own birthweight. The results were consistent across the different sensitivity methods applied,

Table 2 Causal effect estimates of maternal folate and vitamin B12 levels on gestational duration. Causal effects are estimated using five MR models: inverse variance weighted (IVW), weighted median, simple mode, weighted mode and MR Egger regression. Causal effect estimates are the difference in mean gestational duration [in days for the maternal effect and standard deviation (SD) for the fetal effect] per 1 SD higher folate/B12

Method	Folate				B12			
	Number of SNPs	Causal effect estimate[a]	SE	*P*-value	Number of SNPs	Causal effect estimate[a]	SE	*P*-value
Maternal:								
Inverse variance weighted	4	−0.0772	0.1065	0.4685	9	−0.0683	0.0725	0.3457
Weighted median	4	−0.1248	0.1261	0.3225	9	−0.0993	0.0748	0.1844
Simple mode	–	–	–	–	9	−0.1760	0.1349	0.2281
Weighted mode	–	–	–	–	9	−0.1171	0.0885	0.2222
MR Egger	4	−0.1742	0.5498	0.7813	9	−0.2231	0.1512	0.1836
Fetal:								
Inverse variance weighted	4	−0.0233	0.0459	0.6101	8	0.0132	0.0195	0.5007
Weighted median	4	−0.0700	0.0475	0.1407	8	0.0194	0.0212	0.3595
Simple mode	–	–	–	–	8	0.0171	0.0347	0.6363
Weighted mode	–	–	–	–	8	0.0279	0.0270	0.3362
MR Egger	4	0.2572	0.2561	0.4209	8	0.0354	0.0444	0.4557

B12: vitamin B12; SNP: single nucleotide polymorphism; SE: standard error.

[a]Note that these estimates are not maternal- and fetal-specific estimates derived from conditional analyses.

including the analyses with or without the *FUT2* variant. In contrast, we found evidence of a positive causal effect of maternal folate levels on offspring birthweight [0.146 (0.065, 0.227) SD change in birthweight per 1 SD higher folate, which corresponds to an increase in birthweight of 71 g (31 g, 110 g) per 1 SD higher folate]. Although we could not detect any evidence for directional pleiotropy or heterogeneity in these analyses, we have limited capacity to test this due to having only four SNPs associated with folate. We also found some evidence of an inverse effect of fetal folate levels on their own birthweight [−0.051 (−0.100, −0.003) SD change in birthweight per 1 SD higher folate, corresponding to a 25 g [−48 g, −1 g] decrease in birthweight per 1 SD higher folate).

Previous multivariable regression association studies investigating the relationship between maternal B12 levels and offspring birthweight have been inconsistent.[7] Nevertheless, these studies are all relatively small (samples sizes from 51 to 5577 in the meta-analysis presented by Rogne and colleagues[7]) and therefore the effect size estimates from the multivariable regression analyses between maternal B12 levels and offspring birthweight had large confidence intervals. In addition, the meta-analysis combining the results from 12 studies found no evidence of an association between maternal B12 levels and offspring birthweight (N = 9406), with an estimate similar to the results of our MR analysis [5.1 g (−10.9 g, 21.0 g) change in birthweight per 1 SD increase in maternal B12 levels]. Multivariable regression association studies and MR have

different assumptions and sources of bias; however, with regards to maternal B12 levels on offspring birthweight, the effect estimates in the meta-analysis of multivariable regression analyses and our MR are similar, providing additional confidence in our result. In the meta-analysis, Rogne and colleagues[7] observed some heterogeneity between the studies ($I^2 = 30\%$),[7] which was driven by an association between B12 deficiency and birthweight in low- and middle-income countries. They suggested that this association could be explained by preterm birth rather than reduced fetal growth; however, we did not see any evidence for this in our MR analysis with gestational duration. Notably, one of the 10 SNPs from the birthweight analysis was not available for the MR analysis estimating the causal effect of maternal B12 levels on gestational duration. There could be several reasons why we were unable to find any evidence of an effect of B12 levels on offspring birthweight within the normal range. First, there could be a weak effect that we were unable to detect with the current statistical power of our study. Second, folate deficiency can be induced by a B12 deficiency,[17] so there may be a stronger effect of maternal B12 deficiency on offspring birthweight than maternal B12 levels within the normal range. As such, monitoring both B12 and folate levels during pregnancy might be important.

Consistent with previous multivariable regression association studies[8] and randomized controlled trials (RCTs),[14] our MR study suggests a causal effect of higher maternal folate levels on increased offspring birthweight.

Our MR analysis goes one step further than the multivariable regression studies and RCTs, as we have used serum levels of folate, whereas the previous studies were based on the use of folic acid supplements in pregnancy without quantifying the downstream effect that those supplements have on serum levels.[8,14] We found a potential negative causal effect of fetal folate levels, as proxied by the fetal genotype, on their own birthweight, although the effect size was only about a third of the positive effect of maternal folate levels on offspring birthweight. The mechanism underlying this effect is unclear, as folate is actively transported across the placenta by folate transporters, and not produced by the fetus.[43,44] However, folate is metabolized into the active forms both in the mother and in the fetus, so this negative causal effect might indicate that fetal folate metabolism is important. There were only four SNPs included in the folate analysis, three of which are located in the same gene, which was not enough to obtain reliable sensitivity analyses, as partly indicated by the I^2_{GX} of 0.83 for the MR Egger regression. Furthermore, the variant rs1801133 in the *MTHFR* gene has been associated with diastolic blood pressure, which is also known to causally influence birthweight[23] and could therefore indicate potentially pleiotropic pathways [Causal effect estimate from the leave-one-out analysis removing rs1801133: 48 g (0 g, 96 g) increase in birthweight per 1 SD higher folate].

We did not find any evidence of a causal effect of maternal or fetal folate levels on gestational duration, suggesting that our finding of a causal effect of maternal folate levels on offspring birthweight cannot be attributed to gestational duration. However, the sample size in the gestational duration GWAS (Maternal: $N = 43\ 568$; Fetal: $N = 84\ 689$) was smaller than the GWAS of birthweight ($N = 297\ 356$ individuals reporting their own birthweight and 210 248 women reporting their offspring's birthweight) and the maternal SNP effect estimates on gestational duration were not conditioned on fetal genotype.

It is important to examine the findings of a causal relationship between folate and birthweight within the bigger picture. Given that low maternal folate levels during pregnancy are known to cause neural tube defects,[45] we would obviously not argue for stopping the widespread recommendation and use of folic acid supplements during pregnancy and the fortification of foods. Indeed, based on our results, folic acid supplementation and fortification of foods may have contributed to the observed increase in birthweight and reduction of babies born small for gestational age seen over the past few decades (23 g to 46 g, in a 9–18-year time span).[46–48]

Strengths and limitations

One of the strengths of our study was the ability to use the partitioned maternal and fetal genetic effects from the largest GWAS of birthweight ($N = 297\ 356$ individuals reporting their own birthweight and 210 248 women reporting their offspring's birthweight) in a two-sample MR approach to estimate the effect of maternal B12 and folate levels on offspring birthweight, independent of any direct fetal effect. We performed a series of sensitivity analyses to explore potential bias due to horizontal pleiotropy, including comparing findings for B12 levels with and without the *FUT2* genetic instrument which has been shown to have pleiotropic effects. However, we acknowledge that the methods used for the sensitivity analyses work best with more SNPs than we had available in our analyses. For folate, the I^2_{GX} of 0.83, below the suggested cut-off of 0.9, indicates that the MR Egger results could be influenced by measurement error or weak instrument bias.

An important limitation with this study is that the folate and vitamin B12 levels measured and used in the exposure GWAS were from non-pregnant women, as well as men. However, we were able to check that the instruments were valid in a pregnant population by performing an association analysis in the EFSOCH study. We found no evidence of different effect sizes in pregnant women compared with the exposure GWAS used in these analyses, except for in the rs12272669 variant in the *MMACHC* gene. The proxy SNP we used in the EFSOCH study, rs11234541, had low imputation quality, and as such the results should be interpreted with care.

Last, it is important to note that even though we did not find any effect of maternal B12 levels on offspring birthweight, that does not mean that B12 levels do not have an effect on the development of the fetus. B12 levels could influence growth in a particular period of the pregnancy, affect a particular organ or growth of fat, lean or skeletal tissue in different directions, which may not be reflected in an outcome such as birthweight.

Conclusion

In conclusion, evidence is lacking for a causal relationship of maternal vitamin B12 levels on offspring birthweight, indicating that the association detected in previous multivariable regression association studies may have been due to confounding. We found evidence for a causal effect of higher maternal folate levels increasing offspring birthweight, which is consistent with previous multivariable regression association studies and RCTs.

The data underlying the main Mendelian randomization analysis in this manuscript are available at http://

Supplementary data

[Supplementary information](#) is available at *IJE* online.

Author contributions

G.H.M. conceptualized the project, preformed the analysis and wrote the paper. R.N.B. performed analysis and revised the paper. C.S. interpreted the data and revised the paper. B.M.S. performed data collection and revised the paper. D.A.L. interpreted the data and revised the paper. R.M.F. interpreted the data and revised the paper. D.M.E. conceptualized the project, interpreted the data and revised the paper. N.M.W. conceptualized the project, interpreted the data and revised the paper.

Conflict of interest

D.A.L. declares that she receives support from Roche Diagnostics and Medtronic Ltd for research unrelated to this study. All other authors declare that they have no conflict of interest.

References

1. Hales CN, Barker DJ. Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis. *Diabetologia* 1992;**35**:595–601.
2. Roseboom TJ, van der Meulen JH, Ravelli AC, Osmond C, Barker DJ, Bleker OP. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Mol Cell Endocrinol* 2001;**185**:93–98.
3. Gillman MW. Developmental origins of health and disease. *N Engl J Med* 2005;**353**:1848–50.
4. Godfrey KM, Barker DJ. Fetal nutrition and adult disease. *Am J Clin Nutr* 2000;**71**:1344s–52s.
5. Barker DJ, Gluckman PD, Godfrey KM, Harding JE, Owens JA, Robinson JS. Fetal nutrition and cardiovascular disease in adult life. *Lancet* 1993;**341**:938–41.
6. Seghieri G, Anichini R, De Bellis A, Alviggi L, Franconi F, Breschi MC. Relationship between gestational diabetes mellitus and low maternal birth weight. *Diabetes Care* 2002;**25**:1761–65.
7. Rogne T, Tielemans MJ, Chong MF-F *et al.* Associations of maternal vitamin B12 concentration in pregnancy with the risks of preterm birth and low birth weight: a systematic review and meta-analysis of individual participant data. *Am J Epidemiol* 2017;**185**:212–23.
8. Fekete K, Berti C, Trovato M *et al.* Effect of folate intake on health outcomes in pregnancy: a systematic review and meta-analysis on birth weight, placental weight and length of gestation. *Nutr J* 2012;**11**:75.
9. Uitert EM, Steegers-Theunissen RPM. Influence of maternal folate status on human fetal growth parameters. *Mol Nutr Food Res* 2013;**57**:582–95.
10. Mukherjee MD, Sandstead HH, Ratnaparkhi MV, Johnson LK, Milne DB, Stelling HP. Maternal zinc, iron, folic acid, and protein nutriture and outcome of human pregnancy. *Am J Clin Nutr* 1984;**40**:496–507.
11. Grieger JA, Clifton VL. A review of the impact of dietary intakes in human pregnancy on infant birthweight. *Nutrients* 2014;**7**:153–78.
12. Paul L, Selhub J. Interaction between excess folate and low vitamin B12 status. *Mol Aspects Med* 2017;**53**:43–47.

13. Solé-Navais P, Salat-Batlle J, Cavallé-Busquets P *et al.* Early pregnancy folate-cobalamin interactions and their effects on cobalamin status and hematologic variables throughout pregnancy. *Am J Clin Nutr* 2018;**107**:173–82.

14. Lassi ZS, Salam RA, Haider BA, Bhutta ZA. Folic acid supplementation during pregnancy for maternal health and pregnancy outcomes. *Cochrane Database Syst Rev* 2013;**3**:CD006896.

15. Ducker GS, Rabinowitz JD. One-carbon metabolism in health and disease. *Cell Metabol* 2017;**25**:27–42.

16. Bohnsack BL, Hirschi KK. Nutrient regulation of cell cycle progression. *Annu Rev Nutr* 2004;**24**:433–53.

17. Shane B, Stokstad EL. Vitamin B12-folate interrelationships. *Annu Rev Nutr* 1985;**5**:115–41.

18. Carmel R, Green R, Rosenblatt DS, Watkins D. Update on cobalamin, folate, and homocysteine. *Hematol Am Soc Hematol Educ Program* 2003;**2003**:62–81.

19. Davey Smith D, Ebrahim S. 'Randomization': can genetic epidemiology contribute to understanding environmental determinants of disease? *Int J Epidemiol* 2003;**32**:1–22.

20. Warrington NM, Freathy RM, Neale MC, Evans DM. Using structural equation modelling to jointly estimate maternal and fetal effects on birthweight in the UK Biobank. *Int J Epidemiol* 2018;**47**:1229–41.

21. Evans DM, Moen GH, Hwang LD, Lawlor DA, Warrington NM. Elucidating the role of maternal environmental exposures on offspring health and disease using two-sample Mendelian randomization. *Int J Epidemiol* 2019;**48**:861–75.

22. Grarup N, Sulem P, Sandholt CH *et al.* Genetic architecture of Vitamin B12 and folate levels uncovered applying deeply sequenced large datasets. *PLoS Genet* 2013;**9**:e1003530.

23. Warrington NM, Beaumont RN, Horikoshi M *et al.*; EGG Consortium. Maternal and fetal genetic effects on birth weight and their relevance to cardio-metabolic risk factors. *Nat Genet* 2019;**51**:804–14.

24. Yang J, Lee SH, Goddard ME, Visscher PM. Genome-wide complex trait analysis (GCTA): methods, data analyses, and interpretations. *Meth Mol Biol* 2013;**1019**:215–36.

25. Yang J, Lee SH, Goddard ME, Visscher PM. GCTA: a tool for genome-wide complex trait analysis. *Am J Hum Genet* 2011;**88**:76–82.

26. Bycroft C, Freeman C, Petkova D *et al.* The UK Biobank resource with deep phenotyping and genomic data. *Nature* 2018;**562**:203–09.

27. Bowden J, Davey Smith G, Burgess S. Mendelian randomization with invalid instruments: effect estimation and bias detection through Egger regression. *Int J Epidemiol* 2015;**44**:512–25.

28. Bowden J, Del Greco MF, Minelli C, Davey Smith G, Sheehan NA, Thompson JR. Assessing the suitability of summary data for two-sample Mendelian randomization analyses using MR-Egger regression: the role of the I2 statistic. *Int J Epidemiol* 2016;**45**:1961–74.

29. Hemani G, Zheng J, Wade KH, *et al.* MR-Base: a platform for systematic causal inference across the phenome using billions of genetic associations. *bioRxiv*, doi: 10.1101/078972, 16 December 2016, preprint: not peer reviewed.

30. Bowden J, Davey Smith G, Haycock PC, Burgess S. Consistent estimation in Mendelian randomization with some invalid instruments using a weighted median estimator. *Genet Epidemiol* 2016;**40**:304–14.

31. Hartwig FP, Davey Smith G, Bowden J. Robust inference in summary data Mendelian randomization via the zero modal pleiotropy assumption. *Int J Epidemiol* 2017;**46**:1985–98.

32. Annibale B, Capurso G, Delle Fave G. Consequences of Helicobacter pylori infection on the absorption of micronutrients. *Digest Liver Dis* 2002;**34(Suppl 2)**:S72–77.

33. Tamura A, Fujioka T, Nasu M. Relation of Helicobacter pylori infection to plasma vitamin B12, folic acid, and homocysteine levels in patients who underwent diagnostic coronary arteriography. *Am J Gastroenterology* 2002;**97**:861–66.

34. van Oijen MG, Laheij RJ, de Jong CA, Peters WH, Jansen JB. Vitamin B12 status and its association with Helicobacter pylori infection in alcohol dependent patients. *J Nutr Sci Vitaminol* 2004;**50**:305–08.

35. Allin KH, Friedrich N, Pietzner M *et al.* Genetic determinants of serum vitamin B12 and their relation to body mass index. *Eur J Epidemiol* 2017;**32**:125–34.

36. Moen G-H, Qvigstad E, Birkeland KI, Evans DM, Sommer C. Are serum concentrations of vitamin B-12 causally related to cardiometabolic risk factors and disease? A Mendelian randomization study. *Am J Clin Nutr* 2018;**108**:398–404.

37. Relton CL, Davey Smith G. Two-step epigenetic Mendelian randomization: a strategy for establishing the causal role of epigenetic processes in pathways to disease. *Int J Epidemiol* 2012;**41**:161–76.

38. Zhang G, Feenstra B, Bacelis J *et al.* Genetic associations with gestational duration and spontaneous preterm birth. *N Engl J Med* 2017;**377**:1156–67.

39. Liu X, Helenius D, Skotte L *et al.* Variants in the fetal genome near pro-inflammatory cytokine genes on 2q13 associate with gestational duration. *Nat Commun* 2019;**10**:3927.

40. Knight BA, Shields BM, Brook A *et al.* Lower circulating B12 is associated with higher obesity and insulin resistance during pregnancy in a non-diabetic white British population. *PLoS One* 2015;**10**:e0135268.

41. Knight B, Shields BM, Hattersley AT. The Exeter Family Study of Childhood Health (EFSOCH): study protocol and methodology. *Paediatr Perinat Epidemiol* 2006;**20**:172–79.

42. Hughes AE, Nodzenski M, Beaumont RN *et al.* Fetal genotype and maternal glucose have independent and additive effects on birth weight. *Diabetes* 2018;**67**:1024–29.

43. Hutson JR, Stade B, Lehotay DC, Collier CP, Kapur BM. Folic acid transport to the human fetus is decreased in pregnancies with chronic alcohol exposure. *PLoS One* 2012;**7**:e38057.

44. Giugliani ERJ, Jorge SM, Gonçalves AL. Serum and red blood cell folate levels in parturients, in the intervillous space of the placenta and in full-term newborns. *J Perinatal Med* 1985;**13**:55–60.

45. Scholl TO, Johnson WG. Folic acid: influence on the outcome cof pregnancy. *Am J Clin Nutr* 2000;**71(Suppl 5)**:1295s–303s.

46. Hadfield RM, Lain SJ, Simpson JM *et al.* Are babies getting bigger? An analysis of birthweight trends in New South Wales, 1990-2005. *Med J Aust* 2009;**190**:312–15.

47. Kramer MS, Morin I, Yang H *et al.* Why are babies getting bigger? Temporal trends in fetal growth and its determinants. *J Pediatr* 2002;**141**:538–42.

48. Surkan PJ, Hsieh CC, Johansson AL, Dickman PW, Cnattingius S. Reasons for increasing trends in large for gestational age births. *Obstet Gynecol* 2004;**104**:720–26.