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B-Vitamins and One-Carbon Metabolism

Edited by

Kristina Pentieva

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About the Special Issue Editor

Kristina Pentieva received her undergraduate training and PhD in medical science from the Medical University Sofia, Bulgaria. She worked as a postdoctoral fellow at MRC Dunn Nutrition Centre, Cambridge, UK and is currently professor of human nutrition at the Nutrition Innovation Centre for Food and Health, Ulster University, UK. Her research is focused on B-vitamins involved in one-carbon metabolism and their health effects. Of particular research interest in recent years is the area of B-vitamins in pregnancy and fetal programing. Her research has been supported by governmental agencies, the EC and charities. She has published more than 80 scientific papers in high quality journals across several disciplines. Currently she is a member of the Panel on Dietetic Products, Nutrition and Allergies at the European Food Safety Authority (EFSA) and a member of the working group for updating the European Dietary Reference Values for Vitamins set by EFSA.

Preface to "B-Vitamins and One-Carbon Metabolism"

Folate, vitamin B12, vitamin B6 and riboflavin play a key role as coenzymes in one-carbon metabolism which, in turn, is essential for a broad range of fundamental physiological processes, including RNA and DNA synthesis, cell division, tissue growth and methylation. Deficiencies or imbalance of B-vitamins, as well as genetic polymorphisms and environmental factors, are shown to disturb the normal function of one-carbon metabolism with adverse effects on human health. Although a vast volume of research has already been conducted in this area, there are still significant gaps in our knowledge that require further investigation. This prompted the journal Nutrients to publish a Special Issue focused on the novel findings in the area of B-vitamins and one-carbon metabolism. The Special Issue published 26 research papers and reviews on various topics pertinent to one-carbon metabolism.

Several of the published papers examined the relationship between folate and the metabolically related B-vitamins and degenerative diseases in ageing. Porter et al. provided an extensive review on the causes and consequences of low B-vitamin status and the risk for development of cardiovascular disease, cognitive dysfunction and osteoporosis. In a large cohort of French older adults, followed for approximately 10 years, Lefevre-Arbogast et al. demonstrated that higher intake of folate was associated with a decreased risk of dementia. Troesch et al. reviewed the evidence for the role of genetic polymorphisms related to key enzymes of one-carbon metabolism in the aetiology of Alzheimer's Disease in the context of low intake and status of B-vitamins. Vitamin B6 could be an important factor for maintaining cognitive health in ageing. In a prospective study of Irish older adults. Hughes et al. found that lower vitamin B6 intake and status were associated with a greater than expected rate of cognitive decline over a 4-year period. The results of a randomised controlled trial of supplementation with vitamin B12 (500 μg/d) and folic acid (400 μg/d), for 2 years in 2919 older adults with hyperhomocysteinemia, did not reduce depression symptoms, but had some positive effects on health-related quality of life including mobility, self-care and usual activities (De Koning et al.). In the same study, van der Zwaluw et al. found an inverse relationship between homocysteine concentrations and total brain volume assessed by MRI scans.

Another group of studies investigated the prevalence and the determinants of low B-vitamin status in different population groups. A high prevalence of poor vitamin B12 status (17%) was reported in a cross-sectional study of very old adults (85 y and above). However, the authors showed that the high consumers of meat and meat products were less likely to have deficient vitamin B12 status, whereas those who consumed cereal products were at a lower risk to be folate deficient (Mendonca et al.). A study of pregnant women living in Canada showed a high prevalence of plasma B12 concentrations within the deficient range with women of South Asian origin being at a particular risk for B12 deficiency compared to other ethnic groups (Jeruszka-Bielak et al.). This study observed considerable discrepancy in the prevalence of B12 adequacy depending on the biomarker and cut-off used, suggesting the necessity of developing pregnancy-specific cut-offs to be used for assessment of B12 biomarkers in pregnancy. Vitamin B12 deficiency in vegetarians was a subject of a review by Rizzo et al. where the authors discussed suitable food sources for ensuring an adequate dietary intake of vitamin B12. Ho et al. reported for a high prevalence of poor vitamin B6 status in healthy young non-pregnant Canadian women; the study showed that dietary intake and supplement usage, BMI and ethnicity are important predictors of low vitamin B6 status in this cohort.

The relationship between B-vitamins and body weight in pregnancy was investigated in human and animal studies. Bjorke-Monsen et al. showed, in an observational study of 2797 pregnant women

(Norwegian Mother and Child Cohort Study), that a high pre-pregnancy BMI is a risk factor for low B-vitamin status and increased cellular inflammation in the second trimester, which may explain some of the adverse pregnancy outcomes associated with maternal obesity. Similarly, a retrospective case-control study of pregnant women with or without gestational diabetes, by Sukumar et al., found that in both categories of women, BMI was inversely associated with serum vitamin B12 concentrations and that women with vitamin B12 insufficiency were at a higher risk of being obese or suffering from gestational diabetes. In a porcine model of a controlled supplementation throughout gestation with methylation-related micronutrients, including B-vitamins, Oster et al. demonstrated sex-specific phenotypic and transcriptional responses evident by changes in weight and muscular maturation pre- and postnatally in male but not in female offspring.

The topic of B-vitamins and DNA methylation and gene expression was covered by two experimental studies. McKay et al. reported that maternal folate depletion in mice during pregnancy could influence fetal gene expression, but in a highly organ specific manner which is probably related to prioritisation of metabolic pathways and functions essential for survival and protection of the fetus. A study by Kelly et al. showed that excess intake of folic acid in rats induced expression of target genes in the adipose tissue which may exacerbate weight gain, fat accumulation and inflammation caused by consumption of high fat diet.

The link between one-carbon metabolism and antioxidant status was covered in the review by Bueno Dalto et al. The authors examined evidence for the importance of maternal vitamin B6 status for the flow of one-carbon units for the maintenance of both maternal and embryonic glutathione peroxidase, which is a key component of the antioxidant defense. An experimental study by Zhou et al. showed that enhanced supply of methionine during the periparturient period in cows had positive effects on plasma amino acid profile (higher circulating methionine and proportion of methionine in the essential amino acid pool) which, in turn, had an impact on the overall antioxidant status.

The question of metformin treatment and one-carbon metabolism was examined in two papers. Luciano-Mateo et al. reviewed the evidence for the impact of metformin usage on one-carbon metabolic pathways and concluded that metformin may increase the requirements for folate and vitamin B12. This was supported by the systematic review and meta-analysis on the data from randomised controlled trials on metformin treatment which showed that metformin could increase the concentration of plasma homocysteine and should be counterbalanced with folate and vitamin B12 supplementation (Zhang et al.).

In summary, this Special Issue presents novel data on different topics within the area of B-vitamins and one-carbon metabolism and I believe it will be of interest to nutrition scientists working in both basic and applied research.

Kristina Pentieva
Special Issue Editor

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Article

Prevalence and Predictors of Low Vitamin B6 Status in Healthy Young Adult Women in Metro Vancouver

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Abstract: Low periconceptional vitamin B6 (B6) status has been associated with an increased risk of preterm birth and early pregnancy loss. Given many pregnancies are unplanned; it is important for women to maintain an adequate B6 status throughout reproductive years. There is limited data on B6 status in Canadian women. This study aimed to assess the prevalence of B6 deficiency and predictors of B6 status in young adult women in Metro Vancouver. We included a convenience sample of young adult non-pregnant women (19–35 years; n = 202). Vitamin B6 status was determined using fasting plasma concentrations of pyridoxal 5′-phosphate (PLP). Mean (95% confidence interval) plasma PLP concentration was 61.0 (55.2, 67.3) nmol/L. The prevalence of B6 deficiency (plasma PLP < 20 nmol/L) was 1.5% and that of suboptimal B6 status (plasma PLP = 20–30 nmol/L) was 10.9%. Body mass index, South Asian ethnicity, relative dietary B6 intake, and the use of supplemental B6 were significant predictors of plasma PLP. The combined 12.4% prevalence of B6 deficiency and suboptimal status was lower than data reported in US populations and might be due to the high socioeconomic status of our sample. More research is warranted to determine B6 status in the general Canadian population.

Keywords: vitamin B6; pyridoxal 5′-phosphate; suboptimal status; deficiency; ethnicity; dietary intake; supplement use; women; periconceptional

1. Introduction

Vitamin B6 (B6) has an obligatory role in the endocrine system, immune competence, and heme biosynthesis. In the form of pyridoxal 5′-phosphate (PLP), B6 serves as a coenzyme for >140 reactions in human metabolism, including in the interconversion of amino acids, synthesis of neurotransmitters, regulation of energy homeostasis, and formation of heme [1]. In one-carbon metabolism, PLP acts as a coenzyme for the glycine cleavage system, the interconversion of glycine and serine, and the transsulfuration pathway [2,3]. Other coenzyme functions of PLP include reactions in the kynurenine pathway that catabolizes tryptophan [4].

Maternal B6 adequacy is crucial at conception and throughout pregnancy to ensure healthy pregnancy outcomes. In a meta-analysis of maternal B6 interventions (n = 247), maternal B6 supplementation (2.6 to ≥50 mg/day) was associated with a 217 g higher infant birth weight [5]. A lower incidence of preeclampsia was reported in women supplementing with 10 mg/day of

B6 during pregnancy compared to those who did not use supplements [6]. Low maternal B6 status has been associated with a lower Apgar score, an indicator of infant birth health assessed within minutes after delivery [7,8]. In a prospective cohort of 458 young Chinese women, women who experienced an early pregnancy loss had significantly lower periconceptional plasma PLP concentration compared to women with a healthy pregnancy outcome [9]. Also, low periconceptional B6 status (plasma PLP < 30 nmol/L) has been associated with a reduced probability of conception [10]. Given an estimated 50% of pregnancies are unplanned [11], it is important for women to maintain an adequate B6 status throughout reproductive years.

Despite the crucial role of B6 in health, there is no nationally representative data on biochemical B6 status of Canadian women. The Canadian Community Health Survey (CCHS), Cycle 2.2, Nutrition Focus, in 2004 reported that 18% of Canadian adult women did not meet the Estimated Average Requirement (EAR; 1.1 mg/day) of B6 from dietary sources; however, there was no biochemical measurement of B6 status [12]. In the US National Health and Nutrition Examination Survey (NHANES), over 40% of young adult women (21–44 years) had B6 deficiency (defined as having plasma PLP concentration < 20 nmol/L; [13]). In light of these findings, there is an urgent need to investigate the B6 status of Canadian women.

In several other countries (US, Ireland, Norway), various socioeconomic and lifestyle factors have been associated with plasma PLP concentrations, including oral contraceptive use [13], use of supplemental B6 [13,14], smoking [15,16], physical inactivity, and poverty [17]. Finding predictors of low B6 status in a Canadian population will contribute to the identification of vulnerable populations groups for low B6 status and related health consequences in Canada.

This study aimed to determine the prevalence of suboptimal B6 status and B6 deficiency in a convenience sample of young adult women in Metro Vancouver, using plasma PLP, a biochemical marker of B6 status. Demographic, dietary, and lifestyle predictors associated with plasma PLP were also assessed.

2. Experimental Section

2.1. Participants

This study used data from a descriptive cross-sectional study conducted between 2012 and 2013. The original study was designed to determine the prevalence and predictors of low vitamin B12 status in a convenience sample of young adult women of South Asian and European descent. The recruitment and methods of the original study have been described in detail [18]. In brief, a total of 207 healthy, non-pregnant women aged 19 to 35 years of either South Asian or European descent living in Metro Vancouver were recruited by active and passive recruitment using paper and internet-based advertising, community outreach, and word of mouth. Eligibility of the potential research participants was determined by using a screening questionnaire that assessed multiple factors, including age, ethnicity, residence, and health condition to identify individuals who fit the target demographic. Informed written consent was obtained from all participants in the study. The UBC Clinical Research Ethics Board (H11-01216), Vancouver Coastal Health Research Ethics Board, and Fraser Health Research Ethics Board granted human ethics approval.

2.2. Blood Collection and Analysis

Fasting venous blood samples were obtained from all participants during a single clinic visit. Samples were collected into lithium heparin vacutainers. Plasma was separated using centrifugation and stored at –80 °C until analyses. Blood samples of 202 subjects were available for plasma PLP analysis. Plasma PLP was measured by quantification of its semicarbazide derivative using high-performance liquid chromatography (HPLC) with fluorescence detection [19]. The assay showed good agreement and high precision when measuring external controls at low, medium, and high PLP concentrations, with intra- and inter-assay coefficients of variation of 2.6%, 2.2%, and 2.3%,

and 15.7%, 9.5%, and 10.2%, respectively. The cut-off values used for plasma PLP were: Plasma PLP > 30 nmol/L for adequate B6 status, plasma PLP = 20–30 nmol/L for suboptimal B6 status [20], plasma PLP < 20 nmol/L for B6 deficiency [20,21].

2.3. Demographic, Anthropometric, Exercise and Dietary Data

A demographic questionnaire, the "International Physical Activity Questionnaire (IPAQ)—Short Last 7 Day Self-Administered Format" [22,23], and a semi-quantitative Food Frequency Questionnaire (FFQ) "Your dietary intake" [24] were administered under the supervision of research staff during the clinic visit.

The demographic questionnaire collected information on age, ethnicity, immigration, education, household income, oral contraceptive use, and supplement use. South Asian and European ethnicities were defined as having at least three grandparents from a single ethnic group. South Asian ethnicity included Bangladeshi, Bengali, East Indian, Goan, Gujarati, Hindu, Ismaili, Kashmiri, Nepali, Pakistani, Punjabi, Sikh, Sinhalese, Sri Lankan, and Tamil ethnic groups. Because of the low number of participants (n = 8) indicating secondary school education or less than secondary school education, education was dichotomized. Low education was considered as below a bachelor's degree, and high education as equal or higher than bachelor's degree. Due to the low number of participants (n = 36) in the lowest household income bracket, household income was dichotomized into the following categories: Low income, total annual household income < $30,000 if 1–2 people, <$40,000 if 3–4 people, <$60,000 if ≥5 people, and high income, total annual household income ≥ $30,000 if 1–2 people, ≥$40,000 if 3–4 people, ≥$60,000 if ≥5 people. Participants who reported use of oral contraceptives were classified as oral contraceptive (OC) users. Duration and frequency of OC use were recorded. Participants who reported current consumption of any nutritional vitamin or mineral supplements were classified as nutritional supplement users. Participants were asked to bring in nutritional supplement bottles. Brand names and frequencies of intake were recorded and B6 content (yes/no) was obtained retrospectively by researchers from web-based product information. Participants who were taking supplements containing B6 were classified as supplemental B6 users. Subjects were asked about their smoking habit and categorized as non-smoker, former smoker, current occasional smoker (1–9 cigarettes/day), current regular smoker (10–19 cigarettes/day) or current frequent smoker (≥20 cigarettes/day). Due to the high number of non-smokers (n = 174), smoking data were dichotomized to non-smoker and smoker (including current smokers and former smokers).

The IPAQ asked the participants to recall their physical activities in the past seven days categorized as: Walking, moderate-intensity activities, and vigorous intensity activities. Each activity was recorded with its frequency (days per week) and duration (time per day). Participants were categorized into three physical activity levels; low, medium, and high, according to the IPAQ analysis protocol [22].

A semi-quantitative FFQ was used to determine dietary B6 intake. The questionnaire contained 78 food items and has been validated to assess micronutrient intakes in the Canadian population [24,25].

Anthropometric measurements, including height, weight, and waist circumference, were taken by research staff during the clinic visit. Body mass index (BMI) was calculated based on weight and height.

2.4. Statistical Analyses

The primary outcome of the presented analyses is B6 status and described using the direct biomarker plasma PLP and established cut-offs for adequate B6 status, suboptimal B6 status [20], and B6 deficiency [21]. Data were examined for normality by visual histogram assessment. Plasma PLP concentration was log-transformed to carry out statistical analyses and was presented as geometric mean (95% confidence interval (CI)). Relative dietary B6 intakes were presented as quartile ranges.

Bivariate analysis was conducted to identify variables associated with plasma PLP concentration (as continuous variable and as categorical variable using B6 status cutoffs) and relative dietary B6 intake. Two sample t tests were used for dichotomous variables, one-way ANOVA followed by

Tukey's Honest Significance test for categorical variables with more than two levels, and simple linear regression for continuous variables. If the *P* value from bivariate analysis was ≤0.2, the variable was carried forward to the stepwise multiple linear regression model. The full model with plasma PLP concentration as the dependent variable was controlled for relative dietary B6 intake, South Asian ethnicity, first generation immigrant status, BMI, education status, household income status, smoking status, and supplemental B6 use. Backward elimination procedure was used to establish the best fit multiple linear regression model. The estimated percentage change in plasma PLP concentration was presented for each variable after adjustment for other variables. Statistical significance was set at a two-sided *p* value of <0.05. All statistical analyses were performed using R software (3.1.2 windows version; R Foundation for Statistical Computing: Vienna, Austria).

3. Results

3.1. Participant Characteristics

Overall, subjects (n = 202) were highly educated, with 71% having a bachelor's degree or higher (Table 1). The rate of supplemental B6 use was 28% and did not differ based on age, BMI, ethnicity, household income, level of education, level of physical activity, OC use, or smoking status.

Table 1. Demographic and lifestyle characteristics of 202 healthy young adult women in Metro Vancouver.

Variables	Total (n = 202)
Age *, years	26.7 ± 4.2
Body mass index †, kg/m^2	22.7 (22.2, 23.1)
Waist circumference †, cm	74.9 (73.7, 76.1)
Total energy intakes †,‡, kcal/day	1566 (1504, 1629)
Relative dietary vitamin B6 intake ‡, mg/day	
Q1	0–1.1
Q2	1.1–1.4
Q3	1.4–1.7
Q4	>1.7
Ethnicity, n (%)	
European	147 (73)
South Asian	55 (27)
Education, n (%)	
Less than secondary school education	2 (1)
Secondary school diploma	5 (3)
Post-secondary education	51 (25)
Bachelor's degree	87 (43)
University degree or >bachelor's degree	57 (28)
Household income §, n (%)	
Lowest	36 (20)
Lower-middle	44 (24)
Upper-middle	52 (28)
Highest	51 (28)
Physical activity level ǀǀ, n (%)	
Low	8 (4)
Medium	110 (55)
High	82 (41)
Use of oral contraceptives, n (%)	58 (29)
Use of nutritional supplements, n (%)	97 (48)
Use of supplemental vitamin B6, n (%)	56 (28)
Current or former smoker, n (%)	28 (14)

* Values presented as arithmetic mean ± SD. † Values presented as geometric mean (95% confidence interval). ‡ 18 samples were excluded due to plausible misreports in the food frequency questionnaire (n = 184). § Income quartiles are: Lowest: <$15,000 if 1–2 people, <$20,000 if 3–4 people, <$30,000 if ≥5 people, Lower-middle: $15,000–29,999 if 1–2 people, $20,000–39,999 if 3 or 4 people, $30,000 to 50,000 if ≥5 people, Upper-middle: $30,000–59,999 if 1–2 people, $40,000–79,999 if 3–4 people, $60,000–79,999 if ≥5 people, Highest: >$60,000 if 1–2 people, >$80,000 if ≥3 people. Only 183 subjects provided household income information. ǀǀ Only 200 subjects completed physical activity questionnaire.

3.2. Blood Analyses

Mean (95% CI) concentration of plasma PLP was 61.0 (55.2, 67.3) nmol/L (Table 2). The prevalence of B6 deficiency (plasma PLP < 20 nmol/L) was 1.5% and that of suboptimal B6 status (plasma PLP = 20–30 nmol/L) was 10.9%.

Table 2. Differences in plasma pyridoxal 5′-phosphate (PLP) concentration in 202 healthy young adult women by categories of demographic and lifestyle factors.

Variables	Subjects n	Plasma PLP concentration Mean (95% CI) nmol/L	<20 nmol/L n (%) *	20–30 nmol/L n (%) *	>30 nmol/L n (%)
Overall	202	61.0 (55.2, 67.3)	3 (1.5)	22 (11)	177 (88)
Anemia					
Hb > 12 g/dL	166	62.0 (55.5, 69.2)	3 (1.8)	17 (10)	146 (88)
Hb < 12 g/dL	36	56.4 (45.0, 70.8)	0	5 (14)	31 (86)
Ethnicity					
European ‡	147	66.9 (59.2, 75.6) †	2 (1.4)	14 (10)	131 (89)
South Asian	55	47.6 (41.2, 54.8)	1 (1.8)	8 (15)	46 (84)
Education					
Low	7	57.7 (28.5, 116.9)	0	2 (29)	5 (71)
High	195	61.1 (55.3, 67.5)	3 (1.5)	20 (10)	172 (88)
Household income					
Low	80	59.5 (52.1, 68.0)	1 (1.3)	7 (9)	72 (90)
High	103	63.6 (55.3, 73.0)	2 (1.9)	15 (13)	86 (83)
Physical activity level					
Low	8	44.4 (37.8, 52.3)	0	0	8 (100)
Medium	110	60.6 (53.0, 69.4)	2 (1.8)	13 (12)	95 (86)
High	82	63.8 (54.5, 74.8)	1 (1.2)	9 (11)	72 (88)
Oral contraceptive use					
Non-user	144	59.9 (53.5, 67.1)	2 (1.4)	17 (12)	125 (87)
User	58	63.6 (52.0, 77.8)	1 (1.7)	5 (9)	52 (90)
Supplemental vitamin B6 use					
Non-user ‡	146	48.5 (45.0, 52.3)	3 (2.1)	18 (12)	125 (86)
User	56	111.5 (87.2, 139.9) †	0	4 (7)	52 (93)
Smoker					
No	174	61.1 (55.0, 67.8)	2 (1.2)	18 (10)	154 (89)
Yes	28	60.2 (44.0, 82.3)	1 (3.6)	4 (14)	23 (82)

Plasma PLP concentration was presented as geometric mean (95% confidence interval (CI)) and was log-transformed to carry out the following statistical analyses. Hb, hemoglobin. * Chi-squared test revealed no significant difference in the prevalence of B6 deficiency and suboptimal B6 status in all the variables. † p value < 0.05 for two-sample t test comparing the indicated category with the referent category. ‡ Referent category.

Users of supplemental B6 had significantly higher plasma PLP concentration compared to non-users of supplemental B6; mean (95% CI) plasma PLP concentrations were 48.5 (45.0, 52.3) and 111.5 (87.2, 139.9), respectively ($p < 0.001$). Women of South Asian descent had significantly lower plasma PLP concentration (47.6 (41.2, 54.8)) compared with women of European descent (66.9 (59.2, 75.6), $p = 0.002$). There was no significant difference in plasma PLP concentration based on education level, household income, physical activity level, OC use, or smoking status in bivariate analyses. There was no significant difference in the prevalence of B6 deficiency, suboptimal B6 status or both combined (plasma PLP < 30 nmol/L) compared to adequate B6 status based on any demographic or lifestyle factors.

3.3. Dietary Analyses

Quartiles of dietary B6 intake were 0–1.1 mg/day, 1.1–1.4 mg/day, 1.4–1.7 mg/day and >1.7 mg/day, respectively. Individuals with low household income had significantly lower dietary B6

intake compared to individuals with high household income (mean ± SD: 1.4 ± 0.42 vs. 1.5 ± 0.47, respectively; p = 0.045). Plasma PLP concentration and relative dietary B6 intake were positively but weakly correlated (r = 0.17; p = 0.02).

Dietary vitamin B6 intake derived mainly from meat and meat alternatives in this sample of young adult women of South Asian and European descent, as shown by multiple linear regression of dietary B6 intake (Table 3). There was no significant difference in the intake of meat and meat alternatives between women of South Asian and European descent (2.8 versus 3.0 servings; p = 0.2).

Table 3. Food sources of vitamin B6.

Variables	Adjusted change in dietary B6 intake, mg/day (95%CI)	Adjusted *p* value
Grains, serving	0.03 (0.01, 0.04)	<0.001
Fruits and Vegetables, serving	0.10 (0.08, 0.11)	<0.001
Dairy, serving	0.10 (0.07, 0.13)	<0.001
Meat and Alternatives, serving	0.19 (0.16, 0.22)	<0.001

Adjusted changes and p value were from multiple linear regression model controlled for intake of grains, fruits and vegetables, dairy, and meat and alternatives. Number of observation = 184, Model p value < 0.001, R^2: 0.79, Adjusted R^2: 0.79.

3.4. Multivariate Analyses

Relative dietary B6 intake, BMI, ethnicity and the use of supplemental B6 were significant predictors of plasma PLP concentration (Table 4). Relative dietary B6 intake and the use of supplemental B6 were positively associated with plasma PLP concentration; BMI and South Asian ethnicity were negatively associated with plasma PLP concentration. The model explained 32% (R^2) of the variance in plasma PLP concentration.

Table 4. Predictors of plasma PLP concentration from unadjusted and adjusted linear regression models.

Variables	Unadjusted % change in plasma PLP (95% CI) *	Unadjusted *p* value	Adjusted % change in plasma PLP (95% CI) *	Adjusted *p* value
Relative B6 intake				
Q2 (1.1–1.4 mg/day)	7.7 (–20.0, 45.0)	0.62	9.0 (–15.8, 41.2)	0.51
Q3 (1.4–1.7 mg/day)	27.5 (–5.3, 71.6)	0.11	23.1 (–4.4, 58.6)	0.11
Q4 (>1.7 mg/day)	35.6 (0.7, 82.4)	0.045	29.3 (0.3, 66.7)	0.048
BMI, kg/m^2	–3.7 (–6.3, –1.0)	0.007	–2.7 (–5.1, –0.2)	0.034
South Asian descent	–28.9 (–33.6, –24.0)	0.002	–21.1 (–35.7, –3.1)	0.024
Supplemental B-6 use	127.7 (115.6, 140.8)	<0.001	114.5 (75.6, 162.0)	<0.001

Unadjusted percentage changes and p value were from simple linear regression models. Adjusted percentage changes and p value were from multiple linear regression model controlled for relative dietary B-6 intake, South Asian ethnicity, BMI, and supplemental B-6 use. Number of observation = 184, Model p value <0.001, R^2: 0.32, Adjusted R^2: 0.29. * The percentage change in plasma PLP concentration was calculated by $(e^{\beta 1} - 1) \times 100\%$.

Women taking supplemental B6 are expected to have ~113% higher plasma PLP after adjusting for relative dietary B6 intake, BMI and ethnicity (compared to ~127% higher plasma PLP in the unadjusted model) (Table 4). South Asian descent was associated with 21% lower plasma PLP concentration compared to women of European descent (p = 0.026), after adjusting for relative dietary B6 intake, BMI and the use of supplemental B6.

4. Discussion

Since the vitamin B6 status of Canadian women was previously unknown, and the relationship between demographic, dietary, and lifestyle factors and plasma PLP had not been assessed, we conducted a study of B6 status in a convenience sample of 202 healthy young Canadian adult

women. We identified a combined prevalence of B6 deficiency and suboptimal B6 status of 12.4%. We also found that body mass index, South Asian ethnicity, relative dietary B6 intake, and the use of supplemental B6 were significant predictors of plasma PLP.

As defined by the Institute of Medicine, a plasma PLP concentration of <20 nmol/L corresponds with B6 deficiency [21]. The prevalence of B6 deficiency (1.5%) in this sample was much lower than the 40% prevalence of B6 deficiency reported for women aged 21–44 years in the NHANES 2003–2004 [13]. This large discrepancy may partly be due to the high socioeconomic status of the women in our study compared with participants of the NHANES, a representative population-based survey in the US. Low socioeconomic status has been associated with low B6 status [15,17] and overall poorer nutritional intake [26]. Education and income status accounted for 6% of the variation in plasma PLP in the NHANES [15]. The discrepancy between our study and the NHANES may also be attributed to different analytical methods employed for quantification of plasma PLP. The enzymatic assay used for the NHANES 2003–2004 samples has been reported to underestimate plasma PLP concentrations to a degree that would result in a doubling of the prevalence of B6 deficiency compared to the HPLC method we employed [27]. In the recent Alberta Pregnancy Outcomes and Nutrition (APrON) cohort study, none of the 119 pregnant women in the first trimester and only one out of 528 (0.2%) second-trimester pregnant women was B6 deficient; the prevalence of suboptimal B6 status was not reported [28]. Like our study, the APrON cohort is comprised of women of high socioeconomic status and the lack of B6 deficiency observed is likely due to the high rate (>94%) of multivitamin supplement use throughout pregnancy [28].

Suboptimal B6 status has been associated with an increased risk of several chronic diseases, including cardiovascular disease [29], colorectal cancer [30], breast cancer [31] and ovarian cancer [32]. In our study, 11% of our subjects had suboptimal B6 status. A cross-sectional study in Boston reported 18% of suboptimal B6 status among Puerto Rican women aged 45–75 years (n = 1236) [17]. The lower education level and income of subjects in that study may explain the higher prevalence of suboptimal B6 status compared with our study. In light of the limited data on B6 status in Canadians [12,28] and our finding of a substantial rate of suboptimal status in a cohort of healthy women of high socioeconomic status, a representative, population-based study to determine B6 status of Canadians is timely and crucial. We predict a higher prevalence of B6 deficiency and suboptimal B6 status would be observed in a representative sample of Canadian women compared to our study.

The use of supplemental B6 was a strong predictor of plasma PLP concentration both before and after multivariate adjustment. This is consistent with large scale studies that reported higher plasma PLP concentrations and a lower prevalence of B6 deficiency in nutritional supplement users [13] and supplemental B6 users [14] compared with non-users. In this sample, 48% of women used nutritional supplements (i.e., any type of vitamin or mineral supplement) of whom 58% were retrospectively categorized as supplemental B6 users. The prevalence of nutritional supplement use in this sample (48%) was higher than the prevalence reported in the CCHS in 2004 [33] with 35% of Canadian women aged 19–30 years reporting nutritional supplement use.

Relative dietary B6 intake was not a strong predictor of plasma PLP concentration both before and after multivariate adjustment. The weak association may partially be due to the use of a semi-quantitative FFQ. The validation study of the FFQ reported an 11% underestimation of relative dietary B6 intake compared to the use of dietary recalls [24]. Although the FFQ was validated to assess micronutrient intakes in the Canadian population [24], it is possible that it was unable to record certain ethnic specific foods. Further, under-reporting appears to be a factor, as a 1566 kcal/day average daily energy intake is lower than would be anticipated. In the NHANES 2003–2004, a stronger association between total B6 intake (from food sources and supplements combined) and plasma PLP ($r = 0.32$, $p < 0.001$) was observed [13]. We are unable to report quantitative intake of B6 from supplements due to limitations of secondary analysis. The weaker association between plasma PLP and dietary B6 intake in this study compared to the NHANES data might be explained by our data reflecting dietary B6 from food alone and not supplemental B6.

Our study is the first to report that women of South Asian descent may have lower plasma PLP concentrations compared with women of European descent. The difference in B6 status between South Asian and European women was not related to the consumption of different food sources of B6; meat and meat alternatives were the main dietary B6 sources in these healthy adult women. The negative association between South Asian ethnicity and plasma PLP concentrations remained significant after adjustment for relative dietary B6 intake, BMI, and supplemental B6 use. Although the South Asian population was only 56, compared to the European population of 146 in this study, the sample size was sufficient to give over 80% of power with a small effect size of 0.2 and significance level of 0.05 in the multiple linear regression model. Ethnicity has been shown to correlate with nutritional biomarker levels of other micronutrients [34].

Body mass index was inversely associated with plasma PLP concentration after adjustment for relative dietary B6 intake, supplemental B6 use, and South Asian ethnicity. An inverse relationship of BMI and plasma PLP was also reported in the NHANES; every 25% increase in BMI was associated with a 13% decrease in plasma PLP [15]. The volumetric dilution of the blood was suggested as a potential underlying mechanism for the linkage between increasing body mass and decreasing concentrations of B6 as well as other B-vitamins [35]. In addition, plasma PLP concentration has been inversely associated with circulating concentration of C-reactive protein (CRP), a marker of systemic inflammation that is elevated in obesity [36,37].

Compared to other studies, we did not find OC use to be a significant predictor of B6 status. In recent literature, lower-dose OC use was associated with low B6 status [38]. In the NHANES 2003–2004, plasma PLP concentration was also significantly lower in OC users compared to non-users [13]. It was suggested that this may be due to the disruption of tryptophan metabolism by estrogen, independent of B6 status [39]. In our study, the effect of OC use on plasma PLP concentration was small and we had insufficient sample size to detect a statistically significant difference. We also lacked power to detect associations with other variables including physical activity and smoking.

The predictors of B6 status we assessed explained only 32% of the variability in plasma PLP. Our results might be confounded by unexplained biological factors or genetic modifiers. One recent study reported some variants in tissue nonspecific alkaline phosphatase gene influenced plasma PLP concentration, but the clinical implications of these variants were unclear [40].

Some weaknesses of this study include the recruitment of a convenience sample of relatively healthy women of high education and the lack of data on genetic variants. Over 71% of our study participants obtained a bachelor's degree or higher, where a comparatively lower proportion (35%) of women aged 25 to 64 in Metro Vancouver reported this high level of education, according to the National Household Survey [41]. We used plasma PLP as our single biomarker to assess B6 status since it is currently the only established biomarker with cut-offs to define B6 deficiency [21]. However, plasma PLP only reflects the circulating concentration of B6 and is influenced by several factors, such as inflammation, serum albumin and alkaline phosphatase concentrations [42]. The derivation of reference intervals for sensitive functional biomarkers, e.g., plasma cystathionine [43], is crucial for evaluation of intracellular B6 status.

5. Conclusions

We report a 12.4% combined prevalence of B6 deficiency and suboptimal B6 status in healthy young adult women in Metro Vancouver (n = 202). Periconceptional B6 adequacy is crucial for healthy pregnancy outcomes; and women with higher BMI and South Asian ethnicity might be more likely to have low B6 status. The lower prevalence of B6 deficiency and suboptimal B6 status in these women compared to reports from representative samples in other countries may be due to the high socioeconomic status of these women. Given the central roles of B6 in key biological functions and health, more research is warranted to determine B6 status in the general Canadian population.

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Association of Vitamin B12 with Pro-Inflammatory Cytokines and Biochemical Markers Related to Cardiometabolic Risk in Saudi Subjects

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Abstract: Background: This study aimed to examine the relationship between changes in systemic vitamin B12 concentrations with pro-inflammatory cytokines, anthropometric factors and biochemical markers of cardiometabolic risk in a Saudi population. Methods: A total of 364 subjects (224 children, age: 12.99 ± 2.73 (mean ± SD) years; BMI: 20.07 ± 4.92 kg/m^2 and 140 adults, age: 41.87 ± 8.82 years; BMI: 31.65 ± 5.77 kg/m^2) were studied. Fasting blood, anthropometric and biochemical data were collected. Serum cytokines were quantified using multiplex assay kits and B12 concentrations were measured using immunoassay analyzer. Results: Vitamin B12 was negatively associated with TNF-α (r = −0.14, $p < 0.05$), insulin (r = −0.230, $p < 0.01$) and HOMA-IR (r = −0.252, $p < 0.01$) in all subjects. In children, vitamin B12 was negatively associated with serum resistin (r = −0.160, $p < 0.01$), insulin (r = −0.248, $p < 0.01$), HOMA-IR (r = −0.261, $p < 0.01$). In adults, vitamin B12 was negatively associated with TNF-α (r = −0.242, $p < 0.01$) while positively associated with resistin (r = 0.248, $p < 0.01$). Serum resistin was the most significant predictor for circulating vitamin B12 in all subjects ($r^2 = -0.17$, $p < 0.05$) and in children ($r^2 = -0.167$, $p < 0.01$) while HDL-cholesterol was the predictor of B12 in adults ($r^2 = -0.78$, $p < 0.05$). Conclusions: Serum vitamin B12 concentrations were associated with pro-inflammatory cytokines and biochemical markers of cardiometabolic risks in adults. Maintaining adequate vitamin B12 concentrations may lower inflammation-induced cardiometabolic risk in the Saudi adult population.

Keywords: vitamin B12; resistin; TNF-α; cardiometabolic diseases

1. Introduction

Vitamin B12 is an essential micronutrient required for optimal hemopoetic, neurologic and cardiometabolic function [1,2]. Human beings are dependent upon bacterial and protozoal sources to maintain adequate serum concentrations [3]. Vitamin B12 absorption is a complex process and any dysfunction can result in its deficiency, despite adequate dietary intake [4]. Vitamin B12

deficiency affects two principal enzymatic pathways, i.e., methylation process of homocysteine to methionine and the conversion of methylmalonyl coenzyme A (CoA) to succinyl-CoA [2,5], which lead to accumulation of methylmalonic acid (MMA) and/or homocysteine (Hcy) [2,5]. Excessive accumulation of homocysteine in blood (hyperhomocysteinemia) is associated with an increased risk of cardiovascular disease due to its cellular and vasculo-toxic effects [1,5]; whilst, increase in circualting methylmalonic acid (MMA) level is associated with defective fatty acid synthesis of neuronal membranes [3,5].

Current evidence demonstrated that vitamin B12 deficiency is linked to various atherogenic processes, mainly, but not exclusively, through B12 deficiency-induced hyperhomosyteinemia [6]. Hyperhomocysteinemia is a possible independent risk factor for cardiovascular disease, mechanisms of which include reduction of nitric oxide thereby promoting endothelial dysfunction [7,8]. On the other hand, cytokines, such as tumor necrosis factor (TNF-α) and resistin, play an important role in cardiovascular risk and insulin resistance [9,10].

Unfortunately, there is limited information available as to whether circulating vitamin B12 concentrations affect the cardiometabolic status of the Arab population. In fact, while it has been previously observed that vitamin B12 is inversely associated with homocysteine in the general Saudi population [11], homocysteine itself is not a risk factor for endothelial function, at least amongst Saudi Arabs [12]. Therefore, in this study we aimed to examine the associations between serum vitamin B12 concentrations, pro-inflammatory cytokines and biochemical markers of cardiovascular and metabolic risk in the general population of Saudi Arabia.

2. Materials and Methods

2.1. Subjects and Experimental Design

In this cross-sectional study, a total of 364 Saudi subjects (224 children aged 8–17 years and 140 adults aged 30–59 years) were recruited from four Primary Health Care Centers (PHCCs) within Riyadh, Saudi Arabia. Written informed consent was obtained prior to inclusion in the study. Inclusion criteria were apparently healthy children (N = 224) and adults (N = 140), nonsmokers, non-diabetic, non-hypertensive and without morbid obesity. Ethical approval was obtained from the Ethics Committee of the College of Science Research Center (Ethics Approval Code: 8/25/36681), King Saud University, Riyadh, Saudi Arabia.

2.2. Anthropometric Measurements

A generalized questionnaire was given to all participating subjects aimed at seeking demographic information, general health status and past medical history. Physical examination was carried out by a physician who ensured exclusion of subjects with chronic conditions (cardiac, kidney or liver disease, hypertension, psychiatric conditions) or use of medications known to affect body weight (e.g., steroids). Weight and height were recorded to the nearest 0.2 kg and 0.5 cm, respectively, using an appropriate international standard scale (Digital Pearson Scale, ADAM Equipment Inc., Oxford, CT, USA). Both measurements were done while subjects were standing upright and wearing light clothing. Body mass index (BMI) was calculated as weight in kg divided by height in squared meters (kg/m^2). Additionally, waist and hip circumferences were measured using a standardized measuring tape in centimeters (cm) and the waist-to-hip ratio (WHR) was estimated. Blood pressure was measured using an appropriate mercury sphygmomanometer, measured twice with 5-min intervals and the mean of the two readings was recorded.

2.3. Blood Collection and Biochemical Measurements

Fasting blood samples were collected and stored at −20 °C. Serum insulin, leptin, adiponectin, TNF-α and resistin were quantified using multiplex assay kits that utilize fluorescent microbead technology, allowing simultaneous quantification of several target proteins within a single serum

sample of 50–100 μL. These included pre-mixed and fully customized panels that utilized the Luminex's xMAP Technology platform (Luminexcorp, TX, USA). For parameters measured using the multiplex assay, the intra-assay variation was 1.4%–7.9% and inter-assay variation of <21%. Minimum detectable concentrations (MDC) were as follows: insulin, 50.9 pg/mL; leptin, 85.4 pg/mL; adiponectin, 145.4 pg/mL; resistin, 6.7 pg/mL, and TNF-α, 0.14 pg/mL.

Vitamin B12 was measured using Roche Elecsys immunoassay analyzer, Cobas e 411 (Roche Diagnsotics GmbH, Mannheim, Germany) that employs the test principle of competitive electrochemiluminiscence immunoassay. The assay has a linearity range of 30–2000 pg/mL. The assay involves binding of vitamin B12 in the sample to the ruthenium labeled antibody. Streptavidin coated microparticles (solid phase), binds the biotin labeled vitamin B12 and ruthenium labeled antibody. A further wash period leaves the solid phase magnetic microparticles bound with vitamin B12-biotin and ruthenium labeled antibody, which generates chemiluminiscent emission signal that is inversely proportional to the concentration of vitamin B12 present in the patient sample. The inter- and intra-assay coefficients of variation (CV) for Vitamin B12 were 3.0% and 3.7%, respectively.

Fasting serum glucose concentrations and complete lipid profile (triglycerides, total cholesterol, high density lipoprotein (HDL)-cholesterol were determined using a biochemical analyzer) (Konelab, Espoo, Finland). This biochemical analyzer was calibrated routinely prior to the analysis of all serum samples using quality control samples provided by the manufacturer (Thermo-Fisher Scientific, Espoo, Finland). Low density lipoprotein (LDL)-cholesterol was calculated using the Friedman formula.

2.4. Statistical Analyses

Statistical analyses were carried out using the SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. Kolmogorov-Smirnov statistics was performed to test continuous variables for normality. All non-Gaussian parameters were logarithmically or square-root transformed to normalize data. Independent Student T-test was used to compare means between groups of normally distributed data. Pearson correlations between serum vitamin B12 and the rest of the variables were determined. Stepwise linear regression models were performed using vitamin B12 as dependent variable and age, gender, BMI, systolic and diastolic blood pressure, waist and hip circumference, glucose, insulin, HDL, LDL, triglycerides, total cholesterol, leptin, adiponectin, TNF-α, resistin, HOMA-IR as independent variables to determine significant predictors. In all statistical tests, p-values < 0.05 were considered significant.

3. Results

A total of 364 Saudi subjects (224 children and 140 adults) satisfied the inclusion criteria and were included in the analysis. All the anthropometric and laboratory characteristics of the study participants are presented in Table 1.

Table 1. Clinical characteristics, glycemic, lipid and metabolic profiles of subjects.

Parameters	Children	Adults
	Clinical Characteristics	
N	224	140
M/F (%)	122/102 (54.5/45.5)	37/103 (26.4/73.6)
Age (years)	12.99 ± 2.73	41.87 ± 8.82
Body Mass Index (kg/m^2)	20.07 ± 4.92	31.65 ± 5.77
Waist circumference (cm)	66.37 ± 15.50	88.51 ± 15.65
Hip circumference (cm)	82.06 ± 15.59	99.01 ± 15.77
Systolic Blood Pressure	105.54 ± 10.21	114.25 ± 13.53
Diastolic Blood Pressure	68.85 ± 7.12	75.73 ± 8.81
	Glycemic Profile	
Glucose (mmol/L)	5.11 ± 0.61	5.37 ± 0.81
Insulin (IU/mL) #	6.5 (4.1–10.8)	9.3 (7.1–13.2)
HOMA-IR #	1.46 (0.88–2.41)	2.37 (1.50–3.22)

Table 1. *Cont.*

Parameters	Children	Adults
	Lipid Profile	
Triglycerides (mmol/L) #	1.06 ± 0.55	1.54 ± 0.71
Total Cholesterol (mmol/L)	4.23 ± 0.77	4.65 ± 0.98
HDL-Cholesterol (mmol/L)	1.02 ± 0.34	0.87 ± 0.33
LDL-Cholesterol (mmol/L)	2.72 ± 0.69	3.07 ± 0.77
	Metabolic Profile	
Leptin (ng/mL) #	9.10 (1.94–27.62)	15.73 (7.52–25.67)
Adiponectin (mg/mL) #	20.22 (12.66–28.42)	17.37 (5.19–431)
Resistin (ng/mL) #	15.10 (9.97–20.88)	5.01 (2.89–14.47)
TNF-α (pg/mL) #	7.51 (4.91–9.79)	15.34 (5.08–74.02)
Vitamin B12 (Pg/mL) #	421 (290.6–530.6)	371.25 (288.6–496.3)

Note: Data presented as N (%) for frequencies; mean ± standard deviation for normal continuous variables; # denotes continuous variables with non-Gaussian distribution and presented as median (25th–75th Percentile).

Correlations between vitamin B12 and clinical parameters measured are presented in Table 2. In children, vitamin B12 level was negatively associated with age (r = −0.19, $p < 0.01$), hip circumference (r = −0.20, $p < 0.01$), systolic BP (r = −0.21, $p < 0.01$), insulin (r = −0.24, $p < 0.01$), HOMA-IR (r = −0.26, $p < 0.01$) and resistin (r = −0.16, $p < 0.01$). In adults, vitamin B12 was positively associated with serum resistin (r = 0.24, $p < 0.01$), whereas it was negatively associated with TNF-α (r = −0.24, $p < 0.01$). Mean serum concentrations of vitamin B12 were plotted against quartiles of HOMA-IR (supplementary materials Figure S1) and resistin (supplementary materials Figure S2) in children and adults.

Table 2. Associations between vitamin B12 and clinical, glycemic, lipid and metabolic profiles.

Parameters	Coefficients of Correlation (r)	
	Children (N = 224)	Adults (N = 140)
Age (years)	−0.192 **	0.022
Body Mass Index (kg/m^2)	−0.132	0.006
Waist circumference (cm)	−0.105	0.089
Hip circumference (cm)	−0.207 **	0.050
Systolic Blood Pressure (mmHg)	−0.211 **	0.116
Diastolic Blood Pressure (mmHg)	−0.119	−0.029
Glucose (mmol/L)	−0.102	−0.071
Insulin (IU/mL) #	−0.248 **	−0.118
HOMA-IR	−0.261 **	−0.156
Triglyceride (mmol/L) #	−0.104	0.041
Cholesterol (mmol/L)	0.010	0.177
HDL-Cholesterol (mmol/L)	0.139 *	0.030
LDL-Cholesterol (mmol/L)	−0.046	0.117
Leptin (ng/mL) #	−0.130	0.060
Adiponectin (mg/mL) #	0.054	0.036
Resistin (ng/mL) #	−0.160 **	0.248 **
TNF-α (pg/mL) #	−0.062	−0.242 **

Note: Data presented as coefficient (r); * denotes significance at 0.05 level; ** denotes significance at 0.01 level; # log transformed values.

Stepwise linear regression analysis, using vitamin B12 as the dependent variable and all the parameters as independent variables, is presented in Table 3. In adults, HDL-cholesterol was the significant predictor of vitamin B12 ($r^2 = 0.78$, $p = 0.044$) whilst, serum resistin ($r^2 = -0.16$, $p = 0.004$), gender ($r^2 = -0.15$, $p = 0.001$) and age ($r^2 = -0.22$, $p = 0.02$) were independent predictors of vitamin B12 level in children.

Table 3. Stepwise linear regression analysis using vitamin B12 as a dependent variable.

	Predictor	Unstandardized β	SE	*p*-Value
Adults	HDL-cholesterol	0.781	0.169	0.044
Children	Gender	−0.155	0.036	0.001
	Age	−0.022	0.007	0.02
	Resistin	−0.167	0.069	0.004

Note: Independent variables entered: age, gender, BMI, systolic and diastolic blood pressure, waist and hip circumference, glucose, insulin, HDL-cholesterol, LDL-cholesterol, triglycerides, total cholesterol, leptin, adiponectin, TNF-α, resistin, HOMA-IR. Significant at $p < 0.05$.

4. Discussion

The present study represents the first in a Saudi population and highlights a relationship between circulating vitamin B12 concentrations, cytokines and biomarkers associated with cardiometabolic risk. For this study we recruited a total of 364 subjects (224 children, and 140 adults). Fasting blood, anthropometric and biochemical data were collected. Serum cytokines were quantified using multiplex assay kits and B12 concentrations were measured using immunoassay analyzer. Our observations are of considerable clinical importance, as any irregularity in the metabolism of vitamin B12 is known to be associated with a wide spectrum of hematologic, metabolic and cardiovascular disorders and early intervention appears key [1,4,5,9].

TNF-α is a hallmark of inflammation [13] and several lines of evidence suggest the association of vitamin B12 deficiency with an increased incidence of inflammation and associated metabolic complications [3,13]. In the current study we observed a significant inverse correlation between serum vitamin B12 and TNF-α level in adults. However, in children, there was no such correlation. Elevated systemic TNF-α level is observed to be associated with depletion of primary antioxidants that cells use to protect themselves against free radicals' damaging effects [14]. Depletion of antioxidants further induces pro-inflammatory cytokines and other inflammatory by-products like prostaglandins and leukotrienes, known risk factors for metabolic and cardiovascular diseases [14,15]. Earlier studies suggest that consistent chronic inflammation may lead to insulin resistance, type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) [10]. Deficiency of vitamin B12 causes hyperhomocysteinemia (HHcy) [5,16], and elevated homocysteine is known to lead to an inflammatory state, a risk factor for coronary heart disease [16] and insulin resistance [17,18]. In mice, HHcy promotes insulin resistance by directly inducing the expression and secretion of TNF-α and resistin [19]. TNF-α then activates c-Jun N-terminal kinase (JNK), which, in turn, inhibits Akt activity and leads to insulin resistance [20,21].

The study also observed an inverse association between vitamin B12, HOMA-IR and insulin levels in children, indicating that deficiency of vitamin B12 at an early age may cause insulin resistance. Consistent with our observation, a similar association between vitamin B12 and HOMA-IR was observed by Mahalle et al. [1] and Kaya et al. [22], in Indian subjects with coronary artery disease and in women with polycystic ovary syndrome, respectively. Furthermore, Stewart et al. found in Nepalese subjects that low maternal vitamin B12 status was associated with insulin resistance in offspring [23]. Contrary to these, a cross-sectional study conducted in 135 Asian-Indian women found no correlation between serum vitamin B12 and HOMA-IR [24].

Resistin, originally described as an adipocyte-specific hormone, has been suggested to be an important link between obesity, insulin resistance and diabetes [25,26]. It was observed that serum concentrations of resistin are significantly increased in obese and diabetic patients [27,28]. Resistin has also been linked to the development of atherosclerosis and CVD [23,29]. In our study, a significant inverse association between vitamin B12 concentrations and resistin was observed in children, whereas, in adults, it was positively associated. Stepwise linear regression analysis revealed that resistin was the most significant predictor of vitamin B12 in children. In another study, 220 patients with acute coronary syndrome (ACS) had significantly higher serum resistin concentrations than patients

classified as normal [30]. However, research on resistin has also been controversial in understanding their pathological relevance and its association with T2DM and cardiovascular diseases [31,32]. Hence, conclusive data await new strong evidence to elucidate the role of resistin in various disease processes. Nevertheless, resistin has primarily been shown to be relevant to inflammation-related diseases like atherosclerosis and arthritis [33] and may have a role in the regulation of pro-inflammatory cytokines' (IL-6 and TNF-α) expression in human peripheral blood mononuclear cells (PBMC) via NF-κB pathway [33,34]. In yet another study conducted by Stejskal et al., it was observed that persons with clinical signs of severe inflammation showed significantly higher concentrations of resistin than healthy individuals [35]. Since resistin and TNF-α are pro-inflammatory in action, and inflammation has a central role in cardiovascular diseases and insulin resistance, deficiency of vitamin B12 may, therefore, have importance in the development of cardiometabolic diseases in Saudi population.

The limitations of this study include its cross-sectional nature and, thus, we cannot determine causality. Furthermore, information on diet is lacking and would have provided added value as to whether adequate B12 concentrations lead to better cardiometabolic parameters independent of a healthy diet. Lastly, the findings may not be a true representation of the general healthy population, since women were over represented in the current study, and whether the associations are true for those with chronic diseases also cannot be answered in the study's present scope. Despite these limitations, this is the first study particularly from the Arab region, which reflects direct link between vitamin B12 with adipocytokines and biochemical markers of cardiometabolic risks. The present findings warrant further investigation addressing how interventional therapies with vitamin B12 affect cardiovascular and metabolic diseases.

5. Conclusions

In conclusion, our study suggests weak but significant associations between circulating vitamin B12 concentrations with serum insulin and HOMA-IR in children, and inflammatory cytokines such as TNF-α and resistin in adults. Our findings have significant clinical importance, since these biochemical markers and cytokines are independent risk factors for cardiovascular and metabolic diseases in adults. Thus, determination of vitamin B12 status and treatment in cases of deficiency could be a clinically useful strategy in the prevention of cardiometabolic diseases.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/8/9/460/s1, Figure S1: Mean vitamin B12 concentrations according to quartiles of HOMA-IR in children and adults, Figure S2: Mean vitamin B12 concentrations according to quartiles of resistin in children and adults.

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Conflicts of Interest: The authors declare no conflict of interest.

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Excess Folic Acid Increases Lipid Storage, Weight Gain, and Adipose Tissue Inflammation in High Fat Diet-Fed Rats

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Abstract: Folic acid intake has increased to high levels in many countries, raising concerns about possible adverse effects, including disturbances to energy and lipid metabolism. Our aim was to investigate the effects of excess folic acid (EFA) intake compared to adequate folic acid (AFA) intake on metabolic health in a rodent model. We conducted these investigations in the setting of either a 15% energy low fat (LF) diet or 60% energy high fat (HF) diet. There was no difference in weight gain, fat mass, or glucose tolerance in EFA-fed rats compared to AFA-fed rats when they were fed a LF diet. However, rats fed EFA in combination with a HF diet had significantly greater weight gain and fat mass compared to rats fed AFA ($p < 0.05$). Gene expression analysis showed increased mRNA levels of peroxisome proliferator-activated receptor γ (PPARγ) and some of its target genes in adipose tissue of high fat-excess folic acid (HF-EFA) fed rats. Inflammation was increased in HF-EFA fed rats, associated with impaired glucose tolerance compared to high fat-adequate folic acid (HF-AFA) fed rats ($p < 0.05$). In addition, folic acid induced PPARγ expression and triglyceride accumulation in 3T3-L1 cells. Our results suggest that excess folic acid may exacerbate weight gain, fat accumulation, and inflammation caused by consumption of a HF diet.

Keywords: folic acid; obesity; metabolic syndrome; adipose tissue

1. Introduction

The metabolic syndrome, which encompasses excess abdominal adiposity, insulin resistance, dyslipidaemia, and hypertension, represents the largest public health challenge in developed countries [1]. The rise in metabolic syndrome prevalence in recent decades has been mirrored by changes in dietary patterns, reflecting increased nutrient availability [2]. Diets rich in fat and rapidly-digestible carbohydrates have increased total energy intake [2]. At the same time, fortification of staple foods and widespread supplement use has increased folic acid intake in many Western countries, placing importance on investigations into possible adverse effects [3].

Folates are a family of structurally-similar compounds involved in the transfer of one-carbon units for the production of nucleotides used in DNA synthesis; for the methylation of a variety of biological substrates; and for cell division [4]. These functions make folates especially important

during the anabolic stages of foetal and childhood development [4]. Sources of natural folates (pteroylpolyglutamates) include green leafy vegetables, orange juice and legumes [4]. Folic acid (monoglutamate) is a synthetic member of the folate family commonly used in fortified foods and supplements due to its stability and low cost [4]. The current Recommended Daily Allowance (RDA) for folate is 400 μg Dietary Folate Equivalents (DFEs)/day for the general adult population [5]. While mandatory folic acid fortification of grains since 1998 has reduced the incidence of neural tube defects (NTDs) and other developmental disorders in Canada and the USA, population-wide intake of folic acid has increased to unprecedented levels, leading to concern that there may be adverse consequences [3,6,7]. Children and elderly populations are likely to have high folic acid intake because large proportions of their diet typically consist of cereals and bread [3]; while pregnant women are likely to have high intakes due to high supplement use [8]. High folic acid intake by women planning pregnancy is prevalent in many countries, including countries without mandatory folic acid fortification, due to worldwide recommendations for this population to consume 400 μg/day folic acid for the prevention of neural tube defects [9,10]. Human observational evidence has linked high folic acid intake to increased risk of colorectal and prostate cancer [11,12]; impaired immune function [13,14], and impaired cognition [15]. Further observational evidence has linked folate status to obesity, sparking investigations into the relationship between folic acid intake and lipid and energy metabolism [16,17].

Maternal excess folate [17,18] or methyl donor [19] intake during pregnancy in animal models causes weight gain or components of the metabolic syndrome in offspring. These effects may be more pronounced when offspring are fed a high fat diet [20,21]. In humans, high erythrocyte folate status during pregnancy was associated with increased fat mass of children at six years of age [22]. Folic acid appears to influence energy and lipid metabolism by modulating DNA methylation and gene expression patterns [17,18,23]. Diet-gene interactions remain important determinants of health throughout the lifespan [24], and so excess folic acid may continue to promote changes to energy and lipid metabolism in adulthood. However, the effects of excess folic acid intake on metabolic syndrome risk and adiposity in adulthood remains poorly understood.

The effects of excess dietary folic acid intake on the liver, an important site of both folate and lipid metabolism, have been investigated in rodent models [23,25]. Excess folic acid intake may promote changes to one carbon metabolic pathways and gene expression patterns, leading to liver injury [25]. There is evidence that the influence of methyl donors, including folic acid, on gene expression may be tissue-, site-, and gene-specific, and so investigations into the influence of excess folic acid on other tissues (e.g., adipose) are warranted [3].

The aim of our study was to investigate the effects of excess folic acid (EFA) intake compared to adequate folic acid (AFA) intake on metabolic health of rats. We hypothesized that consumption of a diet containing EFA would induce changes to lipid and glucose metabolism. High fat diets are commonly used to study weight gain and components of the metabolic syndrome in animal models. Therefore, we conducted our investigations in the setting of a 15% of energy low fat (LF) and a 60% of energy high fat (HF) diet. Our data suggest that EFA, in combination with a HF diet increases weight gain, adipose tissue mass and markers of inflammation compared to AFA, and that these effects are not seen in the setting of a LF diet. We conducted supporting experiments in vitro, the results of which suggest that folic acid can increase triglyceride accumulation in 3T3-L1 cells by inducing peroxisome proliferator-activated receptor γ (PPARγ).

2. Materials and Methods

2.1. Animals and Diets

All procedures were approved by the University of Alberta's Institutional Animal Care Committee (AUP00000175) in accordance with guidelines of the Canadian Council on Animal Care. All animals had free access to food and water and were housed on a 12-h light-dark cycle. The diet was supplied

by Harlan Teklad, with basal diet formulations designed to AIN-93G specifications. In the first feeding trial, twelve eight-week old male Sprague–Dawley rats were fed a 15% of energy low fat diet with excess folic acid (7.5 mg/kg diet) or control levels of folic acid (0.75 mg/kg diet) (Table 1) [23]. In the second feeding trial, twelve rats were fed a 60% of energy high fat diet containing excess folic acid (7.5 mg/kg) or control levels of folic acid (0.75 mg/kg) (Table 1). Food intake and body weights were recorded three times weekly for the duration of the experiments. Food intake is reported as the average daily intake over the 12-week feeding period. During the eighth weeks of feeding, body composition was analyzed by magnetic resonance imaging (MRI). Animals were euthanized after 12 weeks on diet. Fasting blood was collected in ethylenediaminetetraacetic acid (EDTA)-coated vials by cardiac puncture and plasma was collected after centrifugation of blood at 3000× *g* for 10 min. Tissues were weighed and snap frozen in liquid nitrogen before being stored at −80 °C until analysis.

Table 1. Composition of diets (per kilogram diet).

Ingredients	LF-AFA	LF-EFA	HF-AFA	HF-EFA
Folic acid (mg)	0.75	7.5	0.75	7.5
L-cysteine (g)	3	3	4	4
Corn starch (g)	263.7	263.7	-	-
Sucrose (g)	209.7	209.7	106.3	106.3
Maltodextrin (g)	130	130	160	160
Soybean Oil (g)	60	60	30	30
Lard (g)	-	-	310	310
Cellulose (g)	50	50	20	20
Pectin (g)	50	50	50	50
Succinylsulphathiazole (g)	10	10	10	10
Vitamin-free casein (g)	195	195	265	265
Mineral Mix, AIN-93G (g)	35	35	48	48
Tertiary-Butylhydroquinone (mg)	12	12	3400	3400
Choline bitartrate (g)	2.5	2.5	3	3
Niacin (mg)	30	30	63	63
Calcium pantothenate (mg)	16	16	34	34
Pyridoxine HCl (µg)	7	7	15	15
Thiamin HCl (µg)	6	6	13	13
Riboflavin (mg)	6	6	13	13
Biotin (µg)	200	200	400	400
Vitamin B12 (µg)	25	25	40	40
DL-alpha tocopheryl acetate (500 IU/g) (mg)	150	150	315	315
Vitamin A palmitate (500,000 IU/g) (mg)	8	8	17	17
Cholecalciferol (500,000 IU/g) (mg)	2	2	4	4
Phylloquinone (µg)	800	800	1600	1600

2.2. Histological Analysis of Adipose and Liver Samples

Adipose tissue was collected, dehydrated, and embedded in paraffin. Cross-sections of tissue (5 µm) were prepared and stained with haematoxylin and eosin (H and E). Adipocyte size was estimated using ImageJ software, (the US National Institutes of Health, Bethesda, MD, USA).

2.3. Glucose Tolerance Tests

Eight weeks after initiation of the high fat diet (HFD) feeding trial, rats were fasted overnight before receiving 2 g/kg glucose by intraperitoneal (IP) injection. Blood samples were collected by tail vein bleeding at 15, 30, 60, 90, and 120 min.

2.4. Plasma Measurements

Plasma glucose and alanine aminotransferase (ALT) were measured using commercially available kits (WAKO Diagnostics (Mountain View, CA, USA) and Biotron (Diagnostic Inc., Hemet, CA, USA), respectively). Plasma insulin was measured by ELISA (ALPCO, Salem, NH, USA). Plasma levels of metabolites in the one carbon cycle, including folate, total homocysteine, methionine,

cysteine, *N*,*N*-dimethylglycine, *N*-methylglycine, glycine, serine, cystathionine, α-aminobutyrate, were measured by capillary stable isotope dilution gas chromatography/mass spectrometry, as previously described [26]. Neutral lipids in plasma were quantified by gas-liquid chromatography as described previously [27], with tridecanoin as an internal standard.

2.5. Culture of 3T3L1 Adipocytes

3T3-L1 cells were cultured to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS). At two days post-confluence (designated day 0), cells were induced to differentiate with DMEM supplemented with 10% (*v*/*v*) FBS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, 1 μg/mL insulin. Cells were incubated with 9 μM (standard level) or 20 μM (supplemented) folic acid. Differentiation media was refreshed daily. After 48 h, the media were replaced with DMEM supplemented with 10% FBS and 1 μg/mL insulin and the same level of folic acid that was used during differentiation. The cell media was refreshed every 24 following differentiation.

2.6. Analytical Procedures

Tissue levels of cytokines and chemokines were quantified using ELISA kits, according to manufacturer's instructions (Preprotech, Rocky Hill, NJ, USA or eBioscience, San Diego, CA, USA). To measure triglycerides in 3T3L1 adipocytes, cells were rinsed three times in sterile PBS then collected in 2 mL PBS by scraping. Cells were disrupted by vortexing, followed by sonication 3 × 15 s. Triglycerides were measured by colorimetric assay, according to the manufacturer's instructions (Sekisui Diagnostics, Lexington, MA, USA).

2.7. mRNA Quantification

mRNA was quantified as previously described [28]. Briefly, total RNA was isolated from tissue using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was then reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA, USA). The Universal Probe Library (Roche Diagnostics, Indianapolis, IN, USA) was used to design primers and corresponding probes for each gene being evaluated. Quantitative PCR was run in triplicate on the Biomark system (Fluidigm, South San Francisco, CA, USA) for 40 cycles. Relative mRNA expression for each gene was calculated using the ΔΔCT method, normalized to cyclophilin.

2.8. Statistical Analysis

Data are expressed as the means ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA or Student's *t*-test where appropriate. All analyses were done using GraphPad Prism software version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA). A p-value < 0.05 was taken as statistically significant.

3. Results

3.1. Excess Folic Acid Intake Does Not Influence Body Weight, Body Composition, or Glucose Tolerance on a Low Fat Diet

Rats were fed a LF diet with either AFA or EFA for 12 weeks. LF-AFA and LF-EFA fed rats had similar weight gain, fat mass and lean mass over the 12 weeks study period (Figure 1A–C). Glucose tolerance tests showed no difference in rate of glucose clearance between LF-AFA and LF-EFA fed rats (Figure 1D,E). Plasma ALT concentration, a marker of liver injury, was not different between groups (Figure 1F).

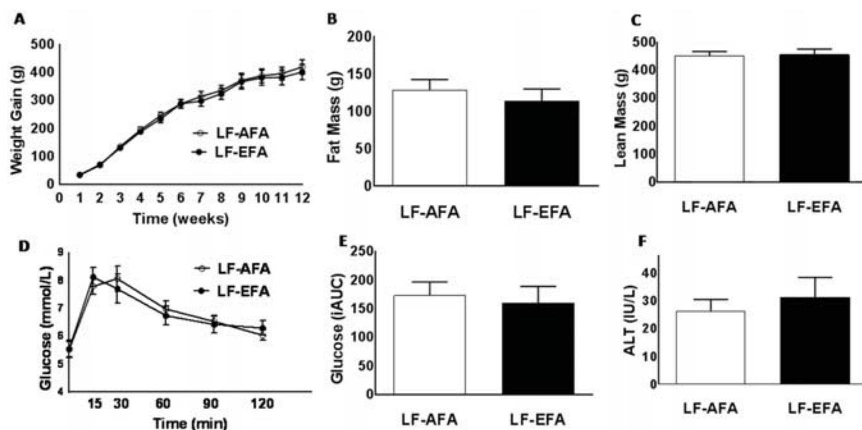

Figure 1. Excess folic acid intake does not influence body weight, body composition or glucose tolerance on a low fat diet (**A**); growth curves (**B**); fat mass (**C**); lean mass (**D**); glucose tolerance (**E**); area under the glucose curve, and (**F**) plasma ALT, of rats fed 15% LF diet with excess or adequate folic acid. Values are means ± SEM, * $p < 0.05$.

3.2. Excess Folic Acid Intake Increases Weight Gain, Fat Mass and Glucose Intolerance on a High Fat Diet

We next investigated the influence of AFA or EFA in rats challenged with a diet containing 60% kilocalories from fat for 12 weeks. Plasma homocysteine concentrations were significantly lower in HF-EFA fed rats (3.28 ± 0.17 compared to 2.650 ± 0.14 μM/L) at the end of the study period (Table 2). Plasma methionine and glycine concentrations were also lower in HF-EFA fed rats, while plasma folate concentration was similar between groups (Table 2). There was no significant difference in plasma concentrations of triglycerides, cholesterol, or cholesterol ester in rats fed HF-AFA or HF-EFA diets (Table 2).

Table 2. HF-EFA fed rats experience alterations in plasma one carbon metabolite profile, while plasma lipids remain unchanged, compared to HF-AFA fed rats.

	HF-AFA	HF-EFA
Plasma one carbon metabolites		
Folate (nmol/L)	41.56 ± 0.50	41.30 ± 0.72
Homocysteine (μM)	3.28 ± 0.17	2.650 ± 0.14 *
Methionine (μM)	69.73 ± 2.93	62.58 ± 1.01 *
Dimethylglycine (μM)	13.10 ± 0.98	15.68 ± 0.95
Methylglycine (μM)	7.19 ± 0.48	6.21 ± 0.31
Glycine (μM)	425.0 ± 21.6	345.5 ± 24.0 *
Serine (μM)	339.2 ± 11.2	364.5 ± 10.9
Cystathionine (nM)	914.7 ± 69.2	763.3 ± 37.2
Cysteine (nM)	311.3 ± 4.5	305.7 ± 9.7
α-aminobutyrate (μM)	27.68 ± 2.6	35.60 ± 4.0
Plasma lipids		
Triglyceride (μg/mL)	292.6 ± 43.26	211.8 ±46.21
Cholesterol Ester (μg/mL)	72.92 ± 5.87	60.18 ± 7.97
Free Cholesterol (μg/mL)	134.4 ± 18.65	118.1 ± 15.41

Values are means ± SEM, * $p < 0.05$.

HF-EFA fed rats had 14% greater weight gain compared to HF-AFA fed controls after 12 weeks (Figure 2A). Estimated daily food intake was similar between groups (Figure 2B). Fat mass accounted

for this difference in weight, with HF-EFA fed rats developing larger peri-renal fat pads (Figure 2C,D). There was no difference in lean body mass between HF-EFA and HF-AFA fed rats (Figure 2E). Fasting plasma glucose and insulin levels were similar between HF-EFA and HF-AFA fed rats (Figure 3A,B). However, IP glucose tolerance tests showed that HF-EFA fed rats had impaired glucose clearance compared to HF-AFA fed rats, as indicated by a significantly greater area under the glucose curve (Figure 3C,D). Therefore, EFA intake exacerbates weight gain, fat mass, and glucose intolerance in rats fed a HF diet.

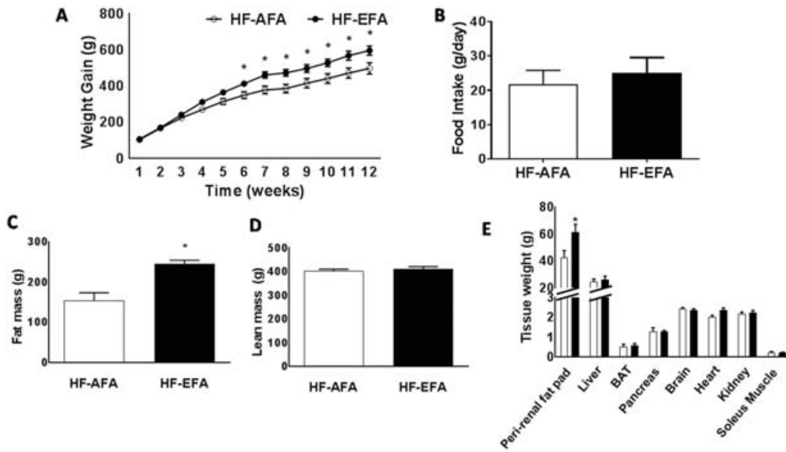

Figure 2. Excess folic acid intake increases weight gain and fat mass on a high fat diet. (**A**) Growth curves; (**B**) food intake; (**C**) fat mass; (**D**) lean mass; and (**E**) tissue weights. Values are means ± SEM, * $p < 0.05$.

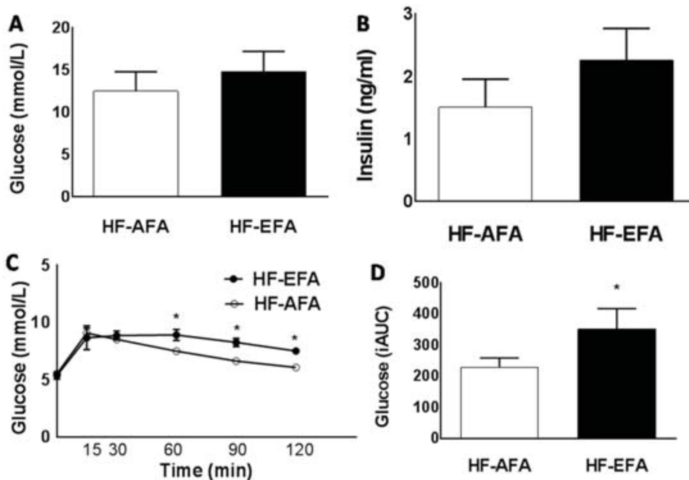

Figure 3. Excess folic acid intake impairs glucose tolerance on a high fat diet. (**A**) Fasting plasma glucose; (**B**) fasting plasma insulin; (**C**) blood glucose concentrations at different time points (15, 30, 60, 90, 120 min) after an intraperitoneal (IP) glucose injection; and (**D**) area under the glucose curve, for male rats fed 60% HF diet with excess or adequate folic acid. Values are means ± SEM, * $p < 0.05$.

3.3. Excess Folic Acid Increases Adipose Tissue Size and Mass By Inducing Lipogenic Genes in High Fat Diet-Fed Rats

Histologic examination of visceral adipose tissue after hematoxylin and eosin (H and E) staining showed increased adipocyte size in HF-EFA fed rats compared to HF-AFA fed controls (Figure 4). To further investigate this increased adiposity, we measured expression of key transcriptional regulators of lipid metabolism (Pparg, Srebf1, Srebf2, Nr1h2, Nr1h3), and lipogenic genes in adipose tissue. PPARγ regulates genes involved in lipid uptake and storage. Adipose tissue PPARγ mRNA was 2.5-fold higher in HF-EFA fed rats compared to HF-AFA fed controls (Figure 5A). Liver X receptor (LXR)-α and -β (encoded by Nr1h3 and Nr1h2) are nuclear transcription factors that have roles in adipose tissue lipid metabolism as well as inflammation [29]. LXR-α and -β mRNA levels were significantly higher in HF-EFA fed rats compared to HF-AFA fed rats (Figure 5A). Furthermore, there was increased mRNA levels of triglyceride synthetic genes (MGAT1, DGAT1 and DGAT2); genes involved in elongation (ELOV5 and ELOV6); and markers of adipogenesis (PLIN2) in adipose tissue of HF-EFA fed rats compared to HF-AFA fed rats (Figure 5B).

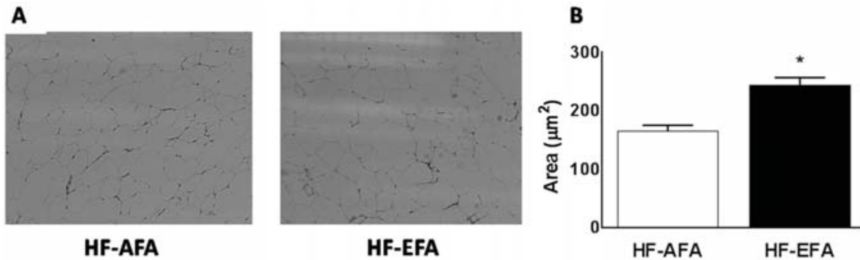

Figure 4. HF-EFA fed rats had larger adipocytes than HF-AFA fed rats. (**A**) Adipose tissue histology after H and E staining; (**B**) adipocyte size was quantified using ImageJ software. Values are means ± SEM, * $p < 0.05$.

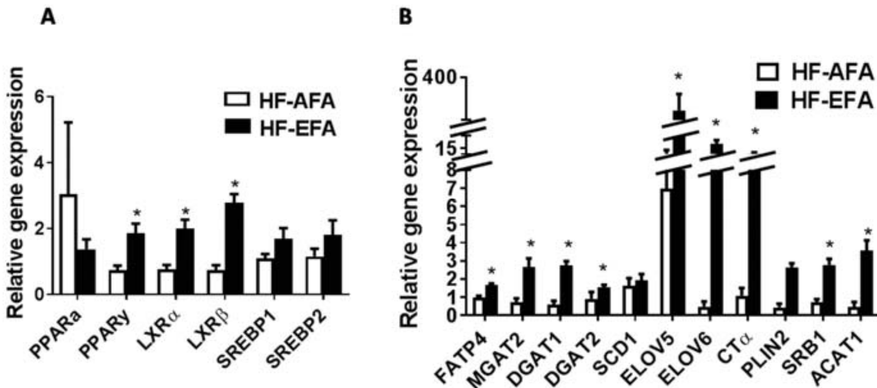

Figure 5. Gene expression analysis in adipose tissue of HF-EFA shows increased levels of lipogenic mediators. Relative mRNA levels of (**A**) transcription factors and (**B**) genes involved in lipid synthesis, storage and transport, in adipose tissue of HF-AFA and HF-EFA fed rats. Values are means ± SEM, * $p < 0.05$.

Increased adipocyte size and number increases demand for phosphatidylcholine (PC), which surrounds adipose tissue lipid droplets in a monolayer. Consistent with this increased demand, mRNA levels of the rate limiting enzyme in PC biosynthesis, cytidine triphosphate: phosphocholine

cytidylyltransferase (CT) α, was increased in adipose tissue of HF-EFA fed rats compared to HF-AFA control rats (Figure 5B). Taken together, these observations indicate that EFA intake may induce lipogenic transcription factors and some of their dependent genes to promote adiposity in the setting of a HF diet.

3.4. Excess Folic Acid Increases Inflammation in White Adipose Tissue

White adipose tissue (WAT), in addition to its role in energy storage, secretes adipocytokines and chemokines which link increased fat mass to local and systemic insulin resistance [30]. In obesity, excess adipose tissue accumulation precedes immune cell infiltration and production of pro-inflammatory cytokines [30]. We measured adipose tissue protein levels of the chemokines monocyte chemoattractant protein-1 (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES), and the cytokines tumor necrosis factor α(TNFα) and interleukin 10 (IL-10), as markers of inflammatory status. MCP-1 is a chemokine involved in monocyte and macrophage recruitment to adipose tissue [31]. Levels of MCP-1 were significantly higher in adipose tissue of HF-EFA fed rats (Figure 6B). Chronic low-grade inflammation after macrophage recruitment to adipose tissue can influence local and systemic insulin sensitivity. Protein and mRNA levels of TNFα, an inflammatory cytokine secreted by macrophages, were significantly higher in adipose tissue of HF-EFA fed rats compared to HF-AFA fed controls (Figure 6A,B). Adipose tissue levels of the cytokine IL-10 were not different between HF-AFA and HF-EFA fed rats (Figure 6B). Transcript levels of the inflammatory markers NADPH oxidase 1 (NOX1) and binding immunoglobulin protein (BiP) were found to be significantly increased in adipose tissue of HF-EFA fed rats after 12 weeks on diet (Figure 6A).

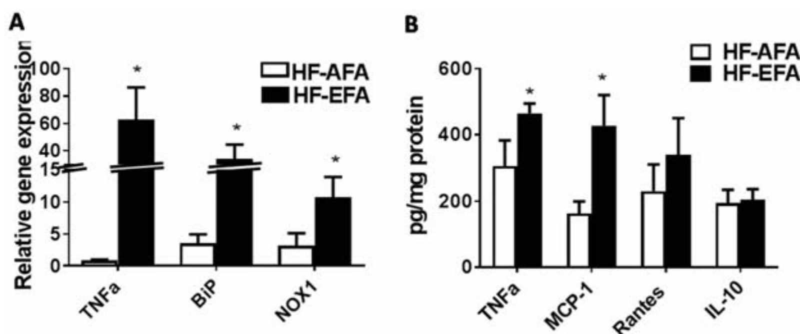

Figure 6. Inflammatory markers are increased in adipose tissue of HF-EFA compared to HF-AFA fed rats. (**A**) Relative mRNA levels of genes related to inflammation; and (**B**) protein levels of TNFα, MCP-1, Rantes, IL-10 in pg/mg protein, in adipose tissue of HF-EFA and HF-AFA fed rats. Values are means ± SEM, * $p < 0.05$.

3.5. Excess Folic Acid Promotes Triglyceride Accumulation in Mature 3T3L1 Adipocytes

We used 3T3-L1 cells to assess the lipogenic capacity of folic acid in vitro (Figure 7). We measured PPARγ mRNA expression as a marker of adipocyte differentiation in undifferentiated and mature 3T3-L1 cells cultured with 9 μM (normal) and 20 μM folic acid (excess). PPARγ was undetectable in undifferentiated 3T3-L1 cells. Treatment of differentiated adipocytes with 9 μM folic acid increased PPARγ expression and triglyceride (TG) accumulation compared to undifferentiated cells. High folic acid (20 μM) further promoted PPARγ expression and TG accumulation, indicating a dose-response to folic acid treatment in 3T3-L1 cells. These results support those found in adipose tissue of rats fed HF-EFA compared to HF-AFA diets.

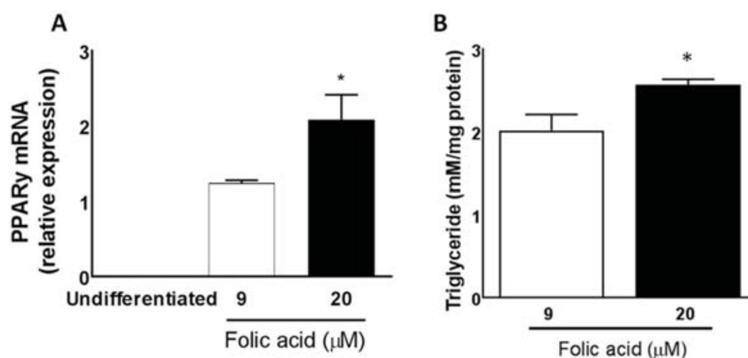

Figure 7. Folic acid increases PPARγ and triglyceride levels in a dose-dependent manner in cultured 3T3-L1 cells. (**A**) Relative PPARγ mRNA levels in cells cultured with 9 μM or 20 μM folic acid and (**B**) Triglyceride levels in cells after treatment with 9 μM compared to 20 μM folic acid. Values are means ± SEM, * $p < 0.05$.

4. Discussion

Our results show that excess dietary folic acid exacerbates fat mass gain, adipose tissue inflammation, and systemic glucose intolerance in rats fed a HFD. These metabolic complications were not observed in rats fed a LF-EFA diet. Energy dense diets have long been implicated in fat mass gain and metabolic syndrome development [2]. Our results suggest that high dietary folic acid may aggravate these effects.

The body weight of rats in the HF-EFA group after 12 weeks was 14% higher than in rats in the HF-AFA group, with increased fat mass accounting for this difference in weight. Histological examination of adipose tissue revealed that HF-EFA fed rats had larger adipocytes. The capacity of folic acid or methyl-rich diets to promote weight gain has been reported previously in young rats fed 5 mg/kg folic acid [23], as well as in maternal rats fed a high fat diet enriched in methyl-containing vitamins [32]. Our data supports these findings and suggests that a high folic acid diet promotes fat gain.

Adipogenesis is a multistep process which is controlled by transcription factors that promote adipocyte development and adipose tissue expansion [33]. PPARγ is an important regulator of adipogenesis and is sensitive to nutrient composition of the diet [34]. Most pro-adipogenic factors appear to function by stimulating PPARγ [34]. Gene expression analysis showed that PPARγ mRNA levels were approximately three times higher in adipose tissue of HF-EFA fed rats compared to HF-AFA fed controls. Folic acid supplementation has previously been shown to decrease PPARγ promoter methylation in rat liver leading to an increase in PPARγ gene expression [35]. Other studies have reported that methylation status influences expression of key genes involved in lipid metabolism [23,36]. Therefore, it is conceivable that the increase in PPARy expression observed in our study is related to changes to methylation status of adipose tissue PPARy, induced by excess folic acid. Addition of folic acid to the medium stimulated PPARγ expression and increased TG storage in 3T3-L1 cells, which further supports the ability of folic acid to stimulate adipogenesis. The capacity of folic acid to stimulate PPARγ expression in adipose tissue has been previously reported [23]. LXRα and LXRβ, transcriptional regulators of lipid and glucose metabolism [29], mRNA levels were increased in HF-EFA compared to HF-AFA fed rats. These data suggest that the increased adiposity observed in HF-EFA fed rats is a result of increased induction of lipogenic transcription factors and their target genes in adipose tissue.

Excessive adiposity is associated with metabolic stress and inflammation which is induced when macrophages migrate to adipose tissue and secrete pro-inflammatory cytokines. HF-EFA fed rats had

higher adipose tissue protein levels of MCP-1, a chemokine that recruits macrophages to obese adipose tissue [37]. Consistent with this observation, protein and mRNA levels of the pro-inflammatory cytokine TNFα were significantly elevated in adipose tissue of HF-EFA fed rats compared to HF-AFA fed controls. Elevated TNFα is linked to insulin resistance in humans and animals. Consistent with significantly increased TNFα protein and mRNA levels in adipose tissue, HF-EFA fed rats had impaired glucose tolerance compared to HF-AFA fed rats. Maternal EFA supplementation has previously been shown to impair glucose metabolism in HF diet fed offspring [20]. Our results support this cross-generational evidence and demonstrate a similar effect in a single-generational study of rats. Thus, our data suggest that the mechanism linking EFA intake to systemic glucose intolerance may be TNFα- mediated inflammation in adipose tissue.

Metabolic complications in adipose tissue generally precede similar complications in other tissues, such as the liver [38,39]. TNFα produced in adipose tissue may migrate into the circulation and exert pro-inflammatory effects on other tissues [38,39]. In addition, when adipose tissue has reached its capacity to store fatty acids there may be a 'spill-over' of fat to other tissues. This amplifies the importance of identifying and controlling factors that influence inflammation and glucose intolerance in adipose tissue as they can ultimately compromise systemic health. After observing increased triglyceride accumulation and inflammation in adipose tissue of HF-EFA fed rats, we examined hepatic neutral lipid accumulation and mRNA levels of inflammatory genes. No significant difference in triglyceride accumulation was observed, and mRNA levels of a select number of inflammatory genes were not increased in the HF-EFA fed groups (data not shown). It has been previously reported that while HFD-induced inflammation can occur relatively quickly in adipose tissue of murine models, hepatic inflammation can take up to 40 weeks to develop, suggesting that our 12 weeks study period may have been too short to observe effects of folic acid in the liver [39]. Twenty-four weeks of supplementary folic acid (20 mg/kg) was sufficient to alter lipid metabolism and cause liver injury in mildly MTHFR-deficient rats [25].

While there is increasing evidence that excess dietary folate intake may have adverse effects, it is important to acknowledge the necessity of adequate levels of folate for good health. The role of dietary folate in NTD prevention is widely acknowledged, but adequate folate is also essential for regulating lipid metabolism, especially in the liver where most methylation reactions occur [40]. Folate deficiency can lead to liver damage and steatosis, with impaired phosphatidylcholine (PC) synthesis and increased expression of lipogenic genes cited as possible mechanisms [40]. Folate is also essential for re-methylation of homocysteine to methionine, which can be used to synthesize protein, or recycled to *S*-adenosylmethionine (*S*-AdoMet), a key methyl donor [4]. Excessively high folic acid intake can increase levels of unmetabolized folic acid in circulation [13], and its transport into tissues [41]. Some of the adverse health effects linked to excessive folic acid intake have been attributed to unmetabolized folic acid. However, the molecular mechanisms involved in processing unmetabolized folic acid within different tissue and cell types, and the biological effects of this, is poorly understood. It is conceivable that the increased adiposity observed in our study is a result of altered gene methylation and expression patterns in adipose tissue by folic acid.

We decided to supplement the rat diet with 10 times the adequate level of folic acid. While this could be considered superphysiological, we have previously reported that a sub-set of pregnant Alberta women consume over 4 mg/day (10 times the RDA for the general population) of folic acid [8]. Rats reduce folic acid to tetrahydrofolate at a rate that far exceeds that seen in humans [42]. Therefore, the observation that 7.5 mg/kg folic acid induced fat mass gain and inflammation in rats despite their relatively high dihydrofolate reductase activity is striking. Interestingly, Burdge et al. reported that folic acid supplementation (5 mg/kg) induced weight gain in juvenile rats fed a high fat diet [23]. Nonentheless, the inter-species differences in capacity to handle high doses of folic acid need to be considered when drawing conclusions from animal studies and places importance on establishing the metabolic effects of excess folic acid in human populations.

5. Conclusions

Folic acid fortification has succeeded in reducing incidence of neural tube defects in North America. However, there is increasing evidence that high folic acid intake may have consequences. Our findings suggest that in adult males the dual insult of a high fat diet combined with excess folic acid may promote fat mass gain, adipose tissue inflammation and systemic glucose intolerance. Obesity and the metabolic syndrome represent major public burdens and the link between excess folic acid and metabolic complications warrants further investigation given the ubiquitous presence of folic acid in the human food chain.

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Intakes of Folate and Vitamin B12 and Biomarkers of Status in the Very Old: The Newcastle 85+ Study

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Abstract: Very old adults are at increased risk of folate and vitamin B12 deficiencies due to reduced food intake and gastrointestinal absorption. The main aim was to determine the association between folate and vitamin B12 intake from total diets and food groups, and status. Folate or vitamin B12 intakes (2×24 h multiple pass recalls) and red blood cell (RBC) folate or plasma vitamin B12 (chemiluminescence immunoassays) concentrations were available at baseline for 731 participants aged 85 from the Newcastle 85+ Study (North-East England). Generalized additive and binary logistic models estimated the associations between folate and vitamin B12 intakes from total diets and food groups, and RBC folate and plasma B12. Folate intake from total diets and cereal and cereal products was strongly associated with RBC folate ($p < 0.001$). Total vitamin B12 intake was weakly associated with plasma vitamin B12 ($p = 0.054$) but those with higher intakes from total diets or meat and meat products were less likely to have deficient status. Women homozygous for the *FUT2* G allele had higher concentrations of plasma vitamin B12. Cereals and cereal products are a very important source of folate in the very old. Higher intakes of folate and vitamin B12 lower the risk of "inadequate" status.

Keywords: 'aged 80 and over'; Newcastle 85+ Study; red blood cell folate; vitamin B12; *FUT2*; *MTHFR*; food groups

1. Introduction

B vitamins, specifically folate and vitamin B12, are essential for one-carbon transfer reactions which include amino acid interconversions, RNA and DNA synthesis and methylation of cell macromolecules [1]. Older adults are at increased risk of B vitamin deficiencies due to decreased food intake and increased malabsorption. Low folate and vitamin B12 status have been associated with adverse health outcomes, including cognitive impairment [2–4], stroke [5,6], fractures [7,8] and cancer in older adults [9]. A review of micronutrient deficiencies in community-dwelling older adults (aged 65 and over) living in western countries reported that 29% and 16% of men and, 30% and 19% of women had intakes below the Nordic Nutrition Recommendations (NNR) estimated average requirement (EAR) for folate (200 μg/day) and vitamin B12 (1.4 μg/day), respectively [10]. The current UK National Diet and Nutrition Survey (NDNS) rolling programme estimated that 1% of older adults (aged 65 and over) were below the UK lower reference nutrient intake (LRNI) for folate (100 μg/day)

and vitamin B12 (1.0 μg/day) but that 7.3% of men and 10.8% of women had red blood cell folate (RBC) concentrations below 340 nmol/L and 5.9% of men and women had serum vitamin B12 concentrations below 150 pmol/L [11]. The complexity of the dose–response relationships between intake and status is influenced by limitations in dietary assessment, food composition data, choice of biomarkers, genotypic variation, bioavailability and complex metabolic pathways. About 10%–30% of older adults have atrophic gastritis (caused by *Helicobacter pylori* infection, long-term use of proton pump inhibitors, H_2 receptor antagonists and biguanides) which leads to hypochlorhydria [12]. This has a detrimental effect onacid–pepsin digestion and favours small bowel bacterial growth resulting in impaired vitamin B12 absorption [13]. In addition, those with autoimmune atrophic gastritis produce antibodies against the intrinsic factor which can lead to pernicious anemia [13]. Therefore, older adults may have adequate vitamin B12 intake but inadequate vitamin B12 plasma concentration. In addition, several single nucleotide polymorphisms (SNP) modulate folate and vitamin B12 status. For example, homozygosity of the T allele (forward orientation) (rs1801133) of the *MTHFR* gene (which encodes methylenetetrahydrofolate reductase) is associated with low folate status [14].

There is conflicting evidence about relationships between folate and vitamin B12 intake and, folate and vitamin B12 status, respectively, in older adults. Some studies report a significant association between folate and vitamin B12 intake and status in older adults [2,15–19] while others do not [20–22]. Differences in folate and vitamin B12 bioavailability from total diets and specific food sources may provide a partial explanation for the observed discrepancies. Folate bioavailability from foods is substantially lower than that from supplements or from foods fortified with folic acid with estimated bioavailability of 50% and 85%, respectively [23]. If intrinsic factor (IF) secretion is intact, approximately 40% of vitamin B12 is absorbed [24].

In light of the concerns about dietary inadequacy, it is imperative to assess folate and vitamin B12 status in older people, particularly the very old (85 years and older). The aims were to determine (i) the prevalence of "inadequate" folate and vitamin B12 intake and status in the Newcastle 85+ Study; (ii) the associations between the top contributing dietary sources of folate and vitamin B12, and status; and (iii) whether high dietary intakes of both vitamins are associated with a reduced risk of "inadequate" status.

2. Material and Methods

2.1. Newcastle 85+ Study

The Newcastle 85+ Study is a longitudinal population-based study of health trajectories and outcomes in the very old which approached all people turning 85 in 2006 (born in 1921) who were registered with participating general practices within Newcastle upon Tyne or North Tyneside primary care trusts (North East England). Details of the study have been reported elsewhere [25–27]. All procedures involving human subjects were approved by the Newcastle and North Tyneside local research ethics committee (06/Q0905/2). Written informed consent was obtained from all participants, and when unable to do so, consent was obtained from a carer or a relative. The recruited cohort was socio-demographically representative of the general UK population [25]. At baseline (2006/2007), multidimensional health assessment, complete general practice (GP) medical records data and complete dietary intake data (without protocol violation) were available for 793 participants [28].

2.2. Dietary Assessment and Food Groups

Dietary intake was collected at baseline using two 24 h Multiple Pass Recalls (24 h-MPR) on two non-consecutive occasions in the participant's usual residence by a trained research nurse and energy, folate and vitamin B12 intakes were estimated using the McCance and Widdowson's Food Composition tables 6th edition [29]. Individual foods were coded and allocated to 15 first level food groups that consisted of: cereals and cereal products, milk and milk products, eggs and egg dishes, oils and fat spreads, meat and meat products, fish and fish dishes, vegetables, potatoes, savoury snacks, nuts

and seeds, fruit, sugar, preserves and confectionery, non-alcoholic beverages, alcoholic beverages and miscellaneous (soups, sauces and remaining foods that did not belong in other food groups) [28]. The top three food group contributors to folate or vitamin B12 intakes (accounted for >50% of total intake) were included in the analysis. These food groups were also widely consumed by this population and, therefore, a possible target for public health policies/fortification. Information on supplement use was limited to type and brand and, therefore, this was only used as a dichotomous covariate (yes/no) [30].

2.3. Nutritional Biomarkers and Single Nucleotide Polymorphisms

Blood samples were taken after an overnight fast at baseline. Forty mL of blood was drawn from the antecubital vein between 7:00 a.m. and 10:30 a.m., placed on ethylenediamine tetraacetic acid (EDTA) tubes and 95% of the samples were taken to the laboratory within 1 h [31]. Red blood cell folate (RBC folate) and plasma vitamin B12 concentrations were determined by chemiluminescence (Microparticle Immunoassay on an Abbott ARCHITECT analyser) and data were available for 731 and 732 participants, respectively. RBC folate was stabilized with ascorbic acid and adjusted for hematocrit. Whole blood DNA was extracted by means of a QiaGEN Amp Maxi DNA Purification Kit. As part of the EU Longevity Genetics Consortium, genome-wide association studies (GWAS) were performed on 765 participants from the Newcastle 85+ Study using Illumina Omni genotyping arrays. Data were obtained from 710 individuals and after quality control, 642 individuals were retained for the final analysis [32]. The single nucleotide polymorphisms (SNP) in the *MTHFR* (rs1801133, chromosome 1, position 11796321), *FUT2* (rs492602, chromosome 19, position 48703160), *MTR* (rs1805087, chromosome 1, position 236885200) and *TCN1* (rs526934, chromosome 11, position 59866020) genes were chosen as candidate modifiers of RBC folate and plasma vitamin B12 concentrations. All SNPs were assessed for deviation from the Hardy–Weinberg equilibrium.

2.4. Statistical Analysis

Normality was assessed graphically with the aid of Q-Q plots and histograms. Linearity and homoscedasticity assumptions were tested with residuals versus predicted values plots. Normally distributed continuous values are presented as means and standard deviations (SD), and non-Gaussian distributed variables as medians and interquartile ranges (IQR). Categorical data are presented as percentages (with corresponding sample size). Sex differences were assessed with the Chi-squared test (χ^2) for categorical variables and by independent *t*-test, and Mann–Whitney U test for parametric and non-parametric continuous data, respectively. Differences between RBC folate and plasma vitamin B12 concentrations according to *MTHFR* (rs1801133), *MTR* (rs1805087), *TCN1* (rs526934) and *FUT2* (rs492602) genotype were assessed using Kruskal–Wallis tests followed by Dunn–Bonferoni tests if the null hypothesis was rejected.

The semi-parametric generalized additive models (gam) were investigated in R version 3.0.1 (R foundation for statistical computing) using the package "gam" to plot the relationship between vitamin intakes (thin plate regression splines for smoothing) and corresponding biomarkers. The generated reference value of zero in the y-axis corresponds to the RBC folate/ plasma vitamin B12 concentrations for the mean intake of folate and vitamin B12, respectively. The odds ratio (OR) (and 95% confidence intervals (CI)) of RBC folate concentrations <600 nmol/L and plasma B12 <148 pmol/L according to quartiles of folate and vitamin B12 intake from total diets and top contributing food groups were computed using binary logistic regression. The commonly used cut-off to define folate deficiency of RBC folate <340 nmol/L [23] could not be used for these models due to the low percentage of deficiency among study participants. Gam and binary logistic regression models were adjusted for sex, energy intake, folic acid or vitamin B12 containing supplement use, folate/vitamin B12 intake from other food groups, *MTHFR* or *FUT2* genotype. The vitamin B12 models were additionally adjusted for H_2 receptor antagonists, biguanides and proton pump inhibitor (PPI) use.

$p < 0.05$ was considered statistically significant. Apart from the gam models, all statistical analyses were conducted using the IBM statistical tool SPSS v22 (IBM, New York, NY, USA).

3. Results

3.1. Folate and Vitamin B12 Intake and Status "Inadequacies"

Although 43% of participants (n = 335) consumed one or more supplements on a regular basis [30], only 4.8% were users of folic acid and vitamin B12 as part of multivitamin supplements (Table 1). A low percentage of participants (3.1%) had folate intakes below the UK LRNI (100 µg/day) [33] or had RBC folate concentrations below the classic cut-off for deficiency of 340 nmol/L (3.6%) [23]. Folate intake and status were "inadequate" in only five participants. Cereals and cereal products, vegetables and fruit and fruit juice were the top food group contributors to folate intake, explaining almost 60% of total folate intake. Vitamin B12 intakes were below the UK LRNI (1 µg/day) [33] in 9.2% (n = 67) of the population while 17.1% (n = 125) were below 148 pmol/L of plasma vitamin B12 [24] (110 of these 125 had also total homocysteine concentrations >15 µmol/L). In addition, 17 participants had "inadequate" intakes and also deficient plasma concentrations of vitamin B12. There were twice as many women with vitamin B12 intakes below the UK LRNI than men (5.0% vs. 12.4%, $p < 0.001$) but not based on plasma vitamin B12 concentrations <148 pmol/L (17.4% vs. 16.9%, $p = 0.238$). Eighty-six percent (n = 628) of the participants had plasma vitamin B12 concentration <400 pmol/L, a concentration that has been associated with high total homocysteine and methylmalonic acid concentrations [34,35]. Intake of the top three food groups (meat, fish and dairy) explained more than 80% of total vitamin B12 intake.

Table 1. Population characteristics, folate and vitamin B12 intakes and biomarkers of one carbon metabolism in the Newcastle 85+ Study.

	All	Men	Women	*p*-Value [1]
Sex (%) (*n*)	732	39 (287)	61 (445)	-
BMI (kg/m^2) (mean ± SD)	24.4 ± 4.3	24.7 ± 3.9	24.3 ± 4.6	0.244 [2]
Smokers (%) (*n*)	5.6 (41)	4.2 (12)	6.5 (29)	0.183
Alcohol Drinkers (%) (*n*)	72 (364)	84 (192)	62 (172)	<0.001
Total Energy Intakes (MJ/day)	6.78 (5.62–8.31)	8.01 (6.65–9.59)	6.26 (5.17–7.38)	<0.001
Folate and vitamin B12 supplement use (%) (*n*)	4.8 (35)	3.8 (11)	5.4 (24)	0.334
H_2 antagonists, PPI and biguanides use (%) (*n*)	26.8 (196)	27.2 (78)	26.5 (118)	0.844
Total Homocysteine (µmol/L)	16.7 (13.5–21.4)	18.0 (14.5–21.9)	16.1 (13.1–21.0)	0.001
>15 µmol/L (%) (*n*)	63.1 (471)	70.3 (206)	58.5 (265)	0.001
Folate				
Intake (µg/day)	209 (157–265)	246 (185–296)	189 (144–242)	<0.001
<100 µg/day (%) (*n*)	3.1 (23)	0.7 (2)	4.7 (21)	0.002
Top food group contributors	Cereals (32%), Vegetables (16%), Fruit (9%)	Cereals (32%), Vegetables (15%), Fruit (8%)	Cereals (31%), Vegetables (17%), Fruit (10%)	-
Red Blood Cell Folate (nmol/L)	863 (451–1287)	868 (596–1282)	854 (614–1287)	0.728
<340 nmol/L (%) (*n*)	3.6 (26)	2.1 (6)	4.5 (20)	0.103
Vitamin B12				
Intake (µg/day)	2.9 (1.9–4.4)	3.5 (2.2–5.2)	2.5 (1.6–3.9)	<0.001
<1.0 µg/day (%) (*n*)	9.2 (67)	4.5 (13)	12.1 (54)	<0.001
Top food group contributors	Meat (53%), Fish (17%), Milk (13%)	Meat (59%), Fish (16%), Milk (10%)	Meat (48%), Fish (19%), Milk (15%)	-
Plasma Vitamin B12 (pmol/L)	232 (170–324)	228 (166–309)	238 (174–337)	0.238
<148 pmol/L (%) (*n*)	17.1 (125)	17.4 (50)	16.9 (75)	0.841

BMI, body mass index; Cereals, Cereals and cereal products; Fruit, Fruit and fruit juice; Meat, Meat and meat products; Fish, Fish and fish dishes; Milk, Milk and milk products; PPI, proton pump inhibitors. Values are medians and IQR unless otherwise stated. [1] No sex difference by Chi-squared test (χ^2) for categorical or Mann–Whitney test for non-parametric continuous variables; [2] Independent *t*-test.

3.2. Folate, Vitamin B12 Status and Genotype

RBC folate and plasma vitamin B12 concentrations according to *FUT2, MTHFR, MTR* and *TCN1* and genotypes are shown in Table 2. Individuals with the *MTHFR* (rs1801133) AG or GA genotype [minor allele frequency (MAF) = 0.33 in the Newcastle 85+ Study vs. 0.32 for the A allele from the 1000 Genomes Project British population phase 3 [36]] had higher RBC folate concentrations than those homozygous for G ($p = 0.024$). Participants with the *FUT2* (rs492602) GG genotype had higher concentrations of plasma vitamin B12 than other *FUT2* genotypes ($p < 0.001$) (MAF = 0.45 in the Newcastle 85+ Study vs. 0.48 for the G allele in residents of England and Scotland [36]). The association between *FUT2* genotype and plasma vitamin B12 concentrations was significant in women ($p < 0.001$) but not in men ($p = 0.140$).

Table 2. Plasma vitamin B12 and RBC folate concentrations by *FUT2, MTHFR*, *MTR*, and *TCN1* genotypes in the Newcastle 85+ Study.

	RBC Folate (nmol/L)	*p*-Value [1]	Plasma Vitamin B12 (pmol/L)	*p*-Value [1]
FUT2 (rs492602)		0.531		<0.001
AA (*n* = 128)	894 (629–1349		216 (146–281)	Ref.
A/G (*n* = 308)	917 (603–1322)		221 (163–309)	0.413
GG (*n* = 187)	835 (595–1206)		277 (209–381)	<0.001
MTHFR (rs1801133)		0.028		0.244
GG (*n* = 276)	871 (614–1275)	Ref.	234 (168–331)	
A/G (*n* = 279)	845 (584–1263)	1.000	230 (164–312)	
AA (*n* = 67)	1010 (693–1626)	0.060	249 (193–339)	
MTR (rs1805087)		0.547		0.277
AA (*n* = 419)	881 (613–1278)		240 (173–337)	
A/G (*n* = 178)	845 (596–1332)		226 (162–297)	
GG (*n* = 26)	1053 (580–1593)		247 (162–310)	
TCN1 (rs526934)		0.065		0.298
AA (*n* = 331)	877 (606–1317)		237 (178–336)	
A/G (*n* = 247)	845 (595–1223)		231 (160–325)	
GG (*n* = 45)	1074 (630–1439)		222 (182–273)	

RBC folate, Red blood cell folate; *FUT2*, Fucosyltrasnferase 2; *MTHFR*, Methylenetetrahydrofolate reductase; *MTR*, Methionine synthase; *TCN1*, Transcobalamin 1. Ref., Reference used for post hoc comparisons. [1] Kruskal–Wallis test followed by Dunn–Bonferroni post-hoc test if the null hypothesis was rejected.

3.3. Association between Folate Intake and Status

The associations between folate intake from all food sources, from cereals and cereal products, from fruit and fruit juice and from vegetables, and RBC folate concentrations are shown in Figure 1 (gam model adjusted for sex, energy intake, *MTHFR* genotype, folate intake from the two other food groups and folic acid supplement use). Total folate intakes were associated with RBC folate ($p < 0.001$). The steepest part of the dose–response curve appeared to be for folate intakes of 50–200 μg per day but RBC folate concentrations continued to increase with increasing folate intake up to ≈500 μg per day. Folate intake from cereals and cereal products, and from fruit and fruit juice were also associated with RBC folate concentrations ($p < 0.001$ and $p = 0.014$, respectively) (Figure 1).

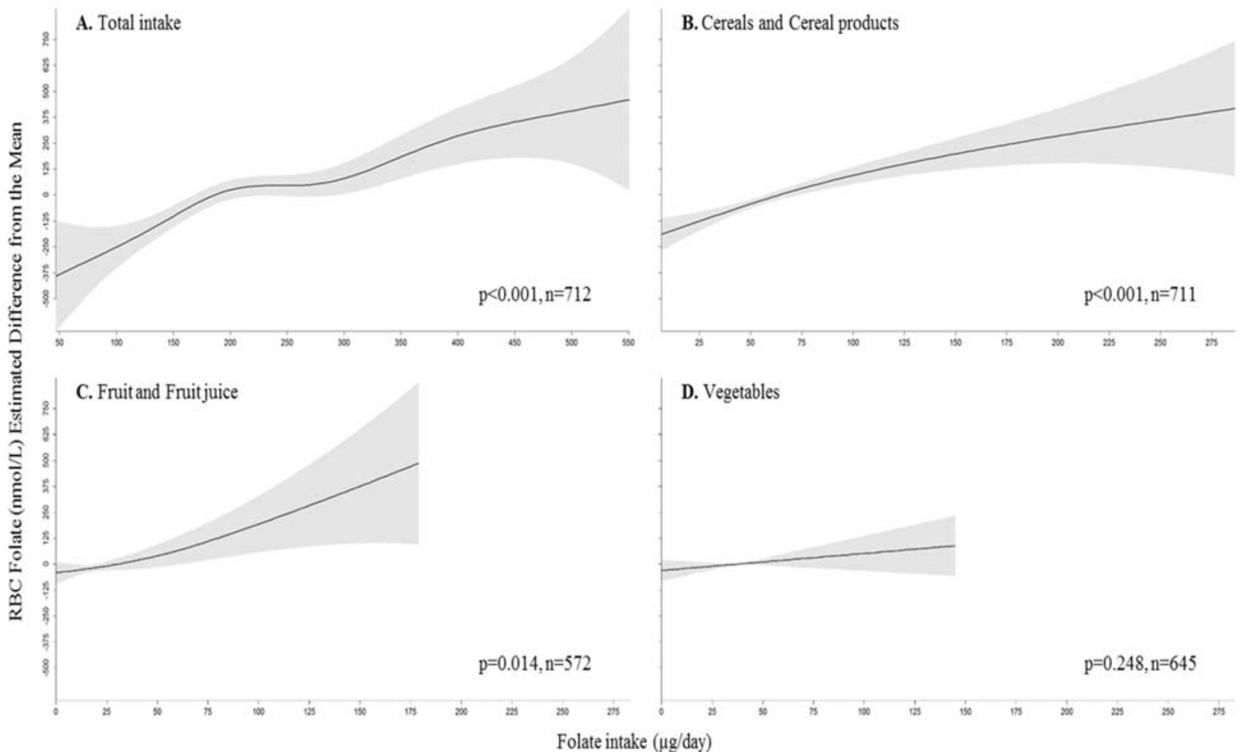

Figure 1. Estimated difference from the mean (and 95% CI) of RBC folate concentration according to folate intake from A. all dietary sources, B. from cereals and cereal products, C. from fruit and fruit juice and D. from vegetables. Generalized additive model (gam) adjusted for sex, energy intake, *MTHFR* genotype, folic acid supplement use and folate intake from the two other food sources. The highest 2.5th percentiles of RBC folate concentrations are not included. Three participants with a folate intake above 150 µg only from vegetables were not included. One participant with a folate intake of 327 µg only from fruit and fruit juice was excluded. *p* values are from the corresponding gam model.

3.4. Risk of Low Folate Status by Folate Intake

Table 3 shows the odds ratio (OR) (and 95% CI) of low RBC folate (<600 nmol/L) according to total, cereals and cereal products, vegetables and, fruit and fruit juice folate intake quartiles. Individuals in the highest quartile of total folate intake (>264 μg/day) were less likely to have RBC folate concentrations <600 nmol/L than those in the lowest quartile (<157 μg/day) in the unadjusted model (OR: 0.58, 95% CI: 0.36, 0.94) and adjusted model (OR: 0.43, 95% CI: 0.23, 0.82). Individuals in higher quartiles of folate intake from cereals and cereal products and from vegetables were also less likely to have low RBC folate concentrations (<600 nmol/L) than those in quartile 1. The same was not true for folate intake from fruit and fruit juice in any model.

Table 3. Odds ratio (95% CI) of low RBC folate concentration according to quartiles of total folate intake and intakes from cereals and cereal products, from vegetables and from fruit and fruit juice in the Newcastle 85+ Study.

Folate Intake	Model 1 (Unadjusted)		Model 2 (Adjusted)	
Total (μg/day)	<600 nmol/L (n = 170)	p	<600 nmol/L (n = 170)	p
<157	1.00 (ref.)	-	1.00 (ref.)	-
157–208	0.64 (0.40, 1.04)	0.071	0.65 (0.38, 1.09)	0.103
209–264	0.72 (0.45, 1.15)	0.173	0.58 (0.34, 1.02)	0.057
>264	0.58 (0.36, 0.94)	0.028	0.43 (0.23, 0.82)	0.010
Cereals and Cereal products (μg/day)	<600 nmol/L (n = 170)	p	<600 nmol/L (n = 170)	p
<36	1.00 (ref.)	-	1.00 (ref.)	-
36–59	0.96 (0.61, 1.49)	0.840	0.84 (0.51, 1.38)	0.493
59–92	0.40 (0.24, 0.66)	<0.001	0.32 (0.18, 0.57)	<0.001
>92	0.41 (0.25, 0.68)	0.001	0.33 (0.18, 0.61)	<0.001
Vegetables (μg/day)	<600 nmol/L (n = 154)	p	<600 nmol/L (n = 154)	p
<15	1.00 (ref.)	-	1.00 (ref.)	-
15–30	0.72 (0.43, 1.21)	0.212	0.49 (0.25, 0.95)	0.035
30–51	0.86 (0.52, 1.41)	0.550	0.59 (0.32, 1.08)	0.089
>51	0.79 (0.48, 1.30)	0.357	0.52 (0.28, 0.99)	0.045
Fruit and Fruit Juice (μg/day)	<600 nmol/L (n = 127)	p	<600 nmol/L (n = 127)	p
<7.3	1.00 (ref.)	-	1.00 (ref.)	-
7.3–16	0.90 (0.53, 1.52)	0.682	1.01 (0.56, 1.83)	0.979
16–34	0.61 (0.35, 1.07)	0.086	0.67 (0.36, 1.25)	0.213
>34	0.76 (0.44, 1.31)	0.329	0.79 (0.43, 1.44)	0.437

RBC folate, Red blood cell folate; p, p-value. Low folate status was defined as RBC folate concentration <600 nmol/L. Binary logistic regression model. Model 1 is unadjusted and Model 2 is adjusted for sex, energy intake, folate intake from the other two food sources (except for total folate), *MTHFR* genotype and folic acid-containing supplement use.

3.5. Association between Vitamin B12 Intake and Status

Total vitamin B12 intake was weakly associated with plasma vitamin B12 concentrations while adjusting for sex, energy intake, vitamin B12 intake from the other two food groups, *FUT2* genotype, vitamin B12 supplement use and H_2 antagonists, biguanides or PPI use (p = 0.054) (Figure 2). Plasma vitamin B12 concentration appeared to decrease when vitamin B12 intake exceeded 10 μg/day but the CI were very wide thereafter. Vitamin B12 intake from meat and meat products, milk and milk products and fish and fish dishes were not associated with plasma vitamin B12 concentration.

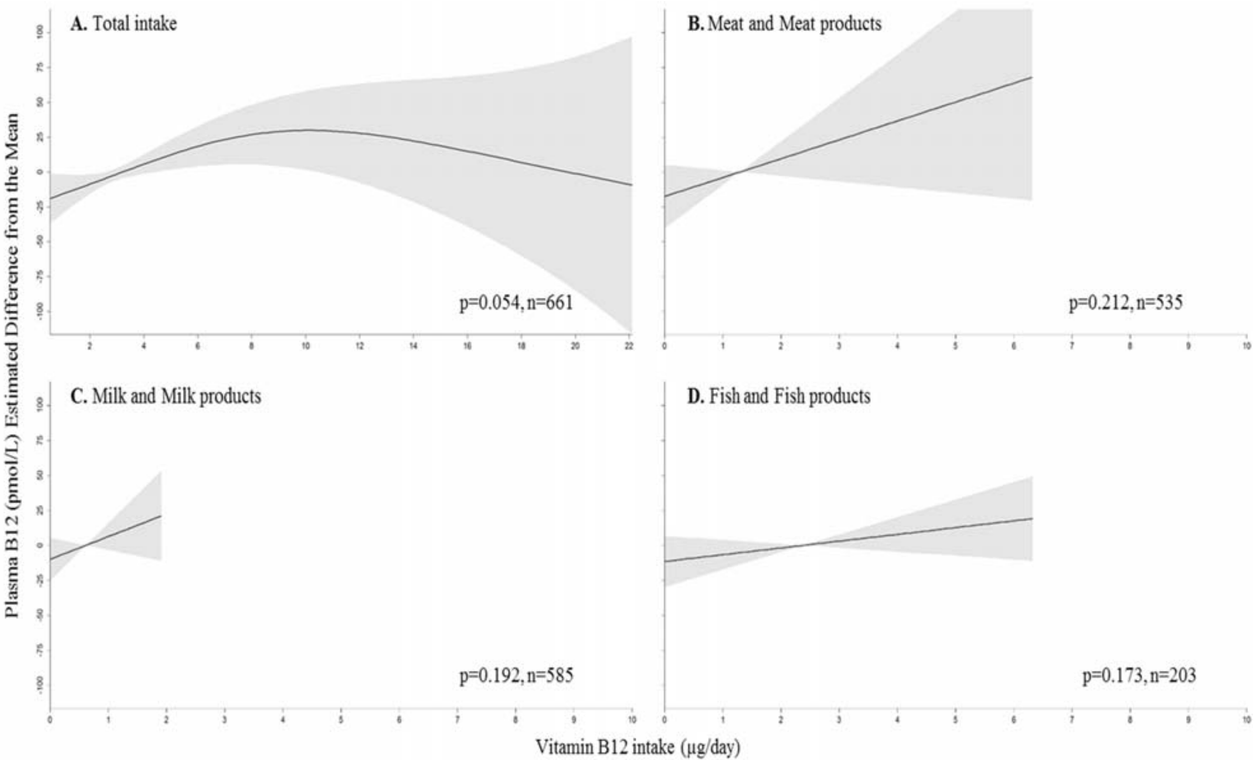

Figure 2. Estimated difference from the mean (and 95% CI) of plasma B12 concentrations according to vitamin B12 intake from A. all dietary sources, B. from meat and meat products, C. from milk and milk products and D. from fish and fish products. Generalized additive model (gam) adjusted for sex, energy intake, *FUT2* genotype, H_2 antagonists, proton pump inhibitors or biguanides use, vitamin B12 supplement use and vitamin B12 intakes from the other two food sources. The lowest and highest 2.5th percentiles of vitamin B12 intakes and plasma vitamin B12 concentrations are not included except for meat and meat products where the highest 5th percentile was excluded. *p* values are from the corresponding gam model.

3.6. Risk of Deficient Vitamin B12 Status by Vitamin B12 Intake

Participants with total vitamin B12 intake above the median (2.88 μg/day) were half as likely to be deficient for plasma B12 as those with the lowest intake (<1.87 μg/day) in the adjusted model (Table 4). Individuals in quartile 4 of vitamin B12 intake from meat and meat products (>2.10 μg/day) were also half as likely to be deficient for plasma vitamin B12 as those in quartile 1 in the unadjusted (OR: 0.55, 95% CI: 0.31–0.98) and adjusted models (OR: 0.41, 95% CI: 0.20–0.81). The same trend was present for milk and milk products but this did not reach statistical significance (p = 0.054).

Table 4. Odds ratio (95% CI) of plasma vitamin B12 deficiency according to quartiles of intake of total vitamin B12 and intakes from meat and meat products, from fish and fish products, and from milk and milk products in the Newcastle 85+ Study.

Vitamin B12 Intake	Model 1 (Unadjusted)		Model 2 (Adjusted)	
Total (μg/day)	<148 pmol/L (n = 125)	p	<148 pmol/L (n = 125)	p
<1.87	1.00 (ref.)	-	1.00 (ref.)	-
1.87–2.88	0.70 (0.42, 1.18)	0.180	0.57 (0.32, 1.01)	0.056
2.88–4.40	0.60 (0.35, 1.02)	0.057	0.50 (0.28, 0.92)	0.026
>4.40	0.53 (0.31, 0.92)	0.024	0.40 (0.21, 0.76)	0.005
Meat and Meat products (μg/day)	<148 pmol/L (n = 118)	p	<148 pmol/L (n = 118)	p
<0.35	1.00 (ref.)	-	1.00 (ref.)	-
0.35–1.03	0.72 (0.42, 1.24)	0.236	0.69 (0.38, 1.25)	0.220
1.03–2.10	0.84 (0.50, 1.44)	0.533	0.78 (0.43, 1.42)	0.422
>2.10	0.55 (0.31, 0.98)	0.043	0.41 (0.20, 0.81)	0.010
Fish and Fish products (μg/day)	<148 pmol/L (n = 43)	p	<148 pmol/L (n = 43)	p
<0.46	1.00 (ref.)	-	1.00 (ref.)	-
0.46–1.06	0.61 (0.23, 1.65)	0.331	0.66 (0.23, 1.91)	0.444
1.06–2.45	0.86 (0.34, 2.15)	0.743	0.66 (0.23, 1.86)	0.427
>2.45	1.00 (0.41, 2.42)	0.992	0.70 (0.25, 1.97)	0.503
Milk and Milk products (μg/day)	<148 pmol/L (n = 102)	p	<148 pmol/L (n = 102)	p
<0.27	1.00 (ref.)	-	1.00 (ref.)	-
0.27–0.53	0.84 (0.47, 1.52)	0.562	0.88 (0.46, 1.71)	0.711
0.53–0.88	1.12 (0.64, 1.96)	0.698	1.28 (0.70, 2.37)	0.425
>0.88	0.58 (0.31, 1.08)	0.086	0.49 (0.24, 1.01)	0.054

p, p-value. Binary logistic regression model. Deficient plasma vitamin B12 concentration was defined as <148 pmol/L. Model 1 is unadjusted and Model 2 is adjusted for sex, energy intake, *FUT2* genotype, vitamin B12 intake from the other two food sources (except total vitamin B12 intake), vitamin B12 containing supplement use, H_2 antagonists, biguanides and proton pump inhibitors use.

4. Discussion

This study found that, in the very old, folate intakes from all food sources and from cereals and cereal products were significantly associated with RBC folate. Individuals with higher total folate intake or intake from cereals and cereal products were less likely to have low concentrations of RBC folate. The association between vitamin B12 intakes and plasma vitamin B12 was weak. Individuals with vitamin B12 intakes from all food sources and from meat and meat products were also less likely to be deficient for plasma vitamin B12. In addition, higher concentrations of RBC folate were found in participants with the *MTHFR* (rs1801133) AA genotype compared with those with A/G or GG genotypes. Women homozygous for the *FUT2* (rs492602) G allele also had higher concentrations of plasma vitamin B12 than those with other *FUT2* genotypes.

4.1. Folate and Vitamin B12 Intake and Status "Inadequacies"

In the Newcastle 85+ Study there was a relatively low prevalence of "inadequate" folate intake (3.1%) and status (3.6%). The NDNS rolling programme estimated that 1% of older adults (aged 65 and over) were below the UK LRNI for folate but that 7.3% of men and 10.8% of women had RBC folate concentrations <340 nmol/L. In the Newcastle 85+ Study, plasma vitamin B12 deficiency (<148 pmol/L) was present in 17.1% of participants and 9.2% were below the UK LRNI (1 μg/day) for vitamin B12 intake whilst the NDNS estimated that 1% were below the LRNI for vitamin B12 but

5.9% had serum vitamin B12 concentrations <150 pmol/L [11]. Age, dietary assessment method (4-day weighted diet record vs. 2 × 24 h-MPR) and other methodological differences are likely explanations for these observed discrepancies. Specifically, the novel method used to assess RBC folate in the NDNS (whole blood folate by a microbiological assay, serum total folate by LC-MS/MS and hematocrit) is likely to give higher estimates of folate "inadequacy" than the one used in this study. The NDNS used a similar method to the Newcastle 85+ Study to assess plasma vitamin B12 (competitive immunoassay with direct chemiluminescence (ADVIA Centaur B12 assay)).

In the post-fortification period in the US, less than 1% of older adults had deficient folate status (RBC folate <340 nmol/L) [37]. In the National Health and Nutrition Examination Survey (NHANES) 2003–2006, it was estimated that 9% of men and 24% of women >70 years old were below the North American EAR for folate (320 DFE/day [38,39]). In the same NHANES edition, 19% of older adults had plasma vitamin B12 concentrations below 221 pmol/L [40]. Moreover, less than 1% of men and 6% of women >70 years were below the EAR for vitamin B12 (2 μg/day) [39]. Almost 30% (n = 235) of the Newcastle 85+ Study participants were below the same EAR for vitamin B12 intake.

4.2. Association between Folate Intake and Status

Folate intake from total diets and from cereals and cereal products but not from vegetables or fruit and fruit juice were associated with RBC folate concentrations in the very old. Vegetables and fruit and fruit juice contributed to 16% and 9%, respectively to folate intake and the relatively lower contribution might explain the lack of associations. Further, folate bioavailability is dependent on the food matrix, stability of labile folates, presence of vitamin C and folate-binding proteins and folate pool sizes [23,41,42]. Nonetheless, there is a consensus that folic acid is better absorbed than dietary folate. Evidence also shows that that folic acid intake is a stronger predictor of RBC folate concentration than total folate intake [43]. The US Institute of Medicine estimated that the absorption efficiency of folic acid in supplements or fortified food was 85% taken with food or 100% from supplements taken on an empty stomach [23], whilst dietary folate absorption efficiency was 50% [23,42]. Breakfast cereals (grouped under cereals and cereal products in this study) have frequently been the target of voluntary fortification in the UK and elsewhere which might explain the stronger association between folate intake from cereals and cereal products and RBC folate concentrations. Cereals and cereal products were also the top contributors to folate intake (32%), suggesting that this is an important source of folate/folic acid in this population group. On the other hand, the incomplete release of dietary folate from plant foods cellular structures may explain a weaker association between folate from vegetables and fruit, and RBC folate.

4.3. Association between Vitamin B12 Intake and Status

Total vitamin B12 was weakly associated with plasma B12 in the very old (p = 0.054) and seemed to saturate at intakes ≈10 μg. The relatively weak association might be due to the low vitamin B12 intakes in relation to the large liver stores (1 μg/g of liver) so that intakes only slowly influence plasma concentrations [24]. Further, vitamin B12 absorption is complex. Bound to protein in food, vitamin B12 has to be released by pepsin and hydrochloric acid in the stomach. The ensuing free form of vitamin B12 binds to haptocorrin, forming a B12-haptocorrin complex. This complex is later broken down in the small intestine by pancreatic proteases which enable vitamin B12 to bind to the glycoprotein IF, be recognized (by cubilin) and absorbed by endocytosis in the enterocytes of the distal ileum. These steps present a problem to older adults as 10%–30% have atrophic gastritis and therefore, reduced gastric acid secretion which is essential to vitamin B12 release from food proteins [12]. The bioavailability of vitamin B12 in any form or dose is estimated to be 40% in healthy adults with intact IF secretion [24]. Meat and meat products, milk and milk products and, fish and fish products were the top sources of vitamin B12 in the Newcastle 85+ Study [30]. However, unlike some findings [44,45], but in agreement with others [46], vitamin B12 intake from meat and meat products was not associated significantly with plasma vitamin B12. Meat and meat products, especially liver (beef liver can reach to as much

as 83 μg per 100 g [29]) and ruminant meat have very high concentrations of vitamin B12 and it is reported that ileal receptors saturate with intakes of 1.5–2.5 μg of vitamin B12 per meal [47]. Only 50% and 5% of vitamin B12 are absorbed with intakes of ~1 and 25 μg, respectively [24]. Others have found that vitamin B12 from dairy products is very bioavailable [48] but also that vitamin B12 in yogurt and cheese is not as bioavailable as that in milk in older individuals [46]. This could explain why vitamin B12 intake from dairy products (that includes yogurt and cheese) was not associated with plasma B12 in this study.

4.4. MTHFR and RBC Folate, and FUT2 and Plasma Vitamin B12

Interestingly, and in contrast to most previous findings [49], participants heterozygous for the A allele of the *MTHFR* gene had higher concentrations of RBC folate than those homozygous for the G allele. This was not a reflection of higher folate or folic acid containing supplements intake. Similar to previous findings [14,49,50], women with the *FUT2* GG genotype had higher concentrations of plasma vitamin B12. *FUT2* encodes galactoside 2-alpha-*L*-fucosyltransferase 2 (EC: 2.4.1.69) which is involved in the regulation of the H antigen and is a precursor of the ABO (H) antigens [50]. *FUT2* variants (from the allele A) are proposed to be protective against *Helicobacter pylori* infection or to increase IF production [14]. Both proposed explanations would explain the higher plasma vitamin B12 concentrations in those homozygous for the G allele.

4.5. Strengths and Weaknesses

The Newcastle 85+ Study is a unique cohort owing to the age group, the large number of participants and the extensive multidimensional health data. The study was socio-demographically representative of the UK but the lack of ethnic diversity warrants caution when generalizing the findings to a non-white population. The rapid processing of blood samples after venepuncture is another strength of this study.

As dietary intake assessment consisted of a 24 h-MPR applied on two non-consecutive days, the possibility of unusually high or low vitamin B12/folate intakes cannot be excluded. For practical reasons, 24 h-MPRs were not conducted during the weekend, therefore food and drink eaten on Fridays and Saturdays was not recalled. Although the MPR method involved several prompts to avoid misreporting, misreporters have been estimated to be 26% of the cohort [28]. Even though the food groups used in the analysis contributed to most of the folate and vitamin B12 intake, other food sources might explain the remaining intake. Intakes of dietary folate equivalents (DFE) and of the crystalline form of vitamin B12 could not be determined because supplement use was collected qualitatively (type and brand but not frequency) and it was not certain which specific foods had been fortified during the dietary collection period (2006/2007). A general limitation of most dietary surveys, including ours, is that assessment of supplement usage may not be accurate by dietary intake records, dietary recalls and other questionnaires [51]. Furthermore, the irregular use of supplements by survey participants, including the alteration of usual patterns of supplement use during the period of dietary data collection, further adds bias to the estimation of true supplement use [51]. It is worth mentioning that the choice of vitamin B12 form used in supplements and fortified foods should also be taken into consideration because of concerns associated with cyanide/thiocyanate from cyanocobalamin [52].

Holotranscobalamin measures the vitamin's active form and because it might better reflect vitamin B12 status than plasma vitamin B12, its use might have yielded different results. There is currently no consensus on the biochemical threshold to use in order to define folate or vitamin B12 "inadequacy", especially in this population. Therefore, results from the binary logistic models might be different if different thresholds were used. Furthermore, atrophic gastritis impairs folate and vitamin B12 absorption. If available, the incidence of atrophic gastritis or a proxy measure, such as *Helicobacter pylori* infection, could have been used as an adjusting factor or to conduct a sensitivity analysis. The list of SNPs used is not exhaustive and other polymorphisms, such as some SNPs in the *TCN2* gene (e.g., rs731991) may influence the folate and vitamin B12 intake–status relationship [53].

5. Conclusions

In summary, almost one-fifth of the 85-year-old participants in the Newcastle 85+ Study had deficient plasma vitamin B12 concentrations but only a few individuals had deficient folate status, according to commonly used biochemical thresholds. Folate and vitamin B12 intakes were associated to RBC folate and plasma B12, respectively and folate intake from cereals and cereal products was strongly associated with RBC folate. This is possibly a consequence of voluntary folic acid fortification of breakfast cereals in the UK, and makes this food group an important source of folate for this population group. Estimates of the bioavailability of folate and vitamin B12 from total diets and from commonly consumed food groups in the very old should be taken into account when setting dietary guidelines.

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Conflicts of Interest: The authors report no conflict of interest.

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Article

Maternal Folate Status and the BHMT c.716G>A Polymorphism Affect the Betaine Dimethylglycine Pathway during Pregnancy

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Abstract: The effect of the betaine: homocysteine methyltransferase BHMT c.716G>A (G: guanosine; A: adenosine) single nucleotide polymorphism (SNP) on the BHMT pathway is unknown during pregnancy. We hypothesised that it impairs betaine to dimethylglycine conversion and that folate status modifies its effect. We studied 612 women from the Reus Tarragona Birth Cohort from ≤12 gestational weeks (GW) throughout pregnancy. The frequency of the variant BHMT c.716A allele was 30.8% (95% confidence interval (CI): 28.3, 33.5). In participants with normal-high plasma folate status (>13.4 nmol/L), least square geometric mean [95% CI] plasma dimethylglycine (pDMG, μmol/L) was lower in the GA (2.35 [2.23, 2.47]) versus GG (2.58 [2.46, 2.70]) genotype at ≤12 GW ($p < 0.05$) and in the GA (2.08 [1.97, 2.19]) and AA (1.94 [1.75, 2.16]) versus GG (2.29 [2.18, 2.40]) genotypes at 15 GW ($p < 0.05$). No differences in pDMG between genotypes were observed in participants with possible folate deficiency (≤13.4 nmol/L) (p for interactions at ≤12 GW: 0.023 and 15 GW: 0.038). PDMG was lower in participants with the AA versus GG genotype at 34 GW (2.01 [1.79, 2.25] versus 2.44 [2.16, 2.76] and at labour, 2.51 [2.39, 2.64] versus 3.00 [2.84, 3.18], ($p < 0.01$)). Possible deficiency compared to normal-high folate status was associated with higher pDMG in multiple linear regression analysis (β coefficients [SEM] ranging from 0.07 [0.04], $p < 0.05$ to 0.20 [0.04], $p < 0.001$ in models from early and mid-late pregnancy) and the AA compared to GG genotype was associated with lower pDMG (β coefficients [SEM] ranging from −0.11 [0.06], $p = 0.055$ to −0.23 [0.06], $p < 0.001$). Conclusion: During pregnancy, the BHMT pathway is affected by folate status and by the variant BHMT c.716A allele.

Keywords: pregnancy; folate; betaine: homocysteine methyltransferase; BHMT c.716G>A; betaine; dimethylglycine

1. Introduction

The importance of one carbon metabolism and homocysteine regulation in foetal development and optimal pregnancy outcome is well established. Homocysteine remethylation contributes not only to homocysteine homeostasis but also to the provision of methyl groups essential for foetal development. Homocysteine is remethylated to methionine by the ubiquitous folate and cobalamin-dependent methionine synthase (MTR; EC 2.1.1.13) and by betaine:homocysteine methyltransferase (BHMT; EC 2.1.1.5) that occurs mainly in the kidney and liver [1]. The estimated consumption of homocysteine by MTR and BHMT is similar in rat liver [2] but globally the MTR reaction is thought to prevail in mammals based on the fact that BHMT activity has only been detected in some organs such as the liver and kidney [3,4] with conflicting reports regarding its presence in the brain [1,5,6]. To the best of our knowledge this information is not available in human studies to date but a study of healthy Dutch adults reported that the inverse association between folate and fasting plasma total homocysteine (tHcy) was stronger than that between betaine and tHcy, suggesting that the MTR reaction prevails under normal circumstances [7]. Some studies also provide indirect evidence for interaction between both remethylation pathways and upregulation of the BHMT pathway when folate status is low [8–12]. Serum folate was positively associated with betaine and inversely associated with dimethylglycine in a study of healthy Dutch adults [8] and serum dimethylglycine was higher in folate-deficient compared to normal status adults in a USA study [9]. Stronger negative associations between betaine and post-methionine load tHcy were observed when serum folate status was low [10] or in the absence of B vitamin supplement use [11]. We reported an apparent shift in the roles of folate and betaine in homocysteine homeostasis as folate status declines with advancing pregnancy and that low plasma folate status was associated with low plasma betaine and high dimethylglycine and a high dimethylglycine/betaine ratio [12].

BHMT c.716G>A (also known as 742G>A, rs3733890) is a common single nucleotide polymorphism (SNP) in which arginine is substituted by glutamine at position 239 [13]. Numerous human studies reported no effect of the SNP on tHcy [14–18]. However, a Norwegian study did report that plasma dimethylglycine decreased with increasing number of A alleles [17].

The BHMT c.716G>A SNP in pregnant women has been associated with increased risk of placental abruption [16]. There are conflicting reports of the effect of the SNP in the foetus on foetal development outcomes. The variant allele was associated with increased risk of grave neural tube defects (NTDs) [19–21] but reduced risk of orofacial cleft [22] and Down syndrome [23,24]. Interestingly, in Liu et al.'s study [19], the risk of NTDs associated with the variant A allele was only observed in pregnancies of women that did not take folic acid supplements.

We hypothesised that the BHMT c.716G>A polymorphism impedes the conversion of betaine to dimethylglycine during pregnancy. We tested how plasma folate status affects plasma betaine and dimethylglycine according to BHMT c.716G>A genotype throughout pregnancy.

2. Experimental Section

2.1. Participants

A total of 612 women that were recruited before November 2014 from the pregnancy phase of the Reus-Tarragona Birth Cohort (RTBC) were studied. The RTBC is an observational longitudinal study of maternal nutritional status and pregnancy that is being carried out by the Area of Preventive Medicine and Public Health, Universitat Rovira i Virgili and the Areas of Obstetrics and Gynaecology of the University Hospitals: Sant Joan, Reus and Joan XXIII, Tarragona (Spain). The study design and participant recruitment have been previously described [12]. Briefly, women attending their first antenatal visit with a viable singleton pregnancy confirmed by ultrasonography that provided a fasting blood sample at or before the 12th gestational week (GW) were eligible to participate in the study. Exclusion criteria included chronic diseases, surgical interventions affecting nutritional status or medication affecting folate or cobalamin metabolism. The study was carried out with ethical approval

from the ethics committees of both participating hospitals and the research has been registered at Clinical Trials. gov: NCT01778205. All participants were informed of the nature and aims of the study and provided signed consent.

Participant age and body mass index (BMI) were recorded at the first antenatal check-up. Data regarding lifestyle, habits and supplement use from periconception throughout pregnancy was collected by the study team using questionnaires at 20 and 32 GW. Socioeconomic status of the participants was defined as low, middle or high according to the family unit income, education level and occupation of both parents [25]. Participants were classified as smokers throughout pregnancy, smokers during the first trimester and non-smokers based on first and second trimester as well as cord plasma cotinine concentrations (see biochemical determinations). Since plasma cotinine is indicative of recent smoking data from questionnaires and prenatal check-ups regarding declared smoking activity by the participants was also considered.

In line with the Spanish Obstetrics and Gynaecology Society recommendations [26], women were advised to take daily prenatal supplements containing 400 μg folic acid during the first trimester. The specific supplements recommended for this study were composed of 400 μg folic acid and 2 μg cyanocobalamin.

2.2. Blood Sample Collection and Biochemical and Genetic Determinations

Fasting blood samples were collected at ≤12, 15, 24–27 and 34 GW and nonfasting samples on admission to hospital with confirmed labour and from the umbilical cord. Maternal blood was drawn from the antecubital vein, and cord blood from the vein, into vacutainers containing EDTA-K_2. Samples were kept at 4 °C for a maximum of 1 h before separating plasma of which aliquots were immediately stored at −80 °C in the Institut d'Investigació Sanitària Pere Virgili biobank (Reus (Tarragona), Spain). Leukocytes were isolated from the blood cells remaining after plasma separation and DNA extracted from these using the Puregene DNA extraction kit (Gentra Systems, Minneapolis, MN, USA). Samples were transported on dry ice to Bevital (www.bevital.no, Bergen, Norway), carrying out biochemical analyses in batches comprising complete pregnancies within 18 months of collection. Plasma folate was determined by microbiological assay with *Lactobacillus casei* [27]. Plasma concentrations of choline, betaine, dimethylglycine, total homocysteine (tHcy) were measured by liquid chromatography–tandem mass spectrometry [28,29]. Plasma cotinine, which is an indicator of recent nicotine exposure, was also determined by liquid chromatography–tandem mass spectrometry [30] and plasma creatinine by modified Jaffé method (Química Clínica Aplicada SA, Amposta, Tarragona, Spain). Classification as current smoker was based on plasma cotinine >10 ng/mL at ≤12 GW (first trimester smoker) and 24–27 GW or in the cord (smoker throughout pregnancy). The maternal BHMT c.716G>A SNP was determined by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF/MS) as previously described [31].

2.3. Statistical Analysis

Previously, we reported a shift in folate status between early and mid-late pregnancy in line with the overall cessation of folic acid supplement use or change in supplement type between the first and second or third trimesters of pregnancy [12]. To account for change in plasma folate status during pregnancy, and to classify plasma folate status according to WHO criteria [32], participants were classified into possibly deficient (plasma folate ≤ 13.4 nmol/L) or normal-high (plasma folate > 13.4 nmol/L) status categories during early pregnancy (based on plasma folate at ≤12 or 15 GW) and again during mid-late pregnancy (based on plasma folate at 24–27 or 34 GW). In the event of falling into different categories at different time points within the corresponding phase of pregnancy, normal-high status was assigned during early pregnancy and the predominant status out of the 3 time points was assigned in mid-late pregnancy. In any analysis using plasma folate status, the category occurring during the corresponding phase of pregnancy was used.

Natural log transformation was applied to normalise the distribution of plasma variables as required for the application of parametric tests. Thus, geometric means (95% CI) are reported for plasma folate, betaine, choline, dimethylglycine and tHcy. In all other cases medians (25th percentile, 75th percentile) are reported and frequencies are reported as % (95% CI). Proportions were compared using the chi-square test and the same test was also used to test the Hardy Weinberg equilibrium of the observed allele frequencies.

Plasma folate, choline, betaine, dimethylglycine and dimethylglycine/betaine ratio and tHcy were compared between the different BHMT c.716G>A genotypes at each gestational period and in the cord by ANCOVA adjusting for gestational age (weeks) at the time of the blood sample, and plasma folate status (in all models except for plasma folate models). The dimethylglycine models were also adjusted for plasma betaine. Interaction between plasma folate status and BHMT c.716G>A genotype in their effects on plasma dimethylglycine, dimethylglycine/betaine ratio and tHcy was tested and if observed, stratified analysis according to folate status was performed.

Changes in plasma concentrations of folate, choline, betaine, dimethylglycine, the dimethylglycine/betaine ratio and tHcy during pregnancy were assessed using 2-factor repeated measures ANOVA (General linear model; Intrasubject factor time of pregnancy and intersubject factor BHMT c.716G>A genotype) with posthoc Bonferroni correction of p values to account for multiple comparisons. The first trimester time point was used as the reference.

Multiple linear regression analysis was used to investigate the effects of plasma folate status and of maternal BHMT c.716G>A genotype on plasma dimethylglycine at each stage of pregnancy and in the cord. The models were adjusted for plasma betaine and gestational age at corresponding time of blood sample. The usual diagnostic techniques for multiple linear regression analysis were applied (Cook's distance to identify influential cases and analysis of residuals). Interaction between plasma folate status and BHMT c.716G>A genotype was also tested. All analyses were carried out with SPSS (SPSS Inc., Chicago, IL, USA) for Windows, version 22.0.

3. Results

Participation in the study by eligible candidates and completion (defined as live birth) is illustrated in Figure 1. Of the women screened at their first prenatal check-up, 42.4% were eligible to participate in the study. Of the 624 participants that entered the study (93.8% of those eligible) first trimester data was collected from 612 and 562 went on to have live births.

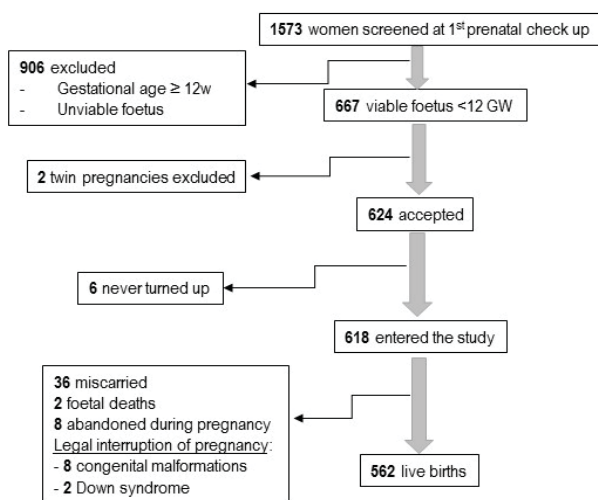

Figure 1. Flow chart of participation in the study.

Participant lifestyle, plasma folate, cobalamin, choline, tHcy and obstetrical characteristics are summarised in Table 1. This data shows that 80.9% of the women reported planning their pregnancy and 34.1% took folic acid supplements before becoming pregnant. Active smoking during the first trimester and throughout pregnancy was observed in 28.1% and in 17% of the participants respectively. The frequency of the BHMT c.716A allele was 30.8% (95% CI: 28.3, 33.5) and the genotypes were in Hardy Weinberg equilibrium (chi square for observed compared to expected genotype frequencies: 1.64).

Table 1. Participant characteristics (*n* = 612).

Age (Year) [1]		**32.0 (29.0, 35.0)**
Body mass index (kg/m^2) [1]		23.0 (20.9, 25.4)
Planned pregnancy [2]		80.9 (77.3, 84.0)
Previous pregnancy [2]		52.6 (48.6, 56.5)
Socioeconomic status [2,3]	High	43.6 (39.6, 47.6)
	Mid	49.6 (45.5, 53.6)
	Low	6.8 (5.1, 9.1)
Smoking during pregnancy [2]	First trimester	28.1 (24.7, 31.8)
	Throughout pregnancy	17.0 (14.2, 20.3)
Folic acid supplement use [2]	Preconception	34.1 (30.2, 38.2)
	First trimester	93.8 (91.5, 95.4)
	Mid-late pregnancy	53.9 (49.6, 58.1)
BHMT c.716G>A [2]	GG	48.9 (45.0, 52.9)
	GA	40.5 (36.6, 44.4)
	AA	10.6 (8.4, 13.3)

G: guanosine; A: adenosine. Values are [1] median (25th percentile, 75th percentile), [2] % (95% confidence interval); [3] based on total income, occupation and education level of both parents.

Plasma folate, choline, betaine and dimethylglycine concentrations, dimethylglycine/betaine ratios and tHcy at each time point of pregnancy and in the cord, according to maternal BHMT c.716G>A genotype are reported in Table 2. Plasma folate, choline, betaine or tHcy did not differ among genotypes at any time of pregnancy. Lower plasma dimethylglycine was observed in the heterozygote compared to homozygote common genotype at ≤12 GW and at labour. This was also true for the homozygote variant genotype during late pregnancy. A lower plasma dimethylglycine/betaine ratio was observed in the homozygote variant compared to heterozygote genotype at ≤12 GW and compared to the homozygote common genotype at 34 GW. This was also true for the heterozygote compared to the homozygote common genotype at 15 GW. Further adjustment of the ANCOVA models for plasma creatinine and smoking habit did not alter the results.

Plasma betaine gradually decreased in all of the genotypes throughout pregnancy. Plasma dimethylglycine fluctuated in a U shape pattern in the homozygote common and heterozygote genotypes where end of pregnancy concentrations were higher than in early pregnancy. However, in the homozygote variant genotype, plasma dimethylglycine concentrations remained lower than in early pregnancy, throughout pregnancy. The plasma dimethylglycine/betaine ratio gradually increased during pregnancy in all genotypes.

Interactions between plasma folate category and the effect of BHMT c.716G>A genotype on dimethylglycine were observed at ≤12 and 15 GW and on the dimethylglycine/betaine ratio at ≤12 GW.

A stratified analysis of plasma dimethylglycine for each genotype according to plasma folate status in early pregnancy is illustrated in Figure 2. In the normal-high category of plasma folate status, lower plasma dimethylglycine was observed in the heterozygote compared to the homozygote common genotype at both ≤12 and 15 GW and in the homozygote variant compared to homozygote common genotype at 15 GW.

Table 2. Plasma folate, choline, betaine, dimethylglycine and tHcy during pregnancy and in the cord according to maternal BHMT c.716G>A genotype.

	BHMT c.716G>A Genotype	≤12 GW [546] [1]	15 GW [440]	24–27 GW [500]	34 GW [485]	Labour [478]	Cord [465]
Folate (nmol/L)	GG	26.8 (24.5, 29.2)	25.6 (23.3, 28.1)	13.3 [a] (12.2, 14.6)	10.9 [a] (9.9, 12.1)	10.8 [a] (9.7, 11.9)	23.9 (22.2, 25.7)
	GA	25.5 (23.2, 28.0)	24.6 (22.3, 27.1)	12.9 [a] (11.7, 14.2)	10.7 [a] (9.7, 11.9)	10.8 [a] (9.7, 12.1)	24.2 (22.4, 26.1)
	AA	24.5 (20.2, 29.6)	25.4 (20.8, 31.1)	12.6 [a] (10.3, 15.3)	12.0 [a] (9.6, 15.1)	10.2 [a] (8.2, 12.9)	22.6 (19.1, 26.6)
	ANCOVA [3] models	NS	NS	NS	NS	NS	NS
Choline (μmo/L)	GG	7.6 (7.4, 7.9)	7.7 (7.5, 7.9)	9.1 [a] (8.9, 9.4)	10.3 [a] (10.1, 10.6)	11.7 [a] (11.3, 12.1)	28.1 (26.8, 29.5)
	GA	7.7 (7.4, 7.9)	7.7 (7.5, 8.0)	9.2 [a] (9.0, 9.5)	10.4 [a] (10.1, 10.7)	11.7 [a] (11.3, 12.2)	28.9 (27.5, 30.4)
	AA	7.6 (7.2, 8.1)	8.0 (7.5, 8.5)	9.5 [a] (9.0, 10.1)	10.5 [a] (9.9, 11.2)	11.6 [a] (10.7, 12.5)	29.8 (26.8, 33.1)
	ANCOVA [3] models	NS	NS	NS	NS	NS	NS
Betaine (μmol/L)	GG	20.9 (20.2, 21.6)	14.6 [a] (14.2, 15.1)	12.8 [a] (12.4, 13.1)	12.8 [a] (12.5, 13.1)	13.1 [a] (12.7, 13.5)	24.7 (24.0, 25.4)
	GA	21.8 (21.0, 22.6)	15.3 [a] (14.8, 15.8)	13.1 [a] (12.7, 13.4)	13.3 [a] (12.9, 13.7)	13.4 [a] (13.0, 13.8)	25.1 (24.3, 25.8)
	AA	20.2 (18.8, 21.8)	14.5 [a] (13.6, 15.5)	12.7 [a] (11.9, 13.4)	13.0 [a] (12.3, 13.8)	12.7 [a] (11.9, 13.5)	25.3 (23.8, 27.0)
	ANCOVA [3] models	NS	NS	NS	NS	NS	NS
Dimethylglycine (μmol/L)	GG	2.58 (2.47, 2.69)	2.29 [a] (2.19, 2.39)	2.22 [a] (2.12, 2.33)	2.51 (2.39, 2.64)	3.00 [a] (2.84, 3.18)	3.73 (3.55, 3.92)
	GA	2.37 (2.26, 2.48) *	2.12 [a] (2.02, 2.22)	2.11 [a] (2.00, 2.22)	2.39 (2.27, 2.52)	2.69 [a] (2.53, 2.86) *	3.54 (3.36, 3.73)
	AA	2.50 (2.28, 2.74)	2.08 [a] (1.88, 2.30)	2.02 [a] (1.82, 2.24)	2.01 [a] (1.79, 2.25) **,†	2.44 (2.16, 2.76) **	3.29 (2.95, 3.67)
	ANCOVA [4] models	$p = 0.026$	$p = 0.040$	NS	$p = 0.002$	$p = 0.002$	NS
	Folate genotype interaction	$p = 0.023$	$p = 0.058$	NS	NS	NS	NS
Dimethylglycine/betaine	GG	0.13 (0.12, 0.14)	0.17 [a] (0.16, 0.18)	0.19 [a] (0.18, 0.21)	0.22 [a] (0.20, 0.24)	0.26 [a] (0.23, 0.28)	0.17 (0.15, 0.18)
	GA	0.12 (0.11, 0.13)	0.15 [a] (0.14, 0.16) *	0.18 [a] (0.16, 0.19)	0.20 [a] (0.18, 0.22)	0.23 [a] (0.21, 0.26)	0.15(0.14, 0.16)
	AA	0.15 (0.13, 0.17) †	0.15 (0.13, 0.17)	0.18 [a] (0.15, 0.22)	0.16 (0.12, 0.20) *	0.22 [a] (0.17, 0.27)	0.14 (0.11, 0.16)
	ANCOVA [5] models	$p = 0.009$	$p = 0.018$	NS	$p = 0.020$	NS	$p = 0.044$
	Folate genotype interaction	$p = 0.038$	NS	NS	NS	NS	NS
Total homocysteine (μmol/L)	GG	5.2 (5.1, 5.4)	4.5 [a] (4.4, 4.7)	4.6 [a] (4.5, 4.8)	5.3 (5.1, 5.4)	6.2 [a] (6.0, 6.5)	4.8 (4.7, 5.0)
	GA	5.3 (5.1, 5.4)	4.4 [a] (4.3, 4.6)	4.7 [a] (4.5, 4.8)	5.4 (5.2, 5.6)	6.2 [a] (5.9, 6.4)	4.9 (4.7, 5.2)
	AA	5.3 (5.0, 5.6)	4.5 [a] (4.3, 4.8)	4.6 [a] (4.3, 4.9)	5.2 (4.9, 5.6)	6.0 [a] (5.6, 6.5)	4.9 (4.5, 5.3)
	ANCOVA [4] models	NS	NS	NS	NS	NS	NS

G: guanosine; A: adenosine; GW: gestational weeks; NS: non-significant. [1] *n* varies between time points due to participant loss due to complications, non-attendance of programmed blood draw or delivery elsewhere or failure to collect blood samples in the labour ward; [2] values are least square geometric means (95% confidence interval); [3] adjusting for plasma folate status and gestational age at time of blood draw; [4] adjusting for plasma folate status, plasma betaine and gestational age at time of blood draw. ANCOVA Bonferroni posthoc: * $p < 0.05$, ** $p < 0.01$ versus GG; † $p < 0.05$, versus GA; Two-factor repeated measures ANOVA versus ≤12 GW (intrasubject factor: gestational age; intersubject factor: BHMT c.716G>A genotype) followed by post hoc Bonferroni correction for multiple comparisons: [a] $p < 0.001$.

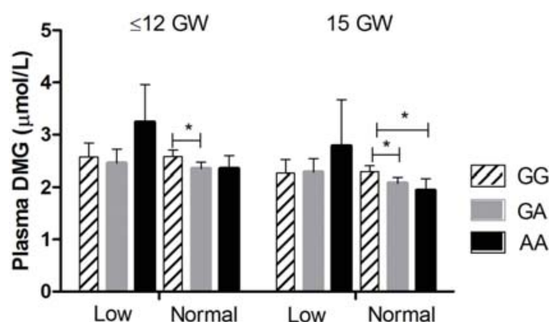

Figure 2. Plasma dimethylglycine according to BHMT c.716G>A genotype and folate status in early pregnancy. G: guanosine; A: adenosine; GW: Gestational weeks. Low: possibly deficient (plasma folate ≤ 13.4 nmol/L). Normal: normal-high (plasma folate > 13.4 nmol/L). At ≤12 GW, Low: GG (n = 57), GA (n = 48), AA (n = 14); Normal: GG (n = 231), GA (n = 193), AA (n = 47). At 15 GW, Low: GG (n = 37), GA (n = 40), AA (n = 6); Normal: GG (n = 169), GA (n = 141), AA (n = 34). The triple screening blood sample at 15 GW is optional and blood samples were available from less participants. Values are least square geometric means. Error bars represent 95% confidence interval. Comparisons between genotypes were made using ANCOVA adjusting for plasma betaine and gestational age at time of blood draw with posthoc Bonferroni correction for multiple comparisons of p values: * $p < 0.05$.

The associations between plasma folate status and BHMT c.716G>A genotype with plasma dimethylglycine throughout pregnancy and in the cord are reported in Table 3. Low compared to normal-high plasma folate status was positively associated with plasma dimethylglycine throughout pregnancy and in the cord. Compared to the homozygous common BHMT GG genotype, both variant GA and AA genotypes were associated with lower plasma dimethylglycine during early and late pregnancy. The observed associations were stronger in the case of the homozygote AA variant genotype during late pregnancy and in the cord. Further adjustment of the multiple linear regression models for plasma creatinine and smoking habit did not alter the results.

Table 3. Change in plasma dimethylglycine throughout pregnancy according to plasma folate category, and BHMT c.716G>A genotype.

		Model [1]		Plasma Folate Category [2]	BHMT c.716G>A Genotype	
		R^2	F (n)	Possibly deficient vs. normal-high	GA vs. GG	AA vs. GG
Early	≤12 GW	15.4	22.5 (592) ***	0.07 (0.04) [3],*	−0.08 (0.03) **	−0.04 (0.05)
	15 GW	12.0	12.6 (427) ***	0.07 (0.04) #	−0.08 (0.03) *	−0.11 (0.06) †
Mid-late	24–27 GW	11.6	14.3 (507) ***	0.13 (0.03) ***	−0.06 (0.04)	−0.10 (0.06)
	34 GW	18.9	23.8 (492) ***	0.20 (0.04) ***	−0.06 (0.04)	−0.23 (0.06) ***
	Labour	13.2	15.5 (477) ***	0.15 (0.04) ***	−0.11 (0.04) *	−0.21 (0.07) **
	Cord	5.0	5.7 (447) ***	0.08 (0.04) *	−0.05 (0.04) *	−0.13 (0.06) *

F: analysis of variance F-test of overall significance; G: guanosine; A: adenosine; GW: Gestational weeks. [1] Multiple linear regression analysis: dependent variable plasma dimethylglycine. Adjusted for plasma betaine and gestational age at time of blood draw; [2] Pregnancy (possibly deficient: ≤13.4 nmol/L; normal-high: >13.4 nmol/L); [3] β coefficient (standard error of the mean). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, † $p = 0.055$, # $p = 0.087$.

4. Discussion

4.1. Principal Findings

This study reports for the first time that the A-allele of the BHMT c.716G>A SNP is associated with lower plasma dimethylglycine during pregnancy and in the cord. Low folate status, on the other

hand is associated with higher plasma dimethylglycine during mid and late pregnancy and in the cord. Folate status and genotype interacted during early pregnancy and stratification by plasma folate status category showed that the effect of the variant BHMT c.716A allele on dimethylglycine was limited to women with normal-high folate status. By late pregnancy, the variant allele's effect was independent of folate status.

4.2. Comparison with Previous Studies and Interpretation

We observed similar frequencies of the BHMT c.716G>A genotypes as previously reported in studies from Europe [14,17,33].

We interpret the lower plasma dimethylglycine concentrations observed in the presence of the variant BHMT c.716A allele to mean that the conversion of betaine to dimethylglycine is low in pregnant women carrying the A-allele. A similar observation was previously reported in a population study [17]. On the other hand, the higher plasma dimethylglycine concentrations when folate status is low, suggests BHMT upregulation when MTR activity is reduced. In early pregnancy when MTR activity was not reduced by low folate availability, the effects of lower BHMT activity in the presence of the BHMT c.716 A-allele are evident. We hypothesised that this effect would be more pronounced at mid-late pregnancy and with low folate status when the BHMT pathway is more active [12]. Our results showed decreased betaine to dimethylglycine conversion in the variant A-allele carriers, but in early pregnancy this effect was limited to women with normal-high folate status. In vitro experiments have shown that BHMT expression and activity may be modified by the molecular environment. SAM has been shown to inhibit BHMT transcription in human cells [34,35] and SAM [36] and to a lesser extent SAH [36,37] to inhibit BHMT activity in rat liver extracts. Methionine has also been shown to inhibit BHMT [38,39]. A study in MTHFR-deficient mice supplemented with very high doses of folic acid showed that despite greater betaine utilisation, likely for homocysteine remethylation, it was insufficient to maintain SAM concentrations [40]. Despite the differences in molecular environment between *in vivo* and *in vitro* studies, they shed light on potential mechanisms that may lead to our observed effects. A speculative suggestion from our data is that high folate status can lead to BHMT inhibition, with the inhibitory effect being stronger on the variant enzyme. Some studies have investigated the effect of folate on BHMT. Liver *BHMT* expression was reported to be lower in the offspring of rat dams supplemented with folic acid during pregnancy [41] but was unaffected by excessive folic acid intake in mice [40]. It has also been suggested that betaine is spared when folate status is replete [42]. It is possible that the combination of both mechanisms, replete folate status and the variant allele, lead to reduced conversion of betaine to dimethylglycine. Cessation of prenatal folic acid supplementation at the end of the first trimester was associated with a sharp reduction in folate status, and from mid pregnancy the genotype effect on dimethylglycine was independent of folate status.

No differences in plasma betaine concentrations between the different genotypes were observed. This is not surprising because a stable betaine pool is maintained even when the BHMT pathway is upregulated [9,43], possibly to spare betaine for its principal functions as an osmolyte [44] and in protein stabilisation [45].

4.3. Implications

BHMT expression and activity in foetal livers has been reported to increase with gestational age in human [5,46] and pig studies [47] which is in line with increased BHMT activity in late pregnancy that we suggest here. Interestingly, foetal liver MTR activity decreases in the third trimester [5]. This supports the idea of complementarity between the BHMT and MTR pathways in late pregnancy and our observations indirectly support this hypothesis.

Here we show that the BHMT c.716G>A polymorphism, previously associated with foetal developmental defects, affects the BHMT pathway during pregnancy. In situations of upregulation of the MTR and BHMT pathways, such as in pregnancy, the effect of the SNP on BHMT activity may

become more evident. Anomalies in homocysteine remethylation affect homocysteine homeostasis as well as methyl group supply to essential epigenetic reactions. Elevated maternal homocysteine has been associated with pregnancy complications affecting both maternal and foetal health as well as foetal development [48] and with lasting developmental effects into childhood [49]. Inhibition of BHMT has been shown to cause hyperhomocysteinaemia in mice [50]. We did not observe an effect of the variant allele on homocysteine. However, the extent of the effect of the SNP on BHMT activity in humans is unknown. Pregnancy itself has a profound effect on homocysteine [51] and may mask any potential effects of the SNP. Comparison of associations between betaine and homocysteine before and after the implementation of mandatory fortification of flour with folic acid in the USA showed that the inverse correlation reported in Framingham study participants with low folate status prior to fortification, was no longer observed post-fortification [52].

Potential modification of effects of folate status by the BHMT c.716G>A SNP should be considered when contrasting/interpreting results from fortified versus non-fortified populations or supplement versus non-supplement users. In addition, the importance of the BHMT pathway in homocysteine homeostasis may be less important in folate-replete populations.

4.4. Strengths and Limitations

A strength of this study is its longitudinal design and follow up from very early pregnancy. It was carried out in the absence of mandatory fortification with folic acid and therefore the effects of prenatal folic acid supplement use were observed. Samples were processed according to specific protocols to prevent artefacts in plasma analyte concentrations caused by temperature and time before separation of blood cells [53].

When folic acid supplementation ceased there was a large drop in plasma folate as we reported previously [12]. Sample power may have been limited in analyses of the effect of the BHMT c.716AA genotype during early pregnancy. However many significant effects of this genotype on dimethylglycine were observed in mid-late pregnancy and in the cord. Another potential limitation to the study was the difference in folic acid supplement use between women. They were recommended to take prenatal supplements containing 400 μg of folic acid during the first trimester. However some took other brands of folic acid containing higher doses or extended their folic acid use beyond the first trimester. We dealt with this in our analysis by classifying the women into plasma folate status categories in early and again in mid-late pregnancy to account for the difference in status that we expected to arise due to differences in patterns of supplement use.

Although there is increasing evidence that BHMT plays an important role in one carbon metabolism many aspects regarding the mechanisms involved are unclear. Information regarding the effect of the common BHMT c.716G>A polymorphism on BHMT activity and how it is affected by variations in molecular environment is lacking.

5. Conclusions

We conclude that dimethylglycine during pregnancy is affected by both folate status and the BHMT c.716G>A polymorphism.

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Author Contributions: J.M.C., S.F.-R., J.D.F.-B. and M.M.M. developed the hypothesis, designed the study and analysed the data. P.C.-B. and M.B. recruited the participants and carried out the pregnancy phase of the study. S.F.-R., P.S.-N. and J.M.C. collected lifestyle and dietary data from the participants and processed samples for biobanking. P.M.U. and K.M. were responsible for the biochemical and genetic determinations. All authors participated in the writing of the paper and approved the final version.

Conflicts of Interest: The authors declare no conflicts of interests.

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Organ-Specific Gene Expression Changes in the Fetal Liver and Placenta in Response to Maternal Folate Depletion

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Abstract: Growing evidence supports the hypothesis that the in utero environment can have profound implications for fetal development and later life offspring health. Current theory suggests conditions experienced in utero prepare, or "programme", the fetus for its anticipated post-natal environment. The mechanisms responsible for these programming events are poorly understood but are likely to involve gene expression changes. Folate is essential for normal fetal development and inadequate maternal folate supply during pregnancy has long term adverse effects for offspring. We tested the hypothesis that folate depletion during pregnancy alters offspring programming through altered gene expression. Female C57BL/6J mice were fed diets containing 2 mg or 0.4 mg folic acid/kg for 4 weeks before mating and throughout pregnancy. At 17.5 day gestation, genome-wide gene expression was measured in male fetal livers and placentas. In the fetal liver, 989 genes were expressed differentially (555 up-regulated, 434 down-regulated) in response to maternal folate depletion, with 460 genes expressed differentially (250 up-regulated, 255 down-regulated) in the placenta. Only 25 differentially expressed genes were common between organs. Maternal folate intake during pregnancy influences fetal gene expression in a highly organ specific manner which may reflect organ-specific functions.

Keywords: transcriptome; programming; developmental origins of health and disease; pregnancy; diet

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1. Introduction

The developmental origins of health and disease (DOHaD) hypothesis proposes that in utero and early life exposures can lead to altered programming of offspring. Such programming events can cause permanent changes in organ development, physiology and metabolism leading to altered disease risk in later life. Suboptimal nutrition, both under- and over-nutrition, during fetal and neonatal development increases susceptibility to a wide range of diseases [1]. These observations indicate a degree of plasticity during development, in which the fetal phenotype may be altered due to environment cues [2] to prepare it for the anticipated post-natal environment [3]. The biological mechanisms underlying this "programming" effect of nutrition during early life are poorly understood, but are likely to involve changes in gene expression.

The protective effect of adequate dietary folate intake and of folic acid supplementation during pregnancy on risk of neural tube defects (NTDs) is well established [4,5]. Further, epidemiological evidence suggests that adequate dietary folate intake or supplementation with folic acid during pregnancy may reduce the risk of other congenital defects [6] and adverse pregnancy outcomes [7], as well enhancing neurodevelopment [8] and reducing the risk of severe language delay [9], autism [10,11] and some cancers (leukaemia [12–14], brain tumours [15,16] and neuroblastoma [17]) [18] in children. In rodent models, folate deficiency during pregnancy can cause spontaneous abortion, teratogenic effects in offspring, reduced litter number, and altered offspring body weight [19,20]. Sufficient maternal folate intake during pregnancy is essential for successful pregnancy outcomes/normal fetal development and for the long-term health of the offspring.

Folate, a B vitamin, is central to one-carbon metabolism which, in addition to formation of the universal methyl donor, *S*-adenosyl-methionine (SAM), interacts with several other cellular pathways including amino acid metabolism and biosynthesis of purine and pyrimidines [21]. SAM is critical for the methylation of biological molecules including DNA, lipids and proteins. Epigenetic mechanisms (including methylation of DNA and of histones) which regulate gene expression are susceptible to modification via altered SAM availability in response to changes in folate intake (reviewed in [22]). Therefore, we hypothesised that inadequate folate supply during pregnancy alters programming of the offspring via changes in gene expression and that this is responsible for the observed adverse effects of this maternal nutritional insult on pregnancy outcomes and offspring health in later life. Mammals respond to inadequate nutrient supply by prioritising allocation of nutrients to specific purposes [23,24] which may result in cell, tissue and organ differences in gene expression. Little is known about such inter-organ differences in fetal gene expression in response to restricted folate supply. We hypothesised that the fetal liver and placenta, which represent organs with long and short-term consequences for the developing animal, would be subject to distinctly different responses to folate depletion. To test these hypotheses, we quantified genome-wide gene expression patterns in fetal liver and placenta in response to maternal folate depletion before, and throughout, pregnancy.

2. Experimental Section

2.1. Animal Husbandry and Experimental Diets

All animal procedures were approved by the Newcastle University Ethics Review Committee and the UK Home Office (Project Licence number 60/3979) and have been described previously [25]. Animals were housed in the Comparative Biology Centre, Newcastle University at 20–22 °C and with 12 h light and dark cycles. Fresh water was available ad libitum. Female C57BL/6J mice were allocated at random to either a low folate (0.4 mg folic acid/kg diet) or normal folate diet (2 mg folic acid/kg diet) (6 g of allocated diet was offered to each mouse per day), and maintained on this diet for 4 weeks prior to mating. Diet compositions were modified from AIN-93G [26] and have been described previously [25]. L-amino acids were used as a protein substitute. All ingredients, other than folic acid, were included in both diets at the same concentrations to avoid potential confounding through other dietary factors. The degree of folate depletion induced by feeding the diet containing 0.4 mg folic acid/kg was sufficient to impose a nutritional stress (evidenced by reduced circulating concentrations of folate) but not so severe as to limit reproduction. The normal folate diet contained 2 mg folic acid/kg diet which is considered sufficient to support breeding and maintenance in this species. Mice were time mated i.e., a male was added to a cage containing two females overnight and removed the following morning. Pregnant females, identified by the presence of a vaginal plug, were re-caged and offered 10 g/day of allocated diet throughout pregnancy. At 17.5 days gestation, dams were killed for collection of blood and organs.

2.2. Sample Collection

Animals were anesthetised using gaseous isoflurane, blood was removed by cardiac puncture and animals were killed by cervical dislocation. Blood was collected and stored in EDTA tubes.

Whole blood 5-methyltetrahydrofolate (THF) and 5–10-methylTHF concentrations were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and data reported previously [25]. The uterus, containing all fetuses and placentas, was removed and placed immediately in ice cold PBS. The liver and placenta of each fetus were removed, weighed and snap frozen in liquid nitrogen and stored at −80 °C until required for RNA extraction.

2.3. RNA Extraction

To avoid any potential influence of sex on resultant data, only male tissues were analysed. Fetal sex was determined by polymerase chain reaction (PCR) of the sex determining region-Y (*SRY*) gene using DNA extracted from embryonic tail tissue [25,27]. RNA was extracted from whole fetal livers and placentas of males only using Tri-reagent (Sigma-Aldrich, Gillingham, Dorset, SP8 4XT, UK) and following the manufacturer's instructions. Briefly, 50 mg tissue was homogenised in 500 μL Tri-reagent on ice. A further 500 μL Tri-reagent and 200 μL chloroform were added, then the sample was mixed by inversion and incubated on ice for 5 min. Samples were then centrifuged at 13,500 rpm for 15 min at 4 °C. The upper aqueous phase was removed, RNA was precipitated by incubation on ice with 500 μL isopropanol for 10 min, after which it was collected by centrifugation at 13,500 rpm for 10 min at 4 °C. The supernatant fluid was removed and the pellet was washed with 1 mL 75% ethanol for 10 min before centrifuging at 6000 rpm for 5 min at 4 °C. The supernatant fluid was removed, and the pellet was allowed to dry for 10 min before resuspending the RNA in 40 μL water. Contaminating DNA was removed using RQ1 DNase (Promega, Southampton, Hampshire, SO16 7NS, UK). RNA integrity was checked on an agarose gel and concentration and purity were measured using a Nanodrop spectrophotometer (Thermo Fisher, Waltham, MA, USA).

2.4. Gene Expression Arrays

RNA from each tissue was pooled for three male fetuses per litter (5 μg/fetus with a final concentration of 500 ng/μL) and hybridised to a single array for a total of 12 litters (n = 6 per dietary group) for each organ. Where a litter had more than three male fetuses, the three fetuses with weights closest to the mean litter weight were analysed.

Genome-wide transcript abundance was determined by ServiceXS (Plesmanlaan 1/D, 2333 BZ Leiden, The Netherlands) on the Affymetrix GeneChip platform with the NuGO mouse array (NuGO_Mm1a520177). This array comprises over 24,000 probe sets, covering the majority of established genes. Before the labelling process, the integrity of all RNA samples (RNA Integrity Number (RIN) > 8) was confirmed using the Agilent 2100 Bioanalyser (Agilent Technologies, Stockport, Cheshire, SK8 3GY, UK). Output data were supplied as Affymetrix CEL files and imported into R (version 2.15.3) using the Affy package [28]. Data from liver and placenta were pre-processed separately using gcRMA background correction and quantile normalisation [29] to correct for batch effects and other technical confounders. To maximize sensitivity and specificity, updated Entrez gene probe-set annotation was used from the BrainArray project [30] (version 14.1.0) resulting in 16,270 re-annotated probesets mapping to unique transcripts. Statistical analysis comparing the two diet groups was performed separately for each organ using the empirical Bayes approach of the Limma package [31] which performs a moderated t-test. On filtering genes for significant changes using False Discovery Rate (FDR) values calculated using the Benjamini and Hochberg method (FDR < 0.05), no genes remained statistically significant. Pathway analysis can in part replace FDR corrections by testing for the regulation of related genes, which suffers less from oversampling. Genes were, therefore, considered to show a differential expression relevant for further analysis in enrichment analysis in response to maternal folate depletion if there was a significant ($p < 0.05$) increase or reduction of at least 1.2 fold. DAVID [32] was used to carry out Gene Ontology enrichment analysis and to investigate KEGG pathways affected by maternal folate depletion. The threshold for significance for Gene Ontology enrichment analysis was set at $p < 0.05$ (corrected for multiple testing), and at $p < 0.05$ (uncorrected) for KEGG pathway enrichment analysis. Additional pathway analysis was carried out using PathVisio 3.2.0 and the curated pathway collection of WikiPathways (download date: 1 September 2015), applying the same parameters for significant

fold-change as stated above, imposing a Z score of 1.9 for significance to filter for probable changed pathways. All raw and processed microarray data have been deposited in the ArrayExpress database (accession ID E-MTAB-3940).

2.5. Validation of Gene Expression Changes Using Real-Time PCR

To confirm the gene expression changes observed in the microarray analysis, real-time PCR was performed on each individual (i.e., not pooled) fetal RNA sample that was analysed by microarray hybridisation, focusing on 13 gene targets. Target genes were selected on the basis of being the most up or down regulated in the liver and placenta. Gene transcripts analysed in placental RNA were *Cyp21a1*, *Hbb-y*, *Slco1b2*, *Ptgs2*, *Vcan*, *Lrrn4*, and *Mettl7b* and in fetal liver RNA were *Asgr1*, *Hamp*, *Actc1*, *Ckm*, *Tnnc2* and *Smpx*.

RNA (1 μg) was reverse transcribed using Quantitect Reverse Transcription kit (Qiagen, Cat No. 205313) according to the manufacturer's instructions. Briefly, 1 μg of RNA was incubated with 2 μL genomic DNA Wipeout buffer in a reaction volume of 14 μL at 42 °C for 2 min to remove any genomic DNA. The mixture was then placed immediately on ice. RNA was incubated with 1 μL Quantiscript reverse transcriptase, 1 μL RT primer mix and 4 μL Quantiscript RT buffer for 15 min at 42 °C, after which time it was incubated at 95 °C for 3 min. The cDNA samples generated were then diluted 1:9 with water for use in real time PCR.

Prior to sample analysis, expression values, linearity and efficiency of each assay were determined through cDNA standard curves completed for each transcript measured. Transcript levels of the genes of interest were measured on a Roche Lightcycler 480 (Roche Applied Science, Burgess Hill, West Sussex, RH15 9RY, UK) in a total reaction volume of 25 μL using 2.5 μL Quantitect sybr green transcript specific primers (Qiagen, see Supplementary Materials Table S1 for details of manufacturer's individual catalogue numbers), 12.5 μL Quantitect SYBR green mix (Qiagen Cat. No. 204145), 1 μL diluted cDNA and 9 μL water and cycling parameters 95 °C for 5 min (1 cycle); 95 °C for 10 s followed by 60 °C for 30 s (40 cycles); followed by a final melt curve analysis and cooling to 40 °C. Using the delta CT method, transcript levels of the genes of interest were normalised to *GAPDH* transcript levels which were determined using the same procedure and parameters described.

2.6. In Silico Analysis of Gene Promoter Regions for Transcription Factor (TF) Binding Sites

Genomatix software (https://www.genomatix.de/v3.5 24 July 2015) was employed to obtain promoter sequences for genes of interest using the Gene2Promoter function. Promoter sequences were analysed for common transcription factor binding sites using the Common TFs function. Lists of TFs belonging to the transcription factor binding site families identified were downloaded from Genomatix and compared with fetal liver gene expression data.

2.7. Statistical Analysis

Statistical analysis of array data is described above. For all other datasets, data distributions were examined by the Kolmogorov-Smirnov test and all datasets were normally distributed. Analysis of variance (Statistical Package for the Social Sciences (SPSS) version 21, IBM, Armonk, New York 10504, NY, USA) was used to examine the effects of diet on placental weight, placental efficiency and gene expression analysis on data obtained from RT PCR analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Influence of Maternal Folate Intake during Pregnancy on Fetal Weight, Liver Weight, Placental Weight and Placental Efficiency

Data on the effects of maternal folate depletion on fetal weight and fetal liver weight have been presented previously [25]. In addition, exposure to the Low Folate diet reduced maternal whole blood

5′methyl THF concentration significantly ($p < 0.001$) with values of 666 (40.9) and 378 (50.0) nmol/L for Normal and Low Folate diets respectively [25]. Maternal folate depletion increased fetal weight but had no effect on weight of the fetal liver. There was no significant influence of maternal folate intake during pregnancy on placental weight (mean placental weights were 107.0 mg and 107.3 mg for normal ($n = 56$) and low ($n = 37$) folate groups respectively ($p = 0.945$)). Placental efficiency, calculated by dividing fetal weight by placental weight, was not influenced by maternal folate depletion (mean placental efficiencies were 7.9 and 9.1 for normal ($n = 56$) and low ($n = 37$) folate groups respectively ($p = 0.200$)).

3.2. Influence of Maternal Folate Intake during Pregnancy on Gene Expression in the Fetal Liver

In the fetal liver, 989 genes were differentially expressed in response to maternal folate depletion, comprising 555 up-regulated genes and 434 down-regulated genes (Supplementary Materials Table S2). All raw and processed microarray data have been deposited in the ArrayExpress database (accession ID E-MTAB-3940). Seventy three (7.4%) of these differentially expressed genes code for known transcription factor proteins (see Supplementary Materials Table S3 for list of genes). Although the majority (95.3%) of the gene expression changes were small (i.e., ranging from a fold change of 1.2–2), the expression of 47 genes was changed by greater than two-fold (Table 1).

Whilst gene ontology analysis found no statistically significant influence on biological processes, KEGG pathway analysis revealed that “Lysosome” and “Tight Junction” pathways were significantly affected in the fetal liver in response to maternal folate depletion (Table 2). Analysis of WikiPathways using PathVisio found 13 pathways in which a significant number of genes had altered expression in the fetal liver in response to maternal folate depletion (Table 3).

3.3. Influence of Maternal Folate Intake during Pregnancy on Gene Expression in the Placenta

In the placenta, 460 genes were differentially expressed (250 up-regulated and 255 down-regulated) in response to maternal folate depletion (Supplementary Materials Table S4). All raw and processed microarray data have been deposited in the ArrayExpress database (accession ID E-MTAB-3940). Twenty two (4.8%) of these differentially expressed genes code for known transcription factor proteins (see Supplementary Materials Table S5 for list of genes). Of all the genes expressed differentially, only three had a change in expression of greater than two-fold (Table 4), with the vast majority (99.4%) of changes being small (i.e., ranging from 1.2 to 2 fold change).

Whilst gene ontology analysis found no statistically significant effects on biological processes in placenta, KEGG pathway analysis revealed that “Amino sugar and nucleotide sugar metabolism”, “Valine, leucine and isoleucine degradation” and “Complement and coagulation cascades” pathways were significantly affected in the placenta in response to maternal folate depletion (Table 5). Interrogation of WikiPathways using PathVisio found 13 pathways which were altered significantly in response to maternal folate depletion (Table 6).

3.4. Comparison of Gene Expression Changes in the Placenta and Fetal Liver in Response to Maternal Folate Depletion

More genes in the fetal liver than in the placenta had altered expression in response to maternal folate intake during pregnancy ($n = 989$ and 460 respectively). Only 25 genes were common to the data-sets for fetal liver and placenta (see Table 7 for gene list). The expected overlap between two random subsets with elements of 989 and 460 respectively picked from a set of 16270 elements (the total number of measured genes) is 28, hence the observed overlap of 25 genes is likely due to chance. Of the genes found to respond to maternal folate in both placenta and fetal liver, only four displayed the same direction of change in both organs, with 21 showing the opposite direction of change (Figure 1), which suggests that finding expression changes in the same genes in the two organs may be due to chance.

Table 1. Genes with greater than two-fold expression change in fetal liver in response to maternal folate depletion [1,2].

Gene Symbol	Gene Name	Ensembl ID	Direction of Change	Fold Change	*p* Value	FDR
Actc1	actin, alpha, cardiac muscle 1	ENSMUSG00000068614	Down	20.5	0.001	0.379
Ckm	creatine kinase, muscle	ENSMUSG00000030399	Down	14.8	0.001	0.379
Tnnc2	troponin C2, fast	ENSMUSG00000017300	Down	14.0	0.015	0.458
Smpx	small muscle protein, X-linked	ENSMUSG00000041476	Down	13.4	0.001	0.379
Tnni2	troponin I, skeletal, fast 2	ENSMUSG00000031097	Down	9.1	0.016	0.460
Actn2	actinin alpha 2	ENSMUSG00000052374	Down	8.6	0.001	0.379
Atp1b4	ATPase, $(Na^+)/K^+$ transporting, beta 4 polypeptide	ENSMUSG00000016327	Down	7.9	0.009	0.443
Tnnt3	troponin T3, skeletal, fast	ENSMUSG00000061723	Down	7.6	0.016	0.461
Eno3	enolase 3, beta muscle	ENSMUSG00000060600	Down	7.5	0.003	0.379
Tnni1	troponin I, skeletal, slow 1	ENSMUSG00000026418	Down	7.2	0.008	0.435
Myh8	myosin, heavy polypeptide 8, skeletal muscle, perinatal	ENSMUSG00000055775	Down	6.9	0.024	0.487
Casq2	calsequestrin 2	ENSMUSG00000027861	Down	6.7	0.001	0.379
Mybpc1	myosin binding protein C, slow-type	ENSMUSG00000020061	Down	6.6	0.022	0.477
Myh3	myosin, heavy polypeptide 3, skeletal muscle, embryonic	ENSMUSG00000020908	Down	6.6	0.015	0.458
Ldb3	LIM domain binding 3	ENSMUSG00000021798	Down	6.3	0.002	0.379
Mylpf	myosin light chain, phosphorylatable, fast skeletal muscle	ENSMUSG00000030672	Down	6.1	0.021	0.477
Sln	Sarcolipin	ENSMUSG00000042045	Down	6.0	0.017	0.465
Myl4	myosin, light polypeptide 4	ENSMUSG00000061086	Down	5.4	0.005	0.407
Ckmt2	creatine kinase, mitochondrial 2	ENSMUSG00000021622	Down	4.9	0.007	0.435
Myot	Myotilin	ENSMUSG00000024471	Down	4.6	0.014	0.451
Csrp3	cysteine and glycine-rich protein 3	ENSMUSG00000030470	Down	4.3	0.017	0.465
Srl	Sarcalumenin	ENSMUSG00000022519	Down	4.1	0.005	0.407
Pgam2	phosphoglycerate mutase 2	ENSMUSG00000020475	Down	3.6	0.015	0.458
Atp2a1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	ENSMUSG00000030730	Down	3.6	0.042	0.545
Tnnt1	troponin T1, skeletal, slow	ENSMUSG00000064179	Down	3.4	0.023	0.481
Sh3bgr	SH3-binding domain glutamic acid-rich protein	ENSMUSG00000040666	Down	3.1	0.002	0.379
Itgb1bp2	integrin beta 1 binding protein 2	ENSMUSG00000031312	Down	2.9	0.023	0.481
Cdh11	cadherin 11	ENSMUSG00000031673	Down	2.6	0.012	0.447
Crh	corticotropin releasing hormone	ENSMUSG00000049796	Down	2.5	0.036	0.529
Fhl1	four and a half LIM domains 1	ENSMUSG00000023092	Down	2.5	0.002	0.379
Apobec2	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 2	ENSMUSG00000040694	Down	2.4	0.009	0.442
Egfl6	EGF-like-domain, multiple 6	ENSMUSG00000000402	Down	2.4	0.004	0.395
Myom1	myomesin 1	ENSMUSG00000024049	Down	2.4	0.029	0.498
Mlf1	myeloid leukemia factor 1	ENSMUSG00000048416	Down	2.3	0.020	0.476
Cav2	caveolin 2	ENSMUSG00000000058	Down	2.3	0.001	0.379
Shh	sonic hedgehog	ENSMUSG00000002633	Down	2.2	0.019	0.465
Slit2	slit homolog 2 (Drosophila)	ENSMUSG00000031558	Down	2.2	0.003	0.379

Table 1. *Cont.*

Gene Symbol	Gene Name	Ensembl ID	Direction of Change	Fold Change	*p* Value	FDR
Rps6ka6	ribosomal protein S6 kinase polypeptide 6	ENSMUSG00000025665	Down	2.1	0.034	0.526
Scn7a	sodium channel, voltage-gated, type VII, alpha	ENSMUSG00000034810	Down	2.1	0.020	0.476
Sparcl1	SPARC-like 1	ENSMUSG00000029309	Down	2.1	0.012	0.447
Nr1h4	nuclear receptor subfamily 1, group H, member 4	ENSMUSG00000047638	Up	2.0	0.001	0.379
Pemt	phosphatidylethanolamine *N*-methyltransferase	ENSMUSG00000000301	Up	2.1	0.021	0.477
Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	ENSMUSG00000037411	Up	2.1	0.001	0.379
LOC100503019	hypothetical protein LOC100503019	NA	Up	2.5	0.018	0.465
Cyp2e1	cytochrome P450, family 2, subfamily e, polypeptide 1	ENSMUSG00000025479	Up	2.7	0.012	0.443
Asgr1	asialoglycoprotein receptor 1	ENSMUSG00000020884	Up	2.8	0.010	0.443
Hamp	hepcidin antimicrobial peptide	ENSMUSG00000050440	Up	3.3	0.001	0.379

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diets.

Table 2. KEGG pathways in the fetal liver altered by maternal folate depletion during pregnancy [1].

KEGG Pathway Term	Pathway Name	Total Genes on Pathway	Number of Altered Genes	Differentially Expressed Genes	*p* Value
mmu04142	Lysosome	119	12	*Ap3m1* (ENSMUSG00000021824), *Gm2a* (ENSMUSG00000000594) *Atp6v0a1* (ENSMUSG00000019302), *Naga* (ENSMUSG00000022453) *Arsg* (ENSMUSG00000020604), *Gba* (ENSMUSG00000028048) *Cd68* (ENSMUSG00000018774), *Ap3s2* (ENSMUSG00000063801) *Gla* (ENSMUSG00000031266), *Dnase2b* (ENSMUSG00000028185) *Galns* (ENSMUSG00000015027), *Gaa* (ENSMUSG00000025579)	0.031
mmu04530	Tight junction	135	13	*Tjp1* (ENSMUSG00000030516), *B230120H23Rik* (ENSMUSG00000004085) *Myh3* (ENSMUSG00000020908), *Hcls1* (ENSMUSG00000022831) *Gnai3* (ENSMUSG00000000001), *Myh8* (ENSMUSG00000055775) *Nras* (ENSMUSG00000027852), *Actn2* (ENSMUSG00000052374) *Ppp2r1a* (ENSMUSG00000007564), *Epb4.1l2* (ENSMUSG00000019978) *Mylpf* (ENSMUSG00000030672), *Spnb2* (ENSMUSG00000020315), *Akt2* (ENSMUSG00000004056)	0.033

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively.

Table 3. WikiPathways in the fetal liver altered by maternal folate depletion during pregnancy [1,2] and identified using PathVisio.

Pathway	Number of Genes Altered on Pathway	Number of Genes Measured on Pathway	Total Number of Genes on Pathway	% Affected	Z Score	*p*-Value (Permuted)
Striated Muscle Contraction	16	32	46	50	9.35	<0.001
Adipogenesis genes	16	111	133	14	2.95	0.005
Fatty Acid Biosynthesis	5	21	26	24	2.93	0.011
Iron Homeostasis	3	10	16	30	2.77	0.013
TGF Beta Signaling Pathway	7	42	53	17	2.36	0.015
miR-1 in cardiac development	1	2	6	50	2.33	0.029
One carbon metabolism and related pathways	6	37	86	16	2.11	0.028
EPO Receptor Signaling	4	21	27	19	2.09	0.038
Spinal Cord Injury	11	85	110	13	2.04	0.035
Alanine and aspartate metabolism	2	8	43	25	1.94	0.038
Methylation	2	8	15	25	1.94	0.045
Osteoblast	2	8	14	25	1.94	0.040

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diets.

Table 4. Genes with greater than two-fold expression change in the placenta in response to maternal low folate intake [1,2].

Gene Symbol	Gene Name	Ensembl ID	Direction of Change	Fold Change	*p* Value	FDR
Cyp21a1	cytochrome P450, family 21, subfamily a, polypeptide 1	ENSMUSG00000024365	Down	2.5	0.002	0.999
Hbb-y	hemoglobin Y, beta-like embryonic chain	ENSMUSG00000052187	Down	2.2	0.035	0.999
Slco1b2	solute carrier organic anion transporter family, member 1b2	ENSMUSG00000030236	Down	2.1	0.011	0.999

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diets.

Table 5. KEGG pathways in the placenta altered by maternal folate depletion during pregnancy [1,2].

KEGG Pathway Term	Pathway Name	Total Genes on Pathway	Number of Altered Genes	Differentially Expressed Genes	*p* Value
mmu00520	Amino sugar and nucleotide sugar metabolism	44	5	*Gnpda2* (ENSMUSG00000029209), *Pgm2* (ENSMUSG00000025791), *Uap1* (ENSMUSG00000026670), *Gmppb* (ENSMUSG00000070284), *Pgm3* (ENSMUSG00000056131)	0.017
mmu00280	Valine, leucine and isoleucine degradation	46	5	*Mccc2* (ENSMUSG00000021646), *Oxct2a* (ENSMUSG00000076436), *Hsd17b10* (ENSMUSG00000025260), *Bckdhb* (ENSMUSG00000032263), *Abat* (ENSMUSG00000057880)	0.019
mmu04610	Complement and coagulation cascades	75	6	*Cd55* (ENSMUSG00000026399), *Serpina1b* (ENSMUSG00000071178), *F11* (ENSMUSG00000031645), *F2* (ENSMUSG00000027249), *C9* (ENSMUSG00000022149), *C3* (ENSMUSG00000024164)	0.026

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diets.

Table 6. WikiPathways in the placenta altered by maternal folate depletion [1,2] during pregnancy and identified using PathVisio.

Pathway	Number of Genes Altered on Pathway	Number of Genes Measured on Pathway	Total Number of Genes on Pathway	% Affected	Z Score	*p*-Value (Permuted)
Complement Activation, Classical Pathway	3	13	19	23	3.77	0.002
Statin Pathway	3	16	29	19	3.26	>0.000
Alanine and aspartate metabolism	2	8	43	25	3.25	0.007
Glucocorticoid & Mineralocorticoid Metabolism	2	9	27	22	3	0.016
Selenium metabolism/Selenoproteins	3	18	49	17	2.98	0.015
Alzheimers Disease	6	54	91	11	2.98	0.006
Nuclear Receptors	4	31	38	13	2.79	0.015
Glucuronidation	2	10	33	20	2.78	0.012
Arachidonate Epoxygenase Epoxide Hydrolase	1	3	13	33	2.76	0.037
Polyol pathway	1	3	12	33	2.76	0.024
Complement and Coagulation Cascades	5	54	64	9	2.24	0.015
Acetylcholine Synthesis	1	5	18	20	1.97	0.050
Aflatoxin B1 metabolism	1	5	11	20	1.97	0.037

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diets.

Table 7. Genes differentially expressed in both fetal liver and placental organs in response to maternal folate depletion [1,2,3].

Gene Symbol	Ensembl ID	Expression fold Change in Fetal Liver	p Value for Fetal Liver	Expression Fold Change in Placenta	p Value for Placenta
A830035A12RIK	NA	1.5	0.025	−1.5	0.019
Aadat	ENSMUSG00000057228	−1.3	0.033	−1.3	0.031
Akr7a5	ENSMUSG00000028743	1.2	0.023	−1.3	0.003
Apon	ENSMUSG00000051716	1.4	0.019	−1.4	0.021
Atp6v0a1	ENSMUSG00000019302	1.2	0.028	−1.3	0.007
Atrnl1	ENSMUSG00000054843	−1.3	0.013	1.3	0.008
Bcas3	ENSMUSG00000059439	1.2	0.034	−1.2	0.035
Col15a1	ENSMUSG00000028339	−1.3	0.013	−1.2	0.029
Cyld	ENSMUSG00000036712	−1.3	0.030	1.3	0.024
E130309D02RIK	ENSMUSG00000039244	1.2	0.015	−1.2	0.027
Fkbp8	ENSMUSG00000019428	1.2	0.045	−1.3	0.020
Inpp1	ENSMUSG00000026102	1.2	0.019	−1.2	0.026
Itgb4	ENSMUSG00000020758	1.3	0.027	−1.2	0.039
Med6	ENSMUSG00000002679	−1.4	0.009	1.3	0.029
Nubpl	ENSMUSG00000035142	1.6	0.014	1.5	0.040
Oxct2a	ENSMUSG00000076436	−1.3	0.024	1.5	0.004
Pgm3	ENSMUSG00000056131	1.2	0.045	−1.3	0.028
Prtg	ENSMUSG00000036030	1.3	0.026	1.2	0.048
Rbp2	ENSMUSG00000032454	1.5	0.036	−1.9	0.001
Sepx1	ENSMUSG00000075705	1.2	0.040	−1.3	0.026
Slc13a3	ENSMUSG00000018459	1.6	0.003	−1.4	0.018
Slc39a5	ENSMUSG00000039878	1.4	0.049	−1.4	0.037
Slc48a1	ENSMUSG00000081534	1.3	0.012	−1.2	0.041
Slc6a13	ENSMUSG00000030108	1.3	0.021	−1.2	0.048
Tomm40	ENSMUSG00000002984	1.4	0.021	−1.5	0.001

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diets; [3] A negative fold change indicates lower expression in offspring from dams fed the Low folate diet.

No common KEGG pathways were significantly influenced by maternal folate intake in both the liver and placenta (Tables 2 and 5). Use of PathVisio to interrogate WikiPathways revealed that "Alanine and aspartate metabolism" may be altered in both the fetal liver and the placenta of offspring of folate depleted mothers (Tables 3 and 6). However, different genes in the pathway were differentially expressed in response to maternal folate depletion in the two tissues. In the fetal liver *Agxt* and *Pcx* were up-regulated in response to maternal folate depletion, whilst in the placenta *Abat* and *Asl* were down regulated.

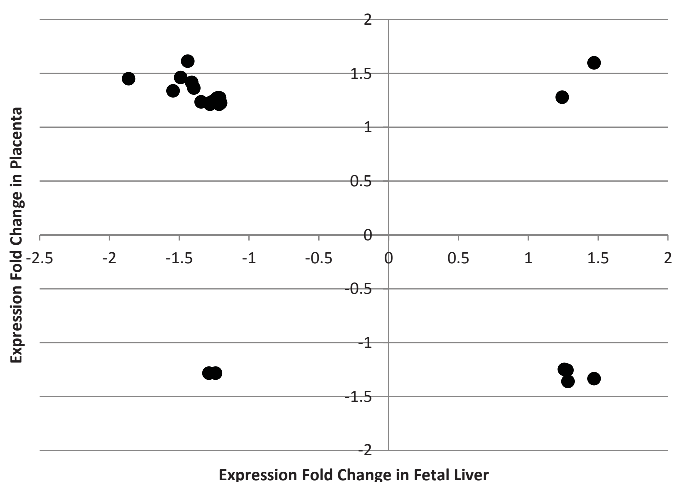

Figure 1. Scatterplot summarising the direction and level of fold change in expression of genes which were altered in both fetal liver and placenta by maternal folate depletion [1,2,3]. [1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diets; [3] A negative fold change indicates lower expression in offspring from dams fed the Low folate diet.

3.5. Validation of Gene Expression Data from Array Analysis by RT-qPCR Analysis

For a subset of genes from both liver and placenta which showed altered expression in response to maternal folate depletion using microarray analysis, data were validated by RT-qPCR. For fetal liver, where the largest fold changes in response to maternal folate depletion were found in down regulated genes, we selected the four most down regulated genes i.e., *Actc1*, *Ckm*, *Tnn2* and *Smpx*. In addition, we analysed the two most up regulated genes i.e., *Hamp* and *Asgr1*. All genes showed the same direction of change using both methods (Table 8) but for 5/6 genes measured by RT-qPCR the change in expression was not statistically significant.

For placenta, fold changes in expression for up- and down-regulated genes in response to maternal folate depletion were similar and so the three genes with the largest fold changes in each direction were selected for validation analysis i.e., *Cyp21a1*, *Hbb-y* and *Slco1b2* for down-regulated genes and *Ptgs2*, *Vcan* and *Lrrn4* for up-regulated genes. In addition, a gene of particular interest, *Mettl7b*, was also selected for RT-PCR analysis. This gene encodes the protein methyltransferase-like 7B, and is likely to have methyltransferase activity (inferred through sequence similarity with other protein methyltransferases (http://www.uniprot.org/uniprot/Q6UX53)), and thus may have a role in epigenetic regulation of gene expression. All seven genes showed the same directional changes in expression in response to maternal folate depletion when assayed by RT-qPCR and by microarray but for 6/7 genes measured by RT-qPCR the change in expression was not statistically significant (Table 9).

Table 8. RT-qPCR expression analysis of selected genes which were differentially expressed in fetal liver in response to maternal folate depletion [1,2] when assayed by microarray.

Gene Name	Mean Expression Low Folate Group	SEM	Mean Expression Normal Folate Group	SEM	*p* Value	Direction of Change in Agreement with Array
Asgr1	0.35	0.07	0.12	0.07	0.050	Yes
Hamp	4.00	0.75	1.73	0.75	0.058	Yes
Actc1	3.63	3.04	8.29	3.04	0.305	Yes
Ckm	1.12	0.95	3.30	0.95	0.134	Yes
Tnnc2	0.51	0.66	2.39	0.66	0.073	Yes
Smpx	0.11	0.09	0.38	0.09	0.066	Yes

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diets. Expression values are in arbitrary units calculated using the delta CT method; *GAPDH* was used as the reference gene. Expression levels were quantified in individual fetuses (n = 18 per group) with mean expression levels calculated for individuals litters (n = 6 per group) for use in statistical analyses to match procedures used for array data.

Table 9. RT-qPCR expression analysis of selected genes which were differentially expressed in placenta in response to maternal folate depletion [1,2] when assayed by microarray. Expression values are in arbitrary units calculated using the delta cycle threshold (CT) method. *GAPDH* was used as the reference gene. Expression levels were quantified in individual fetuses (n = 18 per group) with mean expression levels calculated for individuals litters (n = 6 per group) for use in statistical analyses to match procedures used for array data.

Gene Name	Mean Expression Low Folate Group	SEM	Mean Expression Normal Folate Group	SEM	*p* Value	Direction of Change in Agreement with Array
Cyp21a1	0.55	0.13	0.88	0.13	0.118	Yes
Hbb-y	0.39	0.16	0.83	0.16	0.076	Yes
Slco1b2	0.29	0.08	0.49	0.08	0.106	Yes
Ptgs2	0.31	0.03	0.22	0.03	0.030	Yes
Vcan	0.66	0.09	0.46	0.09	0.144	Yes
Lrrn4	0.05	0.01	0.03	0.01	0.118	Yes
Mettl7b	3.07	0.61	4.83	0.61	0.068	Yes

[1] The Low and Normal folate diets contained 0.4 and 2.0 mg folic acid/kg diet respectively; [2] For each comparison, data were obtained from three male fetuses from each of six litters from dams fed either the Low or Normal folate diet.

3.6. Comparison of Gene Expression Changes in Fetal Liver in Response to Maternal Folate Depletion with Similar Published Transcriptomic Studies

Previous studies have reported gene expression changes at the transcriptomic level in the liver of rodents in response to diets low in folate [33–35]. Although several aspects of design differ between studies (summarised in Table 10), to identify a key set of genes in the liver that respond to changes in folate intake (or more broadly, changes in one carbon metabolism), we compared reported changes in gene expression between our data and three other studies [33–35]. Of the 989 genes which were differentially expressed in the fetal liver in response to maternal folate depletion in our study, 4, 89, 90 and 239 genes were also found to be differentially expressed in CBA mice, BALB/c mice, CBA/Ca mice and Wistar rats respectively [33–35] (full list of these genes for individual studies are in Supplementary Materials Table S6). Comparison of the differentially expressed genes from all studies revealed a set of 21 key genes whose expression was affected by altered folate/methyl donor intake in at least four of the five data sets (see Table 11). Importantly, none of the 25 genes which we found were expressed differentially in both fetal liver and placenta in response to maternal folate depletion are in this list of 21 genes.

Table 10. Overview of experimental designs of studies investigating the influence of folate/methyl donor intake on genome wide gene expression in the liver.

Study	Model	Diets	Period of Exposure	Arrays
This study	Male C57Bl6J mice	2 mg folic acid/kg OR	4 weeks prior to mating to 17.5 days gestation	NuGO mouse array (NuGO_Mm1a520177)
		0.4 mg folic acid/kg		
Champier et al. [33]	Male CBA mice	8 mg folic acid + 10 g of succinylsulfathiazole/kg OR	8–12 weeks of age	CodeLinkUniset Mouse Whole Genome bioarrays
		0 mg folic acid + 10 g of succinylsulfathiazole/kg		
Glen et al. [35]	Male BALB/c & CBA/Ca mice	Low methionine (0.18%, w/w) & lacking choline & folic acid OR	4–12 weeks of age	NimbleGen 12 × 135 K Mouse Expression Array
		Low methionine (0.18%, w/w) & supplemented with choline & folic acid		
Chen et al. [34]	Wistar rats	Standard food OR	1 month before pregnancy to 21 days post-weaning	Agilent Arrays
		Diet without vitamin B_{12} & folate		

Table 11. Genes which were differentially expressed in response to low dietary folate/methyl donor intake in the liver across multiple studies [1].

	Gene Differentially Expressed				
Gene Symbol	**McKay et al.**	**Champier et al. [33]**	**Glen et al. [35] (BALB/c Mice)**	**Glen et al. [35] (CBA/Ca Mice)**	**Chen et al. [34]**
ACOT2	Y	N	Y	Y	Y
COL6A2	Y	N	Y	Y	Y
CSTB	Y	N	Y	Y	Y
DCTD	Y	N	Y	Y	Y
ENTPD2	Y	N	Y	Y	Y
FETUB	Y	N	Y	Y	Y
FGF21	N	Y	Y	Y	Y
IL7R	Y	N	Y	Y	Y
JUB	Y	N	Y	Y	Y
LTBP2	Y	N	Y	Y	Y
OXCT1	Y	N	Y	Y	Y
RHBDF1	Y	N	Y	Y	Y
RUNX3	Y	N	Y	Y	Y
SERPINE1	Y	N	Y	Y	Y
SLC15A3	Y	N	Y	Y	Y
SLC30A10	Y	N	Y	Y	Y
SLC39A5	Y	N	Y	Y	Y
SLC41A3	Y	N	Y	Y	Y
SLPI	Y	N	Y	Y	Y
SORCS2	Y	N	Y	Y	Y
TMIE	Y	N	Y	Y	Y

[1] For details of diet compositions, please see Table 10. Y = Yes; N = No.

3.7. In Silico Analysis of Promoter Regions of Key Folate/Methyl Donor Responsive Genes for Transcription Factor Binding Sites

For these 21 genes found to be affected robustly in liver by altered folate supply (Table 11), 76 unique promoter regions were found for 130 transcripts using the Gene2Promoter function in Genomatix (data not shown). Transcription factor binding sites for five families of transcription factors were present in 90% of the promoters investigated; ETS1 factors (ETSF), Kruppel like transcription factors (KLFS), Nuclear receptor subfamily 2 factors (NR2F), RXR heterodimer binding site (RXRF) and SOX/SRY sex/testis determining and related HMG box factors (SORY). Of these, the RXRF transcription factors had binding sites in all 76 analysed promoters. TFs belonging to the five identified families were compared with TFs found to be differentially expressed in the fetal liver and five TFs were found to overlap viz. *Elk3*, *Nr2c1*, *Nr2f1*, *Rxra*, *and Sox30.*

However the same analysis on randomly-selected sets of 21 genes that were affected by folate supply from each of the five studies plus two sets of 21 genes that did not respond to maternal folate supply revealed similar occurrence rates for these same transcription factor binding sites. Thus, this analysis identified no potential transcriptional regulatory mechanisms which may be responsible exclusively for coordinating the liver-specific changes in expression of these key genes in response to folate supply.

4. Discussion

Here we describe gene expression changes at the transcriptome level in the placenta and fetal liver in response to maternal folate depletion before mating and throughout pregnancy. Ames' triage theory posits that if availability of a specific nutrient is inadequate, Darwinian processes ensure that essential functions, i.e., those required for short-term survival and/or reproduction, dependent on that nutrient that are protected at the expense of those functions that are less essential i.e., where reduced function does not have short term negative consequences. However, such prioritisation of some most critical (short-term) functions may impact adversely on other functions which may have long-term insidious effects that increase risk of diseases associated with ageing [23,24]. In response to inadequate maternal

folate intake during pregnancy, we hypothesised that, due to different organ-specific functions, individual fetal organs would implement differential prioritisation hierarchies and thus display differential gene expression changes in response to folate depletion. We chose to investigate gene expression in the liver and placenta as examples of organs with very different roles in mammalian function across the lifecourse. In addition, the liver represents a major site for folate storage and metabolism, whilst the placenta (containing both maternal and fetal cells) is the route for delivery of nutrients (including folate) from the dam to the fetus during development. Our observations support our hypothesis that specific fetal organs exhibit specific transcriptional responses to maternal folate depletion. It is plausible that these organ-specific changes in gene expression are due to differential pathway prioritisation in each organ which reflect their contrasting functions.

The primary function of the placenta is the transfer of nutrients to the fetus, so it is essential that this function is protected to support fetal growth and development. In response to maternal folate depletion, we saw no changes in pathways associated with nutrient transport, suggesting that placental transfer of nutrients to the fetus was protected despite the reduced maternal folate intake. We observed no teratogenic effects of this reduced nutrient supply on the offspring [25]. It is important to note that we analysed whole placental samples containing both maternal and fetal cell populations and so we are unable to determine the cellular origin of the observed expression changes.

The liver is a highly metabolic organ responsible for a wide range of functions that are important at all life stages and include defence of the body against xenobiotics, assisting with digestion and with metabolism of absorbed nutrients, regulating blood lipids, synthesis of a wide range of secreted proteins and hormonal regulation. As such, during periods of undernutrition, it may not be possible for the liver to maintain all important functions, so that those functions essential for short-term survival and/or reproduction may be prioritised. The sequela is that some functions not critical for short-term survival but which are important for long term health may be de-prioritised leading to impaired function which may have significant long-term adverse effects for the organism. We observed that maternal folate depletion altered several processes and pathways, each of which could impact adversely on long-term liver function and organismal health. These included "adipogenesis genes", "fatty acid biosynthesis" and "iron homeostasis" pathways. Whilst only one time point was investigated here (so that we are unable to comment on long-term gene expression changes or phenotypic outcomes), these observations are consistent with the fact that diets deficient in folate and choline can induce non-alcoholic fatty liver disease (NAFLD) in rodent models [36]. We observed up-regulation of genes involved in "fatty acid biosynthesis" (5/5 differentially expressed genes present on the pathway), and an abundance of up-regulated genes involved in the "adipogenesis genes" pathway (10/16 differentially expressed genes present on the pathway). Up-regulation of these genes may result in overall up-regulation of the pathways that potentially could result in altered hepatic fatty acid metabolism in offspring. Indeed, we observed that when the offspring of folate depleted dams were fed a high fat diet from weaning [37], they had significantly increased plasma triacylglycerol (TAG) concentrations whilst plasma TAG concentrations were unaffected in the offspring of folate replete dams [37]. Taken together, these data suggest that maternal folate depletion results in an altered response to high fat feeding which may be via programmed transcriptional changes in pathways associated with fat metabolism in the liver.

This analysis has focussed on the genes which were differentially expressed in the liver and in the placenta in response to maternal folate depletion. However, it is important to recognise that there are alternative explanations for genes which did not change in response to this nutritional insult. Whilst such unaltered genes may be components of pathways which are especially critical to that particular organ, it is also possible that these genes are simply unresponsive to folate supply. The present study design is unable to distinguish between these alternatives.

It is important to highlight that most of the gene expression changes observed in this study were relatively small and not statistically significant when applying the more stringent FDR value so that these data should be interpreted with caution. However, our observations are consistent with

findings from other studies [33,34] and confirms the subtle, but pervasive, effects on gene expression of folate/methyl donor depletion. Furthermore, it is pertinent to point out that our gene expression studies were undertaken in male offspring only and it is possible, if theoretically unlikely, that effects could be different in females.

Whilst we show clearly that folate responsive gene expression changes are organ specific, the mechanisms behind the observed organ specific expression changes are less clear. A plausible mechanistic explanation could be based on inter-organ differences in expression of transcription factors (TFs) when exposed to inadequate folate intake. We tested this hypothesis by identifying all TFs which were differentially expressed in the liver and in the placenta in response to maternal folate depletion. In the fetal liver, we found that expression of 73 TFs differed in folate replete vs. folate depleted offspring, which accounted for 7.4% of the altered genes. In contrast, in the placenta only 22 TFs (4.8% of altered genes) displayed altered expression. Consistent with the idea that organ-specific changes in gene expression profiles are driven by organ-specific changes in TF expression, there was very little overlap between the TFs that were altered in liver and placenta—only one TF, *Med6*, was common between the organs.

It is plausible that changes in epigenetic processes are responsible for the organ-specific patterns in gene expression in response to folate depletion. Epigenetic marks, including DNA methylation, are modifiable by diet and other environmental factors and constitute an important and flexible system for regulating gene expression [38,39]. Folate is central to one-carbon metabolism and the formation of the universal methyl donor SAM, which is critical for the methylation of biological molecules including DNA. Indeed the influence of dietary folate intake on DNA methylation patterns has been reported widely [25,34,40–42] and may be responsible for the organ specific folate responsive gene expression changes observed here.

Although previous studies have reported gene specific expression changes in the rat placenta in response to increased levels of folate in the maternal diet [42,43], to the best of our knowledge this is the first report describing gene expression changes in response to low maternal folate (or methyl donor) intake in this organ, and the first study to investigate this at the transcriptomic level. However, previous studies have used transcriptomic approaches to investigate gene expression changes in the liver in response to low folate-containing diets [33–36,44,45] so we compared our data with outcomes from these studies. Whilst there were differences in gene expression profiles between studies, likely to be due to differences in rodent models, diet composition, duration and timing of the nutrient insult and the particular transcriptomic approaches employed (Table 10), we uncovered a key set of genes which were differentially expressed in the liver in response to low dietary folate/methyl donor intake across multiple studies (Table 11). Importantly, expression of none of these genes was altered in placenta by maternal folate depletion, indicating that these changes reflect a liver-specific response rather than being genes that are generally malleable in response to perturbation in folate supply. Moreover, this observation substantiates our suggestion that the transcriptome responses of fetal liver and placenta to folate restriction are distinct and physiologically-appropriate. Of the key set of 21 genes differentially expressed across multiple studies (Table 11), the use of DAVID [32] to carry out Gene Ontology enrichment analysis suggested that three genes are involved in wound healing i.e., *JUB*, *ENTPD2* and *SERPINE1* and, thus, altered transcription of genes associated with wound healing may help explain the observed association between low methyl donor intake and liver damage [36]. Furthermore, the discovery of these key folate responsive genes may provide candidate biomarkers for future studies of folate adequacy. Such studies should test the effects of different doses of folate without potential confounding from other dietary factors which may have been an issue in some of the studies included in Table 10.

To investigate potential mechanisms through which expression of these key genes in the liver are altered in response to methyl donor intake, the promoter regions were interrogated for the presence of common transcription factor binding sites. Whilst binding sites for five families of transcription factors were found to be highly represented in the promoters of these 21 genes, similar occurrence rates of

these same transcription factor binding sites were also observed when seven randomly selected sets of genes were analysed. This suggests that this specific set of TFs is not responsible for co-ordinating transcriptional responses to maternal folate/methyl-donor supply in the liver. However, it is possible that there are regulatory regions outside the promoters of the genes investigated here which would merit investigation in future studies.

5. Conclusions

The data presented here suggest that maternal folate status during pregnancy influences gene expression in the fetus in a highly organ-specific manner. We hypothesise that this organ specificity is a rational response to limited nutrient supply which protects the most essential functions of each individual organ for short term survival (and reproduction), at the expense of less immediately essential processes which may lead to long-term adverse health sequelae. However, as we have noted above, there are viable alternative explanations for our observations. Our observations are consistent with the DOHaD hypothesis and suggest a plausible mechanism by which inadequate folate supply during early development leads to changes in gene expression to protect functions of the liver that are appropriate in this particular developmental and nutritional context, but that may contribute to the aetiology of cardiometabolic diseases if the postnatal environment is such that these changes become disadvantageous.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/8/10/661/s1, Table S1: Details of manufacturer's product codes for primers used to determine gene transcript levels by RT-qPCR, Table S2: List of genes differentially expressed in the fetal liver in reponse to low maternal folate. Genes were considered to be significantly differentially expressed in response to low maternal folate if (a) the *p* value for t-test between dietary groups was < 0.05 and (b) if there was a fold change of ±1.2, Table S3: List of genes encoding transcription factors differentially expressed in the fetal liver in reponse to low maternal folate. Genes were considered to be significantly differentially expressed in response to low maternal folate if (a) the *p* value for t-test between dietary groups was < 0.05 and (b) if there was a fold change of ±1.2. B1, Table S4: List of genes differentially expressed in the placenta in reponse to low maternal folate. Genes were considered to be significantly differentially expressed in response to low maternal folate if (a) the p value for t-test between dietary groups was < 0.05 and (b) if there was a fold change of ±1.2.D1, Table S5: List of genes encoding transcription factors differentially expressed in the placenta in reponse to low maternal folate. Genes were considered to be significantly differentially expressed in response to low maternal folate if (a) the *p* value for t-test between dietary groups was < 0.05 and (b) if there was a fold change of ±1.2, Table S6: Lists of genes found in in common with other published data sets. Genes found to be differentially expressed in response to maternal folate depletion in the fetal liver were compared to other published gene lists where diets low in folate were reported to alter gene expression in the liver.

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Review

Novel Approaches to Investigate One-Carbon Metabolism and Related B-Vitamins in Blood Pressure

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Abstract: Hypertension, a major risk factor for heart disease and stroke, is the world's leading cause of preventable, premature death. A common polymorphism (677C$\rightarrow$T) in the gene encoding the folate metabolizing enzyme methylenetetrahydrofolate reductase (MTHFR) is associated with increased blood pressure, and there is accumulating evidence demonstrating that this phenotype can be modulated, specifically in individuals with the *MTHFR* 677TT genotype, by the B-vitamin riboflavin, an essential co-factor for MTHFR. The underlying mechanism that links this polymorphism, and the related gene-nutrient interaction, with hypertension is currently unknown. Previous research has shown that 5-methyltetrahydrofolate, the product of the reaction catalysed by MTHFR, appears to be a positive allosteric modulator of endothelial nitric oxide synthase (eNOS) and may thus increase the production of nitric oxide, a potent vasodilator. Blood pressure follows a circadian pattern, peaking shortly after wakening and falling during the night, a phenomenon known as 'dipping'. Any deviation from this pattern, which can only be identified using ambulatory blood pressure monitoring (ABPM), has been associated with increased cardiovascular disease (CVD) risk. This review will consider the evidence linking this polymorphism and novel gene-nutrient interaction with hypertension and the potential mechanisms that might be involved. The role of ABPM in B-vitamin research and in nutrition research generally will also be reviewed.

Keywords: riboflavin; methylenetetrahydrofolate reductase (MTHFR); hypertension; blood pressure; ABPM; DNA methylation

1. Introduction

Hypertension, defined as a systolic/diastolic blood pressure (BP) of 140/90 mmHg or greater, is the world's leading cause of preventable, premature death and is the most important risk factor for cardiovascular disease (CVD) and stroke. Hypertension is responsible for 7.5 million deaths worldwide and an estimated 12.8% of all deaths annually [1]. It is estimated that by 2025, 1.56 billion people worldwide will suffer with hypertension, and by 2030, costs to the global economy will soar to $274 billion [2–4]. Treating hypertension is highly effective in reducing CVD and mortality [5], with a decrease of as little as 2 mmHg in systolic BP reported to reduce CVD risk by 10% [6]. BP is normally treated using antihypertensive medication, but this is not always effective, and BP control rates can remain poor (even when three or more drugs are co-administered, i.e., polytherapy) [7,8]. Current guidelines to treat hypertension are aimed at achieving a BP of <140/90 mmHg [7]; however, recent evidence suggests that a more intensive treatment regime to reduce BP to as low as <120/80 may be significantly more beneficial, even when BP values fall within the normotensive range [8–10].

There are many well-known modifiable risk factors for hypertension, such as smoking, obesity, high salt intake and physical inactivity, and thus, addressing lifestyle factors is generally the first line of treatment recommended by medical practitioners to reduce BP [7]. Genome-wide association studies (GWAS) have however identified a number of areas in the genome related to the variability in BP, including a region near the *MTHFR* locus [11], a finding replicated by other GWAS [12–14]. Likewise, large meta-analyses of epidemiological studies have shown that adults with the homozygous variant (TT genotype) for the common *MTHFR* C677T polymorphism are at an increased risk of developing hypertension [15–19]. Riboflavin is required as a cofactor for MTHFR, and previous studies at this centre have shown that supplementation with riboflavin significantly reduces BP in adults with this genetic risk factor [20–22]. The mechanism by which riboflavin lowers BP in this genetically at-risk group is unknown; however, some mechanisms have been speculated, and these will be explored below [22,23].

All studies to date investigating this gene-nutrient interaction in hypertension have relied on clinic BP measurements. An alternative, more robust method of BP measurement is ambulatory blood pressure monitoring (ABPM), which can track the circadian pattern of BP, and it is reported to be a better predictor of mortality [24]. Despite the use of ABPM being first reported in the mid-1960s [25], it was not introduced into the relevant UK clinical guidelines to confirm the diagnosis of hypertension until 2011 [7].

2. One-Carbon Metabolism and Related B-Vitamins

In order to be biochemically active, folate needs to be in the fully reduced form as tetrahydrofolate (THF; Figure 1). Thus, folic acid, the synthetic vitamin form as found in supplements and fortified food, requires biological modification (via dihydrofolate reductase (DHFR)) to form THF [26]. This occurs in two consecutive NADPH-dependent reactions, to form dihydrofolate (DHF) and subsequently THF. The reduction of folic acid is, however, a slow process that is influenced by individual variation in DHFR activity [26]. It is possible therefore that exposure to high oral doses of folic acid may result in the appearance of unmetabolised folic acid in the circulation [27], which some have suggested may be associated with adverse health effects [28]. Once THF enters the folate cycle, it gains a methyl group from the conversion of serine to glycine in a vitamin B6-dependent (i.e., pyridoxal 5′-phosphate) reaction to form 5,10-methyleneTHF. Riboflavin also participates in one-carbon metabolism in its active co-factor forms flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). Pyridoxine-phosphate oxidase requires FMN for the formation of the active form of vitamin B6, pyridoxal 5′-phosphate, from pyridoxine phosphate. MTHFR, which requires FAD as a co-factor, converts 5,10-methyleneTHF to 5-methylTHF which is subsequently converted to THF, in a reaction catalysed by methionine synthase, completing the cycle. The latter conversion also requires vitamin B12 (i.e., methylcobalamin) as a co-factor and simultaneously enables the remethylation of homocysteine to methionine and subsequently S-adenosylmethionine (SAM), the universal methyl donor, which is essential for a range of methylation processes, including DNA methylation. DNA methylation involves the addition of a methyl group to the DNA base cytosine, which can alter the transcription of the gene and potentially reduce enzyme production [29]. Thus, apart from folate, three other B-vitamins play essential roles in one-carbon metabolism, as they are required for the activity of the various enzymes within the folate cycle.

It is well established that the common *MTHFR* C677T polymorphism, which causes an amino acid change from alanine to valine in the protein, produces a thermolabile enzyme [30]. Individuals with the *MTHFR* 677TT genotype have 70% reduced activity of MTHFR in comparison to the *MTHFR* 677CC genotype, which in turn reduces the rate of 5-methylTHF production [30] and, potentially, SAM production in cells. The *MTHFR* 677TT genotype is thus associated with increased homocysteine concentration as the primary phenotype [30].

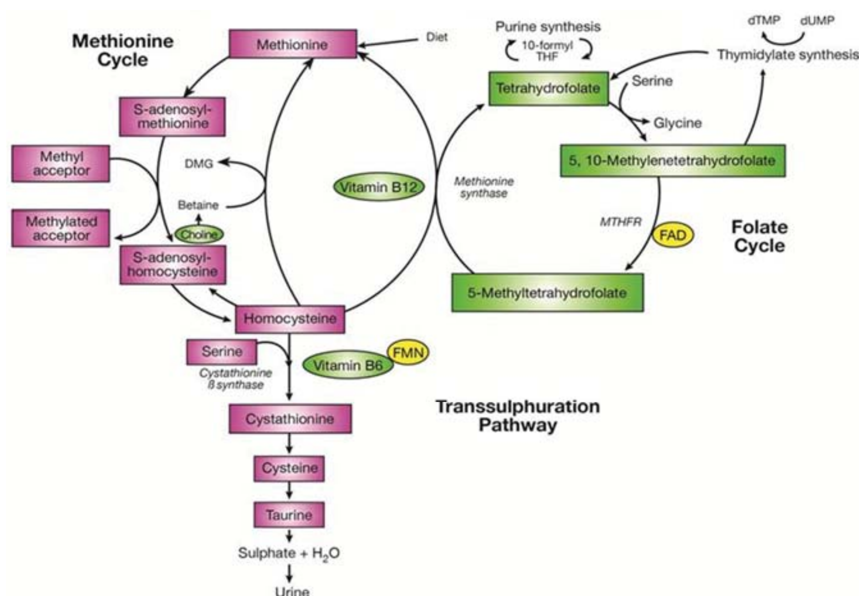

Figure 1. One-carbon metabolism pathway reproduced from Clarke et al. [31]. FAD, flavin adenine dinucleotide; FMN, flavin adenine dinucleotide.

3. B-Vitamins, Cardiovascular Disease and Blood Pressure

Increased plasma homocysteine and/or poor status of the metabolically-related B-vitamins have been linked with an increased risk of CVD over many years, albeit the literature is somewhat controversial [32]. It is well established that intervention with folate, along with vitamin B12 and vitamin B6, can reduce plasma homocysteine concentrations (by about 3 μmol/L) as reported in a large meta-analysis of randomised controlled trials [33]. Of the B-vitamins investigated, folic acid has been shown to be most effective, even at doses as low as 0.2 mg/day when maintained for a long enough period of intervention (i.e., 24 weeks) [33,34]. Separate meta-analyses estimated that a reduction in homocysteine by 25% could reduce the risk of stroke by up to 24% and heart disease by 16% [35,36]. To subsequently establish a cause and effect relationship between elevated homocysteine and CVD outcomes, a number of randomised controlled trials with B-vitamins were conducted; however, despite significant homocysteine lowering, the majority failed to show a reduction in CVD risk in response to intervention with B-vitamins (as extensively reviewed elsewhere; [32,37]). Of note however, a secondary analysis of the Heart Outcomes Prevention Evaluation 2 trial (HOPE 2) and two subsequent meta-analyses demonstrated that interventions with folic acid and related B-vitamins could substantially reduce the risk of stroke [38–40]. This effect was generally confined to trials that were three years in length or greater (suggesting that trials of shorter duration may have underestimated or missed this benefit) and in trials involving participants with no previous history of stroke. However, the vast majority of the previous trials were secondary prevention trials in patients with overt CVD, and as such, participants would be less likely to respond to B-vitamin intervention. Furthermore, it has been reported that antiplatelet drugs, such as aspirin (commonly prescribed to patients after a CVD event) might mask any CVD risk reduction [41]. Of note, a recent primary prevention trial in 20,702 hypertensive Chinese adults reported that folic acid supplementation (0.8 mg/day), for a median duration of 4.5 years, reduced the risk of stroke in those with elevated cholesterol by as much as 31% [42]. The balance of evidence therefore suggests that any beneficial effects of B-vitamins would be most relevant in the prevention rather than the treatment of CVD.

Although most of the previous studies in relation to CVD were designed to examine homocysteine lowering in relation to CVD risk, some also considered the relationship of B-vitamins with BP. One of the largest studies, the VISP (Vitamin Intervention for Stroke Prevention) trial, which involved supplementing 3649 cerebral infarct patients with high or low doses of folic acid for two years, successfully reduced homocysteine, but showed no corresponding effect on BP [43]. Likewise, McMahon and colleagues also reported a significant reduction in homocysteine in response to supplementation with folic acid, B6 and B12 for two years, but no effect on BP [44]. One small study of just 130 individuals who were supplemented with high dose folic acid (5 mg/day) and B6 (250 mg/day) for two years, reported a reduction in homocysteine by 7.8 μmol/L and did achieve a corresponding lowering of systolic BP (−3.7 mmHg) [45]. Somewhat surprisingly, a further study of only 24 male smokers reported a smaller, albeit significant, reduction in homocysteine (2.6 μmol/L) and found a greater lowering of systolic BP (−8 mmHg SBP); however this study was conducted in adults who smoke, and it is well known that smoking increases homocysteine and BP [46,47]. If homocysteine was causally linked with increased BP, intervention studies to lower homocysteine would invariably show a corresponding BP response; however, this is not the case [32,37], suggesting there is no mechanistic association between homocysteine and BP. All of the aforementioned studies reported BP using the clinic BP monitor and have not considered the influence of *MTHFR* genotypes on BP response.

The *MTHFR* 677TT genotype has been independently associated with increased CVD risk [35,48–51]. This association is generally assumed to be owed to homocysteine, which is invariably found to be highest in those with the TT genotype and lowest in the CC genotype, with CT individuals having intermediate concentrations [32,35]. More recently, emerging evidence suggests that the excess genetic risk of CVD owed to this polymorphism may be through an independent association with BP. Furthermore, meta-analyses that have investigated the association between the TT genotype and CVD risk generally show a stronger relationship for stroke [49,51], than for heart disease [35,48,50,52]. Moreover, in studies focusing on the risk of hypertension (as opposed to CVD outcomes), the relationship is stronger again [15–19]. This is of importance as hypertension is a major risk factor for stroke [15–19]. Apart from hypertension in the general population [15,17,19], this polymorphism has also been linked with hypertension in pregnancy [16,18]. Given that hypertension is such a major risk factor for stroke, it is possible that the onset of stroke could be prevented or delayed by modulating the blood pressure phenotype in individuals with the *MTHFR* 677TT genotype.

Among the studies investigating the association between MTHFR and BP, there are differences in the extent of risk linking this polymorphism with CVD including stroke [15–19]. Environmental factors affecting different populations, particularly B-vitamin status, could strongly influence the BP phenotype and thus stroke risk. Within these meta-analyses, the excess risk owed to the *MTHFR* 677TT genotype is found to be considerably higher in Asian populations for both CVD (OR 1.68, 95% CI 1.44–1.97) [51] and hypertension (OR 1.87, 95% CI 1.31–2.68) [16] and lower in North American populations.

Leading on from earlier work, which demonstrated that supplementation with riboflavin could improve MTHFR activity in vivo (evident by decreasing plasma homocysteine concentrations) [53], researchers at this centre investigated the BP lowering effect of riboflavin in adults with the *MTHFR* 677TT genotype. In the first study, Horigan et al. [20] conducted a randomised controlled trial of riboflavin supplementation at 1.6 mg/day in premature CVD patients stratified for the *MTHFR* C677T polymorphism (n = 181). At baseline, BP control rates were poor in the TT genotype group, with 63% failing to reach the target BP of <140/90 mmHg on treatment with antihypertensive drugs. Systolic and diastolic BP were also significantly higher at baseline in the TT compared to the CC or CT genotype groups. In response to intervention with riboflavin (1.6 mg/day for 16 weeks), riboflavin biomarker status improved in all three genotypes in response to intervention ($p < 0.001$; as measured using the erythrocyte glutathione reductase activation coefficient). However, BP was significantly reduced in the TT genotype group only, with one third of patients in this group achieving a reduction in systolic

BP by as much as 20 mmHg. When the *MTHFR* 677TT genotype participants from this first study were subsequently followed up four years later and assigned to further intervention in a cross-over design study (with the original treatments reversed), the BP lowering effects of riboflavin in the TT genotype group were replicated [21]. The BP lowering achieved in response to riboflavin occurred despite a major change in the UK clinical guidelines for the management of hypertension during the four-year follow-up period, resulting in a shift from monotherapy to polytherapy, thus additional medications being prescribed, and a change from β-blockers to angiotensin-converting enzyme (ACE) inhibitors as the drugs of first choice [7]. In a subsequent study, the genotype-specific BP lowering effects of riboflavin were also demonstrated in hypertensive adults without overt CVD [22]; albeit the extent of BP lowering was less pronounced (ranging from 5.6 to 13 mmHg systolic and 6–8 mmHg diastolic). It is not entirely clear why the extent of BP lowering was somewhat less in the last of these trials [22] compared to the previous ones [20,21], but recent evidence from our centre indicates that age and gender may strongly influence the BP phenotype [54]. To date, all studies investigating this gene-nutrient interaction in BP have intervened with riboflavin only. Given the close metabolic interplay between folate and riboflavin and the likelihood that enhancing MTHFR activity increases levels of 5-methylTHF, it is possible that supplementing with 5-methylTHF alone could have beneficial effects on BP in those with the *MTHFR* 677TT genotype, similar to that of riboflavin. Further research is required to investigate the role of 5-methylTHF in individuals with the *MTHFR* 677TT genotype. In any case, these findings have important implications for BP management for subpopulations worldwide given the high frequency of the *MTHFR* 677TT genotype (on average 10%, but as high as 32% in Mexico) [55]. Although biomarker status of riboflavin is rarely measured in populations, deficient and low status appears to commonly occur, especially in countries where intakes of riboflavin-rich food are low [56]. Further studies are clearly required to investigate this phenotype in younger adults in both genders and to consider the potential for preventing the development of hypertension through optimising riboflavin status. Of note, to date, all studies investigating the *MTHFR* C677T polymorphism and BP have utilized clinic BP. Ambulatory blood pressure monitoring (ABPM) offers an alternative method, which measures BP over a 24-h period and will be discussed later.

4. Biological Mechanisms Linking Blood Pressure with One-Carbon Metabolism

When the *MTHFR* C677T polymorphism was first described, Frosst et al. [30] reported that there is a 70% reduced activity in the mutated enzyme as measured in human lymphocytes. Further evidence from in vitro studies in *Escherichia coli* demonstrated that the mutated MTHFR enzyme loses its FAD cofactor at a much greater rate than the wild-type enzyme, reducing the overall functioning of the enzyme [57]. This was supported by a subsequent in vitro study in recombinant human MTHFR, which reported that in low folate compared to optimal folate conditions, the loss of FAD co-factor from the mutated enzyme was exacerbated [58]. As discussed previously, the FAD-dependent enzyme MTHFR is essential for the production of 5-methylTHF within the one-carbon metabolism cycle. Bagely and Selhub [59] observed decreased concentrations of 5-methylTHF and an accumulation of 10-formylTHF in red blood cells of individuals with the TT genotype, indicative of altered red blood cell folate distribution as a result of decreased MTHFR activity. It has also been postulated that the mutated enzyme has an altered active site, therefore reducing the efficacy of the binding of FAD [60]. It is not known how enzyme activity is enhanced by supplementation with riboflavin [53], but it is possible that this is achieved through stabilization of the variant enzyme and potentially increasing the enzyme-substrate binding, thereby improving its function [23].

The potential mechanism underlying the role of MTHFR in hypertension is currently unknown, but may involve endothelial nitric oxide synthase (eNOS). In one study involving patients undergoing cardiac bypass surgery, researchers found that vascular tissue concentrations of 5-methylTHF were positively associated with endothelial function (regardless of *MTHFR* C677T genotype) via the production of nitric oxide, a potent vasodilator [61]. A subsequent study from the same researchers reported that vascular 5-methylTHF is an important regulator of eNOS coupling and nitric oxide

bioavailability [62]. It is possible therefore that supplementation with riboflavin (i.e., the MTHFR cofactor) in individuals with the *MTHFR* 677TT genotype could increase MTHFR enzyme activity in vivo, resulting in an increased cellular production of 5-methylTHF and, thus, lower BP by promoting vasodilation [22,23,61].

A further, and possibly complementary, mechanism to explain this novel gene-nutrient interaction in hypertension may involve epigenetic modification. Epigenetics can change the expression of a gene without altering the underlying DNA sequence by histone modification, RNA interference or DNA methylation [29]. The majority of research into epigenetic modifications is in relation to DNA methylation. Folate, owing to its importance in one-carbon metabolism and the production of SAM, plays a key role in modulating DNA methylation levels, of relevance to diseases, such as cancer [63], and folic acid supplementation during pregnancy has also been reported to alter DNA methylation in the offspring [64]. Furthermore, animal studies have shown that in folate-deficient mice, aberrant DNA methylation occurs at the promoter regions of the *MTHFR* gene, resulting in alterations in gene expression [65]. In support of these findings, the same researchers have found increased methylation in the promoter region of the *MTHFR* gene in paediatric cancerous tissues [65]. One study that investigated the *MTHFR* C677T polymorphism and folate status in relation to DNA methylation found that the TT genotype combined with low folate status was associated with low genomic DNA methylation levels [66]. A reduction in genomic DNA methylation levels could lead to altered gene expression, and optimizing folate status in these individuals could counteract this effect.

One meta-analysis of observational studies examined the relationship between *MTHFR* single nucleotide polymorphisms and genomic DNA methylation. Of the 16 studies included, 10 studies examined the role of the *MTHFR* C677T polymorphism on global DNA methylation and found no significant association. It is important to note however that the studies included in this meta-analysis were designed to examine a number of diseases and were not focused on hypertension alone [67]. Elsewhere, long interspersed element-1 (LINE-1) methylation (an indication of global methylation) has been reported to be significantly lower in individuals with the *MTHFR* 677TT genotype compared to those with the CC genotype [68]. Furthermore, adults with the TT genotype compared to the CC genotype had diminished genomic DNA methylation, and this correlated with folate status [66]. There is also some evidence linking pre-eclampsia (characterised by proteinuria and hypertension) with *MTHFR* gene hypermethylation [69]. However, DNA methylation at *MTHFR* has not been examined to any great extent in relation to hypertension generally. Aberrant LINE-1 and gene-specific DNA methylation profiles have been reported to improve the prediction accuracy of the occurrence of myocardial infarction, when other well-known risk factors for hypertension are taken into account [70]. One randomised controlled trial found that B-vitamin supplementation (folic acid, vitamins B6 and B12) in combination with vitamin D and calcium, increased LINE-1 DNA methylation, but this study did not account for different *MTHFR* genotypes [71].

As MTHFR is required for normal folate recycling and thus the generation of SAM, stabilization of the mutated enzyme by riboflavin supplementation may overcome any possible aberrant DNA methylation owing to this polymorphism. In any case, further studies are required to investigate DNA methylation in relation to MTHFR-mediated blood pressure and to ascertain whether DNA methylation is altered by riboflavin supplementation in the TT genotype group.

5. Ambulatory Blood Pressure Monitoring

BP can be measured in a number of ways in clinical and research settings. The most common and convenient method of BP measurement of patients in the clinic is with a standard sphygmomanometer; however, as a result of BP variability, a one-off clinic BP reading may be a poor representation of an individual's true BP [72]. Out-of-office BP measurement, such as home BP monitoring (HBPM) and ABPM, is recognised as a more accurate measure of BP, as it is not influenced by the clinical environment. ABPM is considered to be more superior to HBPM, as it is a non-invasive and a robust measure of BP owing to its ability to record BP over a defined period of time, usually 24-h. This

continuous monitoring accounts for the daily variability in BP and, as such, is regarded as the gold standard in BP monitoring [73]. The 24-h window of BP monitoring can be further sub-divided into: the white coat window, daytime window, nighttime window and morning increase in BP [74], with each period being independently associated with CVD risk (as will be discussed further below). Despite being first reported in the early 1960s [25], ABPM has only recently been introduced into UK clinical guidelines as a method of confirming a diagnosis of hypertension [7]. The current guidelines recommend that hypertensive patients, once diagnosed, should be monitored on an annual basis using ABPM.

There are a number of important differences between clinic BP and ABPM, as summarised in Table 1, which are discussed in detail below. The most important advantage of ABPM is that it provides a minimum of 14-day time systolic and diastolic BP readings, allowing a more accurate diagnosis of hypertension to be made [75]. These multiple readings improve the prediction of CVD risk and stroke, as clinic BP only provides a 'snap-shot' of the BP [76,77]. Of note, different cut-off values are used for diagnosing hypertension, depending on whether clinic BP or ABPM is used. Clinical guidelines in the UK define hypertension as a clinic BP reading (systolic/diastolic) of ≥140/90 mmHg, whereas using ABPM, the corresponding cut-off values are ≥135/85 mmHg [7].

Table 1. Clinic BP vs. ABPM.

CLINIC BP	ABPM
• Defines hypertension as ≥140/90 mmHg *	• Defines hypertension as ≥135/85 mmHg * (daytime average)
• Convenient, quick, non-invasive method to measure blood pressure • One off measurement of: Systolic and Diastolic BP Pulse Rate	• Robust measure of BP over a 24-h period • Average 24-h/daytime/night-time measurement of: Systolic BP and Diastolic BP Mean Arterial Pressure Pulse Pressure Heart Rate
	• Identifies white-coat/masked/resistant hypertension; • A superior predictor of mortality; • Provides information on: Circadian pattern Morning surge % time in hypertensive state over 24-h Night-time dipping pattern

* Blood pressure is recorded as systolic BP/diastolic BP.

Along with providing multiple measurements of systolic and diastolic BP, continuous monitoring by ABPM provides a wealth of additional relevant information including mean arterial pressure, pulse pressure and heart rate, each of which are independently associated with CVD risk [78]. Although encompassing aspects of systolic and diastolic BP, the relevance of mean arterial pressure (i.e., defined as ⅔ diastolic BP + ⅓ systolic BP), is often underestimated as an independent risk factor for hypertension. In one large study of 11,150 adults, mean arterial pressure was linked with an increased risk of CVD, and this association was strongest in younger males (relative risk 2.52 95% CI 1.87–3.40 compared to 1.43 95% CI 1.07–1.92 in older males) [78]. The relevance of pulse pressure (i.e., defined as the difference between systolic BP and diastolic BP) as a predictor of CVD risk has been more extensively investigated, as it is an indicator of large arterial stiffness and isolated systolic hypertension. As people age, systolic BP increases, and diastolic BP remains steady or may even decrease, ultimately resulting in an increased pulse pressure, with estimates that each 10 mmHg increase in pulse pressure is associated with a 22%–35% increased risk of fatal stroke [79,80] and a 35% increased risk of cardiac events [80]. Thus, increased pulse pressure is now recognised by the European Society of Hypertension as an independent risk factor for cardiovascular events and is distinct from the risk associated with the increase in systolic BP with ageing [81]. Heart rate is another

independent risk factor, which can be measured using ABPM. Increased heart rate has been associated with increasing BP independently of age and sex [82,83]. An increase in heart rate of 10 beats/min is associated with increased risk of cardiac death by as much as 20% and is also related to the severity of atherosclerosis at all ages [83,84]. In contrast, pulse pressure, which increases as people age, is more important for determining CVD risk in the later stages in life. Targeting heart rate could therefore potentially offer a therapeutic option when treating hypertension; however, results to date from trials are inconsistent [85].

5.1. White-Coat Hypertension

ABPM also offers the advantage of enabling the identification of a phenomenon first described by Pickering et al. in 1988, named white-coat hypertension (WCH). This occurs when a patient has BP readings in the hypertensive range as measured in the clinic, but normal BP when monitored throughout the day [86]. WCH is thought to affect up to 30% of the general population [87] and can only be detected by the use of HBPM or ABPM. The main advantage of identifying WCH is to ensure antihypertensive medication is only prescribed to individuals who require it. Furthermore, dietary and lifestyle changes may offer greater benefit to patients with WCH, at least initially [88].

Although earlier studies investigating WCH did not link it with additional CVD risk [89], more recent research has associated WCH with an increased risk of CVD [90,91]. A recent meta-analysis of 29,100 participants reported that patients with WCH (compared to normotensive controls) were at an increased risk of incidence of cardiovascular events (OR 1.73, 95% CI 1.27–2.36) and CVD mortality (OR 2.79, 95% CI 1.62–4.80), although not all-cause mortality or stroke [92]. Two recent studies have also shown that WCH is associated with subclinical arterial wall damage, including thickening of the arterial wall [93,94]. Whilst the association between WCH and CVD risk is not fully understood, it might be due to BP variability, which is associated with increased risk of CVD events and mortality [95]. Randomised controlled trials are required to determine if reducing the BP in WCH patients could reduce CVD risk. Aside from WCH, ABPM also has the ability to detect masked hypertension (i.e., normotensive clinic BP, but hypertensive ABPM) and resistant hypertension (i.e., difficult to treat hypertension). Masked hypertension is generally undetected owing to the appearance of normal BP in the clinic and is therefore associated with a higher risk of cardiovascular events as it remains untreated [90]. In relation to resistant hypertension, ABPM can identify patients that have true resistant hypertension (unresponsive to three or more antihypertensive medications), and is reviewed extensively elsewhere [96,97].

5.2. Circadian Pattern of Blood Pressure

The benefit of being able to trace the circadian pattern of BP is that it enables the identification of those who do not follow the expected pattern, in particular, where BP rises sharply in the morning and falls at night, while sleeping (a phenomenon known as 'dipping'). When first reported, individual patients were categorised into either a dipping or non-dipping pattern [98]. The non-dipping pattern has been independently associated with increased risk of CVD in many studies [99–104] and has also been associated with increased CVD risk in adults without hypertension [105]. It is now more common to further divide non-dippers into three groups; thus, four groups in total referred to as: dippers (considered to have a normal dipping pattern; $\geq$10%–20% fall at night), extreme dippers ($\geq$20%), non-dippers ($\geq$0%–10%) or risers (increase in BP), depending on how much their BP falls at night in comparison to the average daytime BP.

Meta-analyses of studies that have investigated the circadian pattern of BP in relation to CVD events are summarised in Table 2. Salles et al. conducted a large meta-analysis in over three continents to investigate the effect of various dipping patterns on cardiovascular events and stroke [106]. Compared to the normal dipping pattern, individuals with any other dipping pattern at night were found to be at an increased risk of cardiovascular events (OR 1.40, 95% CI 1.20–1.63) and in particular stroke (OR 1.43, 95% CI 1.15–1.77). When the non-dipping pattern was further subdivided, a rising

pattern (increase in BP at night), in comparison to the normal dipping pattern, increased the risk of CVD by 79% and stroke by 89%. An increase in the morning surge of BP has also been associated with increased risk of coronary events [107], suggesting that varying circadian patterns of BP (daytime or nighttime) can increase the risk of CVD. Other large meta-analyses investigating the variation in circadian pattern in different populations support this finding, with estimates of excess CVD risk owing to atypical dipping ranging from 15% to 49% [102,108,109].

Table 2. Meta-analyses of circadian pattern abnormalities and cardiovascular events.

Author	Sample Size (*n*)	Populations	ABPM 24-h Profile	Odds Ratio (95% CI) for Cardiovascular Events
Salles et al. [106]	17,312 hypertensives	South America, Europe and Asia	Non-dippers vs. dippers *	1.40 (1.20–1.63)
Xie et al. [108]	14,133 hypertensives	Europe, South America and Asia	Morning surge	1.24 (0.60–2.53)
Hansen et al. [109]	23,856 hypertensives	Europe, South America and Asia	Non-dippers vs. dippers *	1.25 (1.02–1.52)
	9641 general population			1.15 (1.00–1.33)
Fagard et al. [102]	3468 hypertensives	Europe	Reverse dippers vs. other dipping patterns	1.49 (1.17–1.91)

Definition of dipping patterns (i.e., blood pressure fall at night comparted to daytime average): dippers ≥10%–20% fall in BP; non-dippers ≥0%–10% fall in BP; extreme dippers ≥20% fall in BP; reverse dippers, rise in BP; morning surge in BP is the increase in BP shortly after awakening. * These studies compare the normal dipping patterns of ≥10%–20% (dippers) and all other dipping patterns combined, collectively referred to as 'non-dippers'.

The mechanism for deviation in the circadian dipping pattern is unknown. Speculated mechanisms include increased sympathetic nervous system activity [110,111], decreased baroreceptor reflex sensitivity [112], increased arterial stiffness [113] and endothelial dysfunction [114]. Ageing is also known to play a role in the reduced decline in BP at night as there is an increase in the activity of the sympathetic nervous system and a decrease in baroreceptor reflex sensitivity. Poor quality of sleep has also been associated with increased BP readings at night and therefore could provide inaccurate reporting of dipping status [115]. Measures used to minimize the risk of inaccurate readings include ensuring that the right size of BP cuff is used, that the patient is fully informed of the procedure and a diary of events is kept (including any disturbed sleep). Although the reproducibility of nighttime dipping pattern (based on ABPM carried out on different days) has been questioned by some [116], Keren et al. reported that 71% of individuals reproduced the same dipping pattern when two 24-h readings were taken within 14 weeks [117].

In the clinical setting, ABPM can also be used to monitor the BP response to antihypertensive medication and to detect under- or over-treatment. Some patients present with resistant hypertension (the ineffective response to three or more antihypertensive medications) and chronotherapy can be introduced to potentially overcome this. Chronotherapy, in relation to hypertension treatment, is the administration of medication at a certain time of day, in line with the peaks in BP (as measured by ABPM) to improve the patient's response to treatment [118]. If the non-dipper or riser BP pattern at night is present, ingestion of medication at night, rather than in the morning, may be more beneficial [118,119]. One study reported that changing the time of administrating medication from morning to night significantly reduced BP ($p < 0.001$) in 250 hypertensive adults [118].

In summary, given these clear advantages, ABPM should be used in the clinical setting to diagnose hypertension accurately, to identify those at risk through abnormal circadian patterns and to help identify the optimum time for taking antihypertensive medication to maximise its effect. Further research is required to understand the effects of WCH and the potential effects of lowering BP in this at-risk group.

5.3. Nutrition, B-Vitamins and ABPM

As mentioned above, diet and lifestyle factors are usually suggested as the first line of treatment for the management of hypertension before pharmacological intervention is commenced, unless other CVD risk factors have been noted [7]. Many studies have investigated BP response to nutrition intervention; however, few studies have used ABPM to measure BP response. Those studies that have generally tend to report mean systolic BP and diastolic BP only, with many failing to consider the wealth of additional information that ABPM can contribute. The case for the use of ABPM in nutrition interventions is strong as ABPM has been reported to rule out the placebo effect after the first initial hours of recording BP, providing a more robust measure of potential BP reduction in intervention trials [120,121].

The relationship between obesity and BP as measured by ABPM has been investigated, confirming that BP increases with increasing BMI and showing that the non-dipping pattern is more prevalent in those with a BMI in the obese or severely obese range [122,123]. Micronutrients have also been investigated in relation to BP, and vitamin D deficiency has been associated with increased BP and with the non-dipping BP at night [124,125]. Other studies have reported that higher concentrations of serum calcium, phosphate and parathyroid hormone are associated with the non-dipping pattern in hypertensive patients without renal disease [126]. The majority of other studies failed to report the dipping pattern, mean arterial pressure, heart rate and/or pulse pressure despite the use of ABPM [127–139].

A limited number of randomised controlled trials have examined ABPM response to nutrition interventions, with somewhat inconsistent findings (Table 3). One of the first randomised controlled trials to investigate the ABPM response to a nutrition intervention (and the most comprehensive study to date) involved the Dietary Approaches to Stop Hypertension (DASH) trial [127]. The DASH diet incorporates low fat dairy products along with high intakes of fruit and vegetables and is characterised by being rich in protein and fibre, as well as potassium, magnesium and calcium, and low in saturated fat and cholesterol [140]. In an eight-week study investigating the ABPM response to the DASH diet versus a control diet or a diet rich in fruits and vegetables, the DASH diet was shown to result in the greatest BP lowering, achieving a mean systolic BP lowering of 4.5 mmHg [127]. Two additional DASH studies, also reporting ABPM, confirmed these findings and reported a lowering of systolic BP of between 9.5 and 15 mmHg in response to intervention [128,129]. The greatest lowering achieved (a mean reduction in systolic BP of 15 mmHg) reported by Paula et al. is likely to be explained by the inclusion of an exercise component within this intervention [129].

5.4. Blood Pressure Lowering by B-Vitamins as Measured by ABPM

Few studies to date have investigated the BP response to B-vitamin intervention specifically using ABPM. One small study of 30 postmenopausal women reported that high-dose folate supplementation (in the form of 5-methylTHF, the product of the reaction catalysed by MTHFR), significantly decreased nighttime systolic BP (-4.48 ± 1.8 mmHg; $p = 0.029$), diastolic BP (-5.33 ± 1.3 mmHg; $p < 0.001$) and mean arterial pressure (-5.10 ± 1.1 mmHg; $p = 0.005$), with no effect of daytime BP [141,142]. ABPM has been used in one study that investigated the effect of folic acid supplementation on BP by the *MTHFR* C677T genotype [143]. Although ABPM was reported, the study was confined to hypertensive males, and ABPM was not reported as a primary outcome measure. No BP lowering effect was noted in this study, although a significant decrease in brachial pulse pressure (PP) (4.7 ± 1.6 mmHg; $p < 0.05$) was observed across *MTHFR* genotype groups in response to intervention [143]. To date, the randomised controlled trials investigating this polymorphism in relation to BP have focused on folic acid/folate supplementation, and no previous ABPM studies have considered the BP lowering effects of riboflavin, arguably much more important, given its role in stabilizing MTHFR.

Table 3. Nutritional studies that report blood pressure response using ABPM in randomised controlled trials.

Author	Population	Sample Size	Average Systolic BP Change (mmHg)	Duration of Intervention	ABPM Parameters Reported			
					Dipping Status	Mean Arterial Pressure	Heart Rate	Pulse Pressure
DASH Diet Interventions								
Moore et al. [127]	USA	354	$-4.5^{\neq}$	8 weeks	Yes	No	No	No
Miller et al. [128]	USA	44	$-9.5^{\neq}$	9 weeks	No	No	No	No
Paula et al. * [129]	Brazil	40	$-15.0^{\dagger}$	4 weeks	No	No	Yes	No
Dairy Product Interventions								
Drouin-Chartier et al. [130]	Canada	89	$-2.0^{\dagger}$	4 weeks	No	No	No	No
Machin et al. [131]	USA	49	$-8.0^{\neq}$	4 weeks	No	No	No	Yes
Beetroot Interventions								
Hobbs et al. [132]	Europe	24	-	Acute	No	Yes	Yes	Yes
Jajja et al. [133]	Europe	24	-	3 weeks	No	No	Yes	No
Kapil et al. [134]	Europe	68	$-7.7^{\neq}$	4 weeks	No	No	Yes	No
Coles et al. [135]	Australia	30	$-4.6^{\dagger}$	Acute	No	No	Yes	Yes
Vitamin D Intervention								
Larsen et al. [136]	Europe	112	$-3^{\neq}$	20 weeks	Yes	No	Yes	No
Pilz et al. [137]	Europe	200	-	8 weeks	No	No	No	No
Other								
Brull et al. [138]	Europe	70	$-3.6^{\neq}$	6 weeks	Yes	Yes	Yes	No
Sauder et al. * [139]	USA	30	$-3.5^{\neq}$	4 weeks	Yes	No	Yes	No

* Denotes studies in diabetic patients; acute studies refer to one-off ingestions of beetroot product and effects monitored over 6–24 h. $^{\neq}$ Systolic BP response over 24h; † systolic BP response during daytime. Brull et al. supplemented adults with quercetin-rich onion skin extract; Sauder et al. supplemented adults with pistachio nuts.

6. Conclusions and Future Work

Evidence is accumulating to support the role of the *MTHFR* C677T polymorphism in hypertension and indicates that the BP phenotype may be much more relevant to CVD than the metabolite homocysteine. Riboflavin is an important modulator of the BP phenotype in individuals with the *MTHFR* 677TT genotype, but further work is required to investigate the influence of age and gender on this gene-nutrient interaction in BP. The biological mechanism by which riboflavin lowers BP in individuals with the variant *MTHFR* 677TT genotype is unknown; however, aberrant DNA methylation should be considered, along with other postulated mechanisms. Studies to date investigating the BP-lowering effect of riboflavin on genetically at-risk individuals and in nutrition studies generally have utilized clinic BP, but ABPM is now accepted as being a more robust measure of BP and a better indicator of cardiovascular health and disease risk. Its use in this area and in nutrition research generally should be considered. Given the global burden of hypertension, further research is required to understand the role of the *MTHFR* C677T polymorphism in BP, the modulating effect of riboflavin and the implications of this novel gene-nutrient interaction for the diagnosis and management of hypertension in different populations.

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Conflicts of Interest: There is a patent granted in Europe and pending elsewhere by M.W., H.M. and J.J.S. for the use of riboflavin in the treatment of hypertension.

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Review

Causes, Consequences and Public Health Implications of Low B-Vitamin Status in Ageing

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Abstract: The potential protective roles of folate and the metabolically related B-vitamins (vitamins B12, B6 and riboflavin) in diseases of ageing are of increasing research interest. The most common cause of folate and riboflavin deficiencies in older people is low dietary intake, whereas low B12 status is primarily associated with food-bound malabsorption, while sub-optimal vitamin B6 status is attributed to increased requirements in ageing. Observational evidence links low status of folate and the related B-vitamins (and/or elevated concentrations of homocysteine) with a higher risk of degenerative diseases including cardiovascular disease (CVD), cognitive dysfunction and osteoporosis. Deficient or low status of these B-vitamins alone or in combination with genetic polymorphisms, including the common *MTHFR* 677 C $\rightarrow$ T polymorphism, could contribute to greater disease risk in ageing by causing perturbations in one carbon metabolism. Moreover, interventions with the relevant B-vitamins to optimise status may have beneficial effects in preventing degenerative diseases. The precise mechanisms are unknown but many have been proposed involving the role of folate and the related B-vitamins as co-factors for one-carbon transfer reactions, which are fundamental for DNA and RNA biosynthesis and the maintenance of methylation reactions. This review will examine the evidence linking folate and related B-vitamins with health and disease in ageing, associated mechanisms and public health implications.

Keywords: B-vitamins; ageing; degenerative diseases; cardiovascular disease; cognitive dysfunction; dementia; osteoporosis; methylenetetrahydrofolate reductase (MTHFR)

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1. Introduction

An estimated 900 million people are aged $\geq$65 years globally, equating with 8% of the world's population, and by 2050, this is predicted to exceed two billion (16%) [1]. Approximately one quarter of the total global burden of disease is in older people, with a higher prevalence in high income countries [2]. Hypertension, the leading risk factor of cardiovascular disease (CVD) affects an estimated one billion people worldwide and CVD is the most common cause of death in older people [3]. Globally, osteoporotic fractures affect over nine million older people annually [4], while 46.8 million older people are reported to have dementia worldwide [5]. The prevalence of these diseases of ageing is expected to substantially increase as a result of the ever-increasing ageing population. In addition, these degenerative diseases cause multiple co-morbidities in older people which in turn has important societal and economic consequences. Maintaining good health in older age has therefore become a major public health priority. Poor nutrition is recognised as a modifiable risk factor in the development of degenerative diseases in ageing, and improved nutrition may prevent or delay the onset of adverse health outcomes as people age. In this context, the potential adverse effect of elevated homocysteine and/or the protective roles of folate and the metabolically related B-vitamins (B12 and B6), have received much attention.

This review will examine the emerging evidence linking folate and the metabolically related B-vitamins with ageing, the potential roles of these nutrients in preventing or delaying diseases of ageing and the associated mechanisms. The challenges and opportunities in achieving optimal B-vitamin status in older people will also be considered with particular emphasis on the role of food fortification.

2. Metabolic Role of B-Vitamins in One-Carbon Metabolism

Folate along with vitamins B12, B6 and riboflavin in their co-enzymatic forms are all essential in one-carbon metabolism (Figure 1), a network of reactions involving the transfer of one-carbon units. In the folate cycle, tetrahydrofolate obtains a carbon unit from serine in a vitamin B6 (plasma pyridoxal phosphate; PLP) dependent reaction forming 5,10-methylenetetrahydrofolate which is used for the synthesis of thymidine and purines or converted to 5-methyltetrahydrofolate. 5-methyltetrahydrofolate is the principal circulating form of folate, and this reaction is catalysed by methylenetetrahydrofolate reductase (MTHFR) using riboflavin (flavin adenine dinucleotide, FAD) as a co-factor. At this point, the folate cycle links with the methionine cycle, 5-methyltetrahydrofolate donates its methyl group to homocysteine for the formation of methionine in a reaction catalysed by methionine synthase which uses vitamin B12 (methylcobalamin) as a cofactor. Methionine is the precursor for *S*-adenosyl-methionine (SAM), the universal methyl donor for DNA and RNA, proteins and numerous central nervous system methylation reactions involving neurotransmitters, membrane phospholipid synthesis and myelin methylation [6,7]. SAM is converted to *S*-adenosylhomocysteine and then homocysteine which is either remethylated back to methionine or conversely metabolised in the transsulphuration pathway to form cysteine through another vitamin B6-dependent process [8]. The metabolism of the B-vitamins is closely related; folate and vitamin B12 are both intrinsically linked via the enzyme methionine synthase [9]. In vitamin B12 depletion, methionine synthase activity is reduced and the formation of tetrahydrofolate is blocked, with folate essentially becoming trapped as 5-methyltetrahydrofolate because the conversion by MTHFR is physiologically irreversible [10]. There is also an important metabolic inter-relationship between vitamin B6 and riboflavin. The conversion of dietary vitamin B6 in tissues to its functional enzyme, pyridoxal 5′ phosphate (PLP), requires the enzyme pyridoxine phosphate oxidase (PPO), which is dependent on the riboflavin in its co-factor form, flavin mononucleotide (FMN).

Deficiencies in any of these B-vitamins can perturb the complex regulatory network maintaining one-carbon metabolism resulting in reduced methylation status within the relevant tissue, hyperhomocysteinemia, and/or increased misincorporation of uracil into DNA as a result of thymidylate synthesis being impaired owing to low 5,10-methylene-THF concentrations and thus uracil is inserted instead during DNA synthesis which in turn may contribute to adverse health outcomes in ageing [11,12]. In addition, genetic polymorphisms, including the common 677 C $\rightarrow$ T polymorphism in the gene encoding the folate-metabolising enzyme MTHFR, can interact adversely with sub-optimal status of one or more of the B-vitamins in one-carbon metabolism and thus contribute to a greater disease risk [13]. The *MTHFR* 677TT genotype affects an estimated 10% of individuals worldwide (ranging from 3% to 32% depending on ethnicity) [14] and 12% in Ireland [15].

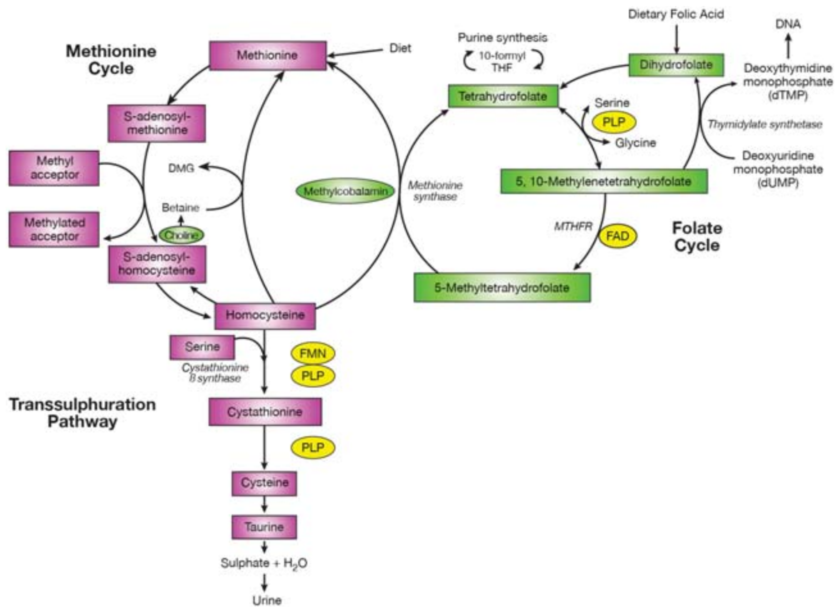

Figure 1. One-carbon metabolism. Abbreviations: PLP, plasma pyridoxal phosphate; MTHFR, methylenetetrahydrofolate reductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide. Adapted from [16].

3. Causes of B-Vitamin Deficiency

Depending on the particular vitamin, there are a number of potential causes of B-vitamin deficiency including inadequate intake, increased requirements, malabsorption, drug–nutrient interactions and others including genetic disorders or medical conditions (Table 1). In addition, the ageing process itself can negatively affect the absorption, transport and metabolism of B-vitamins and thus older people have increased requirements. A recent systematic review in community dwelling older adults in developed Western countries (n = 28,000) reported a high prevalence of low dietary intakes for B-vitamins (i.e., below the estimated average requirement, EAR), including folate (29%–35%), vitamin B6 (24%–31%) and identified riboflavin (31%–41%) among six nutrients of potential public health concern [17]. An estimated 9%–12% of older people in the UK are considered to suffer from folate deficiency [18], with the most common cause being low dietary intake.

Table 1. Causes of B vitamin deficiency.

B Vitamin	Inadequate Intake	Increased Requirement	Malabsorption	Drug–Nutrient Interactions	Other
Folate [19–24]	Common Poor cooking techniques	Elderly Pathological conditions	Intestinal diseases Coeliac disease Crohn's disease Ulcerative Colitis	Phenytoin Phenobarbital/Primidone Trimethoprim Methotrexate Sulfasalazine Metformin	Alcohol abuse Genetic disorders Haemolytic anaemia
B12 [19,21,22,25,26]	Common Vegan diets	Elderly	Intestinal diseases Coeliac disease Crohn's disease Gastric/intestinal resection Atrophic gastritis Bacterial overgrowth Helicobacter pylori Pancreatic insufficiency Pernicious anaemia Zollinger–Ellison Syndrome	Proton pump inhibitors H2-receptor antagonists Metformin Nitrous oxide Colchicine	Alcohol abuse Genetic disorders Tropical or non-tropical sprue
B6 [21,22,25,26]	Rare Chronic dieters	Elderly	HIV	Isoniazid Anti-Convulsants Steroids	Alcohol abuse Genetic disorders Liver disease Renal dialysis Rheumatoid arthritis
B2 [21,25–27]	Common Chronic dieters	Elderly	Diabetes Liver disease Thyroid and renal insufficiency GI and biliary obstruction	Phenothiazines, e.g., chlorpromazine Theophylline	Alcohol abuse Genetic disorders Hypochromic anaemia Metals such as zinc, copper and iron

There is a high prevalence of vitamin B12 deficiency in older people globally [28], with two early studies finding 75% of Asian Indians demonstrating metabolic evidence of vitamin B12 deficiency [29,30]. Numerous population surveys have also identified varying levels of vitamin B12 deficiency and sub-optimal status in older people from the UK (5%–20% deficient) [31–33], US (6% deficient, >20% marginal status) [34,35] Canada (5% deficient) [36], New Zealand (12% deficient, 28% marginal deficiency) [37] and Finland (6% low B12 and 32% borderline) [38]. The variation in prevalence may in part be explained by the diagnostic criteria used and different studies have used different biomarkers and cut off points. Pernicious anaemia, the classic form of B12 deficiency, caused by a lack of intrinsic factor is thought to account for just 1%–2% of cases of insufficiency in older people [39]. The maintenance of vitamin B12 status in older people is not only dependent on adequate dietary intake but more critically on the normal absorption of the vitamin which is dependent on the normal functioning of the gastrointestinal tract, gastric acid secretion and a number of transport proteins including intrinsic factor [40]. Food-bound malabsorption, primarily as a result of atrophic gastritis, an age-related disorder affects up to 30% of older people [41], leads to a reduction in gastric acid secretion which prevents the release of B12 from food and thus absorption. Deficiency is less pronounced and the limitations of the conventional vitamin B12 assays may also mean that a significant proportion of the older population may have low vitamin B12 status which is not detected [40,42].

Medications including proton pump inhibitors (PPI) and H2-receptor antagonists (H_2RA) are commonly prescribed (for conditions such as reflux and peptic ulcers) in older people, resulting in gastric acid suppression, this mimics atrophic gastritis and leads to food-bound malabsorption. There is evidence to suggest that these medications have been associated with up to 4.5 times higher risk of vitamin B12 deficiency in case control studies [43,44]. Recently in the USA, a large community survey (25,956 cases and 184,199 controls) found that the long term use (>2 years) of H_2RAs and PPIs was associated with a 25%–65% greater risk of a subsequent diagnosis of vitamin B12 deficiency [45]. In addition, metformin usage in Type 2 Diabetes can also result in vitamin B12 deficiency, possibly interfering with calcium-dependent membrane action in the terminal ileum required for the absorption of the vitamin B12-intrinsic factor complex [46,47].

Vitamin B6 and riboflavin in older people have not been as widely investigated. There is a high prevalence of vitamin B6 deficiency in older people as evident in three large population based surveys in Europe (Survey in Europe Nutrition and the Elderly, a Concerted Action, SENECA; 23%) [48], the UK (National Diet and Nutrition survey; NDNS; 11%–27% free living, 30%–65% institutionalised) [49], and the US (National Health and Nutrition Examination Survey, NHANES; 15%–23% males, 14%–49% females) [50] This deficiency in older age has been attributed to increased requirements as a result of reduced absorption, increased catabolism and impaired phosphorylation as opposed to inadequate dietary intake [51,52].

Riboflavin deficiency is thought to mainly arise from inadequate dietary intake, particularly in those who do not consume dairy products or fortified foods [17,27,53]. A high prevalence of riboflavin deficiency is acknowledged in the developing world; less well recognised is the emerging evidence to suggest that sub-optimal status is also evident in developed countries. To date, most population based surveys only report dietary intake data, and relatively few include biomarker data. Despite dietary intakes being reported to be sufficient in the NDNS (5% below reference nutrient intake, RNI) [54] and in the Irish National Adult Nutrition Survey (NANS, 13% below EAR) [55], 39%–43% of older people had biochemical deficiency in the NDNS and 19%–22% in NANS. Thus, further population based surveys are still required to investigate the intake and requirements of older people based on robust biomarker data of both vitamin B6 and riboflavin.

4. Assessment of B-Vitamin Biomarker Status

There are a number of direct and functional biomarkers available to determine B-vitamin status each with various strengths and limitations (Table 2). Plasma homocysteine accumulates with folate deficiency, it has been used as a biomarker of status but it lacks specificity as it can also be elevated by

other B-vitamin deficiencies, including vitamins B12 [56], B6 [57] and riboflavin [58]. Serum folate is the earliest indicator of altered folate exposure and reflects recent dietary intake [59]. Red blood cell (RBC) folate is a sensitive indicator of long term folate status (during the preceding 120 days) [60,61]. It parallels liver concentrations this is considered to reflect tissue folate stores [20,62]. One recent meta-analysis demonstrated that both serum folate (27 RCTs) and RBC folate (12 RCTs) respond to interventions with folic acid in a dose-dependent manner and concluded that both indicators were robust measures of folate status [63]. To date, there is no gold standard biomarker for the assessment of vitamin B12 status despite there being a number of direct and functional measures available. Serum B12, although still widely used both clinically and in research, measures the total amount of the vitamin, however only 20% of this is metabolically active [64]. Serum total vitamin B12 can under-report the true prevalence of vitamin B12 deficiency [65,66] and up to 40% of older people can have low serum vitamin B12 but normal metabolic status [41,67]. Holo-transcobalamin (holoTC) measures the biologically active fraction of vitamin B12 and is considered to have superior diagnostic value to total B12 [68,69] although it can be affected by inborn errors altering intracellular vitamin B12 metabolism [68]. Plasma homocysteine can be used as a functional measure of vitamin B12 status but as previously discussed it is not specific to vitamin B12 (Table 2). Methylmalonic acid (MMA) is a more specific and sensitive functional biomarker of vitamin B12 status [31], the conversion of methylmalonyl-CoA to succinyl-CoA is a B12 dependant process, impaired B12 status leads to an accumulation of methymalonyl-CoA which then is metabolised to MMA and excreted in the urine. However, MMA is also elevated in renal dysfunction, which is common in older people [69] and so limits its use. Two reviews have recommended that at least 2 biomarkers are required in the diagnosis of vitamin B12 deficiency [42,70].

Plasma PLP concentration is the most widely used measure of vitamin B6 [71] with good specificity [72]. It is considered to reflect PLP concentrations in the liver [73]. Other markers of B6 are available including 4-pyridoxic acid and erythrocyte pyridoxal-5-phosphate. Erythrocyte glutathione reductase activation (EGRac) assay is the most widely used functional assay for riboflavin status and is generally regarded as the gold standard. EGRac measures glutathione reductase activity in erythrocytes before and after reactivation with its prosthetic group flavin adenine dinucleotide (FAD). A systematic review (14 RCTs) concluded that EGRac was a sensitive biomarker of change in riboflavin intake in populations with status ranging from deficient to normal [74].

In general, the use of different biomarkers or different cut off points for defining deficiency/suboptimal status when assessing individual B-vitamins can influence the interpretation and conclusion of relevant studies. This can make direct comparisons among studies difficult and may explain some inconsistencies in the literature as regards the role of B-vitamins in diseases in ageing.

Table 2. Assessment of B-vitamin biomarker status.

		Biomarker	Strengths	Limitations
Homocysteine [20]	**Functional**	Plasma homocysteine	Sensitive functional biomarker Highly responsive to intervention with B-vitamins Responds within 3–4 weeks of B-vitamin depletion and subsequent repletion Very stable analyte Can be stored frozen for extended periods of time	Lacks specificity as affected by other B-vitamins Requires separation from RBCs within one hour of blood collection, or <8 h if whole blood is kept on ice Influenced by other factors such as lifestyle, genetics, renal insufficiency, age and medications
Folate [20,21,60,63,75]	**Direct**	Serum/Plasma folate	Earliest indicator of altered folate exposure Reflects recent dietary folate intake Requires less time processing at time of blood collection vs. RBC folate Can be measured in the field	Inconsistent use of cut off values makes comparisons across different methods and labs difficult Fasting blood samples are recommended
		Red cell folate	Sensitive indicator of long-term folate status Reflects folate status over half-life of RBCs Reflects tissue folate stores as parallels liver concentrations Highly correlated with habitual intake when expressed as DFEs	Affected by vitamin B12 deficiency Inconsistent use of cut off values makes comparisons across different methods and labs difficult Cannot be measured in the field
B12 [21,34,60,70,75,76]	**Direct**	Serum/Plasma total B12	Serum standard clinical test Variety of assays available Measures all forms of vitamin B12	Does not reflect intracellular vitamin B12 Falsely elevated B12 caused by factors including liver disorders, bacterial overgrowth, renal failure Falsely low B12 caused by factors such as iron deficiency, HIV infection and pregnancy Inconsistent use of cut off values makes comparisons across different methods and labs difficult
		Serum/Plasma Holo-transcobalamin (HoloTC)	Represents metabolically active fraction of B12 Decrease in holoTC can indicate earliest sign of B12 depletion Considered better indicator of B12 status in elderly	Highly sensitive to altered renal function and influenced by factors including genetics
	Functional	Serum/plasma/urine Methylmalonic acid (MMA)	Reflects availability of intracellular B12 Early detection of functional B12 deficiency Not affected by folate deficiency	Lacks sensitivity as can be elevated in those with renal impairment High running costs

Table 2. *Cont.*

		Biomarker	Strengths	Limitations
B6 [21,60,72,77,78]	**Direct**	Plasma Pyridoxal-Phosphate (PLP)	Most widely used Good specificity and reflects PLP content in liver Responds quickly within 1–2 weeks of B6 depletion and subsequent repletion Reference ranges available for younger and older adults Fairly stable at low temperatures	Does not represent PLP content in the muscle which is resistant to B6 depletion Influenced by other factors such as age, sex, pregnancy, protein and alcohol intake PLP declines in samples stored at room temperature and exposure to light Fasting blood samples are recommended Plasma PLP concentrations affected by use of certain drugs
		Erythrocyte PLP	Positively correlated with B6 dietary intake Responds within weeks of B6 depletion and subsequent repletion Appears more responsive than plasma PLP to supplementation May be more reliable marker than plasma PLP under conditions and disease associated with inflammation	Affected by haemoglobin variants Assay is cumbersome, with variable recovery and low precision
B2 [21,60,74,78]	**Direct**	Serum/Plasma/Erythrocyte Riboflavin/Flavine Adenine Dinucleotide (FAD)/Flavin Mononucleotide (FMN)	Riboflavin vitamers are stable for several years when plasma samples are stored at −80 ° CSerum/plasma can be used retrospectively in a hospital setting	Influenced by other factors such as age, sex, pregnancy, protein and alcohol intake Serum/plasma riboflavin concentrations affected by use of certain drugs High variability within and between-subjects compared to the cofactor forms of riboflavin (plasma/erythrocyte)
	Functional	Erythrocyte glutathione reductase activation (EGRac) assay	Most widely used marker of status Measures tissue saturation and long term status Enzyme is stable for several years when erythrocyte lysates are stored at −80 °C	Poor index of optimum riboflavin status Assay is not linear against status Difficult make comparisons across different methods and labs

5. Consequences of B-Vitamin Deficiency

There are established clinical signs of B-vitamin deficiency (Table 3). The haematological manifestation of folate and vitamin B12 deficiency is indistinguishable, as both vitamins are linked through the enzyme methionine synthase [9] which catalyses the remethylation of homocysteine to methionine and thus are metabolically interrelated [79]. Deficiency of either vitamin results in a reduction of the active form of folate which subsequently results in megaloblastic anaemia [80], characterised by megaloblasts in the bone marrow, macrocytes in the peripheral blood and gigantism in the morphology of proliferating cells [25]. Vitamin B12 deficiency can also result in diverse neurological symptoms including irreversible nerve damage and sub-acute combined degeneration of the spinal cord if left untreated, as patients are often asymptomatic [81]. Neuropathy is quite specific to vitamin B12 and does not occur in folate deficiency [79]. Although severe vitamin B6 deficiency is relatively uncommon, it can present with notable symptoms such as anaemia, depression and sores or ulcers of the mouth [22]. The classical signs of riboflavin deficiency are angular stomatitis, cheilosis and glossitis, but these are rarely encountered in isolation and may be as a result of other B-vitamin deficiencies [27].

Apart from clinical deficiency signs, deficient or low status of B-vitamins can be associated with various adverse health outcomes throughout the lifecycle [82]. It should be noted that these can arise in the absence of more classical deficiency signs, and can occur within the range of what may be classed as "normal" within the clinical setting.

Table 3. Consequences of deficient or low status of B-vitamins.

	Clinical Deficiency Signs [19–22]
Folate	Megaloblastic anaemia, clinical features characterised by megaloblasts in the bone marrow macrocytes in the peripheral blood gigantism in the morphology of proliferating cells
B12	Megaloblastic anaemia indistinguishable from folate-related megaloblastic anaemia Irreversible nerve damage/neuropathy Sub-acute combined degeneration of the spinal cord (SCD)
B6	Notable symptoms include: Microcytic anaemia Inflammation of the tongue Sores or ulcers of the mouth Dermatitis Nervous/muscular signs Irritability, fatigue, numbness Headache, muscle twitching Difficulty walking, convulsions Depression and confusion
B2	Classic signs arbioflavinosis, rarely encountered in isolation Anaemia Cheliosis, Angular stomatitis Glossitis Redness and swelling of the lining of the mouth and throat Seborrheic dermatitis particularly affecting the nose, cheeks and forehead Eyes burning and itching Sensitivity to light Loss of visual acuity Gritty sensation under the eyelids
	Health Consequences of Low Status of Folate and/or Other B-Vitamins [83–89]
	Elevated homocysteine CVD and stroke Cognitive decline/dementia/Alzheimer's Osteoporosis and risk of fractures

6. Emerging Roles of B-Vitamin Status in Preventing Diseases of Ageing

6.1. Cardiovascular Health in Ageing

Clinical evidence has linked elevated plasma homocysteine concentrations and/or low folate with an increased risk of CVD. Early meta-analyses of observational studies concluded that lowering homocysteine would reduce the risk of heart disease by 11%–16% [83,84]. Homocysteine may however be a marker of suboptimal B-vitamin status and thus simply reflect a perturbation in one-carbon metabolism, rather than playing a causative role in CVD, but this is not universally accepted [90]. In any case, several secondary prevention randomised controlled trials (RCTs) published between 2004 and 2012 have failed to demonstrate a benefit of homocysteine-lowering by B-vitamin supplementation on CVD events generally [91–97]. These studies, however, typically involved patients with existing optimal B-vitamin status and/or advanced CVD, and thus a significant effect of B-vitamin supplementation on CVD risk may have been unlikely.

The evidence however is generally much stronger for stroke than heart disease, with meta-analyses of earlier observational studies concluding that lowering homocysteine would reduce the risk of stroke by 19%–24% [83,84]. Furthermore, population data from the USA and Canada, reported an improvement in stroke mortality corresponding to the time that mandatory food fortification was introduced; in contrast, no similar improvement was found over the same time period in England and Wales where no mandatory fortification policy exists [98]. Of greatest relevance however are the findings of RCTs in relation to stroke risk, with meta-analyses of folic acid interventions showing a reduced risk of stroke by 18% overall [99], but with much greater reductions in risk noted in studies with longer folic acid treatment duration, with greater homocysteine reduction and in particular in those with no history of stroke [99,100]. More recent evidence including the China Stroke Primary Prevention Trial (CSPPT) [101] and a meta-analysis of 30 RCTs [102] also support a significant beneficial effect of folic acid supplementation on CVD risk (especially stroke), particularly in those with lower baseline folate status and without pre-existing CVD. Thus, optimisation of folate and the related B-vitamins might be beneficial in lowering CVD risk, particularly stroke, and most convincingly in primary prevention.

In addition, genetic studies are now providing stronger evidence for the potential role of folate and the related B-vitamins in CVD, primarily through the investigation of the common 677 C $\rightarrow$ T polymorphism in the gene encoding the folate-metabolising enzyme MTHFR. Epidemiological evidence suggests that this common polymorphism increases the risk of CVD, especially stroke by up to 40% overall [84,103–105]. Furthermore, evidence is emerging to suggest that the excess genetic risk of CVD may be driven by higher blood pressure (rather than higher homocysteine), with meta-analyses of observational studies showing that the *MTHFR* 677 C $\rightarrow$ T polymorphism increases the risk of hypertension by 36%–87% [106,107]. This is also supported by recent evidence from a large study of Irish adults (n = 6069), which estimated that the *MTHFR* 677TT genotype was associated with an almost two-fold increased hypertension risk, a risk which was further increased when the genotype occurred in combination with low riboflavin status [15]. In addition, intervention studies from this centre, conducted in participants with premature CVD or hypertension showed that those with the *MTHFR* 677TT genotype were highly responsive to blood-pressure lowering through riboflavin supplementation, whilst no response was observed in participants with CC or CT genotypes [108–110]. Therefore, sub-populations worldwide with the *MTHFR* 677TT genotype (ranging from 3% to 32%) [14], may particularly be at greater risk of CVD via a blood pressure effect, and could benefit from a more optimal riboflavin status ideally before hypertension has developed.

6.2. Bone Health in Ageing

The role of B-vitamins in bone health has been extensively reviewed [16]. Briefly, observational evidence in older people has found independent associations of elevated homocysteine with bone mineral density (BMD) [111,112] and fracture risk [89,113]. Meta-analyses of observational studies

have confirmed these relationships, with one (n = 14,863) concluding that homocysteine was an independent risk factor [114]. Another dose response meta-analysis (n = 11,511) estimated a 4% increased fracture risk for every 1 μmol/L increase of homocysteine concentration [115]. Low BMD has also been associated with sub-optimal status or dietary intakes of folate [111,116] or vitamin B12 [112,117]. Likewise, studies have also found an increased fracture risk in older people with sub-optimal status/intake of folate [118], vitamin B12 [119,120], or vitamin B6 [121,122]. Only one study to date in coeliac patients (n = 110) has examined riboflavin biomarker status with bone health (BMD), and found no relationship [16]. Although, low riboflavin intake in women (aged ≥55 years, n = 5035) was associated with a 1.8 times increased risk of osteoporotic facture and 2.6 times increased risk of fragility fractures [119]. The evidence is not entirely consistent, however, as some observational studies have shown no relationships with B-vitamin biomarkers on BMD [123] or fracture risk [124]. To date, there is limited RCT evidence linking B-vitamins with bone health and disease. One notable two-year RCT of combined folic acid and vitamin B12 supplementation (n = 628) resulted in a 75% reduction in the risk of hip fractures in older post-stroke Japanese patients, with low baseline folate status [125]. Certain RCTs with B-vitamins, which are not designed to examine bone outcomes (but rather other health outcomes), have reported no significant associations with fracture risk [126,127]. Additionally, interventions involving participants with generally higher baseline folate status have also reported no significant associations with BMD [128] or fracture risk [129]. This suggests that the benefit of interventions with B-vitamins may be confined to at-risk groups such as those with sub-optimal status.

Genetic studies also support the potential role of folate and the related B-vitamins in bone health. The potential influence of *MTHFR* 677TT polymorphism on bone health may be mediated through impaired DNA methylation as a result of impaired DNA structure and stability [130,131], expression and the silencing of genes [132–134]. Epidemiological studies have reported that the *MTHFR* 677TT genotype is associated with significantly lower BMD [135,136] and a 2- to 2.5-fold increased risk of fractures [135,137]. This is also strengthened by one meta-analysis (3525 cases and 17,909 controls) which concluded that the *MTHFR* 677 C → T polymorphism was associated with BMD at multiple sites and individuals with the TT genotype had a 23% increased risk of all fractures [138]. Furthermore, the association appears to be influenced by prevailing B-vitamin intake, an interaction between the *MTHFR* 677TT genotype and low intakes of folate or riboflavin has been linked with lower BMD [139–141], higher bone loss and up to a three-fold increased fracture risk [118,142], although the majority of these studies do not consider B-vitamin biomarker status. This suggests that individuals with the *MTHFR* 677TT genotype and low B-vitamin status may be most vulnerable to poor bone health in later life.

6.3. Brain Health in Ageing

Cognitive Dysfunction

Cognitive dysfunction ranges from mild cognitive impairment (MCI) to dementia and can result in a progressive loss of a number of specific cognitive functions [143,144]. The rate of cognitive decline varies between individuals [145], and it is estimated that 50% of older people with MCI will go on to develop dementia within five years of diagnosis [146]. Dementia has a number of distinct pathologies, although mixed pathologies in individuals have been shown at brain autopsies [147,148]. These include reduced/blocked flow to the brain [149], neuronal loss and damage to the connection between neurones [150] and the presence of Lewy bodies which has also been associated with neurotransmitter depletion [151]. Extra-neuronal β amyloid plaques and intra-neuronal neurofibrillary tangles [152] can also be present. Brain atrophy is a normal part of the ageing process, but with dementia, the rate is significantly higher overall and particularly in the hippocampus region [153,154]. Grey and white matter loss, thinning of cortical gyri and enlarged ventricles can also be evident in those with dementia [155].

Prospective studies make a strong case for elevated homocysteine and/or low B-vitamin status as potential causative factors in cognitive decline [156,157] and dementia [85]. This is further supported by three recent reviews and meta-analyses [87,158,159]. Epidemiological studies have also investigated the role of the relevant B-vitamins, with the focus mainly on folate and vitamin B12. Studies involving populations with lower baseline folate status in general support the role of folate in cognitive dysfunction [160–162] and cognitive decline [163,164], whereas those in countries with food fortification policies and thus overall higher mean concentrations of folate, are generally less supportive for a role in cognitive dysfunction [165,166], cognitive decline [167–169] or dementia [170]. Similarly, a number of large cohort studies have associated low vitamin B12 status (using more sensitive biomarkers of status including MMA and HoloTC) with cognitive dysfunction [160,166,171] and cognitive decline [162,172,173] in older people. However, the evidence for B12 is less convincing, and one meta-analysis has shown no association of vitamin B12 with cognitive decline or dementia (n = 14,325) [174]. This lack of significant association for B12 may be explained the fact that some of the included studies had methodological shortcomings (including limitations in the biomarkers used to determine B12 status). Thus, when sub-analysis of the data was carried out based on studies using more sensitive biomarkers of vitamin B12 status the results indicated that low vitamin B12 was in fact significantly associated with an increased risk of cognitive decline and dementia [174]. Relatively few studies have examined the role of vitamin B6 in cognition and even fewer have examined riboflavin. Low status of vitamin B6 has been shown to contribute to cognitive dysfunction [175,176] and cognitive decline [177]. Low vitamin B6 intake has been associated with a reduced risk of Alzheimer's disease [178] in older Americans. Only one study from Taiwan has investigated the role of riboflavin status and found a significant association with cognitive dysfunction, which was assessed using only a short portable mental status questionnaire and cognition was not the primary outcome [179].

Several RCTs have investigated the potential role of folate alone or in combination, with vitamins B12 and/or B6 in maintaining cognitive health in ageing, although to date most include high pharmacological doses and none include riboflavin (Table 4). One meta-analysis of nine RCTs in healthy people (n = 2835) concluded that folic acid had no effect on cognitive function [180]. However, in this meta-analysis, only two studies had more than 275 participants and were longer than 12 months in duration (five were $\leq$ 6 months). Additionally, one recent controversial meta-analysis in almost 22,000 healthy older people concluded that neither folic acid nor vitamin B12 had a beneficial effect on "cognitive ageing" [181]. The results have been widely criticised, mainly due to the inclusion criteria used to select the trials [182–184]. Only three of the RCTs had final cognitive test scores and two thirds of the baseline results were not available. MMSE, a screening tool that is considered as a crude measure of global cognition, was the main cognitive assessment tool used. Furthermore, this meta-analysis did not identify subgroups or at risk groups in each trial that may have benefited from B-vitamins. The recent B-Vitamins for the Prevention of Osteoporotic Fractures (B-PROOF) study did show a slower rate of global cognitive decline, this was attributed to chance by the authors [185]. In general, greatest cognitive benefits have been demonstrated in RCTs involving participants with lower baseline folate status [186,187], higher homocysteine concentrations [188,189] and lower B-vitamin intake [190]. In contrast, RCTs in individuals with higher baseline folate status [191] or higher cognitive status [185] have shown no significant benefit on cognitive performance. This supports the view that baseline B-vitamin status is a critical consideration in the outcome of trials examining cognition, and further is supported by a systematic review involving 14 RCTs, which found no overall benefit of B-vitamin interventions, but reported a benefit in cognitive function in those with lower baseline folate status [192]. Thus, well designed RCTs are still required, particularly in those with sub-optimal B-vitamin status as they might benefit most in optimising B-vitamin status to maintain cognitive health in ageing.

Table 4. Summary of Randomised Controlled Trials of 2 years or more assessing the effect of B vitamins on cognitive function in ageing.

Author/Year/Trial	Country	Sample Size (*n*)	Age (Years)	Population Studied Plasma tHcy (μmol/L)	Treatment (mg/day)	Duration	Cognitive Outcomes
Questionnaire based assessment							
McMahon 2006 [191]	New Zealand	276	≥65	Healthy tHcy > 13	1.0 FA, 0.5 B12, 10 B6	2 years	No significant effect on cognition
FACIT Durga 2007 [186]	The Netherlands	818	50–70	Healthy tHcy 13–26	0.8 FA or placebo	3 years	Improvement in domains including memory, information-processing and sensorimotor speed No improvement in global cognition or domains of complex speed or word fluency
WAFACS Kang 2008 [190]	USA	2009	≥65	CVD/high risk women tHcy not provided	2.5 FA, 1.0 B12, 50 B6 or placebo	6.6 years	Reduced risk of cognitive decline among women with low baseline dietary intake of B-vitamins Overall no significant effect on rate of cognitive decline
Brady 2009 [193]	USA	659	Mean 67.3	Advanced renal disease tHcy ≥ 15	40 FA, 2.0 B12, 100 B6 or placebo	5 years	No significant effect on cognition
Health in Men Study: sub set Ford 2010 [194]	Australia	299	≥75	Hypertensive men Mean tHcy 13.1–14	2.0 FA, 0.4 B12, 25 B6 or placebo	2 years	No significant effect on cognition
Kwok 2011 [189]	Hong Kong	140	≥60	Dementia diagnosis Mean tHcy 14.1	5.0 FA, 1.0 B12 or placebo	2 years	Improvement in domain of construction No change in global cognitive decline, attention, memory or conceptualisation
Beyond ageing project Walker 2012 [187]	Australia	900	60–74	Elevated psychological distress Mean tHcy 9.7	0.4 FA, 0.1 B12 or placebo	2 years	Improvement in overall global cognition and in domains of immediate and delayed recall scores No significant change in other cognitive domains
VITACOG De Jager 2012 [195]	UK	168	≥70	MCI Mean tHcy 11.3	0.8 FA, 0.5 B12, 20 B6 or placebo	2 years	Slower decline in global cognition and in domains of semantic and episodic memory Clinical benefit in global clinical dementia rating score
BPROOF Van Der Zwaluw 2014 [185]	The Netherlands	2919	≥65	Healthy tHcy 12–50	0.4 FA, 0.5 B12, 0.15 D3 or placebo + D3	2 years	Slower rate of decline in global cognition No change in domains of memory
Brain-imaging assessment							
VITACOG Smith 2010 Douaud 2013 [188,196]	UK	168	≥70	MCI Mean tHcy 11.3	0.8 FA, 0.5 B12, 20 B6 or placebo	2 years	Slowed shrinkage of brain Marked reduction in cerebral atrophy in grey matter regions
BPROOF Van Der Zwaluw 2014 [185]	The Netherlands	2919	≥65	Healthy tHcy 12–50	0.4 FA, 0.5 B12, 0.15 D3 or placebo + D3	2 years	*Awaiting MRI scan results*

Abbreviations: tHcy, homocysteine; FA, folic acid; MMA, methylmalonic acid; holoTC, holo-transcobalamin; EPA, eicosapentaenoic; DHA, docosahexaenoic acid; MCI, mild cognitive impairment; MRI, magnetic resonance imaging; CVD, cardiovascular disease.

Apart from evidence from observational studies and RCTs, genetic studies also provide support for the role of B-vitamins in maintaining cognitive health in ageing. The *MTHFR* 677TT genotype has been associated with poorer cognitive performance in Chinese males [197] and a 42% increased risk of cognitive impairment in older Australian males [198], but not in older Americans [166]. Meta-analyses have also associated the *MTHFR* C677T polymorphism with an increased risk of Alzheimer's disease [199], and dementia albeit in Asian populations only [200,201]. These results may be attributed to the varying prevalence of the *MTHFR* 677TT genotype worldwide and the modulating effect of B-vitamin status. To date, no studies have investigated interactions between this gene and B-vitamin status in relation to cognitive health.

6.4. Future Directions

While questionnaire based tools tests are typically used to assess cognitive performance in observational studies and RCTs, they do have inherent limitations. The use of brain imaging technology is considered a more robust measure of cognitive and brain health as it can overcome the inherent weaknesses of cognitive questionnaire based tools. MRI scans have shown that vitamin B12 status is inversely related to the rate of overall brain atrophy [202], and lower B12 status has been associated with a reduced microstructure integrity of the hippocampus [203], increased rate of brain volume losses [204,205], and a greater severity of white matter lesions [206]. Likewise, lower folate status has also been associated with severe cortical and hippocampal brain atrophy [207] and white matter lesions [161]. Furthermore, the VITACOG trial demonstrated that combined folate, B12 and B6 supplementation over two years markedly reduced (by up to 53%) the rate of brain atrophy. This effect was modulated by baseline homocysteine concentration, with the greatest impact observed in those with concentrations $\geq$ 13 μmol/L [196]. A reduction in cerebral atrophy by as much as seven fold was also observed in grey matter regions, which are the most relevant regions for Alzheimer's disease pathology [188]. More recently, the use of a new functional neuroimaging technique, magnetoencephalography (MEG), has emerged which can map brain activity and assess cognitive processes and specific functions of the brain [208]. MEG is currently being used in some clinical studies, including cognitive studies in ageing, but to date none have examined B-vitamins. Brain imaging could therefore help further elucidate the role and underlying mechanism linking B-vitamins with brain health and disease.

Future studies are therefore warranted to investigate the effects (and interactions) of folate, the related B-vitamins, and relevant genetic variants, particularly in cohorts with lower B-vitamin status. Longitudinal studies are also required to explore differential effects on cognition over time. Targeted RCTs of sufficient duration, with lower doses of B-vitamins at levels achievable through dietary means, are also indicated.

7. Potential Mechanisms Linking B-Vitamins with Diseases of Ageing

Although no clear mechanisms have been established, a variety of biologically plausible mechanisms have been suggested to explain the role of B-vitamins in the diseases of ageing [85,156]. In the brain and other tissues, folate and the metabolically related B-vitamins are required as co-factors for one-carbon transfer reactions, which in turn are fundamental for the synthesis of DNA and RNA nucleotides, the metabolism of amino acids and the maintenance of methylation reactions [6,7]. Thus, the proposed mechanisms by which sub-optimal B-vitamins status or deficiency could contribute to greater risk of cognitive impairment and other diseases of ageing, involve perturbations in the complex regulatory network maintaining one-carbon metabolism [11,12]. This can result in hyperhomocysteinemia [209] and/or reduced methylation status within the relevant tissue [210,211], increased misincorporation of uracil into DNA, and altered RNA [212–214] and neurotransmitter products [215]. Vitamin B6 is important in brain health as pyridoxine is a co-factor in transamination and decarboxylation reactions required for the metabolism of several neurotransmitters, including serotonin, dopamine, and histamine [216]. Elevated homocysteine and/or lower B-vitamins can

also impact on the immune system and cause increased inflammation and antioxidant damage, alone or in synergy, which can have adverse effects on the disease of ageing through the vascular system and atherosclerosis [217–219]. B-vitamins have also been shown to have an indirect role on bone remodelling and protective effects on bone formation which help to maintain bone health in ageing [220–222]. Cognitive deficits and Alzheimer's disease has also been observed in B-vitamin deficient rats and been attributed to abnormal methylation [223,224].

Specific genetic polymorphisms, including the common *MTHFR* 677 C $\rightarrow$ T polymorphism can disrupt normal one-carbon metabolism and thus have an impact on diseases of ageing, independent of homocysteine, as a result of impairments in methylation activity; improved B-vitamin status could help to modulate this risk [13,225,226]. Furthermore, this polymorphism has more recently been associated with blood pressure while riboflavin has emerged as having a novel role in lowering blood pressure in hypertensive patients with the variant *MTHFR* 677TT genotype [110]. The effect of the *MTHFR* 677 C $\rightarrow$ T polymorphism on blood pressure might be mediated by nitric oxide, a potent vasodilator, and riboflavin has been demonstrated as a means to restoring MTHFR activity in vivo [58]. The interaction between the *MTHFR* genotype, B-vitamin status and risk of hypertension requires further exploration.

8. Public Health Implications

Folic acid food fortification was instigated primarily to reduce the prevalence of neural tube defects (NTDs) but it may also have additional benefits for the ageing population. Currently, 80 countries have mandatory folic acid food fortification policies in place, including America and Canada [227]. As a result of fortification, there has been a dramatic fall in NTDs [228] and folate deficiency in the USA and Canada is practically non-existent [34,36,229]. Mandatory folic acid food fortification has also lead to an improvement in stroke mortality in the US and Canada [98] and could have additional beneficial effects in maintaining better cognitive function [230] and bone health [231] in older people. No European country has a mandatory folic acid food fortification policy but most do allow voluntary food fortification. The UK and Ireland have quite liberal voluntary fortification polices and are currently considering mandatory fortification. Voluntary food fortification has resulted in significantly higher dietary intakes and biomarker status of folate and related B-vitamins in Irish adults of all ages [232,233]. The NANS dietary survey however highlighted that the overall 21% of Irish adults who were non-consumers of fortified foods were at higher risk of sub-optimal status of folate and related B-vitamins [233]. Regular fortified food consumption therefore has the potential to improve dietary and biomarker status of B-vitamins but a mandatory food fortification would ensure that the whole population were protected.

Low vitamin B12 status in older people is also a concern. Current dietary recommendations for vitamin B12 range between 2.4 μg/day in the USA [234] and 4 μg/day in Europe [235]. However, the European recommendations do not account for the high prevalence of food-bound malabsorption in older people, whereas people aged $\geq$50 years in the USA are recommended to consume most of their vitamin B12 from crystalline sources (i.e., fortified food and supplements) in order to overcome food-bound B12 malabsorption [234]. The synthetic form of vitamin B12, found in fortified foods and supplements, is freely available and thus has no gastric acid requirement [40]. Furthermore, there was a previous safety concern that the high folic acid intake may potentially mask the anaemia of vitamin B12 deficiency and thus delay diagnosis resulting in irreversible nerve damage [79,236]. Mandatory food fortification in the USA however has not led to these adverse events [237–239]. Therefore, from a public health perspective, there are significant challenges in relation to maintaining vitamin B12 in older people and vitamin B12 food fortification could help address some of these challenges.

Concerns have been raised as regards a possible adverse interaction of high folate with low vitamin B12 leading to an increased risk of cognitive impairment in the USA and Australia [230,240,241], but this is rather controversial. Two studies in the UK and USA failed to detect such an adverse interaction [238,242] and one study in the Netherlands in fact found that this combination (i.e., high

folate and low B12) was associated with a reduced risk of cognitive impairment [243]. Discrepancies among studies in this regard may in part be attributed to the relatively low numbers in most studies with this folate/vitamin B12 combination and the type of biomarker used or the cut off value used to define low status in each study. Additionally, a recent US review concluded that the current observational data was limited and that no RCTs had investigated this folate–B12 interaction in cognitive health [244]. Concerns have also been raised that excessively high folate intakes could have cancer promoting effects in segments of the ageing population [245–247]. A Scientific Advisory Committee on Nutrition (SACN) review in the UK however found insufficient evidence to support the view that mandatory folic acid fortification would promote cancer [248]. Excessive folic acid intakes have also been reported to increase un-metabolized folic acid in the circulation with potential adverse effects on health [34,249], although a recent international review did not find any health concerns even at very high folic acid levels [20]. Suggestions have been made to include both folic acid and vitamin B12 in mandatory food fortification policies, to prevent adverse health outcomes and address food-bound malabsorption but the costs of such an approach may be prohibitive. Furthermore, vitamin B6 and riboflavin status in older people is also of concern and may be much more important than previously appreciated for public health in ageing. Therefore, those contemplating public health issues worldwide need to consider a balanced approach and should endeavour to achieve optimal status of all relevant B-vitamins throughout all stages of life.

9. Conclusions

In summary, folate and the metabolically related B-vitamins, B12, B6 and riboflavin, have a vital role in maintaining one-carbon metabolism and any perturbation in this pathway as a result of low/deficient B-vitamin status can impact on health through a number of related mechanisms. Evidence is accumulating to support the beneficial role of B-vitamins in maintaining cardiovascular, bone and brain health in ageing, with the most at risk sub-populations benefiting from optimising one/more B-vitamins involved in one-carbon metabolism. To date, studies investigating the role of B-vitamins in health and disease have tended to focus predominantly on folate and vitamin B12. Future well designed research is warranted to further investigate the metabolically linked B-vitamins B6 and riboflavin, and should include robust and sensitive measures of B-vitamins as well as novel measures of the health outcome of interest (e.g., imaging techniques to assess brain function). In an era where personalised nutrition has gained much attention, further work is also needed to explore the role of the interaction of the *MTHFR* 677 C $\rightarrow$ T polymorphism with both folate and riboflavin, given that this gene–nutrient interaction has the potential to modulate the risk of disease.

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Review

Nutrition, One-Carbon Metabolism and Neural Tube Defects: A Review

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Abstract: Neural tube defects (NTDs) are a group of severe congenital malformations, induced by the combined effects of genes and the environment. The most valuable finding so far has been the protective effect of folic acid supplementation against NTDs. However, many women do not take folic acid supplements until they are pregnant, which is too late to prevent NTDs effectively. Long-term intake of folic acid–fortified food is a good choice to solve this problem, and mandatory folic acid fortification should be further promoted, especially in Europe, Asia and Africa. Vitamin B2, vitamin B-6, vitamin B-12, choline, betaine and *n*-3 polyunsaturated fatty acids (PUFAs) can also reduce the NTD risk by interacting with the one-carbon metabolism pathway. This suggest that multivitamin B combined with choline, betaine and *n*-3 PUFAs supplementation may have a better protective effect against NTDs than folic acid alone. Genetic polymorphisms involved in one-carbon metabolism are associated with NTD risk, and gene screening for women of childbearing age prior to pregnancy may help prevent NTDs induced by the risk allele. In addition, the consumption of alcohol, tea and coffee, and low intakes of fruit and vegetable are also associated with the increased risk of NTDs, and should be avoided by women of childbearing age.

Keywords: folate; neural tube defects; vitamin B; choline; betaine; one-carbon metabolism; tea; alcohol; coffee

1. Introduction

Neural tube defects (NTDs) are a group of severe congenital malformations. It is estimated that approximately one out of 1000 newborns present with this type of defect [1]. The development and closure of neural tube happen during normal embryogenesis between the 18th and 28th days after fertilization. Failure of the neural tube to close in embryonic development results in NTDs [2]. The etiology of NTDs is still unknown. One possible reason may be the disturbance of the one-carbon metabolism pathway [3,4]. However, there are also folate-resistant NTDs, indicating that folate deficiency is not the unique reason for NTDs, and other potential pathogeneses may also responsible for NTDs. The deficiency of other nutritional factors involved in one-carbon metabolism, such as vitamin B-2, B-6, B-12, choline, betaine, and *n*-3 polyunsaturated fatty acids, may be also associated with NTDs. Genetic factors are another important cause of NTDs. Many loci on genes have been identified as associated with the risk of NTDs, especially on genes involved in the one-carbon metabolism pathway, such as methylenetetrahydrofolate reductase (*MTHFR*) and 5-methyltetrahydrofolate-homocysteine methyltransferase (*MTR*) [5,6].

In this study, we review the research progress on the effect of nutritional factors and genes involved in the one-carbon metabolism pathway on NTDs. This should provide a basis for better nutritional approaches to NTD prevention.

2. B-Vitamins, One-Carbon Metabolism and NTDs

2.1. Folate

Folate, also known as vitamin B-9, plays an important role in the homocysteine (Hcy) metabolism, one-carbon pathway and DNA synthesis (Figure 1). In 1991, the Medical Research Council Vitamin Study Research Group conducted a large-scale randomized controlled trial (RCT) with 1817 women from 33 centers in seven countries to assess the effect of folic acid on the reoccurrence of neural tube defects and found that 4 mg folic acid supplementation per day lowered the risk of NTDs by 72% compared with the control group (without folic acid supplementation) [7]. Another important RCT was conducted by Czeizel et al. in Hungarian women without a history of NTD-affected pregnancy [3]. In that study, no NTD cases occurred in 2104 women who received multivitamins containing 0.8 mg folic acid per day, while six NTD cases occurred in 2052 women who received only trace elements and vitamin C. In 1999, Berry et al. conducted a cohort intervention study in an area of China with high rates of neural tube defects (the northern region) and one with low rates (the southern region), and found that preconceptional supplementation (starting supplementation before the last menstrual period before conception and stopping at the end of the first trimester) of 400 μg folic acid (a synthetic form of folate) per day reduced the NTD risk by 85% in the northern subgroup of 31,960 women and by 40% in the southern subgroup of 215,871 women [4]. These results of intervention studies have provided the most persuasive evidence for the protective effect of folic acid against NTDs.

In 1995, a case-control study by Daly first reported a dose-response relationship between the red blood cell folate concentration of mothers and the risk of NTDs, and that a red blood cell folate concentration ≥906 nmol/L (400 ng/mL) can provide optimal protection against NTDs [8]. In 2014, Crider et al. analyzed data from two intervention studies in Chinese women (400 μg folic acid per day from preconception through the end of the first trimester) by a Bayesian model, and found a dose-response relationship between maternal red blood cell folate and the risk of NTDs: a folate concentration >1000 nmol/L substantially reduced the risk of NTDs [5]. This was consistent with the result of Daly's study. Based on these results, in 2015 the World Health Organization (WHO) recommended a threshold value of the maternal red blood cell folate concentration of 906 nmol/L (400 ng/mL) to prevent NTDs. Folic acid supplementation is a solution for the insufficient dietary intake of folate. A daily intake of 0.4 mg (at least one month before conception through the first three months of conception) folic acid is recommended by the Centers for Disease Control and Prevention for women who do not have a history of a previous NTD-affected pregnancy; this dose can prevent 50% of NTDs [9]. As for women who have had a previous NTD-affected pregnancy, the dose of folic acid is increased to 4 mg per day (at least one month before pregnancy through the first three months of pregnancy) [9]. Although recommendations for preconceptional folic acid supplementation have existed for decades, only a small number of women were actually supplemented with folic acid before conception [10]. Most women started folic acid supplementation when they knew they were pregnant, and this was often too late for the effective prevention of NTDs [11]. Long-term intake of folic acid–fortified foods should complement preconceptional folic acid supplementation. Arth et al. reported that mandatory folic acid fortification of wheat and maize flour prevented 13.2% folic acid–preventable NTDs (35,500 of approximately 268,700 global cases) in 58 countries [12]. Seventy-eight countries have fortified flour with folic acid mandatorily, while most countries in Asia, Europe and Africa do not mandate folic acid fortification [13]. Khoshnood et al. conducted an observational study to assess the long-term trend in NTD prevalence in 19 European countries and found that, without mandatory folic acid fortification, the long-existent recommendation for preconceptional folic acid supplementation and voluntary folic acid fortification did not significantly decrease the prevalence of NTDs [14]. In 2016, an intervention study in 16,648 women in Shanxi,

China (one of the most NTD-affected regions, with a 13.8‰ to 19.9‰ prevalence), found that folic acid–fortified flour decreased the NTD burden by 58.5%, which has informed the future implementation of mandatory folic acid fortification in China [15]. Further promotion of mandatory folic acid fortification is needed to prevent NTDs.

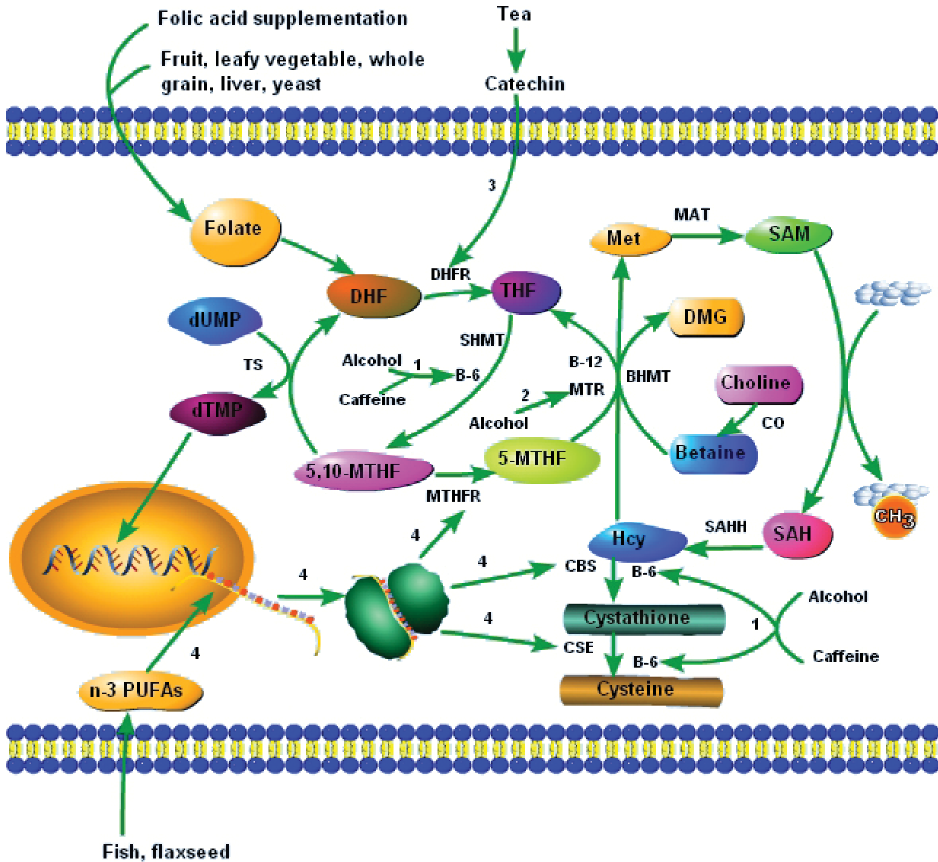

Figure 1. B vitamins and other dietary factors interact with the one-carbon metabolism to influence the development of NTDs. 1, alcohol and caffeine lower vitamin B-6, and thus disturb vitamin B-6–dependent one-carbon metabolism pathways; 2, alcohol reduces the activity of *MTR*, leading to increased Hcy and reduced SAM; 3, catechins in tea reduce the activity of *DHFR*, and hinder the synthesis of THF; 4, *n*-3 PUFAs increase the mRNA expression of enzymes involved in one-carbon metabolism, such as *MTHFR*, *CBS*, and *CSE*. Abbreviations: DHF, dihydrofolate; THF, tetrahydrofolate; 5,10-MTHF, 5,10-methylenetetrahydrofolate; 5-MTHF, 5-methyltetrahydrofolate; dUMP, deoxyuridine monophosphate; dTMP, thymidinemonophosphate; Hcy, homocysteine; Met, methionine; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DMG, dimethylglycine; *DHFR*, dihydrofolate reductase; *SHMT*, serine hydroxymethyltransferase; *MTHFR*, methylenetetrahydrofolate reductase; *MTR*, 5-methyltetrahydrofolate-homocysteine methyltransferase; *TS*, thymidylate synthase; *BHMT*, betaine-homocysteine methyltransferase; *MAT*, methionine adenosyltransferase; *SAHH*, *S*-adenosylhomocysteine hydrolase; *CBS*, cystathionine-beta-synthase; *CSE*, cystathionine-gamma-lyase.

Several genes involved in the folate-dependent one-carbon metabolism have been shown to be associated with the risk of NTDs. One of the most important is the gene encoding *MTHFR*, an enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-MTHF) to 5-methyltetrahydrofolate (5-MTHF) and provides the methyl group needed for remethylation of Hcy to form methionine (Met) (Figure 1) [16]. The T allele of *MTHFR* 677C>T is associated with an increased risk of NTDs in the western population [17–19]. Similar observations are made in Chinese mothers even when they supplement with folic acid [5,16,20]. Two considerations may help explain this association between *MTHFR* 677C>T and NTDs. On the one hand, the TT genotype of *MTHFR* 677C>T can attenuate the plasma and red blood cell (RBC) folate response to folic acid supplementation [21]. In addition, previous studies have reported that the T allele carriers of *MTHFR* 677C>T had lower folate concentrations than non-carriers [18,22]. On the other hand, the *MTHFR* 677C>T mutation is associated with reduced *MTHFR* activity [18,23]. *MTHFR* 1298A>C is yet another mutation associated with decreased *MTHFR* activity [23]. However, the link between *MTHFR* 1298A>C and NTDs remains controversial: one study in Italy reported that the C allele of *MTHFR* 1298A>C was associated with a higher risk of NTDs [24], several other studies found no significant association between *MTHFR* 1298A>C and NTDs [6,25–27], while one study in China even found that the C allele of *MTHFR* 1298A>C had a protective role against NTDs [28].

In addition to *MTHFR*, other genes involved in the folate metabolism have been demonstrated to influence the development of NTDs in the Chinese. *MTR* is an enzyme that catalyzes the remethylation of Hcy to Met and is dependent on the provision of methyl groups from 5-MTHF (Figure 1). The mutation of *MTR* is associated with the increased risk of NTDs [6,19,29,30]. Solute carrier family 19 member 1 (*SCF19M1*) can transport folate into cells. The mutation of *SCF19M1* is associated with increased NTD risk even when mothers are supplemented with folic acid [6]. In addition, mutations on *BHMT* [6,19,31], *CBS* [19,32], *MTRR* [19,33,34], *MTHFD1* [19,35–41], *MTHFD2* [19], *SHMT1* [36], *FOLH1* [42], *RFC1* [43,44], *SARDH* [45], *PEMT* [40], *GART* [40] and *TYMS* [19,36] have also been reported to be associated with the risk of NTDs.

2.2. Vitamins B-2, B-6 and B-12

Vitamin B-12 is the cofactor of *MTR* (Figure 1). Additionally, B-12 deficiency is associated with elevated Hcy [46,47]. In case-control studies, vitamin B-12 status has been found to be protective against NTDs in the Chinese [47,48]. According to a study in 1170 women in northwest China, the prevalence of vitamin B-12 deficiency was 45% [49], indicating that vitamin B-12 supplementation maybe also be needed to prevent NTDs in China. The negative association between vitamin B-12 and the risk of NTDs is also observed in other populations [50–52], and remains significant even in folic acid–fortified populations [53,54]. Transcobalamin II (*TCN2*) is a carrier protein that can bind vitamin B12. The mutation of *TCN2* is associated with an increased NTD risk even when mothers are supplemented with folate [6]. *CUBN* is a gene that encodes the intestinal receptor responsible for the uptake of the vitamin B12–intrinsic factor complex. The mutation of *CUBN* is also associated with the risk of NTDs [40,55]. This genetic evidence also demonstrates the role of vitamin B-12 in the development of NTDs.

Besides folate and vitamin B-12, vitamin B-2 and B-6 are also important enzyme cofactors involved in the one-carbon metabolism. The conversion of 5,10-MTHF to 5-MTHF is catalyzed by *MTHFR* and depends on FADH2, the hydroquinone form of flavin adenine dinucleotide (FAD) (a derivative of vitamin B-2). Hustad et al., in a cross-sectional study, found that plasma vitamin B-2 was negatively associated with Hcy [56]. An intervention study by McNulty et al. in healthy adults found that vitamin B-2 supplementation lowered the concentration of Hcy only in subjects with a TT genotype of *MTHFR* 677C>T, but there was no effect in CC or CT genotypes [57]. In addition, vitamin B-2 can interact with folate to modulate Hcy concentrations. One intervention study found that folic acid supplementation (400 μg/day) had a greater Hcy-lowering effect in subjects with a high plasma level of vitamin B-2, and this effect was unrelated to *MTHFR* 677C>T polymorphism [58]. Several intervention studies

have found that preconceptional multivitamin supplementation (including vitamin B-2 and several other vitamins such as folic acid, vitamin B-6, vitamin B-12, vitamin E, thiamin, vitamin A, vitamin D, nicotinamide, and ascorbic acid) could reduce the risk of NTDs [3,59]. Vitamin B-6 is the cofactor for betaine-Hcy methyltransferase (*BHMT*) (an enzyme that catalyzes the remethylation of Hcy to Met with betaine providing the methyl needed), cystathionine-beta-synthase (*CBS*) (an enzyme that catalyzes the reaction from Hcy to cystathionine) and cystathionine-gamma-lyase (*CSE*) (an enzyme that catalyzes the reaction from cystathionine to cysteine) (Figure 1). Vitamin B-6 deficiency is also associated with increased Hcy [46]. A French case-control study found that maternal plasma vitamin B-6 was negatively associated with NTD risk [33]. Thus, supplementation of vitamin B-2, vitamin B-6 and vitamin B-12 together with folic acid may have a better protective effect against NTDs than folic acid supplementation alone. However, this still needs to be demonstrated by well-designed intervention studies.

2.3. Potential Adverse Effects of B Vitamins

Despite the beneficial effect for NTD prevention, observational studies showed that very high doses of folic acid supplementation during conception also have several adverse effects. However, the evidence is still inconsistent. Observational studies showed that folic acid supplementation during conception was associated with the increased risk of wheezing in children through 18 months of age (dose was not reported) [60] and the increased risk of infant asthma (>72,000 μg·day) [61]. Evidence from observational studies showed that folic acid supplementation was associated with the increased risk of infant clefts (dose was not reported) [62] and spontaneous preterm delivery (mean dose (interquartile range): 313 (167–558) μg/day) [63]. Valera-Gran et al. found that high folic acid supplementation for mothers during conception (>5000 μg/day) had a detrimental effect on the psychomotor development of children [64]. However, Tolarova et al. found that preconceptional supplementation of 10 mg/day folic acid plus multivitamins significantly reduced the risk of infant clefts [65]. One population-based cohort study in China found that preconceptional folic acid supplementation (400 μg/day) significantly reduced the risk of spontaneous preterm delivery [66]. McGarel et al. reported that folic acid supplementation had a beneficial effect on brain development and cognitive performance [67]. The FDA's safe upper limit of folic acid is 1000 μg [68]. Folic acid is the synthetic form of folate. Before entry into the circulation system, folic acid undergoes reduction (by DHFR) and methylation to 5-MTHF, the circulating form of folic acid. However, when folic acid supplementation exceeds a certain dose, other transport mechanisms, such as passive diffusion, will complement the normal absorption mechanism, and thus unaltered folic acid enters the circulation system [69]. That is unmetabolized folic acid. One acute study found that folic acid supplementation of more than 800 μg/day can cause unmetabolized folic acid accumulation in the serum, but when the dose was no more than 400 μg/day, the unmetabolized folic acid in the serum was undetectable [69]. A prospective study of pregnant Canadian women found that unmetabolized folic acid was detectable in more than 90% of maternal and cord plasma samples, which may be a result of excess folic acid supplementation [70]. One RCT found that folic acid supplementation with a dose of 400 μg/day during conception had no significant influence on the unmetabolized folic acid concentration in maternal plasma or newborn cord blood plasma [71]. Therefore, a dose of 400 μg/day is safer than 1000 μg/day for folic acid supplementation.

Bailey reported that a higher vitamin B-6 intake (>1.85 mg/day) of mothers during the last six months of pregnancy was associated with a higher risk of childhood lymphoblastic leukemia [72]. However, one case-control study found that the per 1 mg increase of preconceptional vitamin B-6 intake from food and supplements was associated with a 11% decreased risk of childhood acute lymphoblastic leukemia. As suggested by US authorities, the level of vitamin B-6 with no observed adverse effect is set at 200 mg per day while the safe upper limit is at 100 mg per day [73]. Supplementation of vitamins B-2 and B-12 has not been associated with any adverse effects. Additionally, there is still insufficient evidence to set safe upper intake levels for vitamin B-2 and vitamin B-12 [74].

3. Choline, Betaine, One-Carbon Metabolism and NTDs

Choline is a nutrient associated with NTDs. Food sources of choline are principally those rich in lecithin (phosphatidylcholine) such as eggs and soy beans [75]. Betaine, which may be derived from choline, is found mostly in green leafy vegetables and beets (root vegetables). Choline can be synthesized in vivo by the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) [76]. However, its biosynthesis is limited and dietary intake of choline is necessary [76]. Choline can be metabolized to betaine, catalyzed by choline oxidase (Figure 1). Similar to 5-MTHF, betaine can also provide the methyl needed in the remethylation of Hcy to Met, a reaction catalyzed by *BHMT* (Figure 1). The mutation of *BHMT* has been demonstrated to be associated with the risk of NTDs [6,19,31]. The ability of betaine supplementation to lower Hcy has been reported in the Netherlands [77]. Abnormal choline metabolism can lead to NTDs in the mouse [78]. An observational study found that preconceptional dietary intake of choline and betaine was negatively associated with the risk of fetal NTDs independent of folate intake in Americans [79].

However, choline and betaine intake can adversely affect serum lipid concentrations, such as increase total serum cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triacylglycerol. Through the gut microbiome and trimethylamine production, they can also increase the risk of atherosclerotic vascular disease [80]. The safe upper level of choline may be 3500 mg/day for people with an age $\geq$19 years [81]. The safe upper level of betaine is unknown.

4. Other Dietary Factors Interact with the One-Carbon Metabolism to Influence the Development of NTDs

A case-control study has found that preconceptional tea consumption increases the risk of NTDs in Shanxi, China [82]. Similar findings have been observed in a case-control study in Atlanta [83]. In another case-control study conducted in the US, tea consumption was not associated with the risk of NTDs; however, when subjects were divided into subgroups according to the dose of folic acid intake, tea consumption was associated with an increased risk of NTDs in subjects with a folic acid intake >400 μg, and the authors suggested that tea consumption might interact with the folate metabolism pathway to influence the occurrence of NTDs [84]. The effects of tea consumption on the blood folate level are controversial [85,86]. One study has reported a lowering effect of tea consumption on blood folate [86]. A related case-control study found an association between the polymorphisms of catechol-*O*-methyltransferase (*COMT*) (encoding the enzyme that catalyzes the methylation of catecholamines with S-adenosylmethionine (SAM), a methyl donor) and the risk of NTDs in the Chinese: the mutant homozygotes of rs73,785 or rs4633 had a higher risk of NTDs than the wild homozygotes did, while the heterozygotes of rs4680 had a lower risk of NTDs than the wild homozygotes did, and the rs4680 genotype interacted with tea drinking to alter the risk of NTDs [87]. The effect of tea drinking on the development of NTDs can be explained by an inhibitory effect of tea catechins on dihydrofolatereductase [88]. Dihydrofolatereductase (*DHFR*) is an enzyme that catalyzes the conversion of dihydrofolate (DHF) to tetrahydrofolate (THF), the active form of folate. THF plays an important role in homocysteine metabolism and thymidine monophosphate (dTMP) synthesis. In summary, tea catechins inhibit the activity of *DHFR*, thus blocking DNA synthesis and homocysteine clearance, and this may help explain the association between tea consumption and the risk of NTDs (Figure 1).

One case-control study in Italians found that alcohol, low fruit and vegetable intake, and coffee were associated with an increased risk of NTDs [89]. Alcohol consumption is associated with lower blood folate as well as pyridoxal 5′-phosphate (the active form of vitamin B-6) and higher Hcy [90]. Animal studies showed that alcohol consumption can reduce the level of SAM (the major methyl donor) [91,92] and the activity of *MTR* (the enzyme catalyzing the remethylation of Hcy to Met) (Figure 1) [93]. Fruit and vegetables are rich in folate, and thus a low intake of these foods may be associated with inadequate folate intake (Figure 1). In pregnant Japanese women, it has been found that caffeine intake is associated with elevated Hcy only in subjects with a high intake of vitamin B-6 [94].

The positive association of caffeine with the level of Hcy and the risk of NTDs can be attributed to it having a similar chemical structure to theophylline, which can decrease pyridoxal 5′-phosphate (the active form of vitamin B-6) by acting as an inhibitor of pyridoxal kinase (Figure 1) [95,96].

In addition, *n*-3 PUFAs can lower Hcy [97–100], and upregulate the expression of several enzymes involved in the one-carbon metabolism, such as *MTHFR*, *CBS*, and *CSE* (Figure 1) [101]. Our unpublished case-control study in the Chinese has found that placental C18:3*n*-3, C20:5*n*-3 and C22:5*n*-3 are negatively associated with fetal NTD occurrence. The meta-analysis indicates that *n*-3 PUFAs combined with vitamin B supplementation have a greater Hcy-lowering effect than *n*-3 PUFAs alone [100]. However, there has been a concern that high-dose *n*-3 PUFA supplementation might induce bleeding [102]. However, there is little evidence to support this [103–105]. Previous studies showed that marine-derived *n*-3 PUFAs supplementation can promote LDL oxidation [106], but this is still controversial [107].

5. Perspective and Prospects

Although preconceptional folic acid supplementation has been recommended for decades, its overall ability to reduce the prevalence of NTDs is limited. Many women do not take a folic acid supplement until they are pregnant, which is too late for the effective prevention of NTDs. Long-term intake of folic acid–fortified foods should complement preconceptional folic acid supplementation. However, mandatory folic acid fortification is still not universally in place in Europe, Asia or Africa. However, folate-resistant NTDs exist, so there are other reasons for NTDs other than folate deficiency. In recent years, studies have indicated that the low intake and status of vitamin B-2, B-6, B-12, choline, betaine or *n*-3 PUFAs, and the consumption of alcohol, tea, or coffee are also associated with an increased risk of NTDs. Further cohort and intervention studies are needed to demonstrate whether multivitamin B (folate, vitamin B-2, B-6, B-12) combined with choline, betaine and *n*-3 PUFAs, or simply a biodiverse diet, has a better protective effect against NTDs than folic acid alone. In addition, genetic factors play an important role in the development of NTDs. There are many genetic variants involved in the one-carbon metabolism demonstrated to be associated with the risk of NTDs. Some variants can increase the risk of NTDs regardless of folic acid supplementation. Therefore, gene screening of women of childbearing age prior to pregnancy could enhance efforts to prevent NTDs.

6. Conclusions

Further cohort and intervention studies are needed to demonstrate whether multivitamin B (folate, vitamin B-2, B-6, B-12) combined with choline, betaine and *n*-3 PUFAs supplementation, or a biodiverse diet, has a better protective effect against NTDs than folic acid alone. Mandatory folic acid fortification and nutrition education, targeted at women in the reproductive age group, should be promoted and gene screening for women of childbearing age prior to pregnancy should be made available to prevent NTDs. These strategies would help decrease the burden of this oppressive health problem, especially in high-risk populations.

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Effects of Two-Year Vitamin B_{12} and Folic Acid Supplementation on Depressive Symptoms and Quality of Life in Older Adults with Elevated Homocysteine Concentrations: Additional Results from the B-PROOF Study, an RCT

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Abstract: Lowering elevated plasma homocysteine (Hcy) concentrations by supplementing vitamin B_{12} and folic acid may reduce depressive symptoms and improve health-related quality of life (HR-QoL) in older adults. This study aimed to test this hypothesis in a randomized controlled trial. Participants (N = 2919, ≥65 years, Hcy concentrations ≥12 μmol/L) received either 500 μg vitamin B_{12} and 400 μg folic acid daily or placebo for two years. Both tablets contained 15 μg vitamin D_3. Depressive symptoms were measured with the Geriatric Depression Scale-15 (GDS-15). HR-QoL was assessed with the SF-12 Mental and Physical component summary scores and the EQ-5D Index score and Visual Analogue Scale. Differences in two-year change scores were analyzed with Analysis of Covariance (ANCOVA). Hcy concentrations decreased more in the intervention group, but two-year change scores of the GDS-15 and three of four HR-QoL measures did not differ between groups. The EQ-5D Index score declined less in the intervention group than in the placebo group

(mean change 0.00 vs. −0.02, p = 0.004). In conclusion, two-year supplementation with vitamin B_{12} and folic acid in older adults with hyperhomocysteinemia showed that lowering Hcy concentrations does not reduce depressive symptoms, but it may have a small positive effect on HR-QoL.

Keywords: homocysteine; hyperhomocysteinemia; depressive symptoms; quality of life; vitamin B_{12}; folic acid; older adults; randomized controlled trial

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1. Introduction

Depression is a leading cause of disability worldwide and induces substantial individual and societal burden [1]. In addition, health-related quality of life (HR-QoL) can be compromised by both mental disorders and reduced physical functioning [2,3]. Depression and low HR-QoL increase the risk of institutionalization and mortality [4]. Especially in older adults, management of depression is often suboptimal, for instance due to side-effects of antidepressant medication or interactions with other drugs [5]. Such complexities emphasize the need for simple and safe interventions for both the prevention and treatment of depression.

Elevated plasma homocysteine (Hcy) concentrations are common in older adults [6,7]. Hyperhomocysteinemia has been associated with various adverse health conditions, including cardiovascular disease [6], fractures [7], dementia [8], decreased physical functioning [9], and mortality [10]. In addition, observational studies suggest a link between elevated Hcy concentrations and depressive symptoms [11–14] and lower HR-QoL [15,16]. A meta-analysis of nine observational studies showed that persons with Hcy ≥12.5 μmol/L had a 70% higher risk of prevalent depression than persons with Hcy concentrations <12.5 μmol/L [17]. Supplementation with vitamin B_{12} and folic acid decreases Hcy concentrations [18] and may thereby reduce depressive symptoms and improve HR-QoL. However, trials investigating these effects are scarce and show inconsistent results [16,19–24]. Heterogeneity in study duration, study samples, and supplement dose may explain the observed differences.

Insufficient amounts of folate and vitamin B_{12} limit the conversion of Hcy into methionine. Methionine is a direct precursor of *S*-adenosylmethionine (SAM). SAM plays an important role in the methylation of neurotransmitters involved in depression, such as serotonin, dopamine, and noradrenalin [25,26]. In accordance with this, lower concentrations of SAM and monoamine neurotransmitter metabolites were observed in the cerebrospinal fluid of severely depressed patients who had high Hcy concentrations, compared to similar patients who did not have elevated Hcy concentrations [27].

The current amount of evidence regarding the role of Hcy, vitamin B_{12}, and folic acid in depression and HR-QoL is limited. To gain more insight into these complex relationships, we investigated a large sample of older adults with mildly elevated homocysteine concentrations both cross-sectionally and after two years of supplementation with vitamin B_{12} and folic acid. It was hypothesized that Hcy is positively associated with depressive symptoms, and inversely associated with HR-QoL at baseline, and that supplementation with vitamin B_{12} and folic acid decreases Hcy concentrations, thereby reducing depressive symptoms and improving HR-QoL.

2. Materials and Methods

2.1. Study Design and Participants

Data from the B-vitamins for the PRevention Of Osteoporotic Fractures (B-PROOF) study were used for the present study. The B-PROOF study is a multi-center, randomized, parallel-group, double-blind, placebo-controlled intervention trial investigating the effect of two-year daily vitamin B_{12} (500 μg) and folic acid (400 μg) supplementation versus placebo on fracture incidence in a large sample

of older adults [28,29]. Depressive symptoms and HR-QoL were predefined secondary outcome measures of the B-PROOF study.

The B-PROOF study included 2919 older adults (≥65 years) from the general population. Participants were recruited from three regions in The Netherlands: Wageningen, Amsterdam, and Rotterdam. Participants were included if they had elevated Hcy concentrations (12–50 μmol/L). Exclusion criteria included cancer diagnosis within the last 5 years (except for non-melanoma skin cancer), being bedridden, serum creatinine concentration of >150 μmol/L, current or recent (<4 months) intramuscular injections of vitamin B_{12}, use of high-dose folic acid supplements (>300 μg per day), and participation in other intervention studies. Detailed information concerning recruitment, participants, and study procedures has been extensively reported elsewhere [28].

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Medical Ethics Committee of Wageningen University. Local feasibility was approved by the Medical Ethics Committees of Erasmus Medical Center Rotterdam and VU University Medical Center Amsterdam. Written informed consent was obtained from all participants prior to the start of the intervention. The B-PROOF trial is registered with clinicaltrials.gov as NCT00696514 and with the Netherlands Trial Register as NTR1333.

2.2. Intervention

Participants were randomized in a 1:1 ratio to either the intervention group or the placebo group. Randomization was performed by an independent person by means of computer-generated numbers in stratified permuted blocks of size 4, stratified by sex, age (65–80 years, ≥80 years), study center, and Hcy concentrations (12–18 μmol/L, ≥18 μmol/L). Both groups received a daily oral tablet for a duration of two years. The tablets of the intervention group contained 500 μg vitamin B_{12}, 400 μg folic acid, and 15 μg vitamin D_3 (cholecalciferol). The placebo tablets contained 15 μg vitamin D_3. Tablets were similar in appearance, smell, and taste. Vitamin D_3 was added to both types of tablets to ensure adequate vitamin D concentrations, which was of importance for the primary outcome measure of the B-PROOF study: fracture incidence. At baseline and at the end of the trial, a structured interview took place, including questionnaires and physical measurements.

2.3. Outcomes

2.3.1. Depressive Symptoms

The Geriatric Depression Scale-15 (GDS-15) was used to assess depressive symptoms at baseline and after two years of supplementation. The GDS-15 is a short version of the original 30-item GDS [30] and is a widely used instrument that displays good psychometric properties in various elderly populations [31,32]. Scores range from 0 to 15, with higher scores indicating more symptoms. A score of 5 or higher indicates the presence of clinically relevant depressive symptoms [31].

2.3.2. Health-Related Quality of Life

HR-QoL is a multidimensional construct. We therefore used both the 12-item Short Form Health Survey (SF-12) [3] and the EuroQol 5 Dimensions (EQ-5D) [33] to take into account different aspects of HR-QoL (mental, physical, and general HR-QoL). Both questionnaires are self-rated, widely used, have been validated in various populations, have good test-retest reliability [3,33], and they complement each other [34].

The SF-12 is derived from the SF-36 questionnaire [35] and assesses eight health aspects: physical functioning, bodily pain, role limitations due to physical problems, general health, vitality, social functioning, role limitations due to emotional problems, and mental health. Mental (MCS) and physical (PCS) component summary scores were calculated. These scores were standardized to US general population norms [36], as Dutch norms are currently not available. Scores range from 0 to 100,

with 0 representing lowest HR-QoL and 100 indicating best possible HR-QoL, with a normalized mean around 50 and a standard deviation (SD) of 10.

The EQ-5D covers five dimensions of HR-QoL: mobility, self-care, usual activities, pain/discomfort, and anxiety/depression [33]. A standardized index score (EQ-5D Index) was calculated based on Dutch norm data, ranging from −0.33 to 1, with higher scores indicating better HR-QoL [36]. Finally, the visual analogue scale of the EQ-5D (EQ-5D VAS) was used to indicate current health status on a visual scale ranging from 0 to 100, with higher scores representing better HR-QoL.

2.4. Baseline Characteristics

Information about marital status, highest level of completed education, alcohol intake (light, moderate, excessive/very excessive) [37], smoking habits (never, former, current), and physical activity [38] was obtained through structured questionnaires. Weight was measured using a calibrated scale. Height was measured with a stadiometer. Subsequently, body mass index (BMI) was calculated as weight/height^2 (kg/m^2). The Mini-Mental State Examination (MMSE) was used to assess global cognitive function (score range: 0–30) [39].

2.5. Biochemical Analyses

Venous blood samples were obtained from participants in a fasted state or after a restricted light breakfast. Samples were stored at −80 °C until analysis. Plasma Hcy concentrations were measured at baseline and after two years using either the Architect i2000 RS analyzer (VU University Medical Center; Abbott Diagnostics, Wiesbaden, Germany, intra-assay CV: 2%, inter-assay CV: 4%), HPLC method (Wageningen University, intra assay CV: 3.1%, inter assay CV: 5.9%), or liquid chromatography tandem mass spectrometry (LC-MS/MS) method (Erasmus Medical Center Rotterdam; CV: 3.1%). Cross-calibration showed that the outcomes of the three centers did not differ significantly. Serum folate and three markers of vitamin B_{12} status, i.e., serum vitamin B_{12}, serum holotranscobalamin (HoloTC), and serum methylmalonic acid (MMA), were measured at the Erasmus Medical Center at baseline. Folate and serum vitamin B_{12} were assessed with an electrochemiluminescence immunoassay (Elecsys 2010, Roche GmbH, Mannheim, Germany) (CV folate: 5.9% at 5.7 nmol/L and 2.8% at 23.4 nmol/L; CV vitamin B_{12}: 5.1% at 125 pmol/L and 2.9% at 753 pmol/L). Serum HoloTC was assessed with the AxSYM analyser (Abbott Diagnostics, Wiesbaden, Germany) (CV: <8%) and serum MMA was measured by LC-MS/MS.

2.6. Statistical Analyses

Data were analyzed using SPSS version 22 (SPSS Inc., Chicago, IL, USA). A two-sided *p*-value of <0.05 was regarded as statistically significant. Data are reported as *N* (%), or as median (interquartile range (IQR)). Baseline characteristics were compared between treatment groups using non-parametric Mann-Whitney tests or Pearson Chi-square tests. Participants who dropped out during the study were compared to participants who completed the study with respect to age, sex, baseline Hcy concentration, depressive symptoms, and HR-QoL.

2.6.1. Cross-Sectional Analyses

The GDS-15 and HR-QoL scores were non-normally distributed, even after transformation. Therefore, baseline associations of Hcy concentrations with depressive symptoms and HR-QoL were examined using Cox regression analyses with fixed time points. In this analysis, a constant risk period was assigned (time was fixed to 1). This technique was chosen because the obtained hazard ratio from this analysis can be interpreted as a risk ratio (RR) [40]. In case of a high prevalence of the condition of interest, this is a more conservative estimate of the true effect than an odds ratio (OR). Participants with a GDS-15 score of ≥5, indicating clinically relevant depressive symptoms [31], and participants who scored in the lowest quartile of the HR-QoL measures were considered 'cases'. It was examined whether higher Hcy concentrations were associated with a higher risk of having depressive symptoms

or low HR-QoL. Analyses were performed without adjustments (crude model), adjusted for age, sex, and study center (Model 1), and additional adjustments for education level, smoking behavior, alcohol consumption, creatinine concentrations, BMI, and MMSE (Model 2). These covariates were added because of their possible influence on both the determinant (Hcy) and the outcomes (depressive symptoms, HR-QoL) [2,41,42].

2.6.2. Effect Study

We performed both intention-to-treat analyses, including all participants who completed baseline and follow-up measurements, and per-protocol analyses, including all participants who were compliant with the study protocol (≥80% of tablet intake in two years). The score distributions of the outcome variables violated normality assumptions.

Depressive symptoms—total sample: Due to the high number of null scores on the GDS-15 in the study sample, no change scores were calculated as this would lead to interpretation difficulties. Instead, to examine whether the treatment groups differed in number of persons with depressive symptoms after two years, logistic regression analyses were performed. The dichotomized follow-up GDS-15 score (0–4: no depressive symptoms; ≥5: depressive symptoms) [31] was taken as the outcome measure and treatment group as the independent variable. The dichotomized baseline GDS-15 score was added as a covariate to control for baseline values. Age, sex, study center, and baseline Hcy were added as covariates in a second, adjusted model as randomization was stratified based on these variables.

Depressive symptoms—subsample: To further examine the participants with depressive symptoms, a subgroup analysis was performed. Two-year change scores were calculated for participants who scored GDS-15 ≥5 at baseline and differences in change scores between treatment groups were analyzed using Analysis of Covariance (ANCOVA), with the GDS-15 change scores as the outcome measure, treatment as the fixed between-subjects factor, and baseline GDS-15 scores as a covariate. Age, sex, study center, and baseline Hcy were added as covariates in a second, adjusted model.

HR-QoL: For the HR-QoL analyses, two-year change scores were calculated for the four measures of HR-QoL (SF-12 MCS, SF-12 PCS, EQ-5D Index, and EQ-5D VAS). Subsequently, ANCOVA analyses were performed, similar to the GDS-15 subgroup analyses.

For all outcome measures, predefined interaction terms of treatment with sex, dichotomous age (65–80 and ≥80 years), and dichotomous Hcy concentration (<18 and ≥18 μmol/L) were examined in the adjusted models. The Hcy cutoff value of 18 μmol/L was chosen because stratification of the participants was also performed according to this value. If interaction terms had a *p*-value of <0.10, stratified subgroup analyses were performed. In explorative post-hoc analyses, interactions of the treatment group with plasma concentrations of vitamin B_{12}, HoloTC, and MMA (all indicators of vitamin B_{12} status) and folate were tested to investigate whether these baseline plasma concentrations influenced treatment effects. These variables were dichotomized at the median value (results shown in Table A1 in Appendix A).

As a final check of the effect, it was investigated whether change in Hcy concentration was associated with depressive symptoms and/or HR-QoL over time, irrespective of the treatment group. For this purpose, linear regression analyses were performed with two-year change in Hcy as the predictor and two-year change in the GDS-15 (≥5 subgroup) and HR-QoL measures as the outcome. These analyses were adjusted for baseline values of Hcy and GDS-15/HR-QoL (crude model), additionally adjusted for age, sex, and study center (Model 1), and additionally adjusted for education level, smoking behavior, alcohol consumption, creatinine concentrations, BMI, and MMSE (Model 2).

3. Results

3.1. Sample Characteristics

The baseline characteristics per treatment group are shown in Table 1. Median age of the total group was 73 years (IQR: 69–78 years) and was similar in both treatment groups. A similar number

of women was included in the two groups (50%). Only HoloTC differed significantly between the groups, with the intervention group having slightly higher baseline HoloTC concentrations (median: 65, IQR: 48–86) than the placebo group (median: 63, IQR: 45–84; $p = 0.03$). The median GDS-15 score was 1 (IQR: 0–2), and 7% of the participants had a GDS score ≥5 at baseline ($N = 200$), indicating clinically relevant depressive symptoms (similar numbers in both groups). At follow-up, slightly more participants reported depressive symptoms (9%, $N = 223$, also similar in both groups). At baseline, 210 participants (7.3%) had a vitamin B_{12} deficiency (serum vitamin B_{12} < 150 pmol/L [43]) and 89 participants (3.1%) had a folate deficiency (serum folate <10 nmol/L [34]).

Compliance to the treatment was high; mean tablet intake was 90.1% in two years and 83.8% of participants had a tablet intake of ≥80% (similar in both treatment groups). The dropout percentage was 15.2% ($N = 222$) in the B-vitamin group and 13.7% ($N = 200$) in the placebo group. This group difference was not statistically significant ($p = 0.27$). Persons who dropped out were significantly older ($p < 0.001$), were more often female ($p = 0.01$), had higher baseline Hcy concentrations ($p < 0.001$), more depressive symptoms ($p < 0.001$), and lower scores on all four HR-QoL measures (SF-12 MCS: $p = 0.03$; SF-12 PCS: $p < 0.001$; EQ-5D Index: $p = 0.02$; EQ-5D VAS: $p < 0.001$) compared to persons who completed the study. Detailed information regarding dropout has been extensively reported elsewhere [29].

Table 1. Baseline characteristics of the two treatment groups (total $N = 2919$).

	Intervention (*N* = 1461)	**Placebo (*N* = 1458)**	***p***
Descriptive variables:			
Women	736 (50)	724 (50)	0.70
Age (years)	73 (69–78)	73 (69–78)	0.38
Education (years)	9 (6–15)	9 (6–15)	0.59
Study location:			0.91
WU (Wageningen)	426 (29)	431 (30)	
Erasmus MC (Rotterdam)	649 (44)	636 (44)	
VUmc (Amsterdam)	386 (26)	391 (27)	
Smoking			0.97
Current	139 (10)	142 (10)	
Former	828 (57)	821 (56)	
Never	494 (34)	495 (34)	
Alcohol use:			0.31
Light	994 (68)	972 (67)	
Moderate	417 (29)	422 (29)	
Excessive/very excessive	50 (3)	62 (4)	
Body mass index, kg/m^2	26.7 (24.6–29.2)	26.6 (24.6–29.4)	0.65
Physical activity (kcal/day)	546 (335–823)	556 (347–831)	0.32
MMSE (score 0–30)	28 (27–29)	28 (27–29)	0.10
Depressive symptoms/quality of life measures:			
GDS-15	1 (0–2)	1 (0–2)	0.45
SF12 MCS	57.1 (52.3–59.8)	56.6 (51.6–59.8)	0.29
SF12 PCS	51.3 (43.8–54.2)	50.8 (42.4–54.4)	0.32
EQ-5D Index	0.86 (0.81–1.00)	0.86 (0.81–1.00)	0.84
EQ-5D VAS	80 (75–90)	80 (75–90)	0.50
Biochemical analyses:			
Serum folate (nmol/L)	18.8 (14.9–24.7)	18.9 (14.8–24.5)	0.53
Serum vitamin B_{12} (pmol/L)	267 (213–341)	266 (204–343)	0.27
Serum holoTC (pmol/L)	65 (48–86)	63 (45–84)	0.03
Serum MMA (μmol/L)	0.22 (0.18–0.30)	0.23 (0.18–0.31)	0.26
Plasma Hcy (μmol/L)	14.3 (13.0–16.5)	14.5 (13.0–16.7)	0.46
Serum creatinine (mmol/L)	82.0 (71.0–94.0)	82.0 (71.0–94.0)	0.59

Values are displayed as *N* (%) or median (IQR); $p < 0.05$; *N* varies slightly between variables. MMSE: Mini-Mental State Examination; GDS-15: Geriatric Depression Scale 15-item version; SF-12: 12-item Short-Form Health Survey; MCS: Mental Component Summary score; PCS: Physical Component Summary score; EQ-5D: EuroQol 5 Dimensions; VAS: Visual Analogue Scale; holoTC: holotranscobalamin; MMA: methylmalonic acid; Hcy: homocysteine.

3.2. Cross-Sectional Analyses

Table 2 shows the results of the Cox regression analyses with fixed time points for the baseline association between Hcy concentrations and the GDS-15 and HR-QoL measures. The crude model showed a weak positive association between Hcy and the GDS-15, however, this association disappeared in the fully adjusted model (RR = 1.00; 95% CI: 0.96, 1.03; p = 0.85). For the HR-QoL measures, a similar pattern was observed, with significant crude associations between Hcy and the SF12-PCS, EQ-5D Index, and EQ-5D VAS, but after adjustment for covariates, the significance disappeared.

Table 2. Baseline associations (risk ratios) between Hcy concentrations and the risk of having depressive symptoms (GDS-15 $\geq$ 5) or being in the lowest HR-QoL quartile (intention-to-treat).

	Crude Model		Model 1 [a]		Model 2 (Fully Adjusted) [b]	
	RR (95% CI)	***p***	**RR (95% CI)**	***p***	**RR(95% CI)**	***p***
GDS-15	1.03 (1.00, 1.07)	0.04	1.02 (0.99, 1.06)	0.26	1.00 (0.96, 1.03)	0.85
SF-12 PCS	1.04 (1.02, 1.05)	<0.001	1.02 (1.00, 1.04)	0.04	1.01 (0.99, 1.03)	0.51
SF-12 MCS	1.02 (1.00, 1.04)	0.06	1.02 (1.00, 1.03)	0.13	1.01 (0.99, 1.03)	0.33
EQ-5D Index	1.03 (1.01, 1.05)	<0.001	1.02 (1.00, 1.04)	0.02	1.02 (1.00, 1.04)	0.11
EQ-5D VAS	1.04 (1.02, 1.05)	<0.001	1.03 (1.01, 1.04)	0.01	1.02 (1.00, 1.04)	0.15

[a] adjusted for age and sex; [b] additionally adjusted for study center, education level, smoking, alcohol use, creatinine, body mass index (BMI), and Mini-Mental State Examination (MMSE). *N* varies slightly between the models and variables. Hcy: homocysteine; GDS-15: Geriatric Depression Scale, 15-item version; HR-QoL: health-related quality of life; RR: Risk ratio; SF-12: 12-item Short-Form Health Survey; PCS: Physical Component Summary score; MCS: Mental Component Summary score; EQ-5D: EuroQol 5 Dimensions; VAS: Visual Analogue Scale.

3.3. Effect Study

As expected, Hcy concentrations decreased significantly more (p < 0.001) in the B-vitamin group (mean two-year change: −4.4 μmol/L, SD: 3.3) compared to the placebo group (mean two-year change: −0.2 μmol/L, SD: 4.1).

3.3.1. Depressive Symptoms

Total sample: Logistic regression analyses showed that the number of participants with depressive symptoms did not differ between the two treatment groups after two years, when controlled for baseline depressive symptoms (OR = 1.13. 95% CI: 0.83, 1.53, p = 0.45 in the fully adjusted model; see Table 3).

Subsample (GDS-15 $\geq$ 5): Table 3 also presents the results of the ANCOVA analyses for the subgroup of participants with depressive symptoms at baseline (N = 161). The fully adjusted model did not show significant differences in two-year GDS-15 change scores between the intervention group (mean change: 1.46, 95% CI: 0.71, 2.21) and placebo group (mean change: 1.76, 95% CI: 1.05, 2.47) (p = 0.55). Interaction terms of treatment with age, sex, and Hcy were not significant. Explorative post-hoc interaction analyses with vitamin B_{12} and folate status predictors revealed significant interaction terms for vitamin B_{12}, MMA, and folate, but stratified analyses did not show relevant differences between the intervention and placebo group (Appendix A: Table A1).

Table 3. Effect of the treatment on depressive symptoms, analyzed with logistic regression (total sample) and ANCOVA (subsample with symptoms) (intention-to-treat).

	Baseline	Two-Year Follow-up	Model 1 [a]			Model 2 (Fully Adjusted) [b]		
Logistic regression (total sample, N = 2588)	N with GDS-15 $\geq$ 5 (%)	N with GDS-15 $\geq$ 5 (%)	OR [c] (95% CI)	p		OR [c] (95% CI)	p	
			1.1 (0.8, 1.5)	0.56		1.1 (0.8, 1.5)	0.45	
B-vitamins	101 (7.0)	112 (8.6)						
Placebo	99 (6.8)	111 (8.5)						
ANCOVA (subsample GDS-15 $\geq$ 5, N = 161)	Median GDS-15 score (IQR)	Median GDS-15 score (IQR)	Mean change (95% CI)	F	p	Mean change (95% CI)	F	p
				0.57	0.45		0.36	0.55
B-vitamins	6 (5–8)	5 (3–7)	1.4 (0.7, 2.2)			1.5 (0.7, 2.2)		
Placebo	6 (5–8)	4 (3–6)	1.8 (1.1, 2.5)			1.8 (1.1, 2.5)		

[a] Adjusted for baseline values of the respective outcome variable; [b] additionally adjusted for age, sex, homocysteine, and study center; [c] odds ratio of having clinically relevant depressive symptoms (GDS-15 $\geq$ 5) after two years. GDS-15: Geriatric Depression Scale, 15-item version.

To further examine whether Hcy concentration is associated with depressive symptoms over time, linear regression analyses in the subsample with GDS-15 $\geq$ 5 (irrespective of treatment group) were conducted, with two-year change in Hcy as the predictor and two-year change in GDS-15 as the outcome. The crude and adjusted analyses did not reveal a significant association between the two change scores (Table 4). All per-protocol analyses yielded similar results (data not shown but available on request from the author).

Table 4. Linear regression analyses of the association of the two-year change in Hcy with the two-year change in depressive symptoms and HR-QoL (intention-to-treat).

	Crude Model [a]		Model 1 [b]		Model 2 (Fully Adjusted) [c]	
	β (SE)	*p*	β (SE)	*p*	β (SE)	*p*
GDS-15 (subgroup of ≥5)	0.07 (0.06)	0.27	0.05 (0.06)	0.38	0.05 (0.06)	0.43
SF-12 PCS	−0.14 (0.03)	<0.001	−0.10 (0.03)	0.003	−0.09 (0.03)	0.01
SF-12 MCS	−0.05 (0.04)	0.18	−0.05 (0.04)	0.15	−0.05 (0.04)	0.13
EQ-5D Index	−0.003 (0.001)	<0.001	−0.003 (0.001)	<0.001	−0.002 (0.001)	0.004
EQ-5D VAS	−0.15 (0.06)	0.02	−0.09 (0.06)	0.16	−0.09 0.06)	0.16

[a] Adjusted for baseline values of homocysteine and the respective outcome variable; [b] additionally adjusted for age, sex, and study center; [c] additionally adjusted for education level, smoking, alcohol use, creatinine, BMI, and MMSE. GDS-15: Geriatric Depression Scale, 15-item version; SF-12: 12-item Short-Form Health Survey; PCS: Physical Component Summary score; MCS: Mental Component Summary score; EQ-5D: EuroQol 5 Dimensions; VAS: Visual Analogue Scale.

3.3.2. Health-Related Quality of Life

Two-year change in the mental and physical component summary scales of the SF-12 and in the EQ-5D VAS did not differ significantly between treatment groups (Table 5). The EQ-5D Index score, however, remained stable over time in the intervention group (mean change: 0.00, 95% CI: −0.01, 0.00), whereas this score slightly decreased in the placebo group (mean change: −0.02, 95% CI: −0.03, −0.01) (p = 0.004).

For the EQ-5D VAS, the interaction of treatment with sex was significant (p = 0.05). Stratified analyses, however, did not show significant differences between treatment groups in men or women (Appendix A: Table A1). Exploratory post-hoc interaction analyses with vitamin B_{12} and folate predictors did not reveal significant interaction terms.

Linear regression analyses to investigate associations over time demonstrated that change in Hcy concentration was significantly associated with change in the EQ-5D Index score ($\beta = -0.002$; SE = 0.001; p = 0.004) and the SF-12 PCS score ($\beta = -0.09$; SE = 0.03; p = 0.01) over time (Table 4). This result indicates that these physical and general aspects of HR-QoL decreased as Hcy concentrations increased over time. Results of the per-protocol analyses were similar to the intention-to-treat analyses.

Table 5. Comparison of the two-year change scores of HR-QoL between the two treatment groups with Analysis of Covariance (ANCOVA, intention-to-treat).

			Model 1 [a]			Model 2 (Fully Adjusted) [b]		
	Baseline	Two-Year Follow-up	Change Scores			Change Scores		
	Median (IQR)	Median (IQR)	Mean Change (95% CI)	F	p	Mean Change (95% CI)	F	p
SF-12 PCS (N = 2594)				0.14	0.71		0.09	0.76
B-vitamins	51.6 (44.5–54.3)	51.1 (42.9–54.4)	−0.59 (−0.95, −0.23)			−0.60 (−0.95, −0.25)		
Placebo	51.5 (43.5–54.6)	50.8 (41.8–54.7)	−0.69 (−1.05, −0.32)			−0.68 (−1.03, −0.33)		
SF-12 MCS (N = 2594)				0.14	0.71		0.18	0.67
B-vitamins	57.2 (52.3–59.8)	56.8 (51.8–59.8)	−0.52 (−0.89, −0.15)			−0.53 (−0.90, −0.16)		
Placebo	56.8 (52.0–59.8)	56.6 (51.2–59.8)	−0.42 (−0.79, −0.05)			−0.42 (−0.79, −0.04)		
EQ-5D Index (N = 2617)				8.89	0.003		8.29	0.004
B-vitamins	0.86 (0.81–1.00)	0.89 (0.81–1.00)	0.00 (−0.01, 0.01)			0.00 (−0.01, 0.00)		
Placebo	0.8 (0.81–1.00)	0.84 (0.81–1.00)	−0.02 (−0.03, −0.01)			−0.02 (−0.03, −0.01)		
EQ-5D VAS (N = 2612)				0.20	0.65		0.17	0.68
B-vitamins	80 (75–90)	80 (70–90)	−1.06 (−1.73, −0.39)			−1.07 (−1.73, −0.41)		
Placebo	80 (75–90)	80 (70–90)	−1.28 (−1.95, −0.61)			−1.27 (−1.93, −0.61)		

[a] Adjusted for baseline values of the respective outcome variable; [b] additionally adjusted for age, sex, homocysteine, and study center. HR-QoL: health-related quality of life; SF-12: 12-item Short-Form Health Survey, PCS: Physical Component Summary score; MCS: Mental Component Summary score; EQ-5D: EuroQol 5 Dimensions; VAS: Visual Analogue Scale.

4. Discussion

This study investigated the role of Hcy concentrations and two years of supplementation with vitamin B_{12} and folic acid on depressive symptoms and HR-QoL in a large sample of Dutch older adults with elevated Hcy concentrations. Contrary to our hypotheses, no significant cross-sectional associations between Hcy concentrations and depressive symptoms or HR-QoL were observed, when controlling for confounding variables. Furthermore, supplementation with vitamin B_{12} and folic acid did not reveal significant differences between the two treatment groups (intervention vs. placebo) on depressive symptoms and three out of four HR-QoL measures after two years. The EQ-5D Index (measuring general HR-QoL), however, remained stable over time in the intervention group and decreased slightly but significantly in the placebo group, suggesting that B-vitamin supplementation did have a small positive effect on HR-QoL. Furthermore, the two-year change in Hcy (independent of group assignment) was associated with the two-year change in the EQ-5D Index and the physical component of the SF-12. This implies that a reduction of Hcy concentrations may lead to more stable—as opposed to declining—general and possibly also physical HR-QoL over time.

The results of the present study do not suggest a role of Hcy or vitamin B_{12} and folic acid supplementation in depressive symptoms in persons with mild hyperhomocysteinemia. Several observational studies have shown associations between Hcy and depressive symptoms [44–48], but some studies only found significant results in persons with low folate and/or vitamin B_{12} status [45,47]. Although Hcy concentrations were elevated in all B-PROOF participants, the number of persons with a vitamin B_{12} or folate deficiency was low (7.3% and 3.1%, respectively). Nevertheless, Hintakka et al. observed an association between vitamin B_{12} and depression in persons with vitamin B_{12} concentrations within the normal range [49].

In contrast to the present results, a prospective cohort study with 521 Korean persons (over 65 years old) from the general population observed a significant association of Hcy, vitamin B_{12}, and folate with incident depressive disorder over a follow-up period of 2–3 years [48]. In accordance with our results, previous trials did not observe a beneficial effect of B-vitamin supplementation on depressive symptoms either [50,51]. Supplementation dose and method, however, differed between the trials. The present study did account for some limitations of these previous studies, by including both men and women, as opposed to only men [50], and by using a long follow-up period of two years, in contrast to only four months [51].

Regarding HR-QoL, we observed a beneficial effect of B-vitamin supplementation on the EQ-5D Index score, whereas no effects were observed on the other three HR-QoL measures. The effect, however, was very small (the difference between groups was 0.02, on a scale of -0.33 to 1) and did not reach clinical relevance [52]. Comparison of the present HR-QoL results with other intervention studies is difficult due to the heterogeneity of study designs and samples. A Danish trial studied 140 adults with elevated MMA concentrations and found no effect on the SF-36 mental and physical summary scores after four weeks of vitamin B_{12} injections, but they did observe a positive effect on the general health subscale of this instrument [23]. Although these participants were not selected on the basis of their Hcy concentrations, most participants had mildly elevated Hcy concentrations. Another trial used a drink containing vitamin B_2, B_6, B_{12}, and folic acid that significantly lowered Hcy concentrations and improved HR-QoL (assessed with the General Health Survey) already after six weeks [16]. Other trials, however, did not observe effects on HR-QoL after study periods ranging from one to three years [20–22,24].

The observed significant treatment effect on the EQ-5D Index score, in contrast to the SF-12, was unexpected. In relatively healthy populations such as the B-PROOF sample, the SF-12 is usually a more sensitive instrument than the EQ-5D, as the EQ-5D may display ceiling effects [34,53]. For the scoring of the EQ-5D, Dutch norms were used, whereas the scoring system of the SF-12 was based on the US population because a Dutch sample is not currently available. The EQ-5D scoring system may have been better tailored for the B-PROOF sample, which could partly explain the observed discrepancy.

To the best of our knowledge, this study with almost 3000 participants is one of the largest studies investigating the effects of vitamin B_{12} and folic acid supplementation on mental health and well-being in older persons. The randomized placebo-controlled trial design, the large study sample, and the long follow-up period are major strengths of the B-PROOF study. In this way, relationships between Hcy, depressive symptoms, and HR-QoL could be studied both cross-sectionally and after supplementation. In addition, we were able to adequately control for possible covariates. The instruments that were used for assessing depressive symptoms and HR-QoL are widely used and have satisfactory psychometric properties. Compliance to the treatment was high, which was also reflected by the significantly decreased Hcy concentrations in the intervention group compared to the placebo group.

As depressive symptoms and HR-QoL were secondary outcome measures of the B-PROOF study, relatively few participants had clinically relevant depressive symptoms (7%) and participants generally perceived a high HR-QoL. This may have reduced the power of our analyses, especially in the subgroup of participants with depressive symptoms (N = 161). Furthermore, it should be mentioned that vitamin D_3 was added to both the B-vitamin and placebo tablets. Several observational and experimental studies have suggested that vitamin D supplementation reduces depressive symptoms and improves HR-QoL [54,55], which may have had an attenuating effect on our results.

By including persons with elevated Hcy concentrations, we aimed to include a sensitive sample for the effects of vitamin B_{12} and folic acid treatment. However, although elevated Hcy concentrations are common in the general older population [28], this selection may limit generalizability to the total elderly population. In addition, the restricted range of Hcy concentrations and the absence of participants with normal Hcy concentrations may have attenuated the results of the cross-sectional analyses.

Although the two-year supplementation in this study significantly lowered Hcy concentrations, our vitamin B_{12} dose of 500 μg/day may have been too low to observe an effect on depressive symptoms. A supplementation dose of at least 1000 μg/day may have been more effective [56,57]. Insufficient vitamin B_{12} status by itself has been associated with various neuropsychiatric symptoms, including depression [57]. Although we had only a low number of vitamin B_{12} deficient participants, a Finnish study showed that adult outpatients with clinical depression had a better treatment outcome when their B_{12} status was higher, even though all participants had normal or high vitamin B_{12} concentrations [49].

To further investigate the role of homocysteine in depression and HR-QoL, future research could focus on persons with hyperhomocysteinemia, low vitamin B_{12} or folate status, and on individuals with clinically relevant depressive symptoms and/or lower HR-QoL. These persons may possibly benefit more from B-vitamin supplementation. However, it should be mentioned that participants in the intervention group reported a higher incidence of cancer compared to the placebo group. This adverse effect of the B-vitamin supplementation has been reported previously [29]. Therefore, special caution is warranted in future studies with vitamin B_{12} and folic acid.

5. Conclusions

In conclusion, the results of the present study indicate that reducing Hcy concentrations by supplementation with vitamin B_{12} and folic acid in a generally healthy sample of older adults with mild hyperhomocysteinemia does not reduce depressive symptoms and may have a small positive effect on general HR-QoL.

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Appendix A

Table A1. Stratified analyses for significant interaction terms (stratified at the median, intention-to-treat analyses).

	N	Two-Year Change Scores in the Fully Adjusted Models Mean (95% CI)	*F* [a]	*p*
GDS-15: Stratified analyses for folate:				
Low Folate: (≤18.86 nmol/L)			1.63	0.21
B-vitamins	38	1.87 (0.76, 2.98)		
Placebo	41	0.86 (−0.21, 1.92)		
High Folate: (>18.86 nmol/L)			3.47	0.07
B-vitamins	38	1.19 (0.17, 2.20)		
Placebo	41	2.53 (1.56, 3.51)		
GDS-15: Stratified analyses for MMA:				
Low MMA: (≤0.23 μmol/L)			2.70	0.10
B-vitamins	38	0.63 (−0.50, 1.76)		
Placebo	41	1.95 (0.87, 3.04)		
High MMA: (>0.23 μmol/L)			1.12	0.29
B-vitamins	38	2.28 (1.34, 3.22)		
Placebo	41	1.57 (0.67, 2.48)		
GDS-15: Stratified analyses for Vitamin B_{12}:				
Low Vitamin B_{12}: (≤266.4 pmol/L)			1.07	0.30
B-vitamins	41	2.14 (1.13, 3.16)		
Placebo	39	1.36 (0.32, 2.41)		
High Vitamin B_{12}: (>266.4 pmol/L)			4.52	0.04
B-vitamins	41	0.55 (−0.60, 1.69)		
Placebo	39	2.21 (1.18, 3.24)		
EQ-5D VAS: Stratified analyses for sex:				
Men:			2.75	0.10
B-vitamins	657	−0.86 (−1.73, 0.02)		
Placebo	667	−1.90 (−2.76, −1.03)		
Women:			1.07	0.30
B-vitamins	645	−1.33 (−2.32, −0.33)		
Placebo	643	−0.58 (−1.58, 0.42)		

[a] Differences between the two treatment groups over time were tested with ANCOVA, adjusted for baseline values of the respective outcome variable, age, sex, homocysteine, and study center. GDS-15: Geriatric Depression Scale 15-item version; MMA: methylmalonic acid; EQ-5D VAS: EuroQol 5 Dimensions—Visual Analogue Scale.

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Article

Dietary B Vitamins and a 10-Year Risk of Dementia in Older Persons

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Abstract: B vitamins may lower the risk of dementia, yet epidemiological findings, mostly from countries with folic acid fortification, have remained inconsistent. We evaluated in a large French cohort of older persons the associations between dietary B vitamins and long-term incident dementia. We included 1321 participants from the Three-City Study who completed a 24 h dietary recall, were free of dementia at the time of diet assessment, and were followed for an average of 7.4 years. In Cox proportional hazards models adjusted for multiple potential confounders, including overall diet quality, higher intake of folate was inversely associated with the risk of dementia (p for trend = 0.02), with an approximately 50% lower risk for individuals in the highest compared to the lowest quintile of folate (HR = 0.47; 95% CI 0.28; 0.81). No association was found for vitamins B6 and B12. In conclusion, in a large French cohort with a relatively low baseline folate status (average intake = 278 μg/day), higher folate intakes were associated with a decreased risk of dementia.

Keywords: folate; B vitamins; dementia; aging

1. Introduction

B vitamins have fundamental roles in the function of the central nervous system [1], and have been considered as promising candidates for the prevention of dementia and its major etiology, Alzheimer's disease (AD). B vitamins (B6, folate, and B12) regulate homocysteine (Hcy) levels, and hyperhomocysteinemia is a major vascular risk factor and an established risk factor for dementia [2]. Hyperhomocysteinemia may affect brain function through both vascular mediation and neurotoxicity [3]. B vitamins may also have a direct effect on the brain, independently of homocysteine [4]. Indeed, a lower status in B vitamins decreases *S*-adenosylmethionine, a major intermediate of methylation reactions in the brain which are involved in synaptic transmission and epigenetic regulation [5]. Moreover, tetrahydrofolate, the active form of folate, is involved in DNA repair system [6] and DNA replication, which are both critical for adult hippocampal neurogenesis [7]. Direct mechanistic effects of B vitamins on the brain independent of Hcy may therefore be particularly relevant to folate [8–10].

Prospective epidemiological studies have found associations between higher intakes of B vitamins and a lower risk of dementia and AD; three studies have reported associations with dietary folate (but not with vitamins B6 and B12) [11–13], although inconsistent findings have also been found,

with studies reporting null [14,15] or even harmful associations between folate intakes at high levels and cognitive function, when combined with low vitamin B12 status [16]. Findings have also been inconsistent in studies using blood status in B vitamins (see [17] for a review). Moreover, prevention trials have been inconclusive to date [18], with a few exceptions, including the two European FACIT and VITACOG trials, which evidenced the beneficial effect of folic acid on cognitive function and brain atrophy, respectively [19,20]. Although the reasons for such inconsistencies remain unclear, it is possible that the findings of some studies have been influenced by the folic acid fortification of all flour and cereal-grain products that became mandatory starting in 1998 in the US and Canada, leading to a subsequent dramatic increase in folate status in North American populations [21]. The vast majority of observational studies on B vitamins and cognitive aging have been conducted in the US, and limited research has examined European populations. In the present study, we evaluated in a large cohort of older persons from France—a country not subject to folic acid fortification policy—the association between dietary B vitamins and the risk of dementia and AD over 10 years of follow-up.

2. Materials and Methods

2.1. Study Population

Our analysis was based on the Three-City (3C) study, a French population-based cohort on dementia which started in 1999–2000, including 9294 non-institutionalized community dwellers aged 65 years or older in three French cities: Bordeaux (n = 2104), Dijon (n = 4931), and Montpellier (n = 2259). Face-to-face interviews were performed at the cohort baseline with the collection of sociodemographic, lifestyle, and health-related characteristics, neuropsychological testing, and blood sampling. The protocol of the 3C study has been described in detail previously [22]. The Consultative Committee for the Protection of Persons participating in Biomedical Research at Kremlin-Bicêtre University Hospital (Paris, France) approved the 3C study protocol (Project no. 99-28, june 1999) and all participants provided written informed consent. Follow-up examinations were conducted 2 years (2001–2002), 4 years (2003–2004), 7 years (2006–2007), 10 years (2009–2010), and 12 years (2011–2012) after the cohort baseline. The present study is based on data from Bordeaux, the only 3C center with a dietary assessment.

At the 2-year visit in 2001–2002, a comprehensive dietary survey was conducted by trained dieticians, including a food frequency questionnaire (FFQ) and a 24 h dietary recall (which served as baseline in the present analysis). Figure 1 describes the procedure for the study sample selection. Our final study population included 1321 participants followed for 10 years after the dietary assessment.

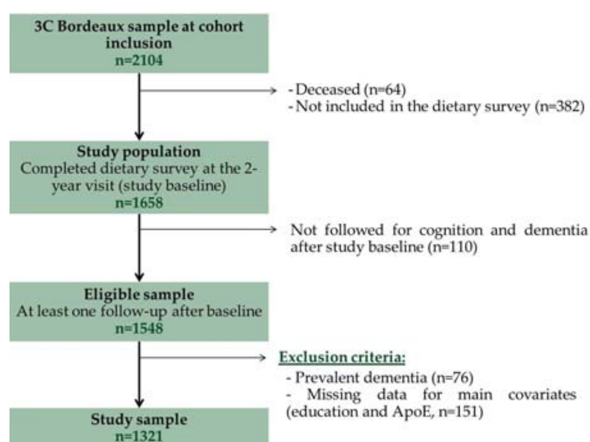

Figure 1. Flow diagram of the study sample selection, the 3C Bordeaux cohort. Abbreviations: 3C: Three-City; ApoE: apolipoprotein genotype.

2.2. Diagnosis of Dementia

Incident dementia was ascertained through a three-stage procedure. First, participants underwent a battery of neuropsychological tests at home conducted by a trained psychologist. Individuals suspected of dementia based on their neuropsychological performances were secondarily examined by a neurologist to establish a clinical diagnosis. Finally, all potential cases of dementia were reviewed by an independent committee of neurologists to set up consensus on dementia diagnosis and etiology, according to the *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition) [23] and the *National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association* Alzheimer's criteria [24].

2.3. Assessment of Diet and Intake of B-Vitamins

The procedure of the 24 h recall data collection was detailed in a previous publication [25]. Briefly, the 24 h dietary recall consisted in reporting all meals and beverages consumed the day prior to the interview (excluding week-end meals). For each food reported, portion sizes were registered using a book of photographs [26] and were used to estimate the quantity (in grams per day) of intake of each food item. Nutrient and total energy intakes were then estimated using several French food composition tables [27–29]. The contribution of supplemental folic acid (from fortified foods or supplements) to total folate intake is very low and virtually all folic acid is of natural origin in French populations [30]. In our analyses, we focused on intakes of B vitamins involved in one-carbon metabolism, i.e., vitamin B6, folate, and vitamin B12.

2.4. Other Variables

Sociodemographic information included age at study baseline, gender, and level of education. ApoEε4 genotype was defined as carrying at least one ε4 allele vs. no ε4 allele. Lifestyle factors included alcohol consumption, tobacco use, and regular exercise. To control for overall diet quality, we also computed a score of adherence to the Mediterranean diet (MeDi); the methodology for computation of the MeDi score has been described in previous publications [31,32]. Cardiovascular risk factors included body mass index (BMI), hypercholesterolemia (total cholesterol $\geq$6.2 mmol/L or anti-cholesterol medication), diabetes (fasting glucose $\geq$7.2 mmol/L or antidiabetic medication), history of cardiovascular diseases (including myocardial infarction and stroke), and hypertension (measured blood pressure higher than 140/90 mm Hg or antihypertensive medication). Depressive

symptomatology was assessed using the Center for Epidemiological Studies-Depression (CES-D) [33] Scale; we defined high depressive symptoms as having a CES-D score higher than 17 in men and 23 in women (over a maximum of 60 [34]) or when the participant was "too depressed to answer". The number of drugs regularly consumed was recorded and used as a general indicator of comorbidities.

2.5. Statistical Analyses

We used Cox proportional hazards models with delayed entry using age as time-scale [35] to evaluate the multivariate associations between intake of B vitamins and the risk of all-cause dementia over 10 years; we secondarily examined AD specifically. Vitamin B6, folate, and vitamin B12 were modeled simultaneously and considered in quintiles. We first adjusted for the main risk factors of dementia (gender, education, and ApoE genotype), energy intake, and season of the 24 h recall (Model 1). We further controlled for lifestyle factors, cardiovascular risk factors, and other comorbidities (Model 2).

We examined linear trends across quintiles of B vitamins by using a continuous variable taking the median intake value of each quintile. Moreover, we investigated potential interactions of biological relevance between (i) folate and alcohol intakes [36]; (ii) folate and vitamin B12 intakes [37,38]; (iii) B vitamins and long-chain omega-3 fatty acid intakes [39]; and (iv) B vitamin intakes and ApoEε4 genotype. Missing data were treated as follows. For most covariates, data were missing for less than 3% of the sample; we thus assigned to missing data the reference category (for categorical variables) or the median value (for continuous variables). Regular exercise was missing for 11.8% of the sample; we thus created a specific missing category for this variable. The proportional hazard assumption was controlled, as well as the log-linearity assumption for continuous covariates.

In sensitivity analyses, we assessed the robustness of our findings by (i) excluding participants with the highest values for vitamin B intakes, i.e., with intakes >95th percentile of at least one of the 3 B vitamins; (ii) adjusting for regular intake of fruits and vegetables instead of the Mediterranean diet score; and (iii) controlling for other potential confounders with very low prevalence, i.e., consumption of B vitamin supplements. We also controlled for potential reverse causation (which occurs when subtle cognitive impairment modifies dietary intakes) by adjusting our multivariate models for global cognitive performances at baseline, represented by the Mini Mental State Examination score [40].

Statistical analyses were performed using SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA) and R software version 3.2.3 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Characteristics of the Sample

Study participants were 75.8 years old on average (range: 67.7–94.9) at the time of the dietary survey and 62.2% were female (Table 1). Nearly 41% of participants had reached high school level or above, and 18% were ApoEε4 carriers; baseline characteristics including lifestyle and cardiovascular risk factors have been provided in Table 1. A total of 197 individuals (15% of the sample) developed dementia over 7.4 years of follow-up on average (range: 0.9–11.2), including 131 classified with possible or probable AD (351 participants died during follow-up and 192 refused or were lost to follow-up). The incidence rate of dementia was 2.0 cases per 100 person-years, and the incidence of AD was 1.3 per 100 person-years.

Table 1. Baseline characteristics and B vitamin intakes of the participants according to incident all-cause dementia over 10 years, the 3C Bordeaux cohort (n = 1321).

Baseline Characteristics	Overall Sample (n = 1321)	Incident Dementia (n = 197)	No Dementia (n = 1124)	p for Risk of Dementia [a]
Age (years)	75.8 ± 4.8	78.3 ± 4.6	75.4 ± 4.7	<0.001
Gender, female	822 (62.2)	140 (71.1)	682 (60.7)	0.30
Education ≥high school	540 (40.9)	72 (36.5)	468 (41.6)	0.24
ApoEε4, carrier	240 (18.2)	49 (24.9)	191 (17.0)	<0.001
Alcohol intake (g/day)	13.9 ± 15.5	12.9 ± 14.0	14.1 ± 15.8	0.86
Tobacco consumption (pack-year)				
0	861 (65.2)	142 (72.1)	719 (64.0)	
<10	156 (11.8)	20 (10.2)	136 (12.1)	
(10–20)	84 (6.4)	10 (5.1)	74 (6.6)	0.75
(20–30)	71 (5.4)	7 (3.6)	64 (5.7)	
≥30	149 (11.3)	18 (9.1)	131 (11.7)	
Regular exercise [b]	412 (35.3)	46 (28.2)	366 (36.5)	0.14
BMI (kg/m^2)	26.6 ± 4.1	26.1 ± 4.3	26.7 ± 4.1	0.23
Hypercholesterolemia	762 (57.7)	125 (63.5)	637 (56.7)	0.16
Diabetes	125 (9.5)	33 (16.8)	92 (8.2)	<0.001
History of cardiovascular diseases	428 (32.4)	63 (32.0)	365 (32.5)	0.94
Hypertension	998 (75.5)	151 (76.6)	847 (75.4)	0.63
High depressive symptomatology	98 (7.4)	22 (11.2)	76 (6.8)	0.10
Number of drugs consumed	4.8 ± 2.9	5.8 ± 3.2	4.6 ± 2.8	<0.001
B vitamin/multivitamin supplement use	19 (1.4)	2 (1.0)	17 (1.5)	0.59
Energy intake (Kcal/day)	1623.0 ± 514.0	1565.0 ± 500.2	1633.0 ± 515.9	0.44
Vitamin B6 intake (mg/day)	1.5 ± 0.6	1.4 ± 0.6	1.5 ± 0.6	0.52
Folate intake (μg/day)	278.3 ± 134.8	251.9 ± 126.1	283.0 ± 135.8	0.01
Vitamin B12 intake (μg/day)	5.7 ± 11.4	4.9 ± 9.9	5.8 ± 11.7	0.30

Values are mean ± SD or number (percentages). [a] Univariate Cox Proportional Hazards models with delayed entry using age as time scale, except for age which use standard Cox Proportional Hazards models; [b] Percentages are of non-missing values. Abbreviations: ApoEε4: allele ε4 for the apolipoprotein E gene; BMI: body mass index.

Mean daily intakes were 1.5 mg/day for vitamin B6, 278.3 μg/day for folate, and 5.7 μg/day for vitamin B12 (Table 1). Compared with individuals who remained free of dementia during follow-up, those who developed the disease had lower B vitamin intakes at baseline, although the difference was statistically significant for folate only in univariate Cox models (p = 0.01 for risk of dementia for each 1SD-increase of folate intake), without any marked difference in daily energy intake across both groups (p = 0.44).

3.2. Multivariate Associations between Intake of B Vitamins and Risk of Dementia

In multivariate analyses, a higher intake of folate was significantly associated with a lower risk of dementia (p for trend $\leq$ 0.02 across increasing quintiles in multivariate models, Table 2). Adjustments for potential confounders did not markedly alter HRs, suggesting a minimal confounding effect. After controlling for a large set of potential confounders in Model 2, compared with individuals in the lowest quintile of folate, those in the highest quintile had a 47% lower risk of dementia over 10 years (HR (95% CI) = 0.47 (0.28, 0.81)). In contrast, we did not find any significant association between vitamin B6 or vitamin B12 intakes and the risk of dementia (p for trend = 0.38 and 0.73, respectively, in Model 2).

Table 2. Multivariate associations between quintiles of B vitamin intake and risk of all-cause dementia over 10 years, the 3C Bordeaux cohort (n = 1321).

	Number of Dementia Cases	Risk of Dementia (HR [95% CI]) [a]	
		Model 1	Model 2
Vitamin B6 (mg/day)			
Q1 <1.0	50	1.0 (reference)	1.0 (reference)
Q2 (1.0–1.2)	36	0.81 (0.51–1.28)	0.86 (0.54–1.36)
Q3 (1.2–1.5)	36	1.02 (0.63–1.65)	1.08 (0.66–1.77)
Q4 (1.5–1.9)	42	1.26 (0.78–2.04)	1.40 (0.85–2.31)
Q5 ≥1.9	33	1.02 (0.58–1.78)	1.08 (0.60–1.94)
p for trend		0.52	0.38
Folate (μg/day)			
Q1 <168.3	56	1.0 (reference)	1.0 (reference)
Q2 (168.3–225.4)	37	0.66 (0.43–1.02)	0.66 (0.42–1.02)
Q3 (225.4–281.4)	40	0.67 (0.43–1.03)	0.73 (0.47–1.15)
Q4 (281.4–375.6)	35	0.69 (0.43–1.10)	0.76 (0.47–1.24)
Q5 ≥375.6	29	0.47 (0.28–0.79)	0.47 (0.28–0.81)
p for trend		0.01	0.02
Vitamin B12 (μg/day)			
Q1 <1.8	38	1.0 (reference)	1.0 (reference)
Q2 (1.8–2.6)	47	1.42 (0.91–2.21)	1.27 (0.81–1.98)
Q3 (2.6–3.7)	40	1.29 (0.81–2.07)	1.15 (0.71–1.86)
Q4 (3.7–5.7)	40	1.30 (0.81–2.10)	1.26 (0.77–2.05)
Q5 ≥5.7	32	1.17 (0.70–1.94)	1.04 (0.61–1.75)
p for trend		0.95	0.73

[a] Cox proportional hazards models with delayed entry using age as time-scale. The three B vitamins were modeled simultaneously. Model 1: adjusted for gender, level of education, ApoEε4, energy intake, and season of the 24 h recall. Model 2: covariates from Model 1 plus alcohol and tobacco consumptions, regular exercise, the Mediterranean Diet score, BMI, hypercholesterolemia, diabetes, history of cardiovascular diseases, hypertension, depressive symptomatology, and number of drugs consumed.

When we examined the risk of AD, we found similar associations of high folate with a lower risk of AD, although the magnitude of associations was slightly weaker than those found with all-cause dementia (p for trend = 0.08 in the first model, HR for higher versus lower quintile of folate = 0.52, 95% CI = 0.28, 0.97; results are not shown in the tables) and relationships were attenuated and no longer statistically significant after further adjustments (p for trend = 0.17 in Model 2).

We did not detect any significant interaction between B vitamins and alcohol, and omega-3 intakes or with ApoEε4 carrier status. Likewise, there was no significant interaction between folate and vitamin B12 intakes on dementia risk.

3.3. Secondary Analyses

When we conducted sensitivity analysis by further adjusting models for baseline cognition, our results remained unchanged, suggesting that findings may not be due to reverse causation by incipient prodromal dementia. Likewise, a sensitivity analysis excluding the n = 155 participants with higher intake values for at least one of the 3 B vitamins did not modify results, suggesting that findings were not driven by a few particular individuals with extreme intake values. Results were also virtually unchanged when adjusting for regular fruit and vegetable consumption instead of the MeDi score, suggesting minimal confounding by other potentially beneficial nutrients contained in the major food source of dietary folate. Very few people (n = 19) consumed B vitamin/multivitamin supplements in this French population, and the inclusion of B vitamin/multivitamin supplementation in our models did not modify findings.

4. Discussion

In this large French cohort of older persons followed for more than a decade, we found that higher folate intakes were inversely associated with the risk of dementia, independently of intakes of other B vitamins, overall diet quality, and a large set of other potential confounders. Compared with individuals with folate intakes in the lowest quintile, those in the higher quintile had an approximately 50% lower risk of dementia. A similar trend was found with AD risk, although associations were weaker and no longer significant after adjustment for the full set of potential confounders (possibly due to less power to detect a significant relationship with AD cases, which represented only 66% of all-cause dementia cases). In contrast to folate, there was no significant association between dietary vitamins B6 and B12 and the risk of dementia or AD in this large cohort.

Our results are consistent with at least three previous US longitudinal studies, in which higher folate intake was associated with a lower risk of dementia or AD, while associations of dietary vitamins B6 and B12 to dementia risk were not significant [11–13]. In contrast, our results are not consistent with two other previous large US cohorts, the Chicago Health and Aging Project (CHAP) and the Cache County Memory Study (CCMS), which did not evidence any association between quintiles of B vitamin intake and risk of AD [14,15]; harmful associations between higher folate and cognition have also been found among persons with low vitamin B12 status in several cohorts [16,37,38]. The reasons for inconsistencies between studies are unclear. The most evident reason may pertain to differences in baseline intakes between French and US populations, with lower intakes reported in France, a country with limited supplement use and no folic acid fortification (e.g., median total folate intake in the upper quintile was 444 μg/day in our 3C sample, compared to 742 μg/day in the CHAP study [16]). It is thus possible that folate is protective for the brain in lower intake ranges (as those observed in France) and becomes inefficient—and even detrimental for those with low B12 status—at higher ranges. Otherwise, findings of US cohorts have remained difficult to interpret due to the fortification policy introduced in the late 1990s. Indeed, part of the dietary data in the CHAP study was collected after folic acid fortification; furthermore, although diet was ascertained in 1995 (i.e., pre-folic acid fortification) in the CCMS, follow-up for cognition and dementia was conducted through 2004, with >70% of the follow-up period for cognition covering post-folic acid fortification. It is thus possible that the increase in folate status in the US population which occurred after folic acid fortification have biased these studies towards the null.

Additionally, although most trials failed to evidence any benefit of B vitamin supplementation on cognitive function, our results are nonetheless consistent with three European trials which found a protective effect of B vitamins on brain aging outcomes among persons with raised Hcy or with low folate status [19,20,41–43]. In Italy, a small pilot trial found a benefit of folic acid supplementation on memory and attention among older persons selected with low initial status in folate [43]. In The Netherlands, the large FACIT trial included participants aged ≥50 years old with high levels of Hcy and found a significant benefit of folic acid supplementation over three years on memory and processing speed [19]; in the UK, the VITACOG study included older persons aged ≥70 years old with mild cognitive impairment (without a priori selection based on Hcy levels) and found an efficacy of B vitamins on brain atrophy [20,41], and on cognition limited to those with raised Hcy at baseline [42]. Finally, in the single largest French study to date based on secondary analyses on cognition from the large SU.FOL.OM3 trial on B vitamins and omega-3 fatty acids for secondary prevention of cardiovascular diseases, a protective effect of B vitamins was evidenced on global cognition and memory in the older age group (65–80 years) [44]. Together with our own study, these results suggest a beneficial effect of B vitamins on cognition in older persons (especially among those with raised Hcy or low folate status).

In contrast to folate, dietary vitamins B6 and B12 were not significantly associated with the risk of dementia or AD in the present study. Although several longitudinal studies found inverse associations between blood markers of vitamin B12 and brain health [45–49] (associations potentially fully mediated by Hcy [47,48]), studies generally found, as in our study, null results when examining dietary B6 and

B12 and cognitive health [11–16]. Indeed, studies based on intake data (including our own study) may not capture food-bound vitamin B12 malabsorption, which is common in older persons and adversely affects vitamin B12 status in healthy older people irrespective of dietary intakes [50]. Thus, circulating vitamin B12 may be a better marker of vitamin B12 deficiency than vitamin B12 intakes in older populations.

A limitation of the present study is that a single 24 h recall was used to assess B vitamin intake. A reported single day of intake does not fully capture intra-individual variation in dietary intakes, and might lead to misclassification, especially for foods consumed occasionally (such as offal, a top source for vitamin B12). However, in large samples, a single 24 h provides acceptable estimations of average intakes in subgroups of a population [51]; accordingly, we observed in our study average intake values similar to other studies in older populations for macronutrients [25] and for micronutrients including B vitamins (e.g., average intake values for B vitamins in our sample were close to those observed among older persons from the French Individual and National Study on Food Consumption (INCA 2) [52]—a national representative survey). In addition, the use of dietary supplements may not have been accurately recorded by our questionnaire [53]. Moreover, as in any observational study, there might be residual confounding. In particular, B vitamin intake may reflect an overall healthy diet, and we did not adjust for other healthy nutrients which may exert protective effects to the brain. However, we were able to control for a well-established index of overall diet quality, the MeDi score, and further adjustment for the MeDi revealed minimal confounding by global diet quality.

Our study also has important strengths. Obvious strengths include a longitudinal design with a decade of follow-up, and a multi-step procedure of diagnosis of dementia including a validation of cases by an independent committee of neurologists. The long lag period between the ascertainment of B vitamin intakes and the diagnosis of dementia minimizes the possibility of reverse causation. Finally, we considered a large range of potential confounders, including cardiovascular and lifestyle factors, such as the overall diet quality, and adjusting for these factors did not markedly decrease our risk estimate, suggesting minimal confounding effect.

5. Conclusions

In conclusion, we found in a large cohort of older persons from France—a country with no folic acid fortification and relatively low average intake levels—a strong association between a higher intake of folate and a lower long-term risk of dementia, while there was no evidence of association of vitamin B6 and B12 intake to dementia risk; however, vitamin B12 malabsorption has not been explored through circulating level data in this cohort of older persons and deserves further research. The protective role of folate in populations with relatively low basal folate status such as France may be worth exploring in future dementia prevention trials.

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nutrients

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Review

Vitamin B12 among Vegetarians: Status, Assessment and Supplementation

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Abstract: Cobalamin is an essential molecule for humans. It acts as a cofactor in one-carbon transfers through methylation and molecular rearrangement. These functions take place in fatty acid, amino acid and nucleic acid metabolic pathways. The deficiency of vitamin B12 is clinically manifested in the blood and nervous system where the cobalamin plays a key role in cell replication and in fatty acid metabolism. Hypovitaminosis arises from inadequate absorption, from genetic defects that alter transport through the body, or from inadequate intake as a result of diet. With the growing adoption of vegetarian eating styles in Western countries, there is growing focus on whether diets that exclude animal foods are adequate. Since food availability in these countries is not a problem, and therefore plant foods are sufficiently adequate, the most delicate issue remains the contribution of cobalamin, which is poorly represented in plants. In this review, we will discuss the status of vitamin B12 among vegetarians, the diagnostic markers for the detection of cobalamin deficiency and appropriate sources for sufficient intake, through the description of the features and functions of vitamin B12 and its absorption mechanism.

Keywords: cobalamin; vitamin B12; vegetarian; vegan; food sources; deficiency; cardiovascular disease; neurological symptoms; supplements; diagnostic markers

1. Introduction

Recently, the vegetarian eating style has increased in popularity, with 10% of the population opting to exclude animal foods from their diet [1]. The decision to undertake such a choice depends primarily on ethical and ecological aspects, but also on health reasons. However, when the reasons are ethical, this may result in a reduced interest in the knowledge of the nutritional aspects of such a choice [2]. The scientific literature shows that the reduction or exclusion of animal foods may reduce the risk of Coronary Heart Disease (CHD) and Type 2 Diabetes (T2D) through modifiable factors such as body mass, serum glucose, blood pressure and serum lipid profile. These disorders contribute

to a high mortality rate in Western countries [3–7]. Nevertheless, the risk of possible nutritional deficiencies in a non-balanced vegetarian diet, due to the absence of nutrients that can nullify these health benefits [8], should not be underestimated. In the European Prospective Investigation into Cancer and Nutrition-Oxford (EPIC-Oxford) cohort study, all-cause mortality among vegetarians in comparison with non-vegetarians was not significantly different [9]. In five prospective study analyses of 24,000 vegetarians among a total of 76,000 men and women, mortality from ischemic heart disease was lower in lacto-ovo-vegetarians than in vegans [10].

In the 2010 American Dietary Guidelines, the United States Department of Agriculture (USDA) proposed vegetarian adaptation patterns, including proposals for a balanced vegan diet [11]. Since 1988, and recently with the position paper of 2009, the American Dietetic Association has established that vegetarian diets are sustainable and safe for all age groups and in all physiological conditions, from childhood to old age, in athletes, and during pregnancy and lactation [12,13]. Sustainability relates to vegetarian diets that do not exclude dairy and eggs, as well as a vegan diet. However, it should be focused on proper planning and supplementation.

Vitamin B12, also called cobalamin (Cbl), is a water-soluble vitamin found in substantial quantities only in animal foods. If the consumption of animal foods is very low or absent, its scarce presence in plant foods makes its introduction essential, either through supplements or fortified foods. This deficiency is common among vegetarians and is the result of a very low intake [14]. Lower Cbl blood concentration can promote hematological shortages, resulting in increased mean corpuscular red cell volume (MCV) and anemia through the alteration of erythropoiesis [15]. Cbl also plays a key role in neuronal health, and a severe deficiency would inhibit the physiological formation of the myelin sheath, altering correct nerve transmission [15]. The slowing down of the degradation pathway of odd-chain fatty acids leads to an unusual incorporation of large quantities of C15 and C17 fatty acids in the nerve sheaths. This is due to an alteration of glial cell synthesis resulting in myelin modification [16].

There is no shared diagnostic consensus for Cbl deficiency, and blood Cbl cutoffs vary from one group to another. In addition, the concentration of Cbl in the blood may not be sensitive enough to detect early signs of a deficiency and should be accompanied by other markers in order to reach a correct diagnosis.

2. Background

Among vegetarians, there are differing styles that are categorized according to the level of animal food exclusion. Vegetarianism can be classified into different plant-based subgroups: lacto-ovo-vegetarian (LOV) which excludes animal flesh but includes eggs and dairy products; ovo-vegetarian (OV), similar to LOV but excluding dairy products; lacto-vegetarian (LV), similar to LOV but excluding eggs; or vegan (VN), which excludes all animal foods, dairy products and eggs. In common language, the term vegetarian is interpreted differently depending on the country (i.e., LOV in Italy or LV in India).

Unbalanced vegetarian diets could be lacking in nutrients that are poorly represented in vegetal foodstuffs or with a low bioavailability (e.g., iron, zinc, vitamin D, ω3 polyunsaturated fatty acids) [17]. However, only Cbl seems to be virtually absent in vegetables and its shortage can have serious implications.

A common mistake is to think that the presence of dairy products and eggs in the diet, as in LOV, can still ensure a proper intake of Cbl, despite excluding animal flesh. In reality, consumption of such foods, despite containing significant amounts of Cbl, would be sufficient neither on a daily basis nor in order to meet vitamin requirements [18]. A Dietary Reference Intake (DRI) of 2.4 μg/day for Cbl in adults is a common chosen value [19,20]. Such an amount is apparently exceeded by American adults, with a mean intake ranging from 4.6 to 6.3 μg/day [21]. However, it is not uncommon to see a moderate deficiency among omnivores in Western countries [22,23]. A recent report by the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies established an

Adequate Intake (AI) of 4 μg/day for adults, with a mean intake in European countries ranging between 4.2 and 8.6 μg/day [24].

The main source of consumption in the general population comes from animal foods with a significant contribution from milk and dairy products [25]. Losses of up to 50% can occur through food processing which involves cooking, pasteurization and exposure to fluorescent light. This limits its availability, together with a drop in absorption capacity and an increase in Cbl concentration in food [26]. Some researchers claim that the currently recommended intake levels may not be sufficient for an adequate daily intake, with particular regard to aging and the physiological reduction in absorptive capacity [27]. With senescence, the epithelial cells of the stomach reduce their ability to biosynthesize the transporter proteins of Cbl. The gastric secretion ability is necessary both for the dissociation of Cbl from foods and for the binding to the carriers [28]. For these reasons, the American Institute of Medicine recommends a supplementation of Cbl for people of 50 years of age and older [19]. The development of blood and cognitive disorders are rather common aspects found among the elderly population [29].

In the vegetarian diet, there are few sources of Cbl, whilst supplement use is frequently resisted.

Although some plant foods seem to represent a significant source of Cbl [30,31], data in the literature are still insufficient to determine whether Cbl is found in the active form, and whether regular consumption of these foods can be sustainable when the variability in the production processes is taken into account.

3. Chemical Properties of Cobalamin and Vitamin Activity

The synthesis of Cbl is a prerogative of some bacteria and archaea [32]. Its presence in animal foodstuffs depends on the process of biomagnification through food chains [30]. Small quantities found in plants and derived from biofilms bound by Cbl cannot be synthesized by either animals or plants [33]. Cbl is the vitamin with the largest steric hindrance (1355.4 Da) [34]. It consists of a tetrapyrrolic corrin ring core with a central cobalt atom, grouped with four nitrogen, one nucleotide base group and one upper ligand. The cobalt atom accepts different ligands on the upper surface: hydroxyl (Hydroxycobalamin-*H*-Cbl), deoxy-5′-adenosine (Deoxy-5′-adenosylcobalamin-Ado-Cbl), metyl (methylcobalamin-Me-Cbl), cyanide (Cyanocobalamin Cn-Cbl). Me-Cbl and Ado-Cbl are the active forms of vitamins used as coenzymes in the cell [35,36]. On the lower surface of the ring, the cobalt atom binds a 5,6-dimethylimidazole nucleotide base (DMB). Naturally occurring corrinoids without vitamin activity bind lower ligands different from DMB such as 2-methyladenylcobamide, methylmercaptoadenylcobamide-2,5-methoxybenzimidazolylcobamide, benzimidazolylcobamide, 5-hydroxybenzimidazolylcobamide, *p*-cresolylcobamide [30].

Me-Cbl is the cofactor of the enzyme methionine synthase participating in the metabolic homocysteine pathway (HCY), which is processed into methionine with the involvement of vitamin B6 and folate [37]. The reaction takes place in the cytosolic environment and the deficiency of vitamin cofactors leads to an increase in HCY blood concentration [38]. This pathway is critical in the regeneration of the methyl donor *S*-adenosylmethionine (SAM) and its dysfunction creates a shortage, affecting DNA synthesis and the physiological processes that require intense cell replication, such as hematopoietic process of the erythrocytes. Ado-Cbl is the vitamin cofactor of the methylmalonyl-CoA mutase enzyme. It works within the mitochondria, in the metabolism of branched-chain amino acids and fatty acid with an odd number of carbon atoms. These atoms are not subject to degradation by a beta-oxidation pathway accepting only two carbon units [39,40]. The shortage of Ado-Cbl leads to an accumulation of methylmalonic acid (MMA), an intermediate molecule of this pathway. A deficiency of this vitamin form is due primarily to neurological effects. The myelin sheaths of neurons are highly dependent on fatty acid metabolism and the low bioavailability of Ado-Cbl in neurons leads to the depletion of the myelin layer with dysfunctional nerve transmission. The role of Cbl in neuropathological lesions seems to be due to interactions with neurotrophic molecules such

as Myelinolytic Tumor Necrosis Factor α (TNF-α), Epidermal Grow Factor (EGF) and Interleukin-6 (IL6) [41]. HCY itself appears to show a neurotoxic effect on synaptic receptors [42].

Cn-Cbl and H-Cbl are provitamin forms that require activation in cofactors Me-Cbl or Ado-Cbl to be utilized by the cells. Cn-Cbl is the form of Cbl first isolated and, although an artifact, it was initially identified as an anti-pernicious anemia factor [43]. Cobalamin performs a secondary function, that of removing potentially harmful molecules from the body. As a result, a small amount of Cbl in the blood stream is present in the Cn-Cbl form that binds cyanide residues. Cyanide is normally present in trace amounts in foods such as cassava, bitter almonds and apricot kernels (responsible for the bitter taste) [44]. H-Cbl is a form of cobalamin highly represented as a physiological intermediate [35]. Other corrinoids are non-vitamin analogs which are capable of engaging the carriers. These become less available to bind the vitamin forms, with an overall antinutrient outcome [45].

In the cell, the Cbl isoforms are metabolized inside the peroxisome by the reactions of dealkylation, decyanation and reduction, and then released in the specific cell compartment as coenzyme Me-Cbl and Ado-Cbl, according to their cytosolic or mitochondrial fate, respectively [46]. This step is crucial in the activation of the provitamin forms. Other corrinoid compounds do not fulfill the vitamin functions, in all probability due to the binding power of the lower ligand with the cobalt, which does not allow peroxisome activation [45]. It seems that all isoforms, provitamins and coenzymes should follow a mandatory route before being assigned to the appropriate cell district. However, the H-Cbl form may be more reactive, and its use can be facilitated by a number of enzymatic processes through non-specific cellular reactions [47]. If this mechanism were confirmed, the use of already active Cbl cofactors would not represent any provitamin advantage [45]. In 1982, Gimsing et al. analyzed Cbl in tissues from patients with pernicious anemia. After administration of H-Cbl or Cn-Cbl, they found that detected plasma Cbl was dependent on the form administered, dominant in the blood pattern. Results from erythrocytes and liver biopsies showed no differences, irrespective of the Cbl form used, indicating that administered Cbl preparations are converted in vivo to the necessary coenzymes [48]. Although current studies have many limitations and altogether there are no significant differences, the retention percentage after oral ingestion may change between different forms [49]. Following the ingestion of 1 μg of Cbl, the retention of Ado-Cbl and H-Cbl is 34% and 56%, respectively. Following the ingestion of 5 μg of Cbl, the retention of Ado-Cbl and Cn-Cbl is 13% and 20%, respectively. Following the ingestion of 25 μg of Cbl, the retention of Cn-Cbl and Ado-Cbl is 6% and 8%, respectively [50]. Updated data are not currently available.

4. Absorption and Transport

The absorption and transport of Cbl occurs via a complex network of proteins, which perform the task of optimizing the management of Cbl through the body districts. The study of rare genetic defects in carriers provided an opportunity to improve our understanding of the transport system and cellular trafficking of cobalamins [47,51]. Cbl is primarily associated with foodstuff proteins, such as Me-Cbl and Ado-Cbl. Free form, called crystalline or protein-unbound, is less represented and derived predominantly from supplements and fortified foods [25]. Transporters accept provitamin and coenzyme forms indifferently [52,53]. Following ingestion, the Cbl binds to the first salivary carrier R-binder or transcobalamin I, belonging to the corrin family of proteins. Its binding is non-specific and it binds various types of corrinoids, including antinutrient forms [52]. The increase of salivation promotes the secretion of R-binder and so the binding to the carrier that will occur after the dissociation from food proteins [54]. The gastric epithelial cells secrete hydrochloric acid and pepsin, responsible for the dissociation of Cbl from food proteins that enables binding to R-binder. The parietal cells also produce intrinsic factor (IF), another much more specific transporter for vitamin forms that binds Cbl released into the duodenum after cleavage from the R-binder through the use of trypsin and other pancreatic enzymes [55]. Thanks to the differing binding specificity, inactive analogs remain linked to R-binder and are excreted, while the vitamin forms continue on the route of absorption [56]. Binding to IF is critical for absorption into the terminal ileum, which is brought about by the membrane protein

cubilin as part of the cubam receptor complex along with an amnion-less protein. The IF-Cbl complex is internalized by receptor-mediated endocytosis on the luminal side of the polarized enterocyte [57]. Inside the enterocyte, the IF-Cbl complex is degraded into the peroxisome with the recycling of cubam protein on the apical side, degrading IF and releasing Cbl from the baseline side due to the MRP1 transport protein. Here Cbl is readily associated with blood carriers [58]. In the blood stream, the cobalamins are transported by the trans-cobalamin II carrier (TCII) in a complex defined as holotranscobalamin II (HTCII) and considered the active Cbl form circulating in blood, as this transporter is highly specific to the molecules with vitamin activity [59]. Presumably, the TCII is secreted by endothelial cells of the cardiovascular system [60]. HTCII represents only a small percentage of the circulating Cbl (5%–20%). The remaining Cbl is linked to another more aspecific carrier named haptocorrin or transcobalamin III (HC), an isoform of the R-binder molecule [61]. While all cells have a specific receptor for HTCII called TCb1R, HC receptors have been discovered only in hepatocytes. It is thought that the function of this transporter is inactive analog removal through reverse transport to the liver and intestinal release from bile salts [62]. The HTCII complex that is not removed from the blood stream by the cell membrane protein TCb1R is filtered by the kidney, and reabsorbed in the proximal tubule through megalin protein-mediated reuptake [63]. The binding specificity of the different carriers for vitamin, high for IF and TCII and low for R-binder and HC, is necessary to reduce the absorption of a non-vitamin or degraded Cbl molecules that may saturate the transport system and affect vitamin absorption. Thus, the function of some carriers acquires the role of scavenger of the non-active corrinoid analogs. The TCb1R-HTCII complex is internalized in peripheral cells and degraded in the peroxisome with receptor recycling and proteolysis of TCII, including the release of Cbl in the cytosol due to transporters LMBD1 and ABCD4 [51,64,65]. Here, chaperon CblC removes upper ligands (methyl, hyroxyl, cyanide, or adenosyl), preparing Cbl for methylation and adenosylation [37,66,67].

Active absorption is severely limited. It is estimated that the absorptive capacity of Cbl is 1.5–2 μg per meal and depends on the maximum saturation of the cubam pool [68]. Conditions that reduce the secretion or binding efficiency can inhibit this amount significantly, as occurs with aging, with the chronic use of proton pump inhibitors (PPI) in Gastro-Esophageal Reflux Disease (GERD) or with gastric dysfunctions such as atrophic gastritis [69]. There is, however, a passive diffusion across the mucosal epithelium. This system is concentration-dependent and it is estimated that about 1% of Cbl follows this route. The aspecific transport is gradient-dependent and may be significant in the use of high oral doses (0.5–1 mg), with a good efficacy also in persons with limited capacity for active absorption, such as in cases of pernicious anemia or gastric atrophy [70,71]. Enterohepatic circulation is essential for the efficient absorption of Cbl. In fact, an excess of Cbl not internalized by cells is also secreted with bile and reabsorbed by the IF transport pathway [72]. Infants born to mothers with an adequate intake of Cbl show reserves of about 25 μg at birth [73]. Due to the intense activity of cell replication in tissue expansion, breast milk should continue to provide Cbl linked to a corrin, from which it becomes separated through the proteolytic action of the child's gastrointestinal enzymes [74].

5. Assessment and Diagnostic Markers

There is no reference method or gold standard regarding Cbl deficiency and the clinical signs could be hematological, neurological and/or biochemical [75]. The deficiency of Cbl is defined by the serum concentrations <110 pmol/L [76,77], <127 pmol/L [78], <148 pmol/L [79,80], <150 pmol/L [81–83], <156 pmol/L [84,85], <220 pmol/L [86], <250 pmol/L [8]. The Institute of Medicine defined 120–180 pmol/L as a depletion range [25]. With such heterogeneous deficiency criteria, there is a risk of ignoring conditions of medical relevance. Moreover, even at concentrations above the cutoffs of 156 pmol/L (a commonly used cutoff), deficiency conditions may already be present [18,87]. The predictive strength of blood concentration of total Cbl is very low and insufficient for a diagnosis of deficiency, which may result in loss of memory, personality disturbance, emotional liability and psychosis even with a low-normal Cbl blood concentration [88]. In fact, total Cbl does

not take into account the ratio between HTCII and Cbl bound to haptocorrin. The detection of HTCII can provide useful information on the immediate availability of dietary Cbl [63]. The blood concentration of HTCII, like those of total Cbl, respond quickly to increased intake and thus can be misleading regardless of cellular restoration while using supplements [75]. HTCII is a more effective diagnostic marker compared to total Cbl but may be affected by the use of contraceptives as well as kidney or liver disease [89,90]. The sufficiency cutoff for HTCII is more homogeneously defined in literature as a plasma concentration above 35 pmol/L [18,82,84,91]. A Cbl deficiency at the cellular level is manifested through an accumulation of the intermediate products of the metabolic pathways, in which it participates as a coenzyme. The increased plasma concentration of HCY and urinary or serum MMA can provide more detailed information on the deficiency condition. As for serum Cbl, the criteria for an excess of HCY are heterogeneous: >15 μmol/L [78,80,81,83,85,92], >12 μmol/L [18,76,77,84,86,91,93,94], >10 μmol/L [8]. In addition, there are different criteria of deficiency for serum MMA cutoffs ranging from >271 nmol/L to >376 nmol/L, with the former being most prevalent [18,81,82,84,85,93–95]. Both blood markers, MMA and HCY, may be altered by renal failure and it is therefore useful to test glomerular function with creatinine [96]. The use of urinary MMA standardized for creatinine, although used less, may reduce the risk of an incorrect diagnosis [97]. Normally, the cutoff used for the assessment of Cbl deficiency by urinary MMA is >4 μg per mg of creatinine [98,99]. The increase of MMA can also be caused by intestinal bacterial overgrowth through the conversion of propionic acid produced by the human intestinal microbiota [100]. Given the high intake of vegetables in a vegetarian diet, it is unlikely that a rise of HCY is dependent on folate deficiency. It is likely that in fortification areas, where flour is enriched with folic acid, the main reason for the rise in plasma HCY is Cbl depletion [79]. The EFSA defined Cbl deficiency with serum Cbl below 140 pmol/L, serum MMA above 750 nmol/L, plasma HCY above 15 μmol/L and serum HTCII below 21–45 pmol/L [24]. In the Third National Health and Nutrition Examination Survey (NHANES III), the cutoffs for the diagnosis of hyperhomocysteinemia were defined as HCY above 11.4 μmol/L and 10.4 μmol for men and women, respectively [101]. The different values of diagnostic markers discussed are summarized in Tables 1–3.

Table 1. Vitamin B12 deficiency criteria.

Value	Country	References
<110 pmol/L	Austria	[76,77]
<127 pmol/L	Italy	[78]
<148 pmol/L	USA	[79]
<148 pmol/L	India	[80]
<150 pmol/L	India	[81]
<150 pmol/L	USA	[82]
<150 pmol/L	Turkey	[83]
<156 pmol/L	Germany/The Netherlands	[84]
<156 pmol/L	Germany	[85]
<220 pmol/L	Slovakia	[86]
<250 pmol/L	Germany	[8]

Table 2. Hyperhomocysteinemia criteria.

Value	Country	References
>15 µmol/L	Italy	[78]
>15 µmol/L	India	[80,81]
>15 µmol/L	Turkey	[83]
>15 µmol/L	Germany	[85]
>15 µmol/L	Taiwan	[92]
>12 µmol/L	Germany/The Netherlands	[18,84,94]
>12 µmol/L	Austria	[76,77]
>12 µmol/L	Slovakia	[86]
>12 µmol/L	Germany	[91,93]
>10 µmol/L	Germany	[8]

Table 3. Methylmalonic acid values for vitamin B12 deficiency.

Value	Country	References
>260 nmol/L	India	[81]
>271 nmol/L	Germany/The Netherlands	[18,84,94]
>271 nmol/L	Germany	[85,93]
>271 nmol/L	Germany/Oman	[95]
>376 nmol/L	USA	[82]

Some hematological markers can help to identify a shortage of vitamins, discriminating against red cell disorders as iron deficiency anemia, heterozygous thalassemia or other chronic diseases. The MCV and red cell distribution width (RDW) may increase in case of reduced availability of Cbl because of underlying hematopoietic alterations [102,103].

Herbert defined various stages of deficiency with the use of multiple markers as summarized in Table 4 [72]:

Table 4. Herbert's stratification for vitamin B12 deficiency [72].

Stage	Markers	Interpretation
I	HTCII	Blood and cellular reserves reduced with low HTCII
II	HC	Low concentrations of HC
III	HCY/MMA	Functional unbalanced with high concentrations of HCY and MMA
IV	MCV/Hb [1]	Clinical signs like high MCV, low Hb and macroovalocytosis

[1] Hb: Hemoglobin.

Unfortunately, some of the markers listed are not part of routine laboratory investigations and it is often difficult to locate diagnostic facilities that can perform all these tests.

Deficiency states could be boosted by impaired absorption. The Schilling test is a method, now rarely used, that was developed in 1953 (thanks to the availability of labeled Cbl) and applied in the diagnosis of food Cbl malabsorption [104]. This method quantifies the fraction of orally ingested labeled Cbl excreted in urine. Even if the Schilling test was considered the gold standard in the diagnosis of a Cbl deficiency, the use of radioactive material and misleading results in patients with antibodies against IF make it less suitable for clinical practice [105].

6. Status among Vegetarians

In Western countries, the intake of Cbl in the general population appears to be above the estimated requirements [19,21,24]. Its need during pregnancy and lactation increase due to the expansion of tissues and delivery to the fetus or the newborn. Thus the recommended dietary allowance has been determined at 2.6 µg/day and 2.8 µg/day for pregnancy and lactation, respectively [19,20].

The EFSA, however, settled on a safer amount of AI at 4.5 μg/day and 5 μg/day for pregnant and breastfeeding women, respectively [24]. A Cbl deficiency may occur by absorption alteration or nutritional insufficiency. Deficiencies are common in the elderly as a result of secondary hypochlorhydria due to drug treatment or a physiological alteration of the gastrointestinal mucosa itself [106]. The malabsorption can take place in cases of gastric or ileal resections, inflammatory bowel disease or for genetic defects in transport and cellular trafficking proteins [47,51,107]. The shortage of Cbl for low or no intake from food has been documented in low-income populations with poor nutritional status or in the case of vegetarians, with the first reports among vegetarian Indian people and Seventh-Day Adventist Church members [108,109]. Recent studies reported low serum cobalamin among vegetarians [103]. A deficiency in 11%–90% of elderly, 62% of pregnant women, 25%–86% of children, and 21%–41% of adolescents has been documented [110]. In a systematic review of literature based on the blood concentration of Cbl among vegetarians, a deficiency was present ranging from 0% to 86.5% among adults and elderly, up to 45% in infants, from 0% to 33.3% in children and adolescents, and from 17% to 39% among pregnant women [111]. The use of supplements or fortified foods seems to prevent deficiencies, indicating that a well-planned plant-based diet has proven to be adequate and sustainable [112,113]. However, despite the use of fortified foods, deficiency over a period of five years could occur, demonstrating a continuing insufficient intake or a possible decline in the absorptive capacity due to aging [114]. In all likelihood, even when supplementation occurs, it is possible that concentrations sufficient to avoid the reduction of body stock in the liver, blood and kidney cannot be reached. The liver is the main reservoir with a capacity of around 1–1.5 mg of Cbl [114].

Supplementation is often avoided due to preconceptions and aversion to products which are thought to be artificial, or due to the myth that the shortage will manifest itself only in rare cases after many years of ceased intake, an idea also supported by some researchers [115]. Although the shortage is documented in the macrobiotic community, many feel reluctant to use supplements, fortified foods, and more generally, processed foodstuffs [116].

The concomitant use of more specific markers enables a more detailed diagnosis. In adult German vegetarians, Cbl deficiency was present in 58%–66% or 61%–72% of participants if both criteria HTCII/MMA or HTCII alone were adopted, respectively [84,91,93,95]. Cbl deficiency leads to the accumulation of HCY, a molecule linked independently to the risk of cardiovascular disease (CVD), endothelial dysfunction and diabetes [117,118]. For each increment of 5 μmol/L of plasma HCY, there was a 20% increase in the risk of coronary heart disease (CHD) [119]. Concentrations of HCY among vegetarians seem to be higher and this correlates negatively with cardiovascular risk [54,76,103]. In a qualitative and quantitative review of the literature, it emerged that hyperhomocysteinemia with or without Cbl hipovitaminosis is a compelling risk factor for dementia [120]. In a meta-analysis on the status of vegetarians compared with omnivores by plasma HCY, six cohort case studies and 11 cross-sectional studies with a total of 3230 participants were analyzed, and an inverse relationship between HCY and Cbl for all diets was detected. Gradual worsening of both markers was evident from omnivores to VN with intermediate values in LV/LOV [54]. The HCY mechanisms of action are still unclear, but several theories have been proposed, including the initiation of the atherogenic process of reactive oxygen species (ROS) formation, inhibition of nitric oxide (NO) synthesis and influences on aortic calcification [121–123]. A recent meta-analysis of genome-wide association studies examined 18 single-nucleotide polymorphisms associated with total HCY concentrations [124]. A Mendelian randomization strategy was used to find no association between total HCY and the risk of coronary artery disease in genetic variants. These data may mitigate a causal correlation between HCY and CVD, but total blood HCY could be a useful marker for Cbl deficiency. When HCY, HTCII or MMA were used alone as diagnostic markers, the prevalence of deficiencies was greater in VN than in LOV/LV [18,76,77,85,91]. Although in the past it was thought that only the VN were at risk of vitamin deficiencies, recent studies indicate that even the LOV are at risk [125–127]. Herrmann et al. found that deficiency rates among LOV/LV and VN were 32% and 43%, respectively [85]. Supplementation in LOV/LV was effective in reducing deficiency rates from 68% to 31%, but the amounts were still

insufficient [18]. Also the increase of MCV and RDW, associated with the lack of Cbl, leads to the increased cardiovascular risk [128,129]. Neurological manifestations of vitamin deficiency can also occur in the absence of anemia [130]. If the repletion comes late, the myelin degeneration caused by deficiency can also be irreversible [131]. In a review of 89 case studies of Cbl deficiency, 13 of which were vegetarianism related, neurological manifestations included progressive spastic paraparesis, acute onset of irrelevant speech and inability to comprehend, involuntary movements of the upper extremities, unsteady gait, acute onset of dizziness, ataxia, vomiting, mildly impaired cognitive functions, disorientation in time and short-term memory loss [132]. Psychiatric abnormalities and dermatological and oral manifestations were also found [132]. In the absence of Cbl sources, the decline of Cbl in blood is already evident during the first five years after the adoption of a vegetarian diet even without taking into account more specific markers, dispelling the myth that the shortage appears only after a very long time [101]. The first signs of a deficiency can occur as early as two years after Cbl intake has ceased [95,97]. The risks related to shortage are also frequent in children born to vegetarian mothers with inadequate reserves [133]. A depleted status among vegetarian women may be responsible for Cbl deficiency in infants, as well as a failure to thrive, low acceptability of solid foods and delays in neurodevelopment [74]. Moreover, there are indications that an inadequate Cbl status during pregnancy will affect fetal programming mechanisms, resulting in an increase of adiposity and insulin resistance in the offspring at six years, as well as for the mother herself [134,135]. The role of one-carbon metabolism of non-communicable diseases in fetal growth has been discussed [136]. The influence of Cbl deficiency during pregnancy and breastfeeding is so considerable that even a dietary restriction in the short term can result in an inadequate state in the infant [137]. In a pooled analysis of 48 cases of infant deficiency, there was a remarkable similarity in clinical symptoms between maternal veganism and maternal pernicious anemia [74].

7. Supplementation and Fortification

Supplements have been demonstrated as efficient in the restoration of Cbl blood concentration [97,138]. Currently, the official position of associations and government agencies is categorical and unequivocal: in the case of a vegetarian diet, including LOV, LV and OV, supplementation of Cbl is required [11,13]. Cbl concentration per 100 g of cow's milk, dairy products and chicken eggs ranged from 0.5 to 0.4 μg, from 4.2 to 3.6 μg, and from 2.5 to 1.1 μg, respectively [139,140]. Taking into account the losses during cooking and the specific absorption rate, these quantities are not sufficient to ensure the daily intake in a balanced diet [141]. The microorganisms generally used for large-scale productions of cobalamin are *Propionibacterium freudenreichii*, *P. shermanii* and *Pseudomonas dentrificans* [142]. The World Health Organization (WHO) and the Food and Agriculture Organization (FAO) Expert Consultation have jointly released a guide for nutrient calculation procedures for the necessary quantities when fortifying foods [143]. In some countries, certain foods are fortified, such as breakfast cereals, with Cbl. However, the quantities used are quite variable and the consumption of such foods cannot guarantee sufficiency in the absence of other sources [25,139]. Some researchers show that the daily intake levels used are insufficient to ensure proper Cbl intake in population subgroups at risk, and they recommended a mandatory program of fortification of flour with folic acid, as is currently the norm in Canada and the USA [144,145]. Although folic acid in the blood seems high among vegetarians, it can bring about a subcellular deficiency as a result of the "folate trap" mechanism, in which the absence of Cbl blocks folate in the form of 5-methyltetrahydrofolate. This occurrence results in the blockage of the methyl group transfer to the substrate. The folate trap can mask a possible silent functional deficiency of folic acid, even with high folate serum concentration [146]. The vegetarian diet, rich in folacin, may mask hematological symptoms, so Cbl deficiency may only be evident due to neurological signs in the late stages, such as neuropsychiatric abnormalities, neuropathy, dementia and, albeit rarely, atrophy of optic nerves [85,130]. Usually hematologic manifestations and anemia precede neurologic signs, which are more severe and mostly irreversible [147,148]. The response to treatment is inversely proportional to the severity of the deficiency state and to the latency of intervention [130]. Cbl used in

fortified foodstuffs and in supplements is in crystalline form. There are different products containing Me-Cbl, Ado-Cbl and H-Cbl, either as a supplement or as pharmaceutical compositions.

Cn-Cbl is the most used form due to its high stability, cost effectiveness and safety of use [45]. At present, a tolerable upper intake level (TUIL) for Cbl from food or supplements was not defined, as the published data are insufficient in determining toxicity events. An accumulation and an excess of absorption are highly unlikely, in fact Cbl is a water-soluble molecule that requires a specific transport system which is easily saturated [19,149]. Its safety has been demonstrated through the use of an ultra-high parenteral dose of 25 mg daily for 10 days followed by 25 mg monthly for five months [150]. Cn-Cbl is the most common form used in the literature and in supplement formulation. Furthermore, it is the only compatible form in fortification thanks to its decent stability when heated [30]. In rare cases of genetic defects in peroxisome activation enzymes, the use of provitamin forms may not be recommendable [45]. At high doses such as 1–2 mg, about 10 μg is absorbed through non-specific internalization, functioning also in malabsorption diseases [151]. The therapeutic administration of oral Cbl has proven to be as effective as intramuscular administration [152]. This is very useful, as intramuscular administration is far more expensive and rather painful for the patient, as well as not being free from complications [153]. Recently, it was debated whether the coenzymes Me-Cbl and Ado-Cbl can be more efficient; that is, as a ready-to-use form that does not require a prior conversion [154,155]. The use of these more expensive formulations is not necessarily justified. In the literature, evidence of efficacy and safety is scarce. Moreover, a more responsive and less stable H-Cbl isoform may not reach the target [45]. If all forms of Cbl follow the same path of reduction-oxidation in the cell, the potential superiority of the coenzyme forms will not exist [45]. Since the crystalline form of Cbl is not bound to food proteins, the bioavailability in supplements is equal, if not superior. The Institute of Medicine (IOM) considers that naturally occurring Cbl in food is absorbed by 50% in healthy adults [25].

The vegetarian diet is very high in fiber, which may reduce the ability to absorb some nutrients efficiently [156]. An excess of fiber in the diet could disrupt the re-absorption of the Cbl mechanism through enterohepatic circulation, although there is no evidence to confirm that this happens in humans [157]. While there is not a higher incidence of anemia among vegetarians than in omnivores, in some cases iron intake may be inadequate [158]. Due to the low bioavailability of inorganic iron, the Institute of Medicine decided that the iron requirement is 1.8 times higher in a vegetarian diet [19]. Lower concentrations of iron can act on the gastric mucosa which favors atrophic gastritis, reducing the absorption capacity of Cbl via the intrinsic factor [110,159]. Although bone fractures among vegetarians are not statistically more frequent than in omnivores, a plant-based diet can result in lower calcium intake, especially among the VN [160]. The internalization of the IF-Cbl complex takes place thanks to a mechanism that requires calcium to function properly. A lower intake of calcium in the diet can inhibit the Cbl endocytosis mechanism in the distal ileum [161]. It was proposed that the metformin pharmacological treatment can alter the Ca-dependent uptake absorption of Cbl [162]. There are some indications that there is a correlation between polyunsaturated fatty acids of the series 3 (*n*3PUFA), especially long-chain (LC), and blood concentrations of HCY [163,164]. From preliminary data, the increased intake of LC *n*3PUFAs reduced plasma HCY with statistical significance [165–167]. Frequently, the vegetarian diet provides a high intake of *n*6PUFA and reduced contributions from *n*3PUFA, especially LC *n*3PUFA [168]. In addition to the possible link with HCY, excess *n*6PUFA can directly increase cardiovascular risk through the generation of proinflammatory eicosanoids and platelet aggregation [169]. For the above reasons, vegetarians should not underestimate Cbl intake through the appropriate use of supplements, while maintaining a balanced diet according to individual needs.

Absorption of Cbl from supplements depends on the dose and frequency of the intake [170]. The absorption capacity depends on the saturable active transport and on the efficiency of the aspecific route. The consumption of oral doses of 1 μg, 10 μg, 50 μg, 500 μg, 1000 μg, are absorbed with an efficiency of 56%, 16%, 3%, 2%, 1.3%, respectively [151]. The presence of Cbl inside the human gut is not

a sufficient amount in terms of a daily intake, since it cannot be absorbed through the specific transport that requires binding to the transporters. In addition, 98% of corrinoid compounds synthesized by the microbiota found in human feces appear to be inactive [171].

Using multivitamins can be inefficient and counterproductive for the supplementation of Cbl. The Cbl can be degraded in the presence of vitamin C and copper with the formation of inactive by-products. These compounds can inhibit the transport system interacting with transporter proteins [172,173]. Nutritional yeast fortified with Cbl is available in the USA, though its use may be less effective than supplements, in the case of deficiency [97].

8. Vegetable Sources of Vitamin B12 and Future Research

The quantification of the Cbl content in foodstuff can be performed by various analytical systems, some of which are not able to distinguish between active and pseudo-Cbl analog forms. The recent use of more accurate systems, such as gas chromatographic methods or IF-based chemiluminescence, clarified that often the content of Cbl in food may have been overestimated [26].

Vegetables like broccoli, asparagus and bean sprouts contain only traces of Cbl [174]. The presence of 0.1–1.2 μg/100 g in tea leaves is not enough to consider tea as an adequate source for daily intake [175]. The most commonly eaten mushrooms in Europe, such as porcini and *pleurotus*, do not contain relevant amounts of Cbl [31]. However, an Italian study has shown that selected types of oyster mushrooms grown in the southern areas of Italy show a wide range of concentrations of Cbl from 0.44 to 1.93 μg/100 g, as detected by ELISA immunoassay. The highest concentration was found in the species *Pleurotus nebrodalis*, typical of the mountain areas in central Sicily [176]. Less common mushrooms such as *Craterellus cornucopioides* or *Cantharellus cibarius* may contain 1.09–2.65 μg/100 g [177]. Shiitake mushrooms, popular among vegetarians, can contain up to 5.61 ± 3.9 μg of Cbl per 100 g of dry weight (mostly in active form), although with great variability [178]. Even in this case, although a portion of 50 g of dried shiitake could be adequate to achieve the daily requirement, it is unlikely that this will happen daily. Among the most widely used edible seaweeds, *Enteromorpha* sp. and *Porphyra* sp. (also known as nori) contain relevant amounts of Cbl ranging from 32.3 to 63.6 μg/100 g [179]. In vitro tests are promising, but there are not enough human clinical trials to consider the use of seaweed as favorable in vitamin provision [180,181]. In a clinical trial of six vegan children, the daily use of nori seaweed seemed to prevent Cbl deficiency, measured via serum Cbl [182]. In disagreement with these data, Dagnelie et al. found no positive effects in using nori seaweed and spirulina on Cbl-deficient children [183]. The Cbl content of other edible macroalgae is negligible and approximately zero [184]. In a pilot study, supplementation with Klamath microalgae (*Aphanizomenon flos-Aque*) improved Cbl status among 15 vegan subjects, assessed by serum Cbl and plasma HCY in a three-month open-label intervention [185]. Klamath contains about 32 μg/100 g of Cbl but Watanabe et al. extracted only a pseudovitamin analog from Klamath [186]. *Chlorella pyrenoidosa* is a microalgae frequently used as a supplement [187]. Corrinoid content in micro and macroalgae depends on an exogenous uptake due to the association with microorganisms responsible for the biosynthesis of Cbl [33]. In a pilot study of 17 LOV/VN, 9 g of *Chlorella* for 30–60 days were effective in mitigating Cbl deficiency, although these quantities are not compatible with a daily intake over the time period and the study was not independent [188]. Cbl in commercial preparations can be highly variable and still lack sufficient clinical trials on humans to verify the viability of use [189]. Several edible cyanobacteria, such as *Spirulina platensis* and *Nostoc* sp., contain significant amounts of corrinoids, many of which appear to be pseudovitamin [190]. Characterization with sensitive methods able to discriminate from different corrinoid compounds showed that the concentration of Cbl in spirulina was 127–244 μg per 100 g of dry weight, about 80% of which were non-vitamin compounds [191]. At present, cyanobacteria cannot be considered a reliable source of Cbl [190,192,193]. Some fermented vegetable foods, such as sauerkraut, natto and tempeh, can have significant amounts of Cbl. It is unlikely that their daily use in Western countries represents a stable source of Cbl. The presence of Cbl in these foods depends on environmental bacteria randomly present in the fermentative microorganism pool [194]. It is very

difficult to standardize the content from one product to another as they are subject to wide variation. Tempeh, for example, during the fermentation of soy beans can develop Cbl in a range between 0.7 to 8 μg per 100 g [195]. Other fermented soy foodstuff has only trace amounts of Cbl [196,197]. In sauerkraut production, the addition of *Proprionibacteria* sp. to cabbage may boost Cbl concentration up to 7.2 μg/100 g [198]. The use of organic fertilizer can increase the content of Cbl in spinach leaves up to 0.14 μg/100 g. However, the quantity of spinach that needs to be ingested in order to satisfy the daily requirement would be prohibitive [199]. Treatment with cyanocobalamin during the sprouting of daikon or through hydroponic techniques in lettuce may increase retention of Cbl and delivery through plant foods [200,201]. Promising data has indicated production of soy yogurt with an enhanced production of Cbl up to 18 μg/L, as detected with the HPLC method [202]. Cbl content in vegetable foods are summarized in Table 5.

Table 5. Cobalamin content in vegetable foods.

Foods	μg/100 g	Assay	References
Tea leaves	0.1–1.2	Microbiological	[175]
Tea leaves	0.046–0.859	IF-Chemiluminescence	[175]
Mushrooms (*Porcini*, *Pleurotus*)	0.01–0.09	LC/ESI-MS/MS	[177]
Mushrooms (*C. cornucopioides*, *C. cibarius*)	1.09–2.65	LC/ESI-MS/MS	[177]
Mushrooms (*Pleurotus* spp. from Sicily)	0.44–1.93	ELISA	[176]
Mushrooms (Shiitake)	3.95–5.61	LC/ESI-MS/MS	[178]
Seaweed (Nori)	32.26–63.58	Microbiological	[179]
Seaweed (Nori)	25.07–69.20	IF-Chemiluminescence	[179]
Microalgae (Klamath)	31.06–34.27	IF-Chemiluminescence	[186]
Microalgae (*Chlorella*)	200.9–211.6	IF-Chemiluminescence	[189]
Cyanobacteria (Spirulina)	127.2–244.3	Microbiological	[191]
Cyanobacteria (Spirulina)	6.2–17.4	IF-Chemiluminescence	[191]
Cyanobacteria (*Nostoc*)	11	HPLC	[190]
Tempeh	0.7–8	Not specified	[195]
Tempeh	0.02–0.7	Microbiological	[196]
Sauerkraut	Up to 7.2	Microbiological	[198]

9. Conclusions

The choice of limiting or removing foods of animal origin from the diet is increasing in popularity due to ethical, environmental and health reasons, posing doubts over whether a number of these restrictions could be detrimental or useful [203]. The vegetarian diet can be sustainable at all stages of life and in all physiological conditions, including infancy, pregnancy, lactation, senescence and sports [13]. Compared to non-vegetarians, vegetarians have reduced body mass index (BMI), serum cholesterol, serum glucose and blood pressure with a lower mortality rate due to ischemic heart disease (IHD) [17,204]. However, underestimating the correct supplementation of cobalamin (Cbl) can nullify these benefits [103]. It is also necessary that the diet be balanced and nutritionally adequate to reduce the risks of other deficiencies which could indirectly affect the absorption of Cbl. Studies on the use of plant foods to increase the Cbl intake are promising, but still require more data. Some seaweed, mushrooms and fermented foods can be a useful source of Cbl, but the data are still insufficient and production is too heterogeneous. The standardization of Cbl-rich plant foods may be useful in preventing vitamin deficiency, overcoming the frequent ideological barriers on supplementation. The use of fortified toothpaste could be a promising alternative to the fortification of flour, resolving the possible reduction of vitamins during cooking [205]. Studies of efficacy in maintaining vitamin sufficiency with different Cbl forms are absent. More trials with vegetarian people using supplements or fortified foods are needed to better explain the efficacy of different strategies of cobalamin uptake, focusing on the best dosages, media or foods, if reliable. Future research on the half-life of Cbl in various human body districts after the intake of different vitamin B12 supplements (H-Cbl, Ado-Cbl, Me-Cbl, Cn-Cbl) would be very useful in helping us understand Cbl utilization and needs, especially

in vegetarians. At present, there is no international consensus for supplementation in vegetarians. According to Carmel, a single oral dose of 50 μg, 500 μg or 1000 μg will be absorbed at an amount of 1.5 μg, 9.7 μg or 13 μg, respectively [151]. To meet the daily requirement of Cbl, one oral dose of 50–100 μg daily or 2000 μg weekly divided into two oral cyanocobalamin doses could be sufficient to meet the needs of 2.4 μg/day for healthy vegetarian adults, taking into account the efficiency of absorption and the passive route. In 1988, Herbert stated that vegetarians' Cbl requirements could be satisfied by 1 μg tablet of vitamin B12 per day, based on human reabsorption through enterohepatic circulation [32]. This quantity is fairly low in respect to the proposed values for Cbl Dietary Reference Intake (DRI) in the general population [19,20]. Cyanocobalamin is the most economical—and historically the most used form—rendering it suitable for safe daily use [45]. There were no apparent substantial differences between the absorption of sublingual and oral forms [152,206]. However, oral dissolution could be critical in the secretion of the salivary R-binder and its subsequent bond. Since the Cbl would not be dissolved, about 88% could be not absorbed [54]. Since the development of a Cbl deficiency can also be observed among the LOV, the use of a supplement is necessary, regardless of the type of vegetarian diet [110]. In cases of malabsorption, such as hypochlorhydria or functional deficit, such amounts might be insufficient [151,207]. With senescence, the lowering of gastric secretion reduces the direct absorption capacities due to reduced release of IF and the impairment of active transport, but not of the passive transport. At the same time, the natural gastric barrier decreases with consequent risk of gastrointestinal bacterial overgrowth and competing for the use of ingested Cbl [69]. If rare genetic defects of cellular trafficking and processing proteins exist, the choice of alternative forms of Cbl, such as Me-Cbl or H-Cbl could improve the effectiveness of supplementation [154,208–210]. It was speculated that the use of Cn-Cbl is unsuitable for supplementation among smokers, because it represents a form preferentially excreted for the purpose of hydrocyanic acid removal [211,212]. In these cases, the use of H-Cbl may be desirable, although it is yet to be confirmed [213,214]. The data currently available do not permit an estimate of a maximum intake level for cobalamin. High levels of administration in particular lead to rare adverse events, mostly dermatological, such as pruritus, rash and skin eruptions [215]. The sufficiency of bodily stocks should not be a reason to delay supplementation, in light of the fact that the manifestation of the deficiency can occur through often irreversible neurological signs. The current data do not support the theory that vitamin deficiency needs 20–30 years to be manifested [125]. Nevertheless, future studies should take into account the possibility of supplementation even in a subclinical condition, as was already done for other deficiencies [216,217], which may become overt during the time-period [218,219]. The use of multiple diagnostic markers can facilitate the correct assessment of the status and allow a more sound decision in relation to the administration plan. The habit of using only one or a few of the multiple markers available implies that the deficiency status of Cbl among vegetarians, as among omnivores, may have sometimes been underestimated. Moreover, cobalamin displays other functions, not strictly metabolic, that could be lacking when deficient. A vitamin B12 deficiency could be related to oxidative stress markers like plasma glutathione, malondialdehyde and serum total antioxidant capacity, which could contribute to a neurophysiological disturbance [220]. Furthermore, Cbl, particularly H-Cbl, can act as a detoxifying agent, removing potentially dangerous cyanide molecules from the body [212].

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Validation of Folate-Enriched Eggs as a Functional Food for Improving Folate Intake in Consumers

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Abstract: Functional foods enriched with folate may be beneficial as a means of optimizing folate status in consumers. We recently developed novel eggs enriched with folate through folic acid supplementation of the hen's feed, but their potential to influence consumer folate status is unknown because the natural folate forms incorporated into the eggs may not necessarily be retained during storage and cooking. This study aimed to determine the stability of natural folates in folate-enriched eggs under typical conditions of storage and cooking. Total folate was determined by microbiological assay following tri-enzyme treatment in folate-enriched eggs and un-enriched (barn and free-range) on the day they were laid, after storage (up to 27 days) and after using four typical cooking methods (boiling, poaching, frying, scrambling) for different durations. On the day of laying, the folate content of enriched eggs was found to be significantly higher than that of un-enriched barn or free-range eggs (mean ± SD; 123.2 ± 12.4 vs. 41.2 ± 2.8 vs. 65.6 ± 18.5 μg/100 g; $p < 0.001$). Storage at refrigerator and room temperature for periods up to the Best Before date resulted in no significant losses to the folate content of folate-enriched eggs. Furthermore, folate in enriched eggs remained stable when cooked by four typical methods for periods up to the maximum cooking time (e.g., 135 ± 22.5, 133.9 ± 23.0 and 132.5 ± 35.1; $p = 0.73$, for raw, scrambled for 50 s and scrambled for 2 min, respectively). Thus, natural folates in folate-enriched eggs remain highly stable with little or no losses following storage and cooking. These findings are important because they demonstrate the feasibility of introducing folate-enriched eggs into the diet of consumers as functional foods with enriched folate content. Further studies will confirm their effectiveness in optimizing the biomarker folate status of consumers.

Keywords: folate-enriched eggs; folate stability; food folate analysis; novel foods; functional foods

1. Introduction

The B-vitamin folate plays an established protective role in the prevention of neural tube defects (NTD) [1,2]. This discovery led to the introduction of worldwide recommendations for women planning a pregnancy to take folic acid (FA) supplements from before conception and up to the 12th gestational week in order to ensure optimal maternal status during the period of neural tube closure in early pregnancy. In more recent years, however, evidence is emerging to suggest that the achievement of optimal folate status may be important not only for women of child-bearing age, but for the general population. This is due to the increased recognition of the potential protective role of folate in the primary prevention of cardiovascular disease, particularly stroke [3,4], age-related cognitive impairment [5,6] and osteoporosis [7,8].

An evaluation of the current folate recommendations for women planning a pregnancy (i.e., based on the use of folic acid supplements) has shown that compliance is low [9,10] and consequently these recommendations have been ineffective in decreasing the incidence of NTDs in all European countries

examined [11]. However, other countries, such as the US and Canada, have addressed this issue by introducing policies requiring mandatory FA fortification of cereal-grain products. This measure has resulted in a substantial increase in folate status of all populations in which such a policy has been introduced [12,13], a subsequent decrease in the incidence of NTDs [14,15] and possibly a reduction in stroke-related deaths [16]. Despite this evidence, many European governments have decided against the introduction of a mandatory FA fortification policy. This is primarily a result of safety concerns regarding chronic exposure of the general population to FA, the synthetic form of the vitamin, which, when consumed in high doses can lead to the appearance of unmetabolized FA in the circulation [17,18]. Previously, the most widely publicised risk of excess FA intake concerned the potential to mask the macrocytic anaemia of vitamin B_{12} deficiency, common in older adults, while allowing the associated irreversible neurological symptoms to progress [19]. However, other safety concerns have also been raised, most notably evidence suggesting the potential of high-dose FA to promote the growth of new or already existing but undiagnosed colorectal adenomas in those with pre-existing lesions [20].

With the lack of success of FA supplementation and concerns regarding FA-fortified foods, alternative approaches to achieving optimal folate status need to be considered. One option is the development of novel functional foods based on animal products enriched with natural forms of folate through the addition of high-dose FA to animal feed. Research has shown that eggs can be successfully enriched with folate by supplementing hens' diets with FA, achieving a maximal folate content of about 2.5 times that of a normal egg [21–23], with a minimal (less than 10%) amount of unmetabolized FA [22].

Although our previous studies demonstrate that eggs can be successfully enriched with natural folates through folic acid supplementation of the hen's feed, the potential of these eggs to influence consumer folate status is unknown because the natural folate forms incorporated into the eggs may not necessarily be retained during storage and cooking. As reduced forms of the vitamin, natural folates are labile and prone to oxidation, thus potentially leading to poor stability during processing and/or cooking [24–26]. The aim of the present study, therefore, was to investigate the effect of both storage and cooking on the stability of natural folates in folate-enriched eggs.

2. Materials and Methods

2.1. Study Design

All eggs used in this study were produced by Skea Eggs (Donaghmore, Northern Ireland) and Devenish Nutrition (Belfast, Northern Ireland) according to the protocol for folate enhancement as previously described [20]. Each batch of eggs was collected from Skea Eggs on the day that they were laid (i.e., day of collection = day 1 for subsequent experiments). All storage and cooking experiments were carried out at the Northern Ireland Centre for Food and Health (Ulster University).

2.1.1. Stability of Folates during Storage

Three types of egg were collected: un-enriched (barn and free-range; $n = 18$ for each type) and folate-enriched (free-range; $n = 54$). Each group of eggs was divided into two sets; set 1 was stored at refrigerator temperature (4–7 °C) and set 2 was stored at room temperature (18–20 °C) for a total of 27 days (the maximum time allowed by Skea Eggs for the 'Best Before' date). On days 1, 7, 14, 21 and 27, samples of enriched eggs ($n = 6$) from each storage condition were weighed without the shell and then analyzed for folate content (Figure 1). Un-enriched eggs (barn and free-range) from each storage condition ($n = 6$) were weighed and analyzed for folate content only on days 1 and 27.

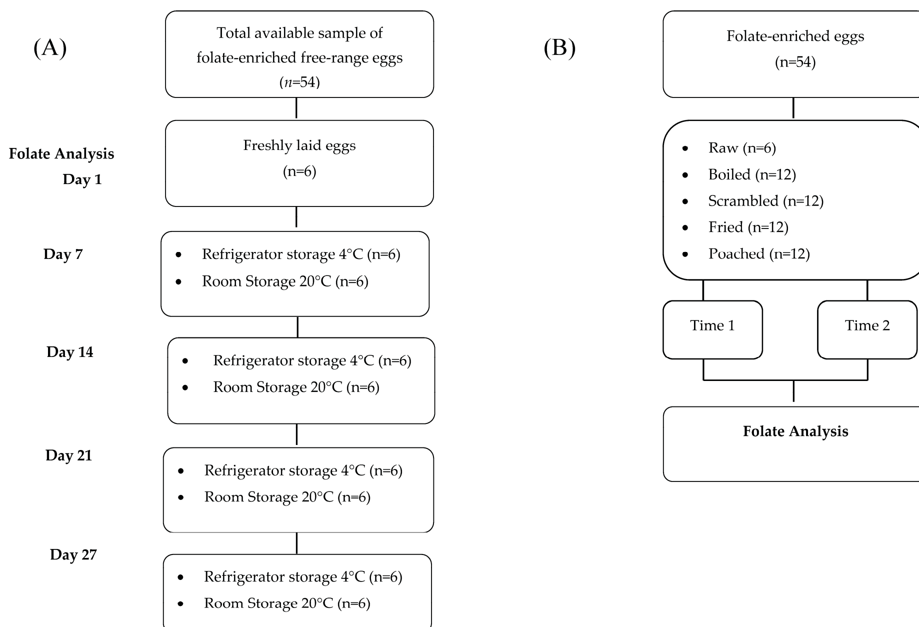

Figure 1. Flowchart of the experiments for the effect of storage (**A**) and cooking (**B**) on the stability of folate in folate-enriched eggs. Time 1 and time 2 refer to sampling times and correspond with the 'soft' and 'hard' consistency of cooked egg.

2.1.2. Stability of Folates during Cooking

Cooking stability experiments were performed on two separate occasions using two separate batches of folate-enriched eggs. On each cooking occasion, a batch of 54 folate-enriched eggs was collected (Figure 1). Eggs were cooked by four typical cooking methods: boiling, poaching, frying, and scrambling, and the folate content was analyzed (n = 6) at two time points, to correspond to the 'soft' and 'hard' cooking of eggs by each cooking method (details below). Preliminary experiments were carried out for each type of cooking method to determine the cooking time (min) required to achieve the corresponding 'soft' and 'hard' consistencies of eggs. Samples of raw egg were also assessed for folate content (n = 6) at the time of each cooking experiment.

Prior to cooking, eggs for frying, poaching and scrambling were weighed without the shell. They were weighed again following cooking in order to take into account changes in weight due to water loss as a result of cooking. Eggs for boiling were weighed without the shell after cooking. For frying, poaching and scrambling experiments, all eggs were cooked individually whereas for boiling experiments the eggs were cooked together. *Boiling*: samples were placed in a stainless-steel saucepan, covered with water and brought to a boil over high heat. Heat was reduced to maintain a simmer and samples were taken out of the water at 3 or 8 min. Following cooking, eggs were drained, rinsed with cold water, and the shell was removed. Once the shell was removed, each egg was weighed. *Frying*: a non-stick frying pan was brought to temperature over high heat, the temperature was reduced to medium, four sprays of non-stick spray oil were applied and an egg was added into the pan. The egg was turned halfway through cooking and samples were collected at 3.5 or 8 min. *Poaching*: water was brought to simmer in a stainless-steel saucepan over medium heat. Once simmering, an egg was added and cooked for 3 or 10 min. Following cooking, eggs were removed and drained well using a slotted spoon. *Scrambling*: a non-stick frying pan was heated over medium heat, four sprays of non-stick spray

oil were applied and an egg was added and stirred using a stainless-steel spoon. Eggs were cooked for 50 s or 2 min.

2.2. Sampling

All raw and cooked samples were homogenised in 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES)/HEPES buffer, using a stand homogeniser. Cooked samples were homogenised in approximately an equal volume of buffer to egg weight and raw samples were homogenised in approximately double the volume of buffer to weight of egg. Homogenates (3 mL aliquots) were purged with N_2 and stored at −80 °C for subsequent folate analysis.

2.3. Determination of Total Folate Content of Samples

All preparative and analytical procedures were performed under golden yellow light and contact with air was minimised by purging with N_2. The total folate content of egg samples was measured in duplicate for all experiments by microbiological assay with *Lactobacillus casei* preceded by thermal extraction with tri-enzyme treatment.

2.4. Thermal Extraction with Tri-Enzyme Treatment

All preparative and analytical procedures were performed as originally described by Tamura [27] and modified by McKillop et al. [26] with the following additional modifications. Solutions of α-amylase and protease (Sigma-Aldrich Company Ltd., Poole, UK) were prepared fresh each day by dissolution in ultrapure water (Purelab Prima, Davidson & Hardy Ltd., Belfast, UK) at concentrations of 50 mg/mL and 2 mg/mL, respectively. Trace levels of folate were removed from the enzyme solution by treatment with activated charcoal and filtration through a 0.2 μm syringe filter.

Thawed samples (500 μL) were mixed in 50 mL Oak Ridge centrifuge tubes (Nalgen, Rochester, NY, USA) with 5 mL CHES/HEPES buffer, pH 7.85, which had been taken to 100 °C by immersion in a boiling water-bath. Samples were boiled at 100 °C for 10 min. Following rapid cooling on ice, 500 μL α-amylase was added to each tube and incubated for 1 h at 37 °C. The enzyme was thermally deactivated, and 500 μL protease was added to each tube and incubated for 2 h at 37 °C. The enzyme was thermally deactivated and samples were centrifuged at 3000× *g* for 10 min. The resulting supernatant was divided into 3 × 1.5 mL aliquots, flushed with N_2, and stored for up to 3 months at −80 °C until folate determination.

2.5. Microbiological Assay with Lactobacillus casei

Total folate content was determined by microbiological assay as previously described by Molloy and Scott [28]. Calibration of the assay was performed using FA (Sigma-Aldrich Company Ltd.) as a standard. For quality-control (QC) purposes, lyophilized pig liver (CRM 487, European Commission, Institute for Reference Materials and Measurements, Belgium) was used. The inter-assay coefficient of variation for folate content of QC samples was 7.1% (n = 12). Samples for all storage conditions were analyzed together and samples from each cooking method in addition to raw samples were analyzed together. All dilutions were carried out in a 0.5% sodium ascorbate solution using an automated dilutor (Hamilton, Bonaduz AG, Bonaduz, Switzerland).

2.6. Statistical Methods

The sample size for this study was estimated by using the results for natural folate content of enriched eggs from our previous published work [22] and considering potential losses of folate during cooking. It was estimated that a sample size of six eggs per group would be required in order to discriminate 15% to 20% difference between the cooked and the raw eggs with a power of 80% at $\alpha = 0.05$.

All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS, version 15.0; SPSS UK Ltd., Chertsey, Surrey, UK). Differences in folate content between egg type,

storage temperature and length of storage were compared using one-way analysis of variance (ANOVA) with the Scheffe post hoc test and independent and paired samples *t* tests. Differences in folate content between cooking methods and cooking time points were established by ANOVA with the Scheffe post hoc test. For normalization purposes, variables were log transformed as appropriate prior to statistical analysis and results were considered significant at $p < 0.05$. Results are expressed as means ± SD.

3. Results

3.1. Effect of Storage on Egg Folate Content

Table 1 presents the total folate content of eggs following storage at either refrigerator (4–7 °C) or room (18–20 °C) temperature for a period of up to 27 days. On day 1, prior to storage, the folate content of the enriched eggs was found to be significantly higher than that of the un-enriched both barn ($p < 0.001$) and free-range ($p < 0.001$) eggs. Furthermore, within the un-enriched eggs, the folate content of the free-range eggs was significantly higher compared to that of the barn eggs ($p < 0.001$). Again, on day 27, there was a significantly higher folate content of the enriched eggs as compared to the un-enriched both barn ($p < 0.001$) and free-range ($p < 0.001$) eggs, with the folate content of the un-enriched free-range eggs also being significantly higher than that of the barn eggs ($p < 0.001$). No changes in the folate content were observed between the un-enriched barn and free-range eggs analyzed on day 1 and those that were stored for 27 days. There was also no significant difference in the folate content of the enriched eggs analyzed on day 1 and those analyzed at day 7, day 14, day 21 and day 27. Un-enriched both barn and free-range eggs stored at refrigerator temperature were not found to differ significantly in folate content compared with the eggs that were stored at room temperature. Moreover, at all time points, there was no significant difference in folate content in the enriched eggs stored at refrigerator temperature and those that were stored at room temperature.

Table 1. Total folate content (μg/100 g) of eggs following 27 days storage at refrigerator temperature (4–7 °C) or room temperature (18–20 °C) [1].

	Folate Concentration (μg/100 g)						
	Un-Enriched Barn		**Un-Enriched Free-Range**		**Folate-Enriched [2]**		***p*-Value [3]**
	4–7 °C	18–20 °C	4–7 °C	18–20 °C	4–7 °C	18–20 °C	
Day 1	41.4 (2.8) [a]		65.6 (18.5) [b]		123.2 (12.4) [c]		<0.001
Day 7	N/A		N/A		107.9 (9.6)	127.0 (26.9)	0.17
Day 14	N/A		N/A		134.0 (16.9)	115.1 (24.3)	0.21
Day 21	N/A		N/A		119.5 (19.7)	108.2 (14.7)	0.28
Day 27	36.6 (11.3) [a]	43.9 (5.9) [a]	70.0 (16.4) [b]	65.9 (7.2) [b]	122.0 (27.7) [c]	123.7 (18.3) [c]	<0.001

[1] Values are presented as mean (standard deviation); $n = 6$ for each type of eggs at each time point; [2] Folate enriched eggs were free-range; [3] Differences in folate content between egg type, storage temperature and length of storage were compared using analysis of variance (ANOVA) with the Scheffe post hoc test and independent and paired samples *t* tests. Different letters denote differences between groups ($p < 0.001$). N/A = Not applicable (not sampled/analyzed).

3.2. Effect of Cooking on Egg Folate Content

Table 2 presents the total folate content of enriched eggs following four typical cooking procedures. No statistically significant difference in folate content was observed between the raw eggs and eggs subjected to boiling, frying, poaching and scrambling. For all cooking methods, it was found that cooking time did not significantly affect folate content. Compared to raw values, no significant difference in folate content was observed in eggs that had been cooked for the minimum time and those that had been cooked for the maximum time.

Table 2. Total folate content (μg/100 g) of folate-enriched eggs following four typical cooking treatments [1].

Cooking Method	Folate Concentration (μg/100 g)			*p*-Value [2]
	Raw (*n* = 6)	Time 1 [3] (*n* = 6) [4]	Time 2 [3] (*n* = 6) [4]	
Boiled	135.7 (22.5)	125.2 (28.6)	145.4 (20.5)	0.402
Fried	135.7 (22.5)	137.2 (11.7)	139.1 (12.1)	0.730
Scrambled	135.7 (22.5)	133.9 (23.0)	132.5 (35.1)	0.616
Poached	135.7 (22.5)	126.9 (10.8)	132.7 (19.1)	0.597

[1] Values are presented as mean (standard deviation); [2] Differences in folate content between cooking methods and cooking time points were compared using analysis of variance (ANOVA) with the Scheffe post hoc test; [3] Time 1 and time 2 refer to sampling times and generally correspond with the 'soft' and 'hard' consistency of the cooked egg, respectively. Time 1 and time 2 differed according to cooking method: for Boiled, 3 and 8 min; for Fried, 3.5 and 8 min; for Scrambled, 50 s and 2 min; for Poached, 3 and 10 min. Times were determined from preliminary cooking experiments (see text); [4] For Boiled, at both Time 1 and Time 2, *n* = 5.

4. Discussion

The purpose of this study was to investigate the effect of storage and typical cooking methods on the stability of natural folates in folate-enriched eggs. The results showed that the folate content was not adversely affected by storage at either refrigerator or room temperature as used by retail outlets and consumers. Moreover, folate in enriched eggs remained stable when cooked by four typical cooking methods, including boiling, frying, poaching and scrambling, even after cooking for prolonged time periods.

Unlike the synthetic vitamin form, FA, natural food folates are chemically unstable [29] and therefore can be prone to losses during storage, processing and cooking [24–26]. The current results however showed that neither the temperature (refrigerator or room), nor the duration of storage for up to 27 days (i.e., from date of laying to Best Before date as applied by producers), resulted in significant folate loss from eggs, whether they were un-enriched or folate-enriched. Similarly, previous reports showed only minimal folate losses during storage for beef liver and a variety of fruits and vegetables [30–33]. Although there is limited evidence specifically relating to the stability of folate in eggs, one previous report—in agreement with the current study—showed that folates remained stable when eggs were stored for 28 days at 4 °C [23].

Of perhaps greater note, the current results showed that cooking of folate-enriched eggs had no significant effect on folate stability, regardless of the method used. Folate in egg yolk from conventional eggs was previously shown to be fairly stable following boiling for 15 min, with folate retention estimated to be 73%–94% [34,35]. The current results on folate-enriched eggs showed even greater stability of the natural folate content (with retention rates of almost 100%), after boiling, frying, poaching or scrambling, even for prolonged time periods (corresponding to 'hard' consistency). In contrast, much older studies reported substantial losses of folates, of up to 70%, from eggs which were boiled, fried, poached or scrambled [36]. This discrepancy is most likely the result of improvements in the methodology for food folate analysis, which has advanced considerably in recent decades, particularly with respect to the extraction buffer. The current study used 2-mercaptoethanol as well as ascorbic acid, whereas older studies used ascorbic acid only in the preparation of the buffer used to extract folates from foods prior to analysis by microbiological assay [36]. The additional use of 2-mercaptoethanol as an antioxidant to protect folates during sample processing analysis was previously reported to achieve greater folate recoveries [37,38].

In contrast to the current findings, showing that boiling even for the maximum cooking time of 8 min resulted in no significant loss of natural folates from eggs, previous research has generally found that processes involving direct contact between the food product and cooking liquid, such as blanching and boiling, can lead to leaching of the vitamin. Thus, considerable losses of folate have been reported with boiling or blanching of green vegetables and legumes, both under domestic conditions and on an industrial scale [24–26,30,39,40]. Longer cooking times have been reported to result in even more substantial folate losses [30,32,41]. The protective presence of the egg shell during boiling is

unlikely to be the explanation for such inconsistencies, since poaching eggs (thus subjecting the egg to direct contact with the cooking water) also resulted in no significant loss of folate in the current study. It is possible that poaching, by causing the proteins in the egg white to coagulate, may provide a protective barrier around the yolk where the majority (99%) of egg folate is located [42]. Another possible explanation for the greater folate stability in egg during boiling may be attributable to its antioxidant content and particularly the amino acid cysteine, which is abundant in eggs and may result in greater folate retention compared to foods deficient in these substances [35]. Likewise, previous reports also showed that folate losses were minimal after boiling, frying or grilling of various animal foods (beef, fish, poultry and liver) known to have a high content of cysteine [26,33,43].

The current findings, that folates in enriched eggs remain stable with storage and cooking, may have important implications for human health. The consumption of one folate-enriched egg per day (containing 75 μg folate per egg) would thus provide an effective means of increasing dietary folate intakes in consumers, thus improving folate status and potentially, the related health benefits. Given that health promotion strategies for increasing folate status based on FA supplements have proven to be ineffective at reducing the occurrence of NTDs in Europe [11], and mandatory FA fortification policies are controversial [10], alternative approaches for optimizing folate status should be considered. Regular consumption of folate-enriched eggs would enhance folate intake without the perceived safety concerns associated with FA fortification. These novel eggs would be particularly beneficial in countries without a mandatory FA fortification policy which ensures more optimal folate intakes on a population-wide basis. As eggs are generally not consumed raw, the current findings demonstrate that consumers would be able to cook folate-enriched eggs by their preferred method, following storage for periods up to the Best Before date, with no risk of folate losses. Although further research is necessary to demonstrate the effectiveness of folate-enriched eggs in increasing biomarker status of folate in consumers, our previous study suggests that egg folate will prove to be highly bioavailable [44].

We previously successfully described the development of novel folate-enriched eggs through the addition of supplemental FA to the hen's diet, increasing the folate content by 2.5-fold, from 32 μg to 75 μg per average sized egg (i.e., 50 to 130 μg/100 g) [22]. This approach relies on the hen's ability to convert high amounts of FA added to the feed into the natural forms of the vitamin (predominantly 5 methyltetrahydrofolate) which then transfer into the egg, with the final product containing a minimal amount (less than 10%) of unmetabolized FA [22]. In the present study, the folate content of folate-enriched eggs was shown to be significantly higher than unenriched eggs, either ree-range or barn eggs, by 1.9-fold and 3-fold, respectively. The greater extent of enrichment (via FA supplementation of the hen's diet) in the latter case was owing to a lower folate content of the unenriched product, with our results showing that the folate content of unenriched free-range eggs was significantly higher than unenriched barn eggs. The finding that conventional (i.e., unenriched) free-range eggs have higher folate content than barn eggs, as observed here for the first time, may possibly be explained by the fact that free-range hens have access to fresh forage which could provide an additional folate source to their daily feed, whereas barn hens are fed only with processed feed of minimal folate content.

The main strength of the study is that the individual experiments were carefully designed to investigate the stability of folates in eggs under the conditions of storage and cooking typically used by retailers and consumers. Prior to the cooking experiments, we carried out preliminary testing for each cooking method in order to identify the specific time required to achieve the 'soft' and 'hard' consistency of eggs, in order to ensure that our results would be directly applicable to consumer practices. In addition, we applied robust analytical methodology (based on *Lactobacillus casei* microbiological assay following thermal extraction and tri-enzyme treatment) to determine the folate content of eggs, and all necessary measures were taken to preserve natural folates during the laboratory analysis, providing confidence in the validity of our results. One limitation is that we investigated the stability of folate-enriched eggs as a food but not when used as an ingredient in composite meals and bakery products. However, given the consistency of our results showing that folates in enriched

eggs are highly stable, we anticipate minimal or no folate losses if enriched eggs were used in meals or bakery products rather than consumed as foods.

5. Conclusions

The current findings demonstrate that natural folates in novel folate-enriched eggs remain highly stable with little or no losses, following storage at refrigerator and room temperature for periods up to the Best Before storage time of 27 days, or when cooked by boiling, frying, poaching and scrambling, even for prolonged periods. These results are particularly relevant given that mandatory FA fortification remains non-existent in the UK and other countries in Europe, resulting in the widespread sub-optimal folate status of populations. The consumption of these novel folate-enriched eggs would offer consumers one alternative and cost efficient means of increasing folate intake and potentially protecting against disease. Current egg consumption of British women is estimated at 3–4 eggs/week [45], however evidence shows that egg consumption can be safely increased to 14 per week, with no unfavorable effect on plasma LDL cholesterol [46]; thus making folate-enriched eggs a potentially viable contributor to the folate intake of consumers. Further research, in the form of human intervention studies, is necessary to demonstrate the effectiveness of folate-enriched eggs in enhancing the biomarker status of folate in consumers.

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Article

Impact of Pre-Pregnancy BMI on B Vitamin and Inflammatory Status in Early Pregnancy: An Observational Cohort Study

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Abstract: Maternal nutrition and inflammation have been suggested as mediators in the development of various adverse pregnancy outcomes associated with maternal obesity. We have investigated the relation between pre-pregnancy BMI, B vitamin status, and inflammatory markers in a group of healthy pregnant women. Cobalamin, folate, pyridoxal 5′-phosphate, and riboflavin; and the metabolic markers homocysteine, methylmalonic acid, and 3-hydroxykynurenine/xanthurenic acid ratio (HK/XA); and markers of cellular inflammation, neopterin and kynurenine/tryptophan ratio (KTR) were determined in pregnancy week 18 and related to pre-pregnancy body mass index (BMI), in 2797 women from the Norwegian Mother and Child Cohort Study (MoBa). Pre-pregnancy BMI was inversely related to folate, cobalamin, pyridoxal 5′-phosphate (PLP), and riboflavin ($p < 0.001$), and associated with increased neopterin and KTR levels ($p < 0.001$). Inflammation seemed to be an independent predictor of low vitamin B6 status, as verified by low PLP and high HK/XA ratio. A high pre-pregnancy BMI is a risk factor for low B vitamin status and increased cellular inflammation. As an optimal micronutrient status is vital for normal fetal development, the observed lower B vitamin levels may contribute to adverse pregnancy outcomes associated with maternal obesity and B vitamin status should be assessed in women with high BMI before they get pregnant.

Keywords: pregnancy; obesity; pre-pregnancy BMI; B vitamins; inflammation

1. Introduction

There is an increasing prevalence of obesity in most parts of the world, also affecting women of childbearing age [1]. Pre-pregnancy obesity is associated with an increased risk of adverse pregnancy outcomes for both mother and child, including subfertility, miscarriage, gestational diabetes, gestational hypertension, preeclampsia, macrosomia, preterm birth, congenital anomalies, and fetal death [2,3]. Epidemiological studies have revealed strong links between nutritional excess during pregnancy and later development of metabolic disease, such as type 2 diabetes and obesity, in adult

life [4]. Maternal metabolic, nutritional, and inflammatory factors have been suggested as mediators in the development of the various negative pregnancy outcomes associated with maternal obesity.

A higher body mass index (BMI) has been associated with an adverse nutritional status in both non-pregnant adults [5–7] and children [8,9]. An inverse relation between pre-pregnancy BMI and several micronutrients like folate, vitamin D, carotenoids, zinc, and essential fatty acids have been reported in pregnant women, and negative pregnancy outcomes related to obesity might be related to impaired micronutrient status [10–13]. An adequate B vitamin status during pregnancy is important for maternal health and normal fetal development [14]. Deficiencies of folate, cobalamin, riboflavin, or vitamin B6 are associated with an increased risk of placental abruption, still-births, low birth weight, preterm deliveries, preeclampsia, as well as fetal malformations [14].

Obesity in itself results in an inflammatory state in metabolic tissues [15], and higher levels of the inflammatory markers C-reactive protein, neopterin, and kynurenine/tryptophan ratio (KTR) are seen in overweight and obese adults [16,17]. Inflammation has been associated with low circulating levels of several micronutrients [18], such as the active form of vitamin B_6, pyridoxal 5′-phosphate (PLP) [19], and vitamin D [20].

The objective of the study was to investigate whether BMI and inflammation are independent determinants of B vitamin status, we investigated the association of pre-pregnancy BMI with plasma B vitamin status and inflammatory markers in pregnancy week 18 in 2797 women from the Norwegian Mother and Child Cohort Study (MoBa).

2. Experimental Section

2.1. Study Population

This study is based on a subsample of 2825 women included in the Norwegian Mother and Child Cohort Study (MoBa), a long-term, prospective study conducted by the Norwegian Institute of Public Health and including more than 100,000 Norwegian pregnant women and their infants during 1999–2008 [21]. The women included in this sub-study of MoBa gave a singleton birth between July 2002 and December 2003, and returned a baseline questionnaire including data on height and pre-pregnancy weight and donated a blood sample in pregnancy week 18, and were registered in the Medical Birth Registry of Norway. A detailed description of this sub population and the sampling procedures has been published [22].

2.2. Ethics

Written informed consent was obtained from each participant, and the study was approved by the Regional Committee for Medical Research Ethics Sør-Øst A, 19/10-2011 (permission number 2009/2593a) and the Norwegian Data Inspectorate. All questions used in the MoBa can be found online at www.fhi.no/morogbarn.

2.3. Blood Sampling and Laboratory Analyses

Maternal non-fasting blood samples were collected in ethylenediamine tetraacetic acid (EDTA) tubes in median pregnancy week 18. The samples were centrifuged within 30 min after collection and stored at 4 °C until shipped overnight to the Norwegian Institute of Public Health in Oslo. On the day of receipt (usually within 1–2 days), plasma was aliquoted into polypropylene microtiter plates and stored at −80 °C until analyses.

Plasma folate was determined by a *Lactobacillus casei* microbiological assay [23] and plasma cobalamin (vitamin B12) by a *Lactobacillus leichmannii* microbiological assay [24]. Concurrent intake of antibiotics may interfere with microbiological assays and cause falsely reduced plasma levels of the vitamins [25], and samples with plasma folate levels <2.33 nmol/L were excluded (n = 28, i.e., the lower one percentile), leaving a total of 2797 samples to be included in the study.

Plasma levels of total homocysteine (tHcy), a marker of folate and cobalamin status, and methylmalonic acid (MMA), a marker of cobalamin status, were assayed using a GC-MS method based on methylchloroformate derivatization [26]. Plasma levels of riboflavin (vitamin B2); pyridoxal 5′-phosphate (PLP; vitamin B6); the ratio between 3-hydroxykynurenine and xanthurenic acid (HK/XA), a marker of vitamin B6 status [27]; and two markers of interferon-gamma mediated cellular immune activation, neopterin [28] and KTR (Kynurenine/Tryptophan $\times$ 1000)—the latter reflecting indoleamine-2,3-dioxygenase (IDO) activation [29]—were analyzed using a liquid chromatography-tandem mass spectrometry assay [30]. All biomarkers were measured at the laboratory of Bevital AS (www.bevital.no).

2.4. Covariates

Data on maternal age at delivery, marital status, and parity were obtained from the Medical Birth Registry of Norway. Data on maternal education, smoking habits, pre-pregnancy BMI, weight increase to pregnancy week 18, and use of supplements were obtained from the self-reported baseline cohort questionnaire. Pre-pregnancy BMI (kg/m^2) was coded as <18.5, 18.5–24.9, 25.0–29.9, 30.0–34.9, and $\geq$35.0. Use of supplements was coded as non-user, use of folic acid or other supplement, and use of folic acid plus other supplement, based on reported intake from four weeks prior to conception up to pregnancy week 18. A B vitamin summary score was calculated as a sum of quintiles for folate, cobalamin, PLP, and riboflavin.

2.5. Statistical Analyses

Values are presented as means with standard deviation (SD) or medians with the interquartile range. Differences between groups were examined by ANOVA, Mann-Whitney U test and the Kruskal Wallis test. Differences in categorical variables were assessed with the Chi-square test. Multiple-linear regression models were used to assess the relation between pre-pregnancy BMI, use of supplements, parity, age, and inflammation with plasma B vitamin status obtained around pregnancy week 18. The odds ratio (ORs) for having a low B vitamin status (quintile 1) according to maternal factors, including the inflammation marker neopterin, was assessed by logistic regression.

To explore non-linearity, we modeled pre-pregnancy BMI versus B vitamins and metabolic markers, using one-dimensional smoothing splines in Generalized Additive Models (GAM), in a model adjusted for adjusted for use of supplements, maternal age, parity, and neopterin.

GAMs were computed using the package mgcv (version 1.4–1) in R (The R Foundation for Statistical Computing, version 2.8.1, Vienna, Austria) [31], and the SPSS/PASW statistical program version 22 (IBM Corp, Armonk, NY, USA).the company, the city, the country) was used for all other statistical analyses. Two-sided p-values < 0.05 were considered statistically significant.

3. Results

3.1. Characteristics of the Study Population According to Pre-Pregnancy BMI

Demographic characteristics according to pre-pregnancy BMI are presented in Table 1. Fifty-eight percent of the women were multipara, and had a mean number of 1.5 (SD 0.7) former children, with no difference according to BMI group (p = 0.14). The majority of the women were healthy, a low percentage (0.7%) had chronic hypertension and 2% had had IVF treatment. The use of multivitamin supplements differed according to BMI group, with an increasing trend for not using any supplements with higher BMI (p < 0.001). Regular use of alcohol was rare and 96% reported drinking alcohol never or less than once a month after becoming pregnant. A total of 231 (8%) women reported daily smoking with a mean number of seven (SD five) cigarettes per day, with no significant difference in number between the smokers in the various BMI groups (p = 0.80). There was an inverse relation between pre-pregnancy BMI and weight gain to pregnancy week 18 (r = −0.24, p < 0.001) and to term (r = −0.16, p < 0.001).

Table 1. Maternal characteristics according to maternal pre-pregnancy BMI, $n = 2797$.

	Pre-pregnancy BMI, Categories					p Value
	<18.5	**18.5–24.9**	**25.0–29.9**	**30.0–34.9**	**≥35.0**	
	***n* = 80 (3%)**	***n* = 1827 (65%)**	***n* = 587 (21%)**	***n* = 213 (8%)**	***n* = 90 (3%)**	
Primipara, *n* (%)	47 (59%)	785 (43%)	231 (39%)	86 (40%)	26 (29%)	0.001 [b]
Age, years, mean (SD)	27.8 (4.9)	29.9 (4.5)	29.9 (4.6)	29.8 (4.6)	29.5 (4.8)	0.003 [a]
Education						
Primary school, *n* (%)	9 (12%)	39 (2%)	15 (3%)	11 (5%)	6 (7%)	
Secondary school, *n* (%)	30 (41%)	623 (36%)	273 (48%)	97 (47%)	48 (55%)	<0.001 [b]
University or college, *n* (%)	35 (47%)	1077 (62%)	286 (50%)	98 (48%)	34 (39%)	
Use of supplements anytime from four weeks before pregnancy up to pregnancy week 18	63 (79%)	1523 (83%)	470 (79%)	156 (72%)	61 (68%)	<0.001 [b]
Use of alcohol (≥1/month), *n* (%)	5 (0.2%)	83 (3%)	15 (0.6%)	1 (0%)	3 (0.1%)	0.009 [b]
Daily smoking, *n* (%)	13 (16%)	140 (8%)	47 (8%)	22 (10%)	9 (10%)	0.04 [b]
Weight increase, kg, mean (SD), (% of pre-pregnancy weight)						
to pregnancy week 18	4.5 (3.1) (9%)	3.3 (2.9) (5%)	2.4 (3.2) (3%)	1.1 (3.5) (1%)	−0.1 (4.3) (−0.1%)	<0.001 [a] <0.001 [a]
to birth	17.3 (6.3) (35%)	15.5 (5.9) (25%)	14.8 (6.7) (20%)	11.5 (7.3) (13%)	8.7 (8.9) (8%)	<0.001 [a] <0.001 [a]
Birth weight, g, mean (SD)	3343 (635)	3575 (588)	3727 (600)	3696 (648)	3758 (755)	<0.001 [a]

[a] ANOVA: A one-way variance analysis; [b] Pearson's Chi Square test. BMI: body mass index; SD: the standard deviation.

Women in the highest pre-pregnancy BMI group (BMI $\geq$ 35) put on less weight during pregnancy, they were more likely to be multipara, not use micronutrient supplements, and to have a lower educational level ($p < 0.01$). The lowest mean birth weight was seen in women with a pre-pregnancy BMI <18.5 (Table 1).

3.2. Plasma B Vitamin Status According to Pre-Pregnancy BMI

There was a trend towards lower levels of B vitamins and higher levels of the metabolic markers with higher pre-pregnancy BMI (Table 2, Figure 1). The best B vitamin status was seen in women with a normal pre-pregnancy BMI (18.5–24.9), as they had the highest levels of folate, PLP, and riboflavin with the lowest tHcy and HK/XA ratio (Table 2). Cobalamin decreased with increasing BMI, but this was not reflected in the MMA levels, which were inversely related to pre-pregnancy BMI.

The circulating B-vitamins were inversely related to their functional markers, as expected. tHcy was inversely related to folate ($r = -0.48$, $p < 0.001$), less to cobalamin ($r = -0.24$, $p < 0.001$), PLP ($r = -0.16$, $p < 0.001$), and riboflavin ($r = -0.15$, $p < 0.001$). MMA was inversely related to cobalamin ($r = -0.17$, $p < 0.001$) and HK/XA to PLP ($r = -0.29$, $p < 0.001$). All B vitamins were positively related to each other ($r > 0.12$, $p < 0.001$).

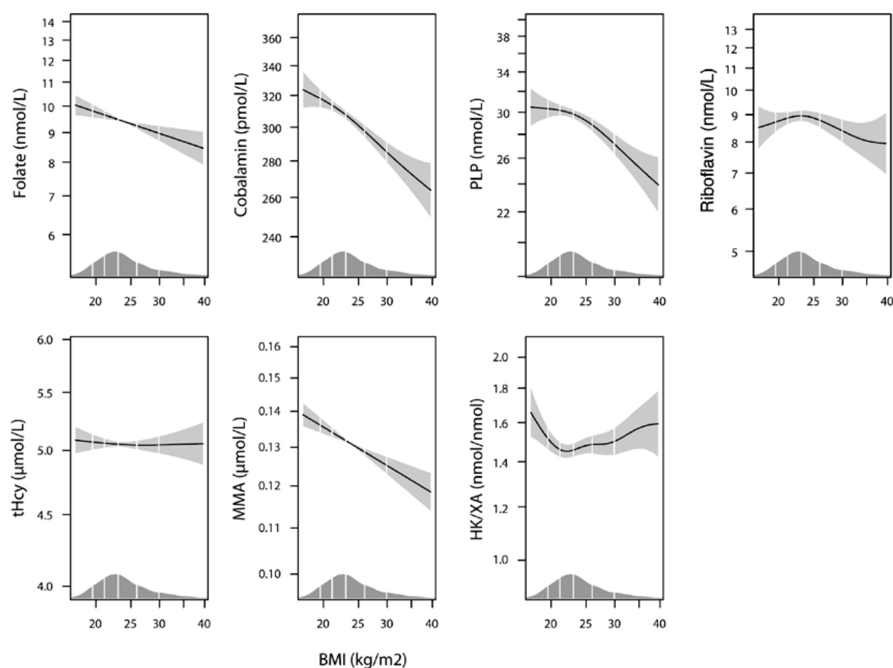

Figure 1. The association of pre-pregnancy BMI with cobalamin, folate, PLP, riboflavin, tHcy, MMA, and HK/XA by generalized additive models (GAM), adjusted for use of multisupplements, maternal age, parity, and neopterin. The solid line shows the fitted model and the shaded areas indicate 95% CIs. PLP, pyridoxal 5′phosphate; tHcy, total homocysteine; MMA, methylmalonic acid, HK/XA, 3-hydroxykynurenine/xanthurenic acid ratio.

Table 2. Maternal plasma levels of vitamins, metabolic and inflammation markers in pregnancy week 18 according to pre-pregnancy BMI, $n = 2797$.

	Pre-pregnancy BMI; Categories					**p Value**
	<18.5	**18.5–24.9**	**25.0–29.9**	**30.0–34.9**	**≥35.0**	
	n = 80	**n = 1827**	**n = 587**	**n = 213**	**n = 90**	
Plasma folate, nmol/L [a]	8.8 (6.0–15.7)	9.2 (6.3–16.0)	8.1 (5.7–12.8)	7.3 (5.1–12.2)	7.3 (5.0–12.1)	<0.001
Plasma cobalamin, pmol/L [a]	328 (243–383)	314 (254–386)	301 (240–363)	271 (220–338)	268 (204–317)	<0.001
Plasma PLP, nmol/L [a]	27.0 (21.4–39.7)	28.3 (21.8–40.7)	26.6 (20.6–35.6)	23.8 (17.4–33.7)	21.5 (17.0–30.1)	<0.001
Plasma riboflavin, nmol/L [a]	7.8 (4.8–14.4)	8.1 (5.6–13.2)	7.8 (5.2–13.9)	7.2 (5.2–11.0)	6.9 (4.4–10.1)	0.004
B vitamin status [a,b]	12.2 (3.7)	12.5 (3.6)	11.7 (3.7)	10.6 (3.7)	10.1 (3.4)	<0.001
Plasma tHcy, µmol/L [a]	5.00 (4.22–5.92)	4.91 (4.27–5.79)	4.94 (4.23–5.78)	5.04 (4.44–6.21)	5.33 (4.43–6.13)	0.03
Plasma MMA, µmol/L [a]	0.13 (0.11–0.16)	0.13 (0.11–0.16)	0.13 (0.10–0.16)	0.12 (0.10–0.15)	0.12 (0.10–0.15)	0.009
Plasma HK/XA [a]	1.6 (1.2–2.1)	1.4 (1.0–2.0)	1.5 (1.1–2.1)	1.7 (1.2–2.2)	1.8 (1.2–2.4)	<0.001
Plasma neopterine, µmol/L [a]	6.7 (6.1–8.1)	7.0 (6.1–8.1)	7.3 (6.3–8.6)	7.9 (7.0–9.1)	8.5 (7.31–9.9)	<0.001
KTR, nmol/µmol [a]	18.1 (16.0–21.5)	18.3 (16.2–20.6)	18.8 (16.8–21.2)	19.6 (17.5–22.8)	21.2 (18.5–24.0)	<0.001

[a] Median (IQR), by Kruskal Wallis test; [b] B vitamin status; summary score based on added quintiles of the 4 B vitamins, Mean (SD), by ANOVA; tHcy, total homocysteine; MMA, methylmalonic acid; HK/XA: 3-hydroxykynurenine/xanthurenic acid; KTR, kynurenine/tryptophan ratio.

3.3. Inflammation Markers According to Pre-Pregnancy BMI

There was a positive correlation between pre-pregnancy BMI and the inflammation markers neopterin ($r = 0.18$, $p < 0.001$) and KTR ($r = 0.13$, $p < 0.001$), as demonstrated in Figure 2. Neopterin and KTR were strongly correlated to each other ($r = 0.48$, $p < 0.001$).

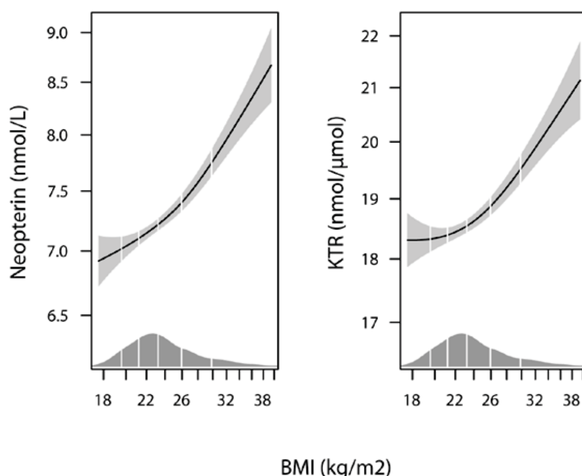

Figure 2. The association of pre-pregnancy BMI with neopterin and KTR by Generalized additive models (GAM), adjusted for use of multisupplements, maternal age, and parity. The solid line shows the fitted model and the shaded areas indicate 95% CIs. KTR, kynurenine/tryptophan ratio.

3.4. Maternal Factors and Inflammation Markers as Determinants of Plasma B Vitamin Status in Pregnancy Week 18

Use of supplements was the most consistent and strongest determinant for maternal B vitamin status, with significant influence on folate, cobalamin, PLP, riboflavin, tHcy, and HK/XA, in a multiple linear regression model, which additionally included pre-pregnancy BMI, age, parity, and neopterin (Table 3). In this model, pre-pregnancy BMI was a significant determinant of all vitamin markers except for riboflavin and tHcy, whereas parity was strongly negatively correlated to folate and PLP status. All parameters in the model were significantly correlated to the calculated B vitamin summary score (Table 3). Neopterin was significantly negative related to PLP, and positively to the metabolic markers tHcy, MMA, and HK/XA. Substituting neopterin with KTR in the model did not essentially change the results (data not shown). Including gestational weight gain in pregnancy week 18 as an additional factor did not change the results.

The OR for having a low calculated B vitamin summary score (quintile 1) was increased with pre-pregnancy BMI >30.0 and higher parity, and reduced with higher maternal age and use of supplements in a logistic regression model, which additionally included neopterin quartiles (Table 4). A high pre-pregnancy BMI was a predictor of low cobalamin and PLP levels, multiparity was a predictor of low folate levels, higher maternal age was associated with better folate and riboflavin status, whereas use of supplements was associated with better status of all four B vitamins. Increased inflammation, evaluated by higher neopterin levels, was only a predictor of low PLP in this model. Substituting neopterin with KTR in the model did not essentially change the results.

Table 3. Determinants of maternal plasma B vitamin status in pregnancy week 18 by multiple linear regression, $n = 2797$.

	Plasma Folate, nmol/L	Plasma Cobalamin, pmol/L	Plasma PLP, nmol/L	Plasma Riboflavin, nmol/L	Plasma tHcy, μmol/L	Plasma MMA, μmol/L	Plasma HK/XA	B Vitamin Score [a]
	B (95% CI)	B (95% CI)	B (95% CI)	B (95% CI)	B (95% CI)	B (95% CI)	B (95% CI)	B (95% CI)
Pre-pregnancy BMI [c]	−0.59 ** (−0.98, −0.20)	−16.7 ** (−22.0, −11.4)	−2.55 ** (−3.72, −1.38)	−0.60 (−1.25, 0.06)	−0.02 (−0.16, 0.12)	−0.006 ** (−0.008, −0.003)	0.11 * (0.04, 0.18)	−0.47 ** (−0.60, −0.35)
Parity [e]	−1.33 ** (−1.72, −0.95)	−3.6 (−8.8, 1.6)	−1.28 * (−2.43, −0.12)	−1.08 * (−1.73, −0.43)	−0.01 (−0.15, 0.13)	−0.001 (−0.004, 0.002)	−0.03 (−0.09, 0.04)	−0.34 ** (−0.47, −0.22)
Age [d]	2.69 ** (2.05, 3.32)	12.3 * (3.6, 20.9)	1.96 * (0.06, 3.86)	0.30 (−0.77, 1.36)	0.04 (−0.19, 0.27)	0.002 (−0.002, 0.007)	−0.12 * (−0.24, −0.01)	0.63 ** (0.43, 0.83)
Use of supplements [b]	5.14 ** (4.35, 5.94)	13.0 * (2.3, 23.7)	8.49 ** (6.12, 10.85)	1.80 * (0.48, 3.13)	−0.75 ** (−1.03, −0.47)	0.001 (−0.004, 0.007)	−0.21 * (−0.35, −0.07)	1.73 ** (1.48, 1.98)
Neopterin [f]	−0.12 (−0.35, 0.11)	−1.9 (−5.0, 1.2)	−1.00 * (−1.68, −0.31)	−0.08 (−0.47, 0.30)	0.10 * (0.02, 0.18)	0.003 ** (0.001, 0.004)	0.19 ** (0.15, 0.23)	−0.12 ** (−0.20, −0.05)

All variables were included in the model; [a] B vitamin score; added quintiles of folate, cobalamin, PLP and riboflavin; [b] No use versus use of supplements any time from four weeks before pregnancy up to pregnancy week 18; [c] Pre-pregnancy BMI, categorized; <18.5, 18.5-24.9, 25.0-29.9, 30.0-34.9, ≥35.0; [d] Age, categorized; <25 years, 25–35 years, >35 years; [e] Parity, categorized; 0, 1, 2, 3+; [f] Neopterin, quintiles; ≤5.84, 5.85–6.68, 6.69–7.50, 7.51–8.67, ≥8.68;* p value < 0.05; ** p value < 0.001. CI: indicates confidence interval.

Table 4. OR for low B vitamin status according to maternal factors, by logistic regression (n = 2797).

Independent Variables	OR (95% CI) for				
	Plasma Folate <5.45 nmol/L (Quintile 1)	Plasma Cobalamin <230 pmol/L (Quintile 1)	Plasma PLP <19.26 µmol/L (Quintile 1)	Plasma Riboflavin <4.91 µmol/L (Quintile 1)	B Vitamin Score [a] (Quintile 1)
Pre-pregnancy BMI (vs. category 2 18.5–24.9)					
<18.5	1.0 (0.5–1.8)	1.1 (0.6–1.9)	1.0 (0.5–1.8)	1.7 (1.0–2.8)	1.1 (0.7–1.9)
25.0–29.9	1.1 (0.9–1.5)	1.3 (1.0–1.6)	1.3 (1.0–1.6)	1.3 (1.0–1.7)	1.5 (1.2–1.8)
30.0–34.9	1.5 (1.0–2.1)	2.1 (1.5–2.9)	2.1 (1.6–3.0)	1.4 (1.0–2.0)	2.7 (2.0–3.7)
≥35.0	1.7 (1.0–2.8)	2.3 (1.4–3.6)	2.2 (1.4–3.5)	1.8 (1.1–3.0)	2.8 (1.7–4.3)
p trend	0.006	<0.001	<0.001	0.001	<0.001
Parity (vs. primipara)					
Para 1	1.2 (1.0–1.6)	1.1 (0.9–1.4)	1.3 (1.0–1.6)	1.3 (1.0–1.6)	1.3 (1.0–1.6)
Para 2	1.7 (1.2–2.3)	1.3 (1.0–1.7)	1.1 (0.8–1.5)	1.2 (0.9–1.6)	1.6 (1.2–2.1)
Para 3+	3.2 (2.1–4.9)	1.6 (1.0–2.4)	1.1 (0.7–1.8)	1.4 (0.9–2.2)	2.1 (1.4–3.1)
p trend	<0.001	0.02	0.36	0.11	<0.001
Maternal age (vs. ≤25 years)					
25–35	0.4 (0.3–0.6)	0.7 (0.6–1.0)	0.7 (0.7–1.0)	0.7 (0.6–1.0)	0.5 (0.4–0.7)
≥35	0.3 (0.2–0.5)	0.8 (0.5–1.2)	0.8 (0.5–1.1)	0.6 (0.4–0.9)	0.4 (0.3–0.6)
p trend	<0.001	0.11	0.11	0.004	<0.001
Use of supplements (vs. non-user)					
User	0.2 (0.2–0.2)	0.8 (0.6–1.0)	0.5 (0.4–0.6)	0.6 (0.5–0.8)	0.3 (0.3–0.4)
p trend	<0.001	0.03	<0.001	<0.001	<0.001
Neopterin (vs. ≤6.20 µmol/L, Quartile 1)					
6.21–7.17	1.1 (0.8–1.4)	1.1 (0.8–1.5)	1.1 (0.8–1.5)	0.9 (0.7–1.2)	1.0 (0.8–1.3)
7.18–8.40	1.3 (1.0–1.8)	1.0 (0.8–1.4)	1.1 (0.8–1.4)	0.9 (0.7–1.1)	1.1 (0.9–1.4)
≥8.41	1.2 (0.9–1.6)	1.2 (0.9–1.6)	1.7 (1.3–2.2)	0.9 (0.7–1.2)	1.2 (1.0–1.6)
p trend	0.11	0.24	<0.001	0.38	0.05

All variables were included in the model; [a] B vitamin score; added quintiles of folate, cobalamin, PLP and riboflavin. OR: Odds ratio.

The relations between pre-pregnancy BMI and B vitamins and metabolic markers, visualized by GAM in a model which additionally corrected for use of supplements, maternal age, parity, and neopterin, are shown in Figure 1. The association curves for folate, cobalamin, PLP, and MMA show a linear negative relation to pre-pregnancy BMI, while the relation between pre-pregnancy BMI and the other biomarkers are inverse U-shaped (riboflavin), null (tHcy), or inverse in the lower range (HK/XA).

The association curves between pre-pregnancy BMI and the inflammation markers, neopterin and KTR, visualized by GAM in a model which also corrected for use of supplements, maternal age, and parity, show a strong linear relation through the whole range of BMI values (Figure 2).

4. Discussion

4.1. Main Findings

In this study of healthy women in pregnancy week 18, use of multi supplements was the strongest maternal determinant for B vitamin status. Pre-pregnancy BMI was inversely related to folate, cobalamin, PLP, and riboflavin levels, and associated with increased markers of cellular immune activation. Inflammation seemed to be an independent predictor of low PLP levels.

4.2. Strength and Limitations

All pregnant women in Norway were invited to participate in the MoBa study, but the attendance rate was only 38.5%, rendering the cohort not representative of the Norwegian population of pregnant women. The women who participated in the MoBa study are reported to be older, more frequently vitamin users, non-smokers, and primiparous [32]. Pre-pregnancy weight was self-reported around pregnancy week 18, and as obesity is an important determinant of underreporting BMI [33], self-reporting without verification is a limitation of the study.

The samples were stored at −80 °C before analyzing. The stability of plasma metabolites according to sample handling and storage conditions has been validated and the biomarkers investigated do not change significantly during storage at −80 °C [34].

C-reactive protein, CRP, an acute phase reactant and a commonly used inflammation marker, might have added additional information about low grade inflammation, but was not analyzed, which is a limitation of the study.

4.3. Interpretation

4.3.1. Effects of Pre-Pregnancy BMI on Gestational Weight Gain and Birth Weight

In a former study of the MoBa cohort including approximately 58,000 mothers, the majority (65%) reported a normal pre-pregnancy BMI (18.5–24.9), whereas 2.9% were underweight (<18.5), and 2.6% were obese ($\geq$35) [35], which is in accordance with the findings in the present substudy.

We observed a significant inverse relation between pre-pregnancy BMI and gestational weight gain up to pregnancy week 18 and throughout pregnancy, and despite this, a higher pre-pregnancy BMI was associated with a higher birth weight, which has been reported previously [35].

4.3.2. Pregnancy Related Changes in Biochemical Parameters

Physiological changes in pregnancy, including hemodilution, altered renal function, hormonal status, and binding-protein concentrations, affect plasma B vitamin levels and the relation to their functional metabolic markers [36,37]. Folate, cobalamin, and PLP tend to decrease, whereas riboflavin is reported to show only minor changes during pregnancy [38–42]. tHcy, a marker of folate and cobalamin deficiency [43], is 30%–60% lower in pregnant compared to nonpregnant women, with the lowest levels seen in the second trimester [44]. MMA, a marker of cobalamin deficiency [45], is reported to be reduced during the first two trimesters, thereafter gradually increasing [46]. We observed a strong

inverse relation between folate and tHcy, and a weaker relation between cobalamin and MMA, as observed by others [36,47,48].

The ratio HK/XA has been shown to have a strong negative correlation with plasma levels of PLP, the active form of vitamin B6, and has been proposed as a potential markers of functional vitamin B6 status [27]. The inverse relation between PLP and HK/XA has not been previously documented in pregnant women, but we observed lower PLP and higher HK/XA ratios with increasing pre-pregnancy BMI, indicating a functional vitamin B6 deficiency, particularly in women with a pre-pregnancy BMI ≥30.

Extensive changes in the maternal immune system are necessary for maintaining a normal pregnancy [49] and both neopterin levels and KTR are reported to increase during pregnancy [50] Neopterin levels correlate with gestational age ($r = 0.28$, $p = 0.001$) and are reported to be higher in pregnancies complicated with preterm birth and preeclampsia [51–53].

4.3.3. Maternal Predictors for B Vitamin Status

An inverse relation between pre-pregnancy BMI and folate, vitamin D, carotenoids has formerly been reported [10,11,13]. Our results have expanded this knowledge by reporting also lower levels of PLP (vitamin B6), riboflavin (vitamin B2), and cobalamin (vitamin B12) with higher pre-pregnancy BMI.

Pre-pregnancy BMI, use of multisupplements, parity, and maternal age were all related to B vitamin status. We observed a slightly U-shaped relation between pre-pregnancy BMI and B vitamin levels in pregnancy week 18, with the best status seen in women with a normal pre-pregnancy BMI (18.5–24.9), slightly lower in underweight women (BMI < 18.5) and lowest in obese women (BMI ≥35.0). The strongest maternal determinant for B vitamin status was use of multisupplements, and the percentage of users was higher in normal weight compared to obese women, which might explain the inverse relation between pre-pregnancy BMI and vitamin status. Use of micronutrient supplements during pregnancy has been associated with better socioeconomic status [48], known to be related to a better diet, maternal health, and pregnancy outcome [54]. Multiparity (≥3) was associated with an increased risk of folate deficiency, while maternal age >25 years was associated with a better folate status. In the MoBa cohort, the 10% who had used supplements regularly from one month before pregnancy throughout the first trimester (as recommended) were more likely to be older, married, nonsmokers, with a higher income and lower parity [54]. Compared to women with a lower pre-pregnancy BMI, obese women (BMI ≥ 35) had not increased their weight in pregnancy week 18, when the blood samples were collected, which might contribute to their lower micronutrient status.

4.3.4. The Complex Relation between Pre-pregnancy BMI, Inflammation, and Vitamin Levels

We observed a linear relation between pre-pregnancy BMI and the markers of cellular immune activation, neopterin and KTR, as previously reported in non-pregnant adults [16]. Inflammation is reported to be associated with lower plasma levels of several micronutrients, particularly vitamin B6 and vitamin D [18–20], but the magnitude of effect varies between different micronutrients and individuals [18,55,56]. The lower PLP levels associated with inflammation have been proposed to be due to increased PLP degradation [57] and to PLP redistribution from plasma to tissues [58], and PLP have been suggested to reflect interferon-γ-mediated immune activation [59].

In this study, neopterin was inversely related to PLP and positively to HK/XA, but not to any other vitamin, in both multiple linear and logistic regression models, which included pre-pregnancy BMI and other maternal factors. The increase in the functional marker, HK/XA, suggests that low PLP does not merely refelect altered distribution, but that inflammation may be an independent risk factor for lower vitamin B6 status.

5. Conclusions

A high pre-pregnancy BMI is associated with lower levels of folate, cobalamin, PLP, and riboflavin; and increased levels of the cellular inflammation markers, neopterin, and KTR. As an optimal

micronutrient status is vital for normal fetal development, the observed lower B vitamin levels may contribute to the negative pregnancy outcomes associated with maternal obesity. Assessment of B vitamin status should be encouraged in women with a high pre-pregnancy BMI when planning a pregnancy.

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Abbreviations

MoBa: the Norwegian Mother and Child Cohort; tHcy: total homocysteine; MMA: methylmalonic acid; HK: 3-hydroxykynurenine; XA: xanthurenic acid; PLP: pyridoxal 5′-phosphate; KTR: kynurenine/tryptophan ratio; IDO: indoleamine-2,3-dioxygenase.

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Article

Vitamin B12 Status among Pregnant Women in the UK and Its Association with Obesity and Gestational Diabetes

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Abstract: Background: To evaluate vitamin B12 and folate status in pregnancy and their relationship with maternal obesity, gestational diabetes mellitus (GDM), and offspring birthweight. Methods: A retrospective case-control study of 344 women (143 GDM, 201 no-GDM) attending a district general hospital and that had B12 and folate levels measured in the early 3rd trimester was performed. Maternal history including early pregnancy body mass index (BMI) and neonatal data (birthweight, sex, and gestational age) was recorded for all subjects. Results: 26% of the cohort had B12 levels <150 pmol/L (32% vs. 22% in the two groups respectively, $p < 0.05$) while 1.5% were folate deficient. After adjusting for confounders, 1st trimester BMI was negatively associated with 3rd trimester B12 levels. Women with B12 insufficiency had higher odds of obesity and GDM (aOR (95% CI) 2.40 (1.31, 4.40), $p = 0.004$, and 2.59 (1.35, 4.98), $p = 0.004$, respectively), although the latter was partly mediated by BMI. In women without GDM, the lowest quartile of B12 and highest quartile of folate had significantly higher adjusted risk of fetal macrosomia (RR 5.3 (1.26, 21.91), $p = 0.02$ and 4.99 (1.15, 21.62), $p = 0.03$ respectively). Conclusion: This is the first study from the UK to show that maternal B12 levels are associated with BMI, risk of GDM, and additionally may have an independent effect on macrosomia. Due to the increasing burden of maternal obesity and GDM, longitudinal studies with B12 measurements in early pregnancy are needed to explore this link.

Keywords: vitamin B12; gestational diabetes; obesity; macrosomia

1. Introduction

The burden of maternal obesity (defined as body mass index (BMI) greater than 30 kg/m^2) is rapidly increasing, affecting nearly 20% of pregnant women in the UK [1]. High BMI is associated with adverse pregnancy outcomes including recurrent miscarriages and maternal deaths [2]. In parallel, the incidence of gestational diabetes mellitus (GDM) has also risen affecting 5%–18% of all pregnancies depending on the diagnostic criteria applied [3,4].

Vitamin B12 (B12) and folate are essential micronutrients required for the synthesis of DNA, protein, and lipids, in a series of cellular reactions collectively known as one-carbon metabolism [5,6]. One step in this process is the conversion of homocysteine (Hcy) to a methyl donor, methionine, for which B12 and folate are necessary cofactors. Additionally, the mitochondrial conversion of methylmalonyl-CoA to succinyl-CoA requires B12 as a coenzyme and in its absence, accumulation

of the former compound inhibits fatty acid oxidation, thereby promoting lipogenesis [7,8]. Therefore it can be postulated that low B12, at a cellular level, may be linked to adipocyte dysfunction and obesity-related complications by modulating lipid metabolism, cellular inflammation [9], and causing hypomethylation of cholesterol biosynthesis pathways [10].

A recent systematic review showed that B12 insufficiency among pregnant women across the world was common in all trimesters (20%–30%) [11]. Low B12 during pregnancy has implications for materno-fetal health including maternal adiposity, maternal and offspring insulin resistance [12–14], and adverse lipid profile in neonates [10,15]. The first two observations were replicated in a cohort of women without GDM from South West England [16] but there are no data available on the role of B12 in GDM in the UK.

Low B12 can have an impact on fetal birthweight by influencing placental development [17], although evidence for association with low birthweight (LBW) is equivocal [18–20]. At the other end of the spectrum, maternal obesity and insulin resistance are well-known to be associated with higher fetal birthweight [21,22]. Since B12 may be inversely associated with maternal BMI [12,16], it is possible that B12 is an independent mediator or a confounder for high birthweight.

The primary aim of our study is to investigate the B12 and folate status of pregnant women in the UK and their relationship with obesity and GDM, and secondarily to assess their relationship with fetal birthweight.

2. Methods

A retrospective case-control study of pregnant women attending the antenatal clinic in a district general hospital in the West Midlands, UK, between 2010 and 2013 was conducted. Using the hospital information database which had routine materno-fetal records of all deliveries during this period, we identified women who had a diagnosis of GDM and those who did not (labelled as no-GDM) and had their B12 levels measured in the 2nd or 3rd trimesters of their pregnancies. The no-GDM group consisted predominantly of women attending the medical obstetrics clinic for varying medical conditions. B12 and folate levels were measured routinely for screening for anaemia by the medical obstetric lead (VP), in addition to haemoglobin and ferritin. The physician (PS) running the antenatal-diabetes clinic measured these micronutrient levels for similar reasons in their first visit after the diagnosis of GDM. The following women were excluded from our analysis: pre-gestational diabetes (Type 1 and 2), multiple pregnancies, and those on vitamin B12 supplements at the time of blood sampling.

Clinical information about the women including medical and pregnancy history, smoking status, and BMI at booking was recorded from the database. Fetal outcomes such as birthweight, sex, and gestation were obtained for the secondary outcome analysis. Analysis of glucose was done by a hexokinase enzymatic method in the hospital laboratory and serum B12 and folate by an electrochemiluminescent immunoassay using a Roche Cobas immunoassay analyser (Roche Diagnostics UK, Burgess Hill, UK).

2.1. Definitions

A selective screening approach was used to screen high-risk women for GDM according to the National Institute for Health and Care Excellence (NICE) guidelines [23] (i.e., BMI > 30 kg/m^2, previous GDM, previous macrosomia, first degree relative with diabetes, and ethnic minority race). This consisted of a 2-h 75 g glucose tolerance test (GTT) between 26 and 28 weeks of gestation. The modified World Health Organisation (WHO) 1999 criteria was used to diagnose GDM (fasting glucose $\geq$ 6.1 mmol/L or 2-h glucose $\geq$ 7.8 mmol/L) during the study period. The reference range for serum B12 was 150–489 pmol/L and for serum folate was 7.0–42.4 nmol/L, respectively. Insufficiency of the two micronutrients were defined as <150 pmol/L and <7 nmol/L, respectively [12,16]. Birthweight percentiles and *z*-scores were calculated using gestational age at delivery and sex-specific reference standards published by the Intergrowth calculator 21st Project [24]. Macrosomia was defined as

birthweight > 4000 g, large for gestational age (LGA) as >90th percentile for sex and gestational age, LBW as <2500 g, and small for gestational age (SGA) as <10th percentile for sex and gestational age.

2.2. Statistical Analysis

Based on the pilot data, the required sample size in each group to demonstrate a 15% difference in mean B12 with 90% power and at 5% significance was calculated to be 144. Statistical analysis was performed using SPSS version 22.0 [25]. Since BMI, serum B12, and folate were not normally distributed, they were log-transformed for statistical purposes. For comparison of GDM and no-GDM mothers, the Student's *t*-test was used for continuous variables (e.g., B12, folate, and BMI) and the Chi-square test for categorical variables. Stepwise multiple linear regression was performed with B12 and folate as the dependent variables with the predictors entered or removed from the model according to the following criteria: Probability-of-F-to-enter ≤ 0.050, Probability-of-F-to-remove ≥ 0.100. Logistic regression was performed to determine the odds of maternal obesity and GDM according to B12/folate insufficiency status and the risk of macrosomia, LGA, LBW, and SGA according to quartiles of B12/folate. The regression models included the following co-variates: age, parity, ethnic origin, smoking, gestation of bloods, BMI, B12, and folate (where appropriate). For macrosomia and LBW, sex and gestational age were additionally added to the models.

Our institution has obtained ethics approval to collect B12 and folate data from pregnant women in an anonymised form (NHS ethics committee reference number 12/LO/0239).

3. Results

Out of approximately 8400 deliveries in the hospital between 2010 and 2013 that were screened, 344 women (143 GDM, 201 no-GDM) who met the inclusion criteria and had B12 levels measured in the 3rd trimester of pregnancy were included. The clinical characteristics of the whole cohort and by GDM status are provided in Table 1. Of the 201 no-GDM women, 45% had GTT as per NICE selective screening criteria [23] and the characteristics of these women are summarised in the Supplementary Materials Table S1.

Table 1. Maternal characteristics according to GDM status.

Variables	Total	GDM	No GDM
Number (%)	344 (100)	143 (41.6)	201 (58.4)
Age (years)	30.3 ± 5.88	31.4 + 5.8	29.6 ± 5.9 **,[a]
BMI (kg/m^2) §	28.8 ± 7.46	31.7 ± 7.0	26.7 ± 7.1 ***
Obesity (BMI > 30 kg/m^2) (%)	38.0	60.6	22.0 ***
Current smokers (%)	18.7	15.2	19.9
Parity	1.1 ± 1.18	1.2 ± 1.18	1.0 ± 1.18
Ethnicity (%)			
European	86.9	86.0	87.6
South Asian	9.3	11.2	8.0
Afro-Caribbean	1.2	0.7	1.5
Other	1.2	1.4	1.0
Gestation of GTT (weeks) [b]	26.6 ± 3.95	26.4 ± 4.40	26.8 ± 3.10
Mean fasting glucose (mmol/L) §	4.9 ± 1.01	5.2 ± 1.15	4.4 ± 0.39 ***
Mean 2 h glucose (mmol/L) §	7.5 ± 1.94	8.7 ± 1.26	5.6 ± 1.13 ***
Gestation of B12 bloods (weeks)	26.9 ± 5.3	28.0 ± 4.3	26.2 + 5.7 **
Vitamin B12 (pmol/L) §	187.5 (146.9, 235.4)	169.0 (140.2, 217.7)	195.6 (157.9, 244.6) **
Vitamin B12 deficiency (<150 pmol/L), *n* (%)	90 (26.2)	46 (32.2)	44 (21.9) *
Serum folate (nmol/L) §	21.3 (14.0, 34.4)	21.5 (13.5, 34.5)	20.8 (14.5, 34.4)
Serum folate deficiency (<7 nmol/L), *n* (%)	5 (1.5)	3 (2.1)	2 (1.0)
Folic acid supplements taken (%)	91.4	90.9	91.5

Continuous variables are mean ± SD (or median (IQR)), categorical variables are percentages; [a] *p*-value as compared to the GDM group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; [b] GTT results available in 90/201 (44.8%) of no-GDM women; § Log-transformed for statistical comparison, GDM: gestational diabetes mellitus, BMI: body mass index, GTT: glucose tolerance test.

For the whole cohort, the mean gestation of serum vitamin B12 and folate measurements was at 26.9 weeks and GTT was at 26.6 weeks. B12 levels were lower in women with GDM (169.0 vs. 195.6 pmol/L, $p < 0.001$) and a significantly higher proportion of women with GDM had B12 insufficiency compared to non-GDM (Table 1). Folate deficiency was rare and 91% of the whole cohort was taking folate supplements. Serum folate levels were not different in the two groups.

3.1. Vitamin B12, Folate Status, Maternal BMI, and GDM

Women with B12 insufficiency had higher 1st trimester BMI than those without (30.9 ± 7.56 vs. 28.0 ± 7.30 kg/m^2, $p < 0.05$). After adjusting for age, parity, ethnicity, smoking status, and gestation of blood tests, BMI was a significant negative predictor of B12 (β coefficient −0.21; 95% CI: −0.47, −0.13; $p = 0.001$) whilst serum folate showed a positive association with B12 (Table 2, Figure 1). BMI was also negatively associated with serum folate after adjustment although the strength of association was weaker (β coefficient −0.12; 95% CI: 0.00, 0.33; $p = 0.05$). Third trimester vitamin B12 insufficiency was additionally associated with a 2.4 times higher odds of first trimester obesity (Table 3).

Table 2. Predictors of vitamin B12 and folate.

Variables	Serum B12 §		Serum Folate §	
	β-Coefficient	*p*-Value	β-Coefficient	*p*-Value
Age	-	NS	0.32	<0.001
Parity	-	NS	−0.24	<0.001
BMI §	−0.21	0.001	−0.12	0.05
Ethnicity	-	NS	-	NS
Smoking	-	NS	-	NS
Gestation of B12/folate bloods	-	NS	−0.28	<0.001
Serum B12 §			0.12	0.05
Serum folate §	0.23	<0.001		
Folic acid supplements	-	NS	-	NS

§ Log-transformed for statistical calculations; NS: non-significant.

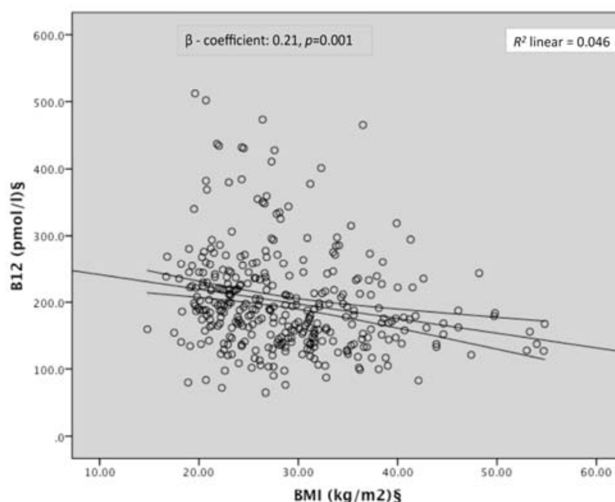

Figure 1. Scatterplot of the correlation between BMI and serum B12. § Log-transformed for statistical comparisons; Regression model included age, parity, ethnicity, smoking, gestation of bloods, folic acid supplements, and serum folate.

Table 3. Relationship of maternal B12 and folate with obesity and gestational diabetes.

	n (%)	Obesity, *n* (%)	GDM, *n* (%)
Vitamin B12 deficiency			
Yes	90	44 (49.4)	46 (51.1)
No	254	86 (34.0)	97 (38.2)
Model 1 OR (95% CI) [a]		2.40 (1.31, 4.40)	2.59 (1.35, 4.98)
adjusted *p*		0.004	0.004
Model 2 OR (95% CI) [b]		N/A	2.05 (1.03, 4.10)
adjusted *p*		N/A	0.042
Folate deficiency			
Yes	5	4 (80.0)	3 (60.0)
No	332	125 (37.9)	139 (41.9)
Model 1 OR (95% CI) [a]		6.29 (0.48, 82.79)	1.93 (0.17, 22.23)
adjusted *p*		NS	NS
Model 2 OR (95% CI) [b]		N/A	0.89 (0.07, 11.38)
adjusted *p*		N/A	NS

Table showing the proportions and odds ratio of obesity and development of GDM according to the thresholds of B12 and folate (reference categories are 'No B12/folate deficiency'); [a] Model 1 adjusted for age, parity, ethnic origin, smoking, gestation of bloods, and serum folate (or B12, respectively); [b] as for Model 1 plus gestational BMI; N/A: not applicable; NS: non-significant.

B12 deficient women were at 2.59-times higher odds of having a diagnosis of GDM after adjusting for age, parity, ethnic origin, smoking, gestation of bloods, and serum folate (Table 3). The effect size was weaker when maternal BMI was added into the model (aOR 2.05, $p = 0.04$). Folate deficiency was not significantly associated with a risk of GDM. There was also no association seen between folate thresholds and obesity.

3.2. Vitamin B12, Folate, and Birth Outcomes

Birth outcome data were available in 335 women (97% of total cohort) and one baby born at less than 32 weeks gestation was excluded from this analysis. 54.5% of the babies were male and the mean birthweight was 3353 g. GDM women delivered 10 days earlier than no-GDM women and their mean offspring birthweight was 180 g lower (3250 vs. 3428 g, $p < 0.01$) (Supplementary Materials Table S2). Due to the likely confounding effects of treatment in GDM women, the relationship between maternal B12 and folate and birth outcomes were analysed only in no-GDM women (Table 4). Women in the lowest quartile of B12 had higher rates of macrosomic babies compared to the highest quartile (22.9% vs. 8.0%) (Table 4). After adjustment for age, parity, ethnicity, smoking, serum folate, gestation of B12 bloods, and newborn sex and gestational age, the relative risk (RR) of fetal macrosomia was higher in women in the lowest quartile (RR 5.26, 95% CI: 1.26, 21.91, $p = 0.02$). The significance was attenuated when gestational BMI was added to the model (Table 4). A similar trend for the risk of LGA was observed although the result did not reach statistical significance. There was no association between B12 thresholds and the outcomes of LBW or SGA. The impact of serum folate on fetal macrosomia showed the reverse pattern for all of these outcome measures. Women in the highest quartile of folate had significantly higher risk of fetal macrosomia compared to those in the lowest quartile (RR 4.99, 95% CI: 1.15, 21.62, $p = 0.03$), which remained significant after adjusting for maternal BMI (RR 6.60, 95% CI: 1.42, 30.71, $p = 0.02$) (Table 4).

Table 4. Relationship between maternal B12 on birth outcome measures in no-GDM women.

	n	Range of Values (pmol/L)	Macrosomia, *n* (%)	LGA, *n* (%)	LBW, *n* (%)	SGA, *n* (%)
Vitamin B12 (quartiles)						
1	48	71.6, 157.2	11 (22.9)	12 (25.0)	1 (2.1)	4 (8.3)
2	48	158.7, 195.6	10 (20.8)	12 (25.0)	2 (4.2)	2 (4.2)
3	47	196.3, 244.3	9 (19.1)	10 (21.3)	3 (6.4)	3 (6.4)
4	50	245.0, 512.2	4 (8.0)	5 (10.0)	3 (6.0)	5 (10.0)
Relative risk (95% CI) [a]			5.26 (1.26, 21.91)	3.18 (0.96, 10.56)	0.10 (0.002, 5.75)	1.35 (0.28, 6.47)
p [b]			0.02	0.06	0.27	0.71
p [c]			0.05	0.13	0.37	0.52
Folate (quartiles)						
1	44	4.5, 14.3	5 (11.4)	7 (15.9)	4 (9.1)	4 (9.1)
2	47	14.5, 20.6	7 (14.9)	9 (19.1)	1 (2.1)	2 (4.3)
3	48	20.8, 34.2	11 (22.9)	10 (20.8)	1 (2.1)	3 (6.3)
4	48	34.4, 45.3	10 (20.8)	12 (25.0)	3 (6.3)	5 (10.4)
Relative risk (95% CI) [a]			4.99 (1.15, 21.62)	2.32 (0.74, 7.34)	0.21 (0.01, 9.64)	1.52 (0.26, 8.93)
p [b]			0.03	0.15	0.42	0.64
p [c]			0.02	0.06	0.41	0.90

[a] Relative risk of birthweight outcome in quartile 1 vs. quartile 4 of B12 and quartile 4 vs. quartile 1 of folate; [b] adjusted for age, parity, ethnic origin, smoking, gestation of bloods, and serum folate (or B12, respectively), plus sex and gestational age for macrosomia and LBW; [c] as for Model b plus gestational BMI; LGA: large for gestational age; LBW: low birthweight; SGA: small for gestational age.

4. Discussion

Our study, although retrospective in nature, showed three key findings. Firstly, it is the first study to show that low B12 status in pregnancy is associated with a higher risk of GDM in a UK population. Secondly, higher first trimester BMI was an independent predictor of later B12 insufficiency. Thirdly, low B12 levels were associated with macrosomia in the subgroup of no-GDM women, which seems to be partly mediated by maternal BMI.

The only other study that examined the link between B12 and GDM by Krishnaveni et al. was in an Indian cohort [12]. The magnitude of association found in that study was similar to ours, but the significance was lost after adjusting for maternal BMI. In our study, although the effect size was reduced when adjusted for BMI (aOR 2.59 vs. 2.05; Table 3), the significance persisted, suggesting a potential independent effect of B12. Higher numbers of women with GDM in our cohort and a 'case-control' design might explain the larger effect size. The recent finding by Knight et al., albeit in no-GDM women, also supports the inverse link between B12 levels and insulin resistance in pregnant White Caucasian women [16]. Indeed, higher insulin resistance in the context of low B12 has been shown by other authors in obese adolescents [26], non-pregnant adults [27,28], as well as in women with polycystic ovarian syndrome [28]. Prospective longitudinal studies are needed to investigate whether the presence of low B12 status in early pregnancy independently increases the risk of incident GDM.

The aetiology of the inverse relationship between B12 and BMI found in our study is an intriguing one. While confounding factors such as dietary habits, socioeconomic status, and hemodilution may be present, other studies that have corrected for these still show an independent link between B12 and BMI [12,16]. Interestingly, the frying and roasting of meat products reduces the bioavailability of B12 by 20%–40% [29], so higher consumption of processed foods may increase the risk of both B12 insufficiency and metabolic diseases. Additionally, B12 has been shown to be negatively associated with other markers of obesity such as triglycerides [7], blood pressure [30], and the metabolic syndrome [31], which lends support to a possible pathological association between them. In one

trial, the supplementation of B12 and folate in adults with metabolic syndrome improved insulin resistance by ameliorating endothelial dysfunction, providing further insight into how these conditions may be linked [32]. Further studies are needed to determine the direction of association and a potential reverse causality.

This is the first study that has demonstrated a relationship between maternal B12 and macrosomia, which seem to be mediated in part by maternal obesity. We demonstrated this only in no-GDM women as the treatment of GDM is a major confounder for macrosomia. Unfortunately, we did not have adiposity measures or a bigger sample size to assess the interactions between B12 status and maternal BMI/adiposity with offspring size and adiposity.

The rates of B12 insufficiency observed in our no-GDM population was similar to that observed by Knight et al. [16] (22% vs. 20%), suggesting that such higher rates of insufficiency are not limited to Indian populations [11]. It must be noted that a fall in B12 during pregnancy may be physiological due to a decrease in the fraction bound to inactive haptocorrin [33], but the evidence is equivocal with regards to whether there is also a fall in the active form, holotranscobalamin [34,35]. In the absence of specific cut-off values to define B12 deficiency in pregnancy, we used the non-pregnant reference range (<150 pmmol/L). It is noteworthy that associations with adverse maternal metabolic outcomes [12] and elevation in Hcy during pregnancy [36] were found by other authors at B12 thresholds similar to this.

It was reassuring to see that folate deficiency was rare, albeit in this selected hospital-based cohort. However, the combination of low B12 and high folate has been shown to be associated with lower neonatal birthweight [37] as well as central adiposity and insulin resistance in 6-year old offspring [14]. Whilst our sample size was not large enough to perform a detailed subgroup analysis, we observed that women in the lowest quartile of B12 and highest quartile of folate had similar risks of macrosomia (aRR of 5.3 and 4.99; Table 4). Therefore, it is possible that the women with such a B12-folate imbalance are particularly at high risk of having larger babies. This phenomenon (high folate/low B12), is increasingly common in populations with mandatory folic acid fortification such as in the USA and Canada [38,39], and is related to adverse clinical outcomes in the elderly population [40].

Although we have identified associations between B12, maternal obesity, risk of GDM, and fetal macrosomia, our study does not prove causation or the direction of the relationship between these factors. Some of the important limitations were that this was a single-centre, retrospective study involving pregnant women attending a hospital clinic. Therefore it was not possible to obtain early pregnancy B12/folate levels. We adjusted for the gestation of bloods in all the regression analyses, to reduce some of the bias due to longitudinal changes in B12 during pregnancy. We did not have markers of adiposity, and therefore it was not possible to study the potential differential association of low B12 status with obesity and adiposity in pregnant women as well as their offspring. Lack of functional measures of B12 insufficiency, such as Hcy and methylmalonic acid (MMA), or holotranscobalamin, which is the active fraction of B12 available for uptake by tissues, limits the ability to study the thresholds of B12 sufficiency during pregnancy and should be measured in future studies.

5. Conclusions

We have shown for the first time in a UK population that B12 deficiency in pregnancy is common particularly in obese women, is independently associated with GDM, and may contribute to macrosomia. As the prevalence of maternal obesity and GDM is rapidly increasing, our findings warrant longitudinal cohort studies to understand the interplay between B12 and these outcomes. If early pregnancy B12 status is found to be independently predictive of incident GDM, such findings could potentially offer simple interventions to improve the metabolic health of pregnant women and their offspring.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/8/12/768/s1, Table S1: Table of characteristics of no-GDM women who did and did not undergo GTT, Table S2: Table of birth outcomes of offspring according to maternal GDM status.

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Review

Metformin Treatment and Homocysteine: A Systematic Review and Meta-Analysis of Randomized Controlled Trials

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Abstract: The aim of this systematic review is to assess whether metformin could change the concentration of serum homocysteine (Hcy) with and without simultaneous supplementation of B-group vitamins or folic acid. A literature search was conducted in PubMed, EmBase, and Cochrane Central Register of Controlled Trials (CENTRAL) to identify randomized controlled trials (RCTs) reporting the concentration of serum Hcy in metformin-treated adults. Meta-analysis was applied to assess the association between metformin and the changes of Hcy concentration. Twelve publications were included in this study. In the overall analysis, metformin administration was not statistically associated with the change of Hcy when compared with the control treatment (mean difference (MD), 0.40 μmol/L; 95% confidence interval (CI), −0.07~0.87 μmol/L, p = 0.10). In the subgroup analysis, metformin was significantly associated with an increased concentration of Hcy in the absence of exogenous supplementation of folic acid or B-group vitamins (MD, 2.02 μmol/L; 95% CI, 1.37~2.67 μmol/L, p < 0.00001), but with a decreased concentration of serum Hcy in the presence of these exogenous supplementations (MD, −0.74 μmol/L; 95% CI, −1.19~−0.30 μmol/L, p = 0.001). Therefore, although the overall effect of metformin on the concentration of serum Hcy was neutral, our results suggested that metformin could increase the concentration of Hcy when exogenous B-group vitamins or folic acid supplementation was not given.

Keywords: metformin; homocysteine; vitamin B_{12}; folic acid; systematic review; meta-analysis

1. Introduction

Metformin, a first-line drug for type 2 diabetes mellitus (T2DM) recommended by most guidelines of diabetes, is also widely used in patients with polycystic ovary syndrome (PCOS), pre-diabetes, and other diseases involving insulin resistance [1,2]. However, Vitamin B_{12} deficiency was noted to be a potential disadvantage of metformin by the latest American Diabetes Association (ADA) guidelines [1]. A previous meta-analysis demonstrated that metformin treatment was associated with a decreased concentration of serum Vitamin B_{12} in a dose-dependent manner [3].

Homocysteine (Hcy) is a key component in the one-carbon pathway of methionine metabolism, which plays a dominant role in DNA methylation. The accumulation of Hcy, known as hyperhomocysteinemia (HHcy), is often resulted from Vitamin B_{12} deficiency [4], and is associated with an increased risk of cardiovascular diseases, cognitive impairment, cancer, chronic renal failure and other chronic diseases [4–11]. However, no consensus was reached on whether metformin

could induce Hcy elevation. This systematic review aimed to assess the association between metformin administration and the changes of serum Hcy concentration with and without simultaneous supplementation of B-group vitamins or folic acid.

2. Materials and Methods

2.1. Literature Search and Study Selection

This systematic review was conducted and reported according to the Cochrane Handbook for Systematic Reviews of Interventions and the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) statement. Literature search was conducted systematically in PubMed, EmBase, and Cochrane Central Register of Controlled Trials (CENTRAL) until January, 2016 by two authors (Q.Z. and S.L.). 'Metformin' and 'homocysteine' were used as keywords in the literature search. The references of original studies were also screened to ensure that potentially eligible publications were included. A detailed search strategy was presented in Supplementary Materials Figure S1.

We included studies which met the following criteria: (1) metformin was given as intervention while non-biguanide agents as control; (2) reporting serum Hcy concentrations as one of the outcomes; (3) designed as randomized controlled trials (RCTs). Studies with inadequate outcome data of interest, published in non-English languages, and duplicated reports were excluded. Two authors (Q.Z. and S.L.) reviewed all searched papers independently. Disagreements were settled by consensus between the two reviewers or by discussion with a third author (J.L.). The detailed inclusion and exclusion criteria were presented in Supplementary Materials Table S1.

2.2. Data Extraction and Quality Assessment

Data were obtained by two authors (Q.Z. and S.L.) independently from each included study using a predefined form. Disagreements were resolved by discussion with a third author (J.L.). The following information was extracted: title, date of publication time, author names, participant characteristics, intervention strategy, treatment received before study, background treatment (used in both groups together with the intervention), study outcomes, and method for Hcy assay. The risk of bias for each included RCT was assessed using the Cochrane Handbook for Systematic Reviews of Interventions [12].

2.3. Statistical Methods

A meta-analysis was conducted to assess the association between metformin and the changes of Hcy concentration. Considering the significant clinical heterogeneity, a random effects model was used. Subgroup analyses were conducted based on pre-defined parameters: gender, disease type, dosage of metformin, background treatment, pre-study treatment, control medication, duration of follow-up, and test method of Hcy. Pooled mean differences (MDs) and their 95% confidence intervals (CIs) were used for all continuous data. All absolute values and changes of serum Hcy concentration were unified and recorded as μmol/L. Funnel graph was also presented to evaluate the publication bias. Review Manager (RevMan 5.3 from the Cochrane Collaboration, Oxford, UK) was used for statistical analysis.

3. Results

3.1. Search Results

Twelve papers, involving 1311 participants, 1156 of whom completed the studies, were included in the pooled analysis after screening of 1227 articles (Figure 1). Reasons for excluding each paper during full-text screening were presented in Supplementary Materials Table S2 [13–25]. Among included studies, 11 were parallel trials and two were 2×2 factorial designed trials. The mean age of participants ranged from 24.8 to 61.4 years. The mean duration of follow-up ranged from 6 to 224 weeks. Detailed characteristics of included studies were presented in Tables 1 and 2. Additionally, primary

and secondary outcomes of each included study and strategies of intervention were summarized in Supplementary Materials Tables S3 and S4.

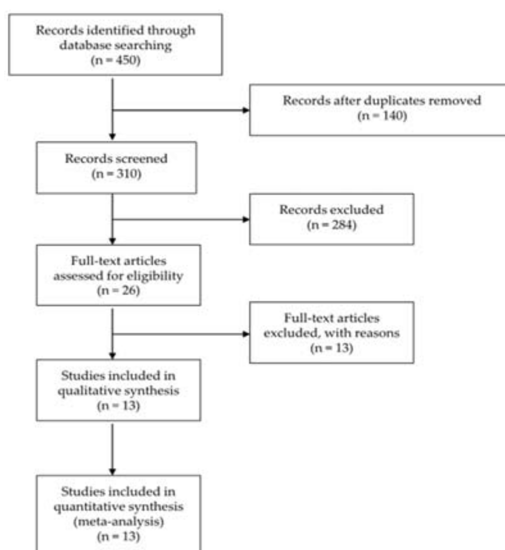

Figure 1. Flow diagram for study identification and inclusion.

Table 1. Baseline characteristics of each included study.

Study	Country	Patients, *N* (I/C)	BMI, (kg/m^2)	Age (Years)	Participants	Women (%)
Carlsen 1997 [26]	Norway	29/30	NA	53	CHD without diabetes	All men
Carlsen 2007a [27]	Norway and Turkey	31/32	NA	NA	PCOS, infertile	All women
Carlsen 2007b [27]	Norway and Turkey	16/18	NA	NA	PCOS, pregnant	All women
de Jager 2010 * [28] Wulffele 2003 * [15]	The Netherlands	131/146	30	61.4	Insulin-treated T2DM	75.5
Kilic 2011a [29]	Turkey	24/25	29.6	28.9	PCOS with IGT, BMI > 25 kg/m^2	All women
Kilic 2011b [29]	Turkey	23/24	22.4	26.5	PCOS with IGT, BMI < 25 kg/m^2	All women
Kilicdag 2005 [30]	Turkey	15/15	27.7	24.8	PCOS	All women
Sahin 2007 [31]	Turkey	74/36	28.9	58.6	Newly diagnosed T2DM	58.2
Schachter 2007a [32]	Israel	28/23	NA	NA	PCOS with IR	All women
Schachter 2007b [32]	Israel	27/24	NA	NA	PCOS with IR	All women
Derosa 2003 [33]	Italy	49/53	25.0	53.6	Drug naïve T2DM	50.0
Ghazeeri 2015 [34]	Lebanon	18/19	NA	25.8	PCOS	All women
Derosa 2004 [35]	Italy	75/73	27.9	NA	Newly diagnosed T2DM	51.2
Erem 2014 [36]	Turkey	19/19	32.4	52.4	Newly diagnosed T2DM	71.1
Hassan 2015 [37]	Egypt	30/30	27.3	NA	Newly diagnosed T2DM	All men

I/C, intervention/control group; BMI, body mass index; CHD, coronary heart disease; PCOS, polycystic ovary syndrome; T2DM, type 2 diabetes mellitus; IGT, impaired glucose tolerance; NA, not available; IR, insulin resistance. * These two analyses were short-term and long-term outcomes of the same trial, respectively. The long-term follow-up data (de Jager, 2010 [28]) were included in the quantitative analysis.

Table 2. Study characteristics of each included study.

Study ID	Intervention	Control	Primary Treatment	Washout Period	Background Treatment	Follow-up	Assay Method of Hcy	B_{12} Supplement	Folic Acid Supplement
Carlsen 1997 [26]	Metformin (2000 mg/day) [1]	Blank	Coronary artery bypass surgery or angioplasty	Lifestyle intervention and lovastatin, 40 mg daily	Lovastatin, 40 mg daily	40 weeks	HPLC	No	No
Carlsen 2007a [2] [27]	Metformin (2000 mg/day)	Placebo	NS	No	Lifestyle intervention, folic acid 0.4 mg/day and a daily multivitamin tablet	16 weeks	HPLC	1 μg/day	0.4 mg/day
Carlsen 2007b [2] [27]	Metformin (1700 mg/day)	Placebo	NS	No	Lifestyle intervention, folate 1 mg/day and a daily multi-vitamin tablet	16 weeks	HPLC	1 μg/day	1 mg/day
de Jager 2010 [28]/ Wulffele 2003 [3] [15]	Metformin (2550 mg/day) [4]	Placebo	Insulin	Insulin (12 weeks)	Insulin	224 weeks	Chromsystems kit	No	No
Kilic 2011a [5] [29]	Metformin (1700 mg/day)	Oral contraceptive	NS	No	B-group vitamins	24 weeks	CLI	2000 mg/day	No
Kilic 2011b [5] [29]	Metformin (1700 mg/day)	Oral contraceptive	NS	No	B-group vitamins	24 weeks	CLI	2000 mg/day	No
Kilicdag 2005 [30]	Metformin (1700 mg/day)	Rosiglitazone (4 mg/day)	NS	No	No	12 weeks	FPI	No	No
Sahin 2007 [31]	Metformin (1700 mg/day)	Blank	Lifestyle intervention	Lifestyle intervention (4 weeks)	Lifestyle intervention	6 weeks	CLI	No	No
Schachter 2007a [6] [32]	Metformin (1700 mg/day)	Blank	NS	No	Infertility treatment and folic acid 0.4 mg daily	Three cycles of treatment [7]	FPI	No	0.4 mg/day
Schachter 2007b [6] [32]	Metformin (1700 mg/day)	Blank	NS	No	Infertility treatment and B-group vitamins	Three cycles of treatment [7]	FPI	0.5 mg/day	0.4 mg/day
Derosa 2003 [33]	Metformin (1500–2500 mg/day) [8]	Repaglinide (2–4 mg/day) [8]	NS	Placebo	Lifestyle intervention	60 weeks	HPLC	No	No

Table 2. *Cont.*

Study ID	Intervention	Control	Primary Treatment	Washout Period	Background Treatment	Follow-up	Assay Method of Hcy	B_{12} Supplement	Folic Acid Supplement
Ghazeeri 2015 [34]	Metformin (1700 mg/day)	placebo	NS	3 months of rosuvastatin (10 mg/day)	Rosuvastatin (10 mg/day)	24 weeks	NA	No	No
Derosa 2004 [35]	Metformin (1000–3000 mg/day) [9]	Glimepiride (1–4 mg/day) [9]	NS	No	Lifestyle intervention	56 weeks	HPLC and fluorescence detection	No	No
Erem 2014 [36]	Metformin (2000 mg/day)	Pioglitazone (15–45 mg/day) [10]	No	No	Lifestyle intervention	12 months	ELISA	No	No
Hassan 2015 [37]	Metformin (1000 mg/day)	moderately calorie-restricted diet and an active lifestyle	No	No	No	3 months	enzyme-linked immunoassay and an automated fluorescence polarization analyzer	No	No

HPLC, High pressure liquid chromatography; NS, Not significant; CLI, Chemiluminescence immunoassay; FPI, Fluorescence polarization immunoassay. [1] The average daily intake of metformin was 1707 mg at week 4, 1759 mg at week 12, and 1741 mg at week 40; [2] This article included two independent RCTs; [3] These two analyses were short-term and long-term outcomes of the same trial, respectively. The long-term follow-up data (de Jager, 2010 [28]) were included in the quantitative analysis; [4] Each patient in this group was given his or her maximally tolerated daily dose (one, two, or three tablets of 850 mg) during the trial. The actual mean dose in the metformin-treated group was 2050 mg/day; [5,6] These are 2 × 2 factorial designed trials with four treatment arms in each trial; [7] This study did not report the exact duration of follow-up; [8] The average daily intake of metformin was 2000 mg, and that of repaglinide was 3 mg; [9] The average daily intake of metformin was 2500 mg, and that of glimepiride was 3 mg; [10] Each patient in this group was given his or her maximally tolerated daily dose during the trial (15 mg/day in six patients, 30 mg/day in twelve patients, and 45 mg/day in one patient).

3.2. Quality Assessment

The risk of bias of the included studies was demonstrated in Supplementary Materials Figures S2 and S3. The procedures of random sequence generation in seven studies [26,27,31,33,35–37], of allocation concealment in nine studies [26–28,31,33–37], and of blinding of participants and personnel in five studies [30,32–34,36] were not clearly described. Selective reporting risk was unclear in one study [32]. One study was at high risk of bias in allocation concealment [32], and one study provided incomplete outcome data [32]. Quality assessment of each included study was shown in Supplementary Materials Table S5.

Potential publication bias was suspected from the funnel graph analyses, which was presented in Supplementary Materials Figure S4.

3.3. Metformin and Homocysteine

The results of the overall analysis showed that metformin did not have a statistically significant effect on the concentration of Hcy when compared with the control treatment (MD, 0.40 μmol/L; 95% CI, −0.07~0.87 μmol/L, p = 0.10; Figure 2). Subgroup analyses according to whether the patients received folic acid or B-group vitamins supplementation showed that administration of metformin was associated with a decreased concentration of serum Hcy in the patients receiving regular folic acid or B-group vitamins (MD, −0.74 μmol/L; 95% CI, −1.19~−0.30 μmol/L, p = 0.001), while with an elevated concentration of serum Hcy in the patients without any supplementation (MD, 2.02 μmol/L; 95% CI, 1.37~2.67 μmol/L, p < 0.00001).

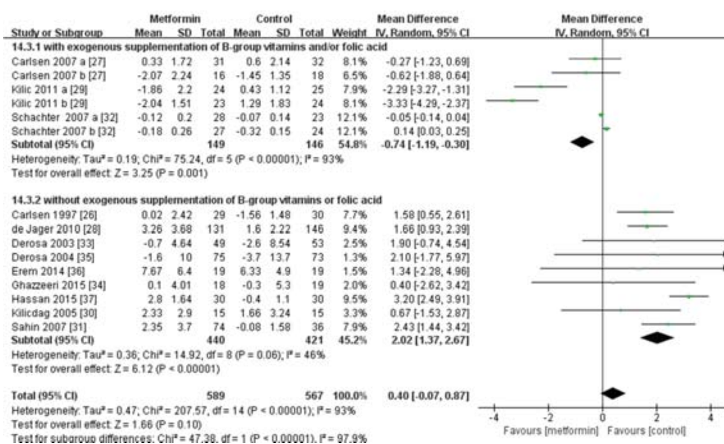

Figure 2. Overall analysis on Hcy concentration and subgroup analysis on Hcy concentration of patients with or without folic acid or B-group vitamins supplementation. The changes from baseline (Mean ± SD) between the two groups were compared. SD, standard deviation; CI, confidence interval; IV, inverse variance.

Subgroup analyses were conducted according to gender, disease type, dosage of metformin, background treatment, pre-study treatment, control treatment, duration of follow-up, change of Vitamin B_{12} concentration, and assay method of Hcy. Detailed results were presented in Supplementary Materials Table S6. It was demonstrated that the administration of metformin was associated with a significant reduction of serum Hcy among young female patients with PCOS. In addition, subgroup analysis based on the dosage of metformin showed that higher dosages of metformin (≥2000 mg daily) were associated with an elevation of serum Hcy, when compared with dosages less than 2000 mg daily (MD, 1.07 μmol/L; 95% CI, −0.17~2.30 μmol/L, p = 0.09).

3.4. Adverse Events

Five studies reported that more patients in the metformin group suffered gastrointestinal side effects when compared with those in the control [27,28,30,33,35]. Also, five papers did not provide any information about adverse events [26,29,31,32,34]. No death was reported. Details were shown in Supplementary Materials Table S7.

4. Discussion

Our study did not find significant association between metformin treatment and the change of serum Hcy concentration in the overall population. However, the subgroup analyses noted that metformin administration was associated with elevation of Hcy in the patients without supplementation of folic acid or B-group vitamins, which indicated that metformin might induce HHcy in the absence of exogenous folic acid or B-group vitamins supplementation.

Hcy is a sulfur amino acid with a free sulfhydryl group as the final metabolite of methionine and Vitamin B_{12} serves as a cofactor in the degeneration of Hcy to methionine. The insufficiency in Vitamin B_{12} results in the accumulation of Hcy, which is known as HHcy. HHcy is a well-established risk factor for cardiovascular diseases, cognitive impairment, and chronic renal failure [4–9], moreover, Hcy has been found to be an independent predictor of all-cause and vascular mortality [38,39]. Metformin has been demonstrated to be associated with reduction of serum Vitamin B_{12} concentration [3,40]. It has been shown that metformin could induce Vitamin B_{12} malabsorption by enhancing bacterial overgrowth, altering bacterial flora in enteric canal, and binding to the Vitamin B_{12}-intrinsic factor (IF). This malabsorption ultimately leads to a reduction of serum Vitamin B_{12} [41–45]. Hence, some researchers were calling attention to the monitoring of Vitamin B_{12} concentration in the diabetic patients treated with metformin, and suggested Vitamin B_{12} supplementation could be considered in patients with Vitamin B_{12} deficiency [3,46,47], although some authors still doubt the clinical significance of this reduction [48,49]. A recent clinical trial suggested that the metformin-associated reduction of the serum Vitamin B_{12} was due to the increased transportation and utility of Vitamin B_{12} by cells stimulated by metformin [50]. One of our subgroup analyses showed that metformin raised serum Hcy in the patients without folic acid or Vitamin B_{12} supplementation, but reduced Hcy when folic acid or Vitamin B_{12} was supplemented, indicating that metformin-associated Vitamin B_{12} reduction might be responsible for Hcy elevation, and exogenous folic acid and Vitamin B_{12} may rescue the methionine metabolic disturbance in metformin-treated patients. Considering Hcy as an important biomarker of a series of diseases and the few adverse effects of folic acid and Vitamin B_{12}, exogenous supplementation of these two vitamins could be necessary for metformin-treated patients, which is consistent with the recommendation of regular Vitamin B_{12} supplementation in the current American Association of Clinical Endocrinologists (AACE) guideline [51]. However, this recommendation had not yet been supported by well-designed randomized trials.

Our subgroup analyses also demonstrated that, the administration of metformin might cause a significant reduction of serum Hcy in young women with PCOS. However, it must be noted that, most of the young women enrolled received exogenous folic acid or B-group vitamins supplementation, and hence the observed Hcy reduction might be partially caused by the effects of exogenous folic acid or B-group vitamins. Meanwhile, estrogen, progestin, and age may also have some effects on the concentration of Hcy [5,8]. Experimental studies are required to further explain this difference.

The increase of serum Hcy concentration in the metformin-treated patients was confirmed by a series of observational studies [16–18,20,25,52–56]. These studies indicated that metformin was associated with an elevated concentration of serum Hcy compared with control treatment. Moreover, in Yilmaz's trial [20], where all the included patients were young women with PCOS but without Vitamin B_{12} or folic acid supplementation, Hcy was found to be elevated, while Vitamin B_{12} was reduced in the metformin-treated patients. In addition, Carlsen and colleagues [27] noticed that Hcy was reduced in pregnant women but not in infertile women. No explanation has been established currently, but further investigations on the pseudo reduction of Vitamin B_{12} during pregnancy and

the effect of estrogen and progestin on the concentration of Hcy might help us better understand the underlying mechanism. In Carlsen's and Kilic's trials [27,29], all participants received exogenous folic acid or B-group vitamins and, interestingly, metformin-treated patients had a lower concentration of Hcy compared with controls. A possible explanation was that exogenous folic acid or B-group vitamins might counteract the reduction of the Vitamin B_{12} absorption caused by metformin [46]. In Schachter's trial [32], no matter whether the patients were treated with metformin or not, the reduction of Hcy in the patients receiving both Vitamin B_{12} and folic acid was greater than that in the patients receiving folic acid only (metformin-treated: -0.18 versus -0.12 μmol/L; metformin-untreated: -0.32 versus -0.07 μmol/L). It indicated that Vitamin B_{12} was critical in reducing serum Hcy, which could be explained by the vital role of Vitamin B_{12} in the metabolism of methionine [5].

Our study has several limitations. Firstly, the heterogeneity among the included studies was significant. Although subgroup analyses were conducted to explore possible sources of heterogeneity, factors such as weight, age, gender, and race might still influence the results of our study. Particularly, the dosage and the follow-up duration of included studies varied largely, although subgroup analyses did not find significant effects of these factors on the results. Secondly, in our subgroup analysis concerning exogenous B-group vitamins or folic acid supplementation, most patients receiving exogenous B-group vitamins or folic acid were diagnosed with PCOS or infertility, which could induce some potential biases. Further studies are required to demonstrate the interaction between metformin and B-group vitamins in patients with PCOS or infertility. Thirdly, long-term outcomes such as mortality and cardiovascular events were not studied in our analysis. Finally, the strength of the pooled results was restricted by the generally high risk of bias of included studies.

5. Conclusions

Although there is no significant change of the concentration serum Hcy between metformin-treated and non-biguanide-treated patients in the overall pooled analysis, our subgroup analysis suggested that metformin may induce an elevation of serum Hcy concentration in the absence of B-group vitamins or folic acid supplementation. Nevertheless, given the supplementation of B-group vitamins or folic acid, metformin could even be associated with reduced concentration of serum Hcy. Since HHcy is a risk factor for a series of adverse clinical outcomes, supplementation of B-group vitamins or folic acid might be necessary in metformin-treated patients, regardless of the background diseases. However, further investigations are still required to demonstrate the effects and long-term outcomes of Vitamin B_{12} or folic acid supplementation in the metformin-treated patients.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/8/12/798/s1; Table S1: Detailed inclusion and exclusion criteria of each included studies in the meta-analysis, Table S2: Rationale for excluding studies after full-text screening, Table S3: Primary and secondary outcomes of each included study, Table S4: Intervention strategy of each included study, Table S5: Rationale of quality assessment for each included study, Table S6: Summary of subgroup analysis, Table S7: Adverse events, Figure S1: Risk of bias graph: review authors' judgements about each risk of bias item presented as percentages across all included studies, Figure S2: Risk of bias summary: review authors' judgements about each risk of bias item for each included study, Figure S3: Funnel plot of comparisons.

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Author Contributions: S.L. and J.L. designed this study; Q.Z., Q.L. and K.R. extracted the data; X.S. and L.L. are both biostatisticians from the Chinese Evidence-Based Medicine Center, West China Hospital, Sichuan University, China; X.S. provided methodological guidance of this manuscript as well as reviewed the statistics; L.L. and Q.Z. performed the data analysis and statistical procedure; Q.Z. and S.L. drafted the manuscript and all authors reviewed and revised the manuscript; J.L. is responsible for the integrity of the study as a whole.

Conflicts of Interest: The authors declare no conflict of interest.

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Review

Potential Links between Impaired One-Carbon Metabolism Due to Polymorphisms, Inadequate B-Vitamin Status, and the Development of Alzheimer's Disease

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Abstract: Alzheimer's disease (AD) is the major cause of dementia and no preventive or effective treatment has been established to date. The etiology of AD is poorly understood, but genetic and environmental factors seem to play a role in its onset and progression. In particular, factors affecting the one-carbon metabolism (OCM) are thought to be important and elevated homocysteine (Hcy) levels, indicating impaired OCM, have been associated with AD. We aimed at evaluating the role of polymorphisms of key OCM enzymes in the etiology of AD, particularly when intakes of relevant B-vitamins are inadequate. Our review indicates that a range of compensatory mechanisms exist to maintain a metabolic balance. However, these become overwhelmed if the activity of more than one enzyme is reduced due to genetic factors or insufficient folate, riboflavin, vitamin B6 and/or vitamin B12 levels. Consequences include increased Hcy levels and reduced capacity to synthetize, methylate and repair DNA, and/or modulated neurotransmission. This seems to favor the development of hallmarks of AD particularly when combined with increased oxidative stress e.g., in apolipoprotein E (ApoE) ε4 carriers. However, as these effects can be compensated at least partially by adequate intakes of B-vitamins, achieving optimal B-vitamin status for the general population should be a public health priority.

Keywords: homocysteine; dementia; Alzheimer's disease; nutrition; one-carbon metabolism; B-vitamins; polymorphism; prevention; therapy

1. Introduction

Alzheimer's disease (AD) is the most frequent type of dementia, causing around two-thirds of cases [1]. The condition becomes more common with increasing age, affecting between 5% and 8%, 15% and 20%, and 25% and 50% of those in the age groups ≥65 years, ≥75 years and ≥85 years, respectively [1]. The number of people aged ≥65 years is estimated to increase from ~500 million in 2008 to ~1.3 billion in 2040 [2]. A significant increase in absolute numbers, but also in the proportion of the population affected by the disease, is expected for the coming decades. In Europe, the predicted increase in numbers of individuals with dementia from ~36 million in 2010 to ~115 million in 2050 will result in an around €250 billion health care cost with respect to the condition by 2030 [1]. In the U.S., the cost is projected to exceed $1 trillion by 2050 if the disease continues to progress at its current pace [3]. Even more importantly, despite intensive research, there is currently no treatment available to cure or reverse AD [4]. This is reflected in the alarming mortality rates: for diseases such as human immunodeficiency virus, cardiovascular disease (CVD) and some cancers, important decreases in the death rate were achieved between 2000 and 2010, while for AD, the death rate increased by nearly 70% during the same period [5].

While many questions remain concerning its etiology, treatment is further complicated by the early onset of neuro-pathological changes: postmortem studies have revealed specific hallmarks of AD such as amyloid plaque formations in cognitively normal elderly [6]. It has been postulated that they develop decades before even mild symptoms of dementia manifest [7]. By the time the disease is diagnosed, cellular damage and amyloid plaque deposition might therefore already be too advanced for treatment to be successful [8,9]. Consequently, preventing the development o AD seems to be a promising avenue for improving health and quality of life for the elderly and to reduce the burden for society.

For a preventive approach to be successful, a better understanding of risk factors for AD is crucial. Some rare genetic mutations have been identified as the cause of early onset of the disease [10], but only a relatively small fraction of cases falls into this category. We will therefore concentrate on the significantly more common late-onset type, which is thought to be triggered by a combination of genetic, epigenetic and environmental factors [11]. It has been well established that apolipoprotein E (ApoE) is a very important genetic risk factor for age-dependent chronic diseases, including CVD and AD [12]. Due to two major polymorphisms on the encoding exon 4 of this gene, three major protein isoforms, ApoE ε2, ApoE ε3 and ApoE ε4 exist [13]. It has been shown that homozygous carriers of the ApoE ε4 allele have a more than 10-fold increased risk of developing AD, possibly due to increased cholesterol levels, altered brain development early in life [12] or increased oxidative brain damage [14].

Environmental factors such as nutrition seem to play a role in the development of the disease [11]. In particular, some B-vitamins are thought to be implicated, even though the mechanism linking low status of B-vitamins and the development of AD is poorly understood. However, it seems that elevated levels of homocysteine (Hcy), a non-protein sulfur-containing amino acid implicated in the etiology of a range of medical conditions such as CVD [15], play an important role. Insufficiency of B-vitamins may also affect the development of the diseases via their role in DNA methylation [16], synthesis and/or repair [17] or in modulating neurotransmission [18]. Polymorphisms in genes encoding for specific enzymes can significantly affect their activity [19]. Therefore, studying mutations at critical steps in the metabolism of B-vitamins might help resolve some of the inconsistencies reported for their protective effect on the development of AD. Our aim is to evaluate the role of common polymorphisms of key enzymes in one-carbon metabolism (OCM; See Table 1) in the etiology of AD, particularly when intakes of the relevant B-vitamins are inadequate.

Table 1. Polymorphisms relating to key enzymes in the one-carbon metabolism that are potentially relevant to the development of Alzheimer's disease (AD).

Enzyme	Polymorphism	Reference
MTHFR	C677T	Schwahn and Rozen 2001 [20], Yamada et al., 2001 [21], Guenther et al., 1999 [22]
	A1298C	Weisberg et al., 2001 [23]
	T1317C	Weisberg et al., 1998 [24]
MS	A2756G	Leclerc et al., 1996 [25], Chen et al., 1997 [26]
MSR	A66G	Olteanu et al., 2002 [27]
	C524T	Olteanu et al., 2002 [27]
CBS	68 bp insertion at exon 8	Sebastio et al., 1995 [28]
	G9276A	Nienaber-Rousseau et al., 2013 [29]
	31 bp variable number of tandem repeats	Lievers et al., 2001 [30]
SHMT	C1420T	Heil et al., 2001 [31]

bp: base pairs; CBS: Cystathionine β-synthase; MSR: Methionine synthase reductase; MS: Methionine synthase; MTHFR: Methylenetetrahydrofolate reductase; SHMT: Serine hydroxymethyltransferase.

2. Evidence Linking B-Vitamins, Hcy and the Pathogenesis of AD

2.1. Observational Trials

Epidemiological studies provide evidence that AD patients tend to have higher Hcy plasma levels than controls, while there are trends for lower levels of B-vitamins [9,32]. Follow-up of a cohort with initially dementia-free elderly for a median of eight years found that plasma Hcy level >14 μmol/L at baseline doubled the risk of developing AD [33]. In addition, the inverse association between Hcy levels and cognitive decline seemed to exist even when the former was in what is generally accepted as the normal range (≤15 μmol/ L) [34]. A meta-analysis in 2011 concluded that Hcy levels were clearly higher in AD patients compared to controls [32]. However, based on the available evidence, they could not establish that hyperhomocysteinemia preceded AD [32]. McCaddon and Miller [35] concluded that the available evidence showed a strong and coherent relationship, biological plausibility, dose-response relationship and temporality, and therefore, most of the criteria necessary to establish causality between elevated Hcy and dementia were fulfilled. In the studies they reviewed, elevated Hcy predated hallmarks of AD such as dementia, brain atrophy or neurofibrillary tangles by 5–35 years [35]. They felt what was needed were well-designed intervention trials showing a clear effect of Hcy lowering on cognitive decline [35] (see below). A more recent meta-analysis concluded that the relative risk of developing AD due to elevated Hcy levels or low folate levels were ~1.8 (95% confidence interval 1.37–2.16) and ~2.1 (95% confidence interval 1.51–2.71), respectively, while the data for vitamin B12 and AD was inconclusive, even though AD patients had lower serum levels than controls [36].

2.2. Evidence from Supplementation with B-Vitamins

While somewhat inconsistent, some of the studies supplementing B vitamins show promising results: In a randomized placebo-controlled trial in elderly men, supplementation with 2 mg folic acid, 25 mg vitamin B6 and 400 μg vitamin B12 daily for 2 years decreased Hcy levels and reduced the rate of increase in circulating levels of amyloid-β1-40, an indicator for AD [37]. Supplementation with 800 μg folic acid daily for 3 years also led to a reduced progression of cognitive decline in parallel with a decrease in Hcy plasma levels compared to a control group receiving a placebo [38]. Moreover, an intervention with B-vitamins (800 μg folic acid, 500 μg vitamin B12 and 20 mg vitamin B6 per day for 2 years) in elderly with mild cognitive impairment was shown to slow down the progression of brain atrophy and reduce Hcy levels, both of which were associated with improved cognitive performance [39]. More specifically, this intervention decelerated shrinkage of the grey matter regions of the brain that are particularly affected by AD and the protective effect of the B-vitamins was limited to those with elevated Hcy levels [40]. Doses of these vitamins well above the recommended daily intakes in elderly men (aged ≥75 years) who were not specifically selected for elevated Hcy levels led to an improvement in vitamin status and Hcy levels [37]. In addition, these doses slowed the increase in circulating levels of amyloid beta, a proposed indicator for amyloid plaque formation, even though it did not reach statistical significance [37]. An intervention with supplements in a similar range improved memory and reduced the rate of atrophy in regions particularly affected by AD in elderly with mild cognitive impairment, particularly if they had elevated Hcy levels [39,40].

However, despite some encouraging results, a study on the benefits of Hcy lowering on heart health concluded that the evidence did not support the recommendation of routine supplementation with B-vitamins [41]. Similarly, despite lowering Hcy by around 25%, B-vitamin supplementation only had a marginal effect on cognitive aging [42]. McCaddon and Miller [35] pointed out that most individuals included did not actually experience such a decline and they highlighted the need for further studies specifically designed to assess such an effect.

3. Role of Key Polymorphisms in the OCM

3.1. Overview of the Enzymes of the OCM

The OCM is a complex metabolic pathway in which reduced tetrahydrofolate (THF), the active form of folate, acts as co-enzyme in the transfer of methyl groups [43]. It consists of three interrelated cycles, which are the methionine, thymidylate and purine cycles [44] (Figure 1A). Hcy can either be fed into the methionine or the thymidylate cycle (Figure 1A–C): When methionine levels are low, Hcy is remethylated into methionine (Figure 1B,D). For this, a methyl group is transferred from methylenetetrahydrofolate (MTHF) to Hcy by the methionine synthase (MS), resulting in THF and methionine. The latter can be further metabolized into *S*-adenosylmethionine (SAM), which plays a crucial role as a methyl-donor in other metabolic pathways such as DNA methylation or synthesis of neurotransmitters, phospholipids and myelin [44].

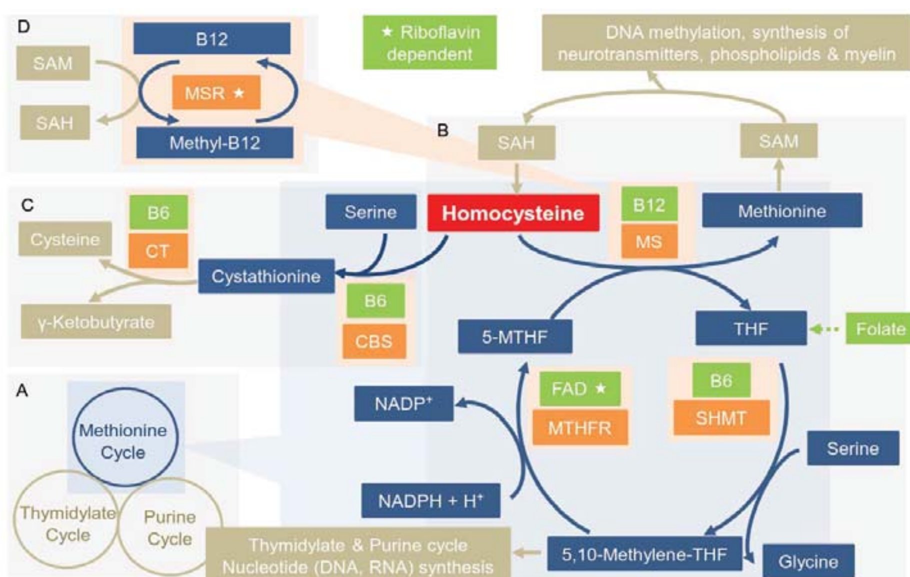

Figure 1. Metabolic pathways of the one-carbon metabolism: (**A**) Overview of the three cycles; (**B**) Methionine cycle: remethylation of homocysteine to methionine; (**C**) Transsulfuartion pathway: Irreversible conversion of homocysteine into cysteine; (**D**) Remethylation of vitamin B12 to its active form; CBS: cystathionine β-synthase; CT: γ-cystathionase; FAD: flavin adenine dinucleotide; MTHF: methylenetetrahydrofolate; MTHFR: methylenetetrahydrofolate reductase; MS: methionine synthase; MSR: methionine synthase reductase; NADP(H): (Hydroxy) Nicotinamide adenine dinucleotide phosphate; SAH: *S*-adenosylhomocysteine; SAM: *S*-adenosylmethionine; SHMT: serine hydroxymethyltransferase THF: tetrahydrofolate.

S-adenosylhomocysteine (SAH), remaining after the one-carbon transfer from SAM, is then hydrolyzed back to Hcy [45]. Serine and THF are turned into glycine and 5,10-methylene-THF in a reaction catalyzed by the serine hydroxymethyltransferase (SHMT) [46]. Then, 5,10-methylene-THF is reduced to 5-methylenetetrahydrofolate (MTHF) by the action of the methylenetetrahydrofolate reductase (MTHFR) [47], closing the cycle. If sufficient methionine is available or Hcy is accumulating, Hcy condenses with serine to form cystathionine and subsequently cysteine [45]. This reaction is called the transsulfuration pathway and is mediated by two vitamin B6-dependent enzymes (cystathionine β-synthase (CBS) and γ-cystathionase) [45].

MS depends on methyl-cobalamin, the active form of vitamin B12 [44], as an intermediate methyl carrier, and consequently, adequate amounts of the nutrient are essential to keep the cycle going [48]. Vitamin B12 is regenerated into its active form by the methionine synthase reductase (MSR) through remethylation with one-carbon units from SAM [49,50]. MSR is a flavoprotein [51] and therefore riboflavin dependent. SHMT consists of four subunits and each of those uses pyridoxal-5′-phosphate, the active form of vitamin B6, as a cofactor [52]. MTHFR also uses flavin adenine dinucleotide (FAD), derived from riboflavin, as a cofactor [51]. This highlights the important role B-vitamins play in the OCM and how deficiencies of each of them are likely to disturb its balance in specific ways.

3.2. Polymorphisms in Key Enzymes of the OCM

The relationship between B-vitamins, relevant polymorphisms and AD has not been studied in great detail and the potential mechanisms are poorly understood. The association between MTHFR C677T and AD has been studied in most detail, while for the other polymorphism the available evidence is very limited. In addition, a great shortcoming of the majority of studies is that no information on nutritional status in general and on B-vitamins more specifically is provided. We will also review how reduced enzymatic activity due to polymorphisms combined with lack of cofactors caused by inadequate dietary intake might unbalance these metabolic processes, thereby potentially favoring the development of AD.

3.2.1. Methylenetetrahydrofolate Reductase (MTHFR) Polymorphisms

MTHFR is by far the most widely studied enzyme in regard to polymorphisms affecting the OCM and their effect on Hcy levels. While deficiency is relatively rare in humans [53], three common mutations of the MTHFR gene, namely C677T, A1298C, and T1317C, have been proposed for an association with various pathological conditions. However, the T1317C mutation appears to be a silent polymorphism [24]; very limited evidence is available and none of it shows any association with Hcy levels or B-vitamin intakes [54,55], let alone AD and this will therefore not be discussed further in this review.

Globally, the frequency of population homozygote for the MTHFR 677TT mutation is thought to range from close to 0% in Sub-Saharan Africans to 32% in Mexicans [56,57]. Homozygotes for the polymorphism were reported to be more likely to have elevated Hcy levels compared to the population average [58–68] and the mutation constitutes the most frequent cause of moderate hyperhomocysteinemia due to genetic factors [20]. There is some evidence for gender-specific differences: one study found men who were homozygous carriers of the mutation had a much higher risk for significantly elevated Hcy levels than women [61]. In addition, a French study corroborated the above results by showing that genotype affected Hcy levels in men, but not in women [69]. Interestingly, one study found the age-dependent increase in Hcy masked the effect of the mutation and only showed a significant association in the older participants [70].

The enzyme activity seems to be reduced by up to 50% [20] due to reduced stability of the association with its cofactor FAD [21,22]. The addition of folate derivatives was shown to stabilize the FAD-MTHFR-folate complex in *Escherichia coli* with the 677TT mutation [22]. In line with this, the effect of the mutation on Hcy levels was more pronounced if folate levels were low [48,54,60,61,63,70–72] and it was not apparent in persons with high intakes from supplements ($\geq$400 μg/day) [66]. It was shown that the odds ratio for elevated Hcy levels in this genotype increased from 15 to 175 if plasma folate was $\leq$3.7 nmol/L [73]. Moreover, hyperhomocysteinemia in persons homozygous for the 677T mutation could be reversed or reduced with folic acid supplementation [47,73], while this had no effect in persons carrying the wild type allele [47]. A folate depletion–repletion study in elderly women showed a more pronounced decrease in serum folate levels accompanied by a steeper increase of Hcy levels in homozygous carriers of the mutation compared to the wild type [74]. After repletion, both serum folate and Hcy levels normalized to levels comparable with those of the participants with the wild type [74]. All this indicates that individuals with the MTHFR 677TT genotype may have

higher folate requirements and might benefit even more from supplementation [71] as increasing folate intakes could compensate for the reduced activity of the MTHFR. Multivitamin supplements showed a positive impact on levels of other B-vitamins such as vitamin B12 and pyridoxal 5′-phosphate [47] and might therefore also beneficially affect Hcy levels. Riboflavin status was also negatively associated with Hcy levels in carriers of at least one copy of this polymorphism [75]. In particular, Hcy levels were increased in persons homozygous for 677TT with marginal or low riboflavin status compared to heterozygous and wild types, which was not the case if the vitamin status was adequate [72]. In line with this, daily supplementation with 1.6 mg improved riboflavin status in all subjects with low levels at baseline, but Hcy levels only decreased significantly (by 40%) in subjects who were homozygous carriers of the mutation [76]. Moreover, the impact of riboflavin status on Hcy levels was more important in homozygous carriers of the 677TT mutation with low folate status [77]. Consequently, these data indicate that both riboflavin and folate can independently compensate the decreased MTHFR enzymatic activity due to the mutation.

While the effect of vitamin B6 on Hcy was found to be inconsistent, Hustad and colleagues [78] suggest that the effect is particularly evident in persons homozygous for the MTHFR 677T mutation and that interactions with other B vitamins might further complicate the relationship. Hcy levels were found to be inversely associated with vitamin B6 status if riboflavin levels were adequate, but plasma folate levels were low [72]. If re-methylation of Hcy via the methionine cycle (Figure 1B) is not possible due to lack of folate, the alternative pathway for Hcy is condensation with serine to cystathionine and this is catalyzed by the vitamin B6-dependent CBS (Figure 1C). However, if this pathway is also disturbed due to insufficient levels of vitamin B6, Hcy seems to accumulate. The role of vitamin B12 in persons with 677TT genotype is not completely clear: Vitamin B12 levels did not seem to have any effect on the risk of hyperhomocysteinemia in the 677TT genotype in some studies [60,71], while others reported a negative association between Hcy and serum vitamin B12 levels, particularly in person homozygous for 677T [54,79]. Moreover, Hcy levels were found to be higher in homozygous carriers of the mutation with low vitamin B12 levels, particularly if they did not take folate supplements [66]. The mechanism for such an effect is unknown, but it has been suggested it might be due to a coexisting mutation within the OCM [48].

The A1298C mutation in MTHFR gene was reported in approximately 10% of Canadians [24]. The prevalence seems to differ between ethnic groups: while non-Hispanic whites in the U.S. showed a similar prevalence of homozygous carriers (~12%) as that reported for Canadians, in Mexican Americans, the prevalence was ~20% and in non-Hispanic blacks it was just over 1% [66]. In itself, the A1298C mutation was not associated with elevated Hcy levels in either heterozygotes or homozygotes in most studies [54,61–63,66,70,80,81] and the reduction in MTHFR activity is lower than for the 677TTTT mutation (~70% of wild type) [23]. However, in vitro studies indicate a synergistic effect for the two mutations [23], Hcy levels were found to be highest [61,82] and the corresponding red cell folate level lowest in individuals with both mutations [61]. No significant effects were found for the combinations of genotypes and serum folate or vitamin B12, but this might be due to the low prevalence of the recombinant genotype [61,82]. In other studies, the activity of MTHFR was further reduced than what would have been expected from C677T alone in individuals heterozygous for C677T and A1298C (none of the subjects was homozygous for both mutations), which was accompanied by increased Hcy and decreased plasma folate [23,24,83]. Carriers of the wild type for both polymorphisms on the other hand were found to have the lowest Hcy levels compared with other combinations of the genotypes [84]. Again, the effect seems to be more pronounced in individuals with low folate levels [23].

In addition, plasma vitamin B6 levels were lower in individuals heterozygous for C677T carrying at least one copy of the mutation for A1298C compared to those who were homozygous for A1298C, but this might have been due to differences in supplement use between the groups [23]. Moreover, in doubly heterozygous subjects, plasma vitamin B12 was a significant predictor of Hcy levels, which was not the case for those who were wild type for at least one of the polymorphisms [48]. The authors

conclude that these people would benefit from an increase in vitamin B12 status, as this would help reducing or normalizing Hcy levels [48].

3.2.2. Methionine Synthase (MS) Polymorphism

Several polymorphisms in MS have been identified, which might potentially be relevant for the Hcy metabolism [25,26]. The most prevalent is the A2756G polymorphism with an allele frequency of around 20% [25,26,85–87]. A number of studies in both healthy and sick individuals of different age and gender groups assessed its effect on Hcy or B-vitamin levels and found no or only marginal effects that failed to reach statistical significance [61,86–91]. Given the relatively low prevalence of homozygous mutation in the gene encoding for MS, larger studies might be able to shed more light on the relationship between the different genotypes, levels of B-vitamins and Hcy concentrations. One study in more than 1200 healthy men between the ages of 50 and 61 years found that carriers homozygous for the more common AA genotype had higher Hcy levels than those with at least one copy of the G mutation, independent of folate or vitamin B12 status [60]. Similarly, fasting and post-methionine load Hcy levels were lower in individuals with at least one copy of the 2756G allele [59]. Moreover, an additive effect on Hcy levels was reported in carriers who have at least one copy of the MTHFR 677T allele and who are homozygous for MS 2756A [60].

In addition, Ma and colleagues report a trend towards a protective effect of the GG genotype for colorectal cancer despite the lack of association with Hcy, indicating an effect via a different mechanism, possibly via DNA methylation [91]. In line with this, the AG genotype was associated with increased erythrocyte folate and lower risk for myocardial infarction, but not Hcy or vitamin B12 levels, compared to the wild type (only one patient was homozygous for the mutation and was therefore not included in the analysis) [92]. Reduced activity of MS and the resulting decrease in SAM could affect DNA methylation and/or synthesis of neurotransmitters, phospholipids and myelin (Figure 1B), which in turn could contribute to the development of the AD. Hcy levels could still be kept in the normal range by condensing it with serine to cystathionine (Figure 1C). This is in line with the finding that the mutation correlated with cystathionine levels [70], indicating a preference for transsulfuration rather than re-methylation in the 2756AG/GG genotype. In line with this, one study found moderately increased Hcy levels in persons with the AA genotype, which increased with decreasing levels of vitamin B6, but seemed independent of folate and vitamin B12 status [93].

Moreover, a study in American men aged ~40 to 80 years found that Hcy levels decreased with increasing number of copies of the 2756G allele in healthy controls, but not in cases with a history of myocardial infarction [94]. It has been proposed that in conditions of elevated oxidative stress, functional vitamin B12 deficiency arises as the recycling into its active form cannot keep up with the rate of its oxidation [95]. Consequently, it can be speculated that the effect of the polymorphism on enzyme activity in the above-mentioned patients is masked by the stronger effect of vitamin B12 oxidation. Whether this underlying mechanism is relevant for the etiology of AD needs to be established. However, it is conceivable to assume such a link given the elevated levels of oxidative stress found in AD patients' brains.

3.2.3. Methionine Synthase Reductase (MSR) Polymorphism

Another relatively common mutation affecting an enzyme of the OCM is the A66G mutation in the gene encoding for the MSR. In a range of studies, it was reported that ~25% to 30% of Caucasians were homozygous carriers of the mutation [61,72,96]. Data from case-control studies indicate a great range between countries (for a review see [97]), but also between ethnic groups within one country: In the U.S. ~30% non-Hispanic whites were found to be homozygous for the mutation compared to ~20% Ashkenazi Jews, ~8% to 10% non-Hispanic blacks and ~7% Mexican Americans [66,98], while in Muslims in India, ~50% were carriers of two copies of the mutation [97].

It was reported that the mutation lead to a less efficient regeneration of vitamin B12 [27] and it has been proposed as a risk factor for elevated Hcy levels [51] as it reduces its conversion into

methionine. Another consequence of this impairment of the OCM is the reduced availability of SAM for DNA methylation [97]. However, the majority of studies does not confirm an effect on Hcy levels [66,70,82,88,96,99–101] and only two studies found significantly [49,102] and borderline significantly higher Hcy [61] levels in homozygous carriers of the mutation.

Given the role of MSR in recycling vitamin B12 and thereby contributing to the remethylation of Hcy, the decreased activity caused by the mutation can be assumed to be particularly critical if vitamin B12 levels are low. This was confirmed by a study showing that in persons with low plasma cobalamin levels (≤273 pmol/L), Hcy levels were higher in carriers of the mutant allele, if their riboflavin status was adequate [72]. In other words, adequate vitamin B12 levels seem to be able to compensate the reduced enzymatic activity, and the impairment due to inadequate riboflavin levels masks that due to genetic variation. If Hcy cannot be turned into methionine, the transsulfuration pathway involving the vitamin B6-dependent CBS will be activated to regulate its levels (Figure 1C). It is therefore not surprising that vitamin B6 status has an impact on the effect of the A66G polymorphism on Hcy levels [72].

There might also be an interaction between the different genotypes: in non-Hispanic whites homozygous for MTHFR 677T, there was a significant trend towards lower Hcy levels with increasing numbers of copies of 66G, which was not the case for the 677CC or CT genotypes [66]. While the MSR genotype in itself had no effect on Hcy in a study in healthy women, there seems to be an effect in combination with the MTHFR 677TT genotype [82]. However, the authors conclude that due to the small sample size in the MTHFR 677TT/MSR 66AA and GG groups, they failed to detect potential differences in plasma Hcy between these groups.

Further research is needed to assess the effect of this mutation, particularly in combination with other polymorphisms affecting the OCM and/or in individuals with inadequate status of one or more of the relevant B-vitamins. Brown and colleagues [100] showed an effect of the mutation on the risk for coronary artery disease, but not on Hcy levels, indicating that a mechanism other than elevated Hcy levels might be relevant. Given that vascular diseases seem to increase the risk of developing AD [103], this link should be further investigated.

Less is known about the C524T mutation in the gene encoding for MSR, for which ~14% homozygous carriers were found in a group of healthy Spaniards, while nearly 60% had at least one copy of the mutation [72]. It seems to affect the enzyme structure in the region between the binding domains for flavin mononucleotide and FAD/(Hydroxy) Nicotinamide adenine dinucleotide phosphate (NADPH), respectively [27]. As for the A66G variant, this mutation reduced the efficacy of B12 regeneration by MSR [27]. In carriers of the C524T mutation, Hcy was significantly higher than in controls if vitamin B12 levels were low, while riboflavin status had no clear effect [72]. For both mutations of the MSR, vitamin B6 levels were inversely associated with Hcy levels in persons with optimal riboflavin and vitamin B12 levels [72], highlighting again the importance of the transsulfuration pathway for keeping Hcy in the normal range when re-methylation is impaired.

3.2.4. Cystathionine β-Synthase (CBS) Polymorphism

Relatively rare mutations of the gene encoding for CBS are frequently found in patients with homocystinuria, but they do not seem to be more common in persons with moderately elevated plasma Hcy levels and were consequently considered to be of minor importance as risk factors for the general population [90,104–107]. In addition, some rare mutations in the gene encoding for CBS have been reported to have beneficial, albeit statistically not significant effects on Hcy levels. These mutations, however, were not regarded as significant due to their low prevalence [108]. However, a more common 68 base pair (bp) insertion in exon 8 in the gene encoding for CBS [28] might be relevant. The prevalence of this insertion in healthy men and women from Northern Ireland was around 18% [61] and around 12% in healthy US controls [109]. In the US, allele frequency has been reported to be significantly higher in non-Hispanic blacks (~26%) compared to non-Hispanic whites (~8%) or Mexican American individuals (~6%) [66]. It had been proposed that insertion had no effect on the enzyme activity [109]

and assessed on its own, its effect on Hcy is inconsistent: while some found no effect on Hcy [61,70], others showed a trend towards lower levels at least in specific subpopulations [59,60,66,89,110].

A few studies that assessed the effect of combined polymorphisms found that the insertion is capable of compensating the negative effect of MTHFR 677TT and MS 2756 AA [59,60,108]. However, in another study, a combination of homozygous 677T and 68 bd led to a further increase in Hcy levels, albeit in a very small sample ($n = 4$) [70]. While in black South Africans, the insertion itself had no effect on Hcy, in combination with MTHFR 677TT, those without the insertion had the highest Hcy levels [29]. Similarly, a different mutation of CBS (9276 GA genotype compared to 9276 GG, no 9276 AA in the study) led to an increase in Hcy levels in individuals homozygous for the MTHFR 677T mutation compared to other genotypes [29]. Moreover, increasing numbers of repeat units of the 31-bp *variable number of tandem repeats* polymorphism in the non-coding sequence of CBS at the boundary of exon 13 to intron 13 were found to decrease CBS activity and increase Hcy levels [30]. Frequency and position of these seem to vary between different ethnic groups [111]. Albeit inconsistent, these results highlight the importance of the transsulfuration pathway as an alternative to catabolize Hcy if remethylation is impaired. In line with this, the protective effect of the insertion on Hcy appears to be independent of folate and vitamin B12 status [60].

3.2.5. Serine Hydroxymethyltransferase (SHMT) Polymorphism

For SHMT, a polymorphism has been described at the position C1420T. While it has not been studied extensively, one study reports that women with the 1420CC genotype had significantly increased Hcy and decreased red cell and plasma folate levels [31]. In another study in patients with coronary artery disease, the mutation was also associated with lower levels of Hcy, higher plasma folate concentrations and decreased markers of oxidative stress [112].

4. Proposed Mechanisms Linking Polymorphisms, Hcy, B-Vitamins and AD

The data presented on polymorphisms in the genes encoding for key enzymes in the OCM and their interaction with various B-vitamins highlights the complex relationship between the various steps of these metabolic pathways. Genetic factors affecting the OCM alone are likely to play a relatively minor role in the overall risk of developing AD: 9% of variation in Hcy levels could be explained by differences in the polymorphisms for MTHFR, MS, MTR and CBS, while folate and vitamin B12 status are thought to be responsible for 35% of the variance [61]. Combining these genetic and nutritional factors increased the effect to 42%in relatively young subjects (20–25 years of age) [61]. The authors chose this age group as they expected the genetic effects to be less masked by a range of environmental influences that accumulate over a lifetime [61]. They suggest that more subtle genetic effects might only manifest in combination with longer-term exposure to other factors such as smoking [61]. In any case, polymorphisms help to understand the complexity of the metabolic system and explain some of the inconsistencies encountered in studies trying to link nutritional factors with risks for diseases.

The evidence presented above shows that lack of substrate or reduced enzymatic activity in one step in the OCM can be compensated at least partially or results in a shift to a different pathway. Consequently, it seems that health is only affected if these copying mechanisms fail due to a combination of more than one polymorphisms and/or inadequate supply of relevant vitamins (Figure 2). It is therefore not surprising that studies concentrating on a single polymorphism and its association with AD failed to show a consistent picture: A number of studies did not detect a difference in the frequency of the MTHFR C677T genotype in AD patients and controls [113–126], which is probably only partially due to the small sample sizes. Other studies and meta-analyses found an effect of the C677T polymorphism on AD [127–129], but there seemed to be some differences between the ethnicities [127,130]. Unfortunately, no study actually took into account the different polymorphisms of key enzymes in the OCM in combination with B-vitamin status. Importantly, one study showed that despite a lack of difference in C677T genotypes or Hcy levels between patients and controls, plasma Hcy concentrations were significantly higher in patients with dementia who were

either TT or CT and had low folate levels (<5.7 nmol/L) compared to those with adequate folate levels or CC genotype [131].

AD is a multifactorial disease, which is poorly understood and a range of hypothesis have been proposed for its etiology, which are reviewed in detail elsewhere [9,132]. According to the authors of a recent review, low folate and vitamin B12 status contribute to the development of cognitive impairment directly and via elevated Hcy levels [16]. These mechanisms will be discussed in the following sections.

4.1. Proposed Mechanism Linking Hcy & AD

A range of mechanisms have been proposed for the link between elevated Hcy and AD and preclinical studies show that hyperhomocysteinemia, induced by genetic manipulation or by B-vitamin deficiency, causes known hallmarks of AD such as accumulation of amyloid-β peptide [133–136] and intensified tau protein hyperphosphorylation in the brain [137]. An autopsy study showed a clear association between Hcy levels and neurofibrillary tangles, a known hallmark of AD, with an odds ratio of having such deposits of 2.60 (95% confidence interval 1.28–5.28) when comparing the top with the bottom Hcy quartile [138]. A prospective study showed greater brain atrophy in AD patients with higher Hcy levels [116] and this association between Hcy and grey matter atrophy has been confirmed by a range of studies (See review by Smith and Refsum [16]).

Amyloid plaque formation is thought to be an important event in the etiology of AD [139,140] and there is evidence that elevated levels of Hcy can impact the plaque formation by reducing the clearing rate of amyloid-β in the brain of mice [141]. Moreover, amyloid-β levels increased in rats after injection of Hcy into their brain and this was accompanied by loss in spatial memory [142]. Folate and vitamin B12 supplementation was able to lessen these effects [142]. A further piece of the puzzle is the finding that Hcy can bind to amyloid-β in vivo and in vitro, thereby triggering the formation of interconnections and subsequently aggregates [143]. Moreover, these deposits can induce oxidative stress, another important element in the etiology of AD [144,145].

The effect of elevated Hcy on brain capillaries is a further mechanism through which an impaired OCM might facilitate the development of AD [146] (Figure 2C). It has been postulated that elevated Hcy levels due to genetics or dietary inadequacies may compromise vascular health, thereby contributing to dementia and AD [131]. Hcy is thought to affect endothelial integrity by promoting the generation of peroxides, but also by reducing the availability of nitric oxide through a reduction of intracellular glutathione peroxide levels [147]. Moreover, while these vascular effects might be more prominent in individuals who are not otherwise genetically predisposed to AD, it has been speculated that there might be a more direct effect on brain cells in those with the ApoE ε4 genotype [146].

Increased levels of Hcy were shown to be a risk factor for shrinkage of specific brain regions including the bilateral hippocampus and parahippocampal gyrus, retrosplenial precuneus, lingual and fusiform gyrus, which is a key component of the AD process and is associated with cognitive decline [40]. In rats, it has been shown that exposure to Hcy leads to apoptosis in hippocampal neurons by inducing a cascade that results in DNA damage, decline of mitochondrial membrane potential and eventually nuclear disintegration, possibly triggered by nicotinamide adenine dinucleotide and adenosine triphosphate depletion [148]. Hcy was shown to accumulate in neurons as it is rapidly taken up via specific membrane transporters [149]. These changes might then increase the vulnerability of neuronal cells to oxidative stress and further contribute to the development of AD [148].

Evidence from animal studies also indicates that Hcy is likely to contribute to cognitive decline, but also that its levels further increase as a result of neurodegeneration [150]. The authors conclude that dietary intake or supplementation with B-vitamins might be able to break this vicious cycle. Moreover, in many conditions that are related to oxidative stress, including neurodegenerative diseases, a simultaneous elevation of Hcy and reduced level of B-vitamins, particularly folate, has been reported [151]. It has consequently been proposed that folate requirements might be increased due to irreversible oxidation and that hyperhomocysteinemia might be a consequence of the pro-oxidative environment and not just a result of inadequate intakes [151,152].

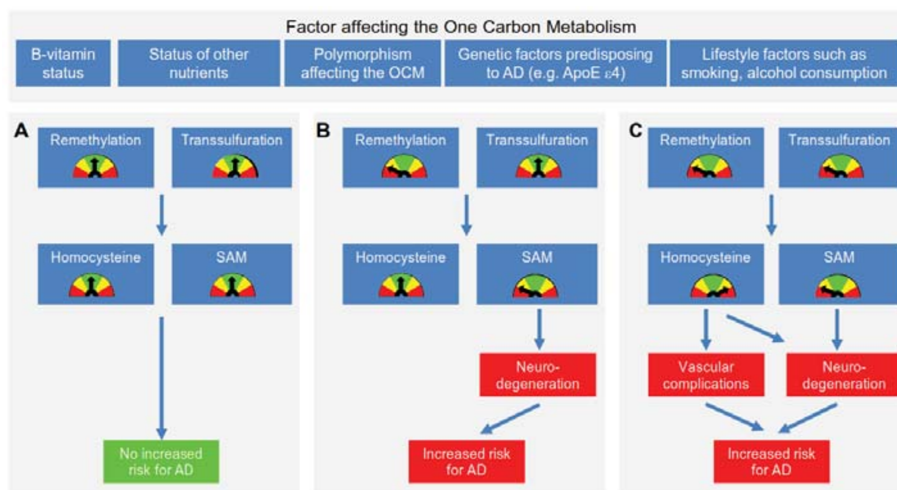

Figure 2. Proposed framework for the effect on genetic, nutritional and lifestyle factors on the development of Alzheimer's disease: (**A**) Balance between remethylation and transsulfuration results in adequate levels of homocysteine, DNA synthesis, repair and methylation as well as synthesis of neurotransmitter, phospholipids and myelin and consequently no increase in the risk of Alzheimer's disease; (**B**) Remethylation is decreased, while homocysteine is still kept in the normal range via transsulfuration, resulting in reduced DNA synthesis, repair and methylation as well as synthesis of neurotransmitter, phospholipids and myelin and consequently, an increase in the risk of Alzheimer's, but not vascular disease; (**C**) Remethylation and transsulfuration are decreased, resulting in reduced DNA synthesis, repair and methylation as well as synthesis of neurotransmitter, phospholipids and myelin and consequently, an increase in the risk of Alzheimer's, also due to compromised vascular health; AD: Alzheimer's disease; ApoE: Apolipoprotein E; OCM: One-carbon metabolism; SAM: *S*-adenosylmethionine.

4.2. Further Mechanisms Linking an Impaired OCM to the Development of AD

As the framework in Figure 2 shows, Hcy levels in the normal range do not necessarily mean that there is no disturbance of the OCM. Mechanisms such as DNA repair can be reduced due to specific polymorphisms alone or in combination with low levels of folate, riboflavin and/or vitamin B12, while Hcy is catabolized via transsulfuration. This pathway has been known to be upregulated if methionine recycling is reduced in order to keep Hcy levels low [153]. However, as the switch affects the substrates or methyl donors for essential pathways, such imbalances not only compromise DNA synthesis, repair and methylation, but also the availability of neurotransmitters, phospholipids and myelin (See below). Hcy can further be re-methylated to methionine via the betaine pathway in the liver or kidney [154], but not in the brain [155]. The balance between these pathways depends on an elaborate feedback loop, but also on the availability of nutrients such as folate, vitamin B6, B12 and methionine as well as the methyl-donors choline and betaine [154]. Interestingly, one study found that choline was a strong positive predictor of Hcy levels in Mexican American men with the MTHFR 677TT, but not the 677CC, genotype who had low folate levels [156].

It has also been shown that decreased activity in one enzyme of the OCM can trigger downregulation in the gene expression for key enzymes in alternative pathways, thereby affecting the balance, e.g., between DNA methylation and synthesis [157]. In addition, there is evidence that during folate deficiency, mechanisms are in place to preserve thymidylate and consequently DNA synthesis at the expense of Hcy remethylation [158]. Even though one has to be careful to draw causative conclusions from associations, it is conceivable that such imbalances contribute to the development of

AD as SAM levels in postmortem brains of AD patients were reduced compared to non-demented controls [159] and changed methylation patterns were found in postmortem analysis of specific brain regions of AD patients [160].

Van Driel and colleagues argue that the ratio of SAM to SAH might be a more relevant predictor of health outcomes due to impaired OCM [161] and this might also apply in the case of AD. SAM plays a crucial role as methyl-donor in other metabolic pathways such as DNA methylation or synthesis of neurotransmitters, phospholipids and myelin [44] (See Figure 1B). In the brain, SAM-dependent methylations are of particular importance [162–165] and a lack seems to favor the accumulation of amyloid precursor protein and phosphorylated tau protein, validated hallmarks of AD [133,137,166–168]. SAM is the major methyl-group donor for DNA methylation; it is involved in the regulation of enzymes necessary for these processes, such as the DNA methyltransferase, and inadequate availability of SAM is thought to play a role in the development of neurodegenerative diseases such as AD (For review see Fuso 2013 [169]). Evidence from transgenic mouse model of amyloid deposition shows that folate deficiency decreased SAM levels and DNA methyltransferase activity in the hippocampus and consequently increased activity of genes thought to be involved in the formation of amyloid plaque [170].

Polyunsaturated fatty acids, docosahexaenoic acid (DHA) in particular, play an important role in cognitive health as they are implicated in synaptic functions and signaling pathways, but also for the structure of membranes in the brain and imbalances are thought to be implicated in a range of neuropsychiatric diseases including AD (See review by Liu and colleagues [171]). A crucial step to ensure adequate supply with essential fatty acids to tissues such as the brain includes the methylation of phosphatidylethanolamine to phosphatidylcholine, which requires the phosphatidylethanolamine methyltransferase (PEMT) [172]. PEMT is thought to be regulated by SAM and SAH concentrations [173] and an impaired OCM can therefore be expected to limit the availability of essential fatty acids such as DHA to the brain. This is in line with the findings of a study that found significantly decreased DHA mobilization from the liver likely due to elevated levels of Hcy and SAH AD patients compared to healthy controls [18]. Moreover, DHA levels in the brains of AD patients were lower than in those of controls and at least in some regions of the brain (temporal and mix-frontal cortex, but not cerebellum) they were negatively correlated with the degree of cognitive decline [174].

It has been postulated that early on in the disease, oxidative stress levels increase due to mechanisms most likely unrelated to the OCM [95]. As a consequence, functional vitamin B12 deficiency can develop if the rate of oxidation surpasses its recycling [95]. This effect is likely more pronounced if the activity of MSR is reduced due to a polymorphism. Elevated Hcy levels would therefore be a consequence of changes occurring due to the AD pathophysiology, but they might then also contribute to its progression [95]. The MSR A66G was found to be correlated not only with Hcy levels, but also with markers of oxidative stress [112]. It has been suggested that the reduction in MS activity due to lack of vitamin B12 might mask the more subtle decrease in activity due to an MS polymorphism, thereby further complicating the association between the genotype and AD [175].

Dorszewska and colleagues [176] report an increase of markers for oxidative stress as well as Hcy levels in AD patients, while in elderly controls, there was an age-related, but less pronounced increase in the latter, but not the former. Moreover, even though ApoE ε4 itself does not seem to be linked to elevated Hcy levels [115], the increased level of oxidative damage thought to be linked to the ApoE ε4 genotype might be aggravated if the OCM is disturbed. Studies in mice demonstrate that folate might play an important role in countering the effect of elevated oxidative stress prevalent in brains of ApoE ε4carriers [177,178]. Markers of oxidative stress in the central nervous system of ApoE knockout mice only increased following an iron challenge if folate was deficient [178]. Folate deficiency was associated with increased Hcy and a reduced ability to counter oxidative stress as it was shown to decrease the activity of key antioxidant enzymes, namely the Cu-Zn superoxide dismutase and the glutathione peroxidase [179]. While it was long assumed that the oxidative pentose phosphate pathway was the main source of NADPH, it was recently shown that the OCM is equally important

in providing this important reducing agent [180]. An impaired OCM can therefore be expected to increase the vulnerability to oxidative stress by decreasing the cell's oxidative defense mechanisms. Wakutani and colleagues therefore propose that an impaired folate metabolism due to the MTHFR polymorphism or inadequate dietary supply might enhance the adverse effect of ApoE ε4 on the etiology of AD [181].

In addition, studies in mice that are not genetically predisposed to AD show that inadequate supply with B vitamins in itself can cause cognitive decline [146]. One potential mechanism is that impaired DNA repair due to deficiency of folate seems to increase oxidative neuronal damage induced by amyloid beta-peptide [182]. It is postulated that damage to mitochondrial DNA accumulating with age leads to increased oxidative stress, which—in the absence of efficient repair mechanisms—causes neurodegeneration (for a review see Swerdlow et al. 2014 [17]). The activity of CBS is thought to increase in response to oxidative stress [183], which might result in a further imbalance of the OCM that could potentially contribute to the development of the disease.

Age itself might further contribute to impaired OCM as there was an age-dependent decrease in THF accompanied by an increase in products of its oxidation, which are biologically inactive [184]. Hcy levels were elevated in both dementia patients and elderly controls when compared to a younger group of neurological patients without dementia [131]. Similarly, an association between age and Hcy levels as well as a negative association between the former and serum folate and vitamin B12 concentrations were found in the combined as well as stratified analysis of AD patients and controls [10]. In addition to the aging process, lifestyle factors such as alcohol consumption and smoking might also influence the interaction between B-vitamins, polymorphisms of the OCM and AD [60,91]. A vicious circle between impaired OCM and oxidative stress seems to develop in the elderly, particularly in certain genotypes prone to impaired cognitive health. It was suggest that carriers of the 677T mutation might still be able to compensate the imbalance in the folate metabolism under normal circumstances, but if vitamin B12 supply is also inadequate, an effective compensation might no longer be possible [185]. This ties in with the findings of another study that reported a weak, but significant association between the MSR 66G mutation and the risk for AD as well as with the severity of the disease, particularly in combination with the ApoEε4 genotype [123].

Many questions remain concerning the proposed mechanisms and given their key roles in a range of processes pertinent to brain health, it is likely that more than one is relevant for the etiology of AD.

5. Dietary Intake of B-Vitamins

Current intake recommendations do not take into account potentially increased needs due to reduced enzymatic activity caused by a polymorphism, as the mechanisms are not understood well enough to adapt them accordingly. Until this is possible, it is advisable to assure intakes of B-vitamins in all age groups are in line with the available recommendations to reduce the risk of the developments that eventually result in AD.

Worryingly, for part of the general population even in affluent countries, this is not the case. A relatively recent analysis of data from the National Health and Nutrition Examination Survey reported elevated Hcy levels in ~6% of the U.S. population aged ≥19 years, with levels ranging from >3% in 19–39 year-olds to ~18% in those ≥60 years old [186]. Despite mandatory folic acid fortification, around 5% and 15% of men and women, respectively, in the age range of ≥19years have folate intakes below the Estimated Average Requirement (EAR) in the United States [187]. Similar figures were given by Agarwal and colleagues [188], who also report intakes of vitamin B6 to be low for ~15%. In different European countries, intakes below EAR range from 0% to 40% for vitamin B12 and from 10% to just over 90% for folate in adults aged 19–64 years [189]. In Ireland, voluntary fortification as well as dietary supplements significantly contributed to achieving adequate folate intakes, but still, nearly 70% of women aged 18–50 years had suboptimal serum folate levels [190]. Vitamin B12 deficiency is typically seen as a problem of the elderly due to malabsorption [191–194].

However, Bailey and colleagues argue that even if the proportions of people with deficiencies in the general population are not very high, the absolute number of affected persons is still significant [186].

As is often the case in nutrition, B-vitamins can only function properly if the supply of other essential nutrients is assured: It has been shown that an intervention with B-vitamins in elderly with mild cognitive impairment only showed beneficial effects if their omega-3 fatty acid status, particularly DHA, was adequate [195]. Worryingly, it has been shown that intakes of DHA are low in many regions of the world [196]. Other nutritional inadequacies likely also play a role in the development of AD, which further highlights the importance of a diet that supplies all essential nutrients in adequate amounts through.

6. Conclusions

The evidence presented shows that persons with specific genotypes are more susceptible to imbalances in the OCM, resulting in increased levels of Hcy, insufficient DNA repair, methylation and/or synthesis as well as reduced availability of neurotransmitters, phospholipids and myelin. This can facilitate the development of AD via a range of—as of yet—poorly understood mechanisms, particularly, but not exclusively, if other risk factors such as the ApoE ε4 polymorphism predispose an individual to the disease. The reduced enzymatic activity can be compensated at least to some degree by adequate intakes of the relevant B-vitamins. Even though supplementation with folate, vitamin B6 and B12 might be able to slow the progression of dementia at an early stage [37,39,40], by the time overt clinical signs appear it might be too late to reverse the decline [197]. This emphasizes the importance of a life-long adequate intake of B-vitamins for prevention of cognitive decline and dementia.

The relationship between polymorphisms of the OCM, intakes of B-vitamins and AD can only be resolved with well-designed long-term cohort studies with detailed neuropsychological and vascular measurements. Given the long latency period between the occurrence of elevated Hcy as well as oxidative stress levels and the first symptoms of cognitive decline, studies should be initiated with healthy, middle-aged subjects. Moreover, these indicators have to be assessed at regular intervals to allow for a more in-depth understanding of the mechanisms eventually leading to AD. Until these issues are resolved, efforts should be made to ensure adequate intakes of all B-vitamins via the diet, fortified foods and possibly dietary supplements.

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Article

Folate and Vitamin B_{12}-Related Biomarkers in Relation to Brain Volumes

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Abstract: Aim: We investigated cross-sectional associations between circulating homocysteine, folate, biomarkers of vitamin B_{12} status and brain volumes. We furthermore compared brain volumes of participants who received daily folic acid and vitamin B_{12} supplementation with participants who did not. Methods: Participants of the B-PROOF study (n = 2919) were assigned to 400 µg folic acid and 500 µg vitamin B_{12}, or a placebo. After two years of intervention, T_1-weighted magnetic resonance imaging (MRI) scans were made in a random subsample (n = 218) to obtain grey and white matter volume, and total brain volume (TBV). Plasma homocysteine, serum folate, vitamin B_{12}, holotranscobalamin, and methylmalonic acid concentrations were measured. Results: Multiple linear regression analyses showed inverse associations between plasma homocysteine with TBV (β = −0.91, 95% CI −1.85–0.03; p = 0.06) and between serum folate and TBV (β = −0.20, 95% CI −0.38, −0.02; p = 0.03). No significant associations were observed for serum vitamin B_{12} and holotranscobalamin. Fully adjusted ANCOVA models showed that the group that received B-vitamins had a lower TBV (adjusted mean 1064, 95% CI 1058–1069 mL) than the non-supplemented group (1072, 95% CI 1067–1078 mL, p = 0.03). Conclusions: Results were contradictory, with higher Hcy levels associated with lower TBV, but also with higher folate levels associated with lower TBV. In addition, the lack of a baseline measurement withholds us from giving recommendations on whether folic acid and vitamin B_{12} supplementation will be beneficial above and beyond normal dietary intake for brain health.

Keywords: homocysteine; vitamin B_{12}; folate; holotranscobalamin; methylmalonic acid; brain volume; grey matter; white matter

1. Introduction

Elevated homocysteine (Hcy) levels have been associated with faster cognitive decline, cognitive impairment and dementia [1], by neurotoxicity or via other, probably vascular, pathways [2]. Remethylation of Hcy into methionine is dependent of vitamin B_{12} and folate. Low intake or low status of these vitamins can result in elevated Hcy levels, and as such, negatively affect cognitive health. A direct negative effect of low vitamin B_{12} status is also possible, as is for instance observed in people with a vitamin B_{12} deficiency, resulting in neurological problems. A low vitamin B_{12} status is characterized by low levels of serum B_{12} and holotranscobalamin (holoTC), and high levels of methylmalonic acid (MMA) and Hcy [3].

Observational studies have shown associations between vitamin B_{12} and folate with cognitive performance. Most of the intervention studies, however, did not show an effect of supplementation with vitamin B_{12} and folic acid on cognitive performance, despite a lowering effect on Hcy concentrations [4]. A possible explanation of the lack of findings after supplementation is that the classical neuropsychological paper-and-pencil tests are not sensitive enough to detect subtle changes in cognitive performance induced by supplementation. Another, relatively new, method to investigate the role of vitamin B_{12}, folate and Hcy in brain health, is by studying brain volumes. Cognitive decline has been associated with brain atrophy as measured by magnetic resonance imaging (MRI) [5]. Furthermore, associations or effects of nutrients observed in structural MRI scans may be expected to have long-term consequences on central nervous system functions [6]. Until now, only a few studies have investigated the associations of Hcy, vitamin B_{12} and folic acid with brain volumes. Cross-sectional studies have shown positive associations of Hcy levels with ventricle-brain ratios [2] and white-matter lesions as an index of the integrity of white matter [7]. Additionally, inverse associations were observed between vitamin B_{12} status and white-matter lesions [7,8]. Positive associations were observed between vitamin B_{12} status and total brain volume, cross-sectionally [2,9] and prospectively [10]. Higher folate levels have been associated with less white matter lesions, but not with more hippocampal or amygdalar volume [11].

We investigated the associations of levels of plasma Hcy, serum folate and three markers related to vitamin B_{12} status (serum B_{12}, MMA, and holoTC) with volumes of grey and white matter, and total brain volume as a derivative, measured by MRI in the B-PROOF study. We also studied the difference between participants who received a daily supplement with folic acid and vitamin B_{12} for two years and those who did not receive this supplement.

2. Materials and Methods

2.1. Study Design and Participants

This study was part of the B-PROOF (B-vitamins for the PRevention Of Osteoporotic Fractures) study that was conducted between October 2008 and April 2013 in three research centers in The Netherlands; VU medical center, Erasmus Medical Centre, and Wageningen University. Primary objective of this randomized, double-blind, placebo-controlled intervention study was to assess the efficacy of lowering homocysteine levels, by two years oral supplementation with 400 μg folic acid and 500 μg vitamin B_{12}, in the prevention of osteoporotic fractures. Both the placebo tablet and the B-vitamin tablet contained 15 μg (600 IU) vitamin D. Participants were people aged ≥65 years with mildly elevated plasma Hcy levels (12–50 μmol/L). Participant selection has been extensively described elsewhere [12,13]. MRI scans were made in a random subsample of the population of Wageningen University.

Participants who came for their two-year follow-up measurement between July 2012 and April 2013 and who had not dropped out during the intervention period were invited to participate in

the MRI study (see Figure 1 for the participant flow). MRI scans were made within one month after completion of the intervention period, and before unblinding the treatment allocation. Participants were carefully screened on contra-indications for MRI. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human participants were approved by the Wageningen University Medical Ethical Committee. All participants gave their written informed consent. This trial is registered at clinicaltrials.gov as NCT00696514 and at Netherlands Trial Register as NTR1333.

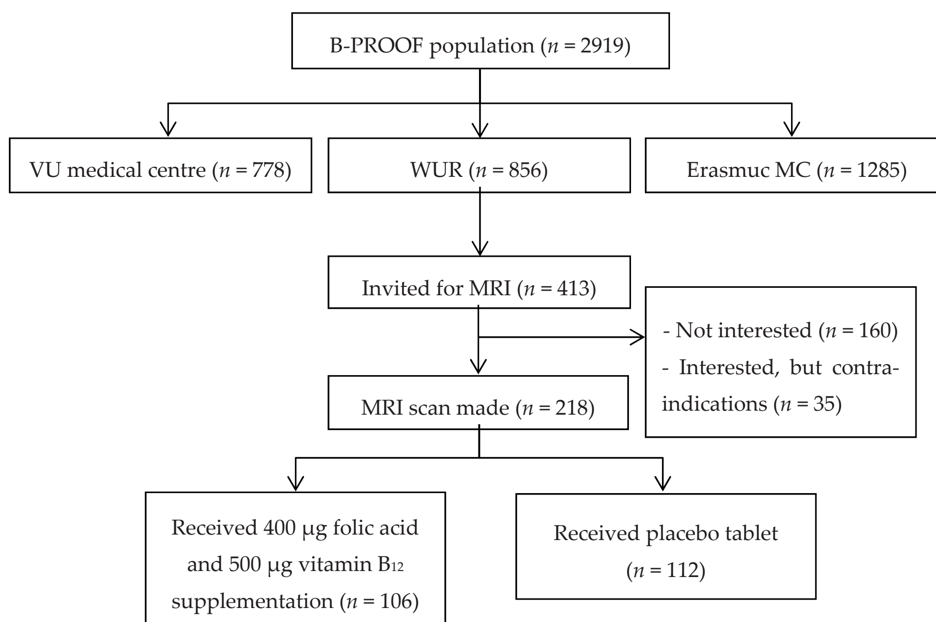

Figure 1. Flowchart of participants that were enrolled in the magnetic resonance imaging (MRI) study, a sub-study of the B-PROOF trial.

2.2. Descriptive Measures

Global cognitive performance was measured with the Mini-Mental State Examination (MMSE) [14]. Depressive symptoms were assessed with the 15-item Geriatric Depression Scale (GDS-15) [15], in which a score $\geq$5 is indicated as being likely to be depressed. Standing height was measured with a stadiometer to the nearest 0.1 cm and weight was measured to the nearest 0.5 kg with a calibrated scale (Seca, Deventer, The Netherlands). Body mass index (BMI) was calculated by dividing the weight by the squared height (kg/m^2). Information about highest educational level, smoking habits, alcohol intake (Garrett index [16]), marital status, and living situation was obtained by structured questionnaires. Physical activity was measured with the LASA Physical Activity Questionnaire (LAPAQ) [17]. Self-reported frequency and duration of activities during the past two weeks were checked by a research assistant and were used to calculate physical activity in kcal/day.

2.3. MRI Scans

Cranial volumetric MRI scans were made after two years of intervention at the Hospital Gelderse Vallei (Ede, The Netherlands) on a 3-Tesla Siemens Magnetom Verio (Siemens, Erlangen, Germany), with a 32-channel head coil. Here, we analyzed the T_1-weighted scan (MPRAGE, repetition time = 2300 ms, echo time = 3.0 ms, inversion time = 900 ms, 9° flip angle, field of view = 256 × 256 mm, 192 sagittal slices, voxel size = 1 × 1 × 1 mm, acceleration factor (GRAPPA) = 2).

The voxel-based morphometry (VBM8) toolbox within the SPM8 software (Wellcome Department of Imaging Neuroscience, London, UK) and FSL-VBM v6.0 (FMRIB Software Library, Oxford, UK) [18] were used for segmentation. T_1-weighted images were first reoriented to match the standard template images in FSL-VBM. VBM8 spatially normalizes participants' brain images to a standard space, and then automated segments into grey matter, white matter, and cerebrospinal fluid, using a unified tissue segmentation approach [19]. These three measures were summed to calculate intracranial volume. Grey and white matter volumes were summed to calculate total brain volume.

2.4. Blood Measurements

Blood samples were obtained in the morning, when participants were fasted or had consumed a restricted breakfast. For the current study, follow-up levels of serum folate and vitamin B_{12} biomarkers were used. Plasma Hcy was measured using the HPLC method (intra assay CV = 3.1%, inter assay CV = 5.9%). Serum vitamin B_{12} and serum folate were analyzed by using electrochemiluminescence immunoassay (Elecsys, 2010, Roche GmbH, Mannheim, Germany) (CV vitamin B_{12} 5.1% at 125 pmol/L and 2.9% at 753 pmol/L; CV folate: 5.9% at 5.7 nmol/L and 2.8% at 23.4 nmol/L). Serum holoTC was determined by the AxSYM analyser (Abbott Diagnostics, Hoofddorp, The Netherlands) (intra assay CV < 8%) and serum MMA was measured by LC-MS/MS (intra assay CV = 8.1% at 0.18 μmol/L, inter assay CV = 1.6% at 0.24 μmol/L). DNA was isolated from buffy coats to determine the genotype for methylenetetrahydrofolate reductase (MTHFR 677TT) using the Illumina Omni-express array and to determine Apolipoprotein E (ApoE) genotype by using Taqman. All analyses were done in the biochemical laboratory of Erasmus MC, except Hcy, which was measured at Wageningen University.

2.5. Statistics

Population characteristics are reported as *n* (%), means ± SD, or as median (interquartile range; IQR) for non-normally distributed data. Comparisons between treatment groups were made using Chi-square test, independent-samples *t*-test or Mann–Whitney non-parametric test.

Linear regression analyses were used to examine the associations between serum folate, different markers of vitamin B_{12} status (serum B_{12}, holoTC, MMA), and plasma Hcy, with volumes of grey and white matter and total brain volume. The crude model was adjusted for intracranial volume, age and sex, and model 1 included the covariates of the first model plus BMI, smoking, alcohol, education, and physical activity.

Differences between treatment groups were analyzed using analyses of covariance (ANCOVA), in which the brain volumes acted as dependent factor, treatment as fixed between-subject factor, and intracranial volume, age and sex as covariates (crude model). Model 1 included the covariates from first model plus BMI, smoking, alcohol, education, and physical activity.

Results are presented as β-coefficients with 95% confidence interval (95% CI) for the regression analyses, and as adjusted means with 95% CI for the ANCOVA analyses. Alpha was set at 0.05 and two-tailed analyses were performed. All statistical analyses were performed using SPSS Statistics v24 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Participants

The general characteristics of the total subgroup at time of the MRI measurement are presented in Table 1. The group that received two years of B-vitamin supplementation (*n* = 106) and the group that did not receive B-vitamins (*n* = 112) included both 57% men. Participants in the B-vitamin group were significantly younger and had a slightly higher MMSE score (age: 72.7 ± 5.3 year; median MMSE 29, IQR 27–30, range 19–30) than the non-supplemented group (age: 74.5 ± 6.3 year, $p = 0.02$; median MMSE 28, IQR 27–29, range 24–30, $p = 0.09$). Concentrations of folate, vitamin B_{12} and holoTC were higher in the group that received B-vitamin supplementation than in the group that did not, whereas MMA and Hcy were lower (all *p*-values < 0.001), as a result of the two-year supplementation.

Table 1. Population characteristics at time of MRI measurements in participants who received two years of B-vitamin supplementation and participants who did not receive this supplementation.

Variable	Total Population (*n* = 218)	With B-Vitamin Supplementation (*n* = 106)	Without B-Vitamin Supplementation (*n* = 112)	*p*-Value
Age	73.6 ± 5.9	72.7 ± 5.3	74.5 ± 6.3	0.02
Sex, men, *n* (%)	124 (57%)	60 (57%)	64 (57%)	0.94
Body mass index, kg/m^2	27.7 ± 4.2	27.3 ± 4.4	28.1 ± 4.1	0.21
Blood pressure, systolic, mmHg	147 ± 18	146 ± 19	148 ± 17	0.42
Blood pressure, diastolic, mmHg	78 ± 10	78 ± 10	78 ± 10	0.76
Education, *n* (%)				0.12
Low	90 (41%)	49 (46%)	41 (37%)	
Medium	52 (24%)	19 (18%)	33 (30%)	
High	76 (35%)	38 (36%)	39 (34%)	
Smoking, *n* (%)				0.67
Never	64 (29%)	27 (26%)	37 (33%)	
Former	139 (64%)	70 (66%)	69 (62%)	
Current	15 (7%)	9 (9%)	6 (5%)	
Alcohol, *n* (%)				0.72
Light	152 (70%)	73 (69%)	79 (71%)	
Moderate	60 (28%)	31 (29%)	29 (26%)	
Excessive	6 (3%)	2 (2%)	4 (3%)	
Self-initiated supplement use, vitamin B_{12}, *n* (%)	27 (8%)	11 (7%)	16 (10%)	0.43
Self-initiated supplement use, folic acid, *n* (%)	30 (12%)	12 (9%)	18 (15%)	0.37
MMSE, max 30 points	28 [27–29]	29 [27–30]	28 [27–29]	0.09
Low MMSE < 25, *n* (%)	6 (3%)	4 (4%)	2 (2%)	0.37
GDS, max 15 points	1 (0–2)	1 (0–2)	1 (0–2)	0.89
Physical activity (kcal/day)	664 ± 416	642 ± 288	683 ± 440	0.47
ApoE-ε4, carrier, *n* (%)	60 (28%)	32 (30%)	28 (25%)	0.45
MTHFR, 677TT, *n* (%)	28 (13%)	17 (15%)	11 (10%)	0.40
Grey matter (mL)	574 ± 56	577 ± 57	572 ± 55	0.51
Grey matter/ICV	0.42 ± 0.02	0.42 ± 0.02	0.42 ± 0.02	0.98
White matter (mL)	493 ± 61	495 ± 63	492 ± 60	0.73
White matter/ICV	0.36 ± 0.02	0.36 ± 0.02	0.36 ± 0.02	0.74
Cerebrospinal fluid (mL)	304 ± 52	306 ± 51	302 ± 53	0.62
Cerebrospinal fluid/ICV	0.22 ± 0.03	0.22 ± 0.03	0.22 ± 0.03	0.81
Serum folate (nmol/L)	36.7 [23.4–54.2]	53.1 [42.5–68.3]	24.1 [19.5–31.8]	<0.001
Serum vitamine B_{12} (pmol/L)	404 [262–558]	558 [459–715]	274 [222–373]	<0.001
Vitamin B_{12} < 258 pmol/L, *n* (%)	49 (23%)	1 (1%)	48 (43%)	<0.001
Serum holotranscobalamin (pmol/L)	81 [56–111]	111 [89–147] [a]	63 [44–80]	<0.001
Holotranscobalamin < 30 pmol/L, *n* (%)	12 (6%)	1 (1%)	11 (10%)	0.01
Serum methylmalonic acid (µmol/L)	0.20 [0.16–0.25]	0.17 [0.15–0.21]	0.24 [0.19–0.30]	<0.001
Methylmalonic acid > 0.30 µmol/L, *n* (%)	35 (16%)	5 (5%)	30 (27%)	<0.001
Plasma homocysteine (µmol/L)	11.4 [8.9–14.4]	9.1 [7.8–10.6] [b]	13.9 [12.0–16.3] [c]	<0.001

Values are reported as mean ± SD, median [interquartile range], or as *n* (%); MMSE, Mini-Mental State Examination; GDS, Geriatric Depression Scale; ApoE-ε4, Apolipoprotein E; MTHFR, methylenetetrahydrofolate reductase; ICV, intracranial volume; [a] *n* = 96; [b] *n* = 103; [c] *n* = 109.

3.2. B-Vitamin Status and Brain MRI Volumes

Fully adjusted linear regression models of biomarkers and brain volumes in the total population (Table 2) showed an inverse association for serum folate with total brain volume (β = −0.20, 95% CI −0.34–−0.02; p = 0.03), and a borderline significant association with white matter volume (β = −0.17, 95% CI −0.34–0.01). Furthermore, plasma Hcy was borderline significantly inversely associated with total brain volume (β = −0.91, 95% CI −1.85–0.03). No significant associations were observed for serum B_{12}, holoTC, or MMA with any of the brain volumes in the fully adjusted models.

Associations differed between the group that received B-vitamin supplementation and the group that did not (Table 2), more specifically, interaction terms with treatment were significant or tended towards significance for MMA and grey matter (p = 0.04), MMA and total brain volume (p = 0.10), and Hcy and total brain volume (p = 0.10). In both groups, serum MMA was inversely associated with total brain volume, but only in the group that received B-vitamin supplementation the association remained significant after adjustment for covariates (B-vitamin group: β = −127.1, 95% CI −232.2–−21.9; p = 0.02; group without B-vitamins: β = −27.1, 95% CI −57.7, 3.5). Only in the B-vitamin group, MMA was also associated with grey matter volume (β = −136.2, 95% CI −241.6–−30.8; p = 0.01). Inverse associations were observed for Hcy and total brain volume in both groups, with stronger associations in the supplemented group (β = −4.9, 95% CI −6.73–−1.44; $p < 0.01$) than in the non-supplemented group (β = −1.88, 95% CI−3.21–−0.55; p = 0.01). In the B-vitamin group, a trend was observed for Hcy and grey matter volume (β = −2.53, 95% CI −5.24–0.19), but not for white matter volume. In the non-supplemented group, a trend was observed for Hcy and white matter volume (β = −1.30, 95% CI −2.50–0.10), but not for grey matter volume.

3.3. Differences between Supplementation Groups

Table 3 presents the differences in brain volumes between the group that received B-vitamin supplementation and the group that did not. Unadjusted analyses did not show a difference between groups (data not shown). The fully adjusted model, however, revealed a lower total brain volume in the B-vitamin group (1063.6, 95% CI 1058.2–1069.0 mL) compared to the non-supplemented group (1072.3, 95% CI 1067.2–1077.5 mL; p = 0.03). This was also reflected by a non-significant (p = 0.07) lower volume of white matter in the B-vitamin group (490.2, 95% CI 485.2–495.3 mL) compared to the non-supplemented group (496.7, 95% CI 491.7–501.6 mL). Grey matter volume did not differ between groups.

Table 2. Associations between follow-up blood values and brain MRI measures in the total population (n = 218) and stratified for the group with (n = 106) and without a history of B-vitamin supplementation (n = 112) (β-coefficients (95% CI confidence interval).

Variable	Total Population (n = 218)		Without B-Vitamin Supplementation (n = 112)		With B-Vitamin Supplementation (n = 106)	
	Crude	Model 1	Crude	Model 1 [f]	Crude	Model 1 [i]
Serum folate, nmol/L						
Grey matter	−0.05 (−0.22, 0.13)	−0.03 (−0.21, 0.15) [a]	0.29 (−0.18, 0.75)	0.19 (−0.28, 0.65)	−0.07 (−0.38, 0.24)	−0.03 (−0.36, 0.31)
White matter	−0.14 (−0.31, 0.02)	−0.17 (−0.34, 0.01) [a]	−0.08 (−0.50, 0.34)	−0.06 (−0.49, 0.37)	−0.14 (−0.43, 0.16)	−0.24 (−0.56, 0.09)
Total brain volume	−0.19 (−0.37, −0.01)	−0.20 (−0.38, −0.02) [a],*	0.20 (−0.27, 0.68)	0.11 (−0.46, 0.68)	−0.20 (−0.51, 0.11)	−0.24 (−0.57, 0.09)
Serum vitamin B_{12}, pmol/L						
Grey matter	−0.01 (−0.02, 0.01)	−0.01 (−0.02, 0.01)	0.02 (−0.03, 0.06)	0.01 (−0.04, 0.06)	−0.01 (−0.04, 0.02)	−0.01 (−0.04, 0.02)
White matter	0.00 (0.01, 0.02)	−0.00 (−0.02 0.02)	0.02 (−0.02, 0.07)	0.02 (−0.02, 0.07)	0.02 (−0.01, 0.04)	0.02 (−0.01, 0.04)
Total brain volume	−0.01 (−0.02, 0.01)	−0.01 (−0.03, 0.01)	0.04 (−0.01, 0.09)	0.03 (−0.02, 0.08)	0.01 (−0.02, 0.04)	0.00 (−0.02, 0.03)
Serum holotranscobalamin, pmol/L						
Grey matter	−0.00 (−0.09, 0.09) [b]	0.01 (−0.08, 0.09) [c]	0.00 (−0.21, 0.21)	−0.04 (−0.25, 0.18)	0.05 (−0.09, 0.18) [j]	0.06 (−0.08, 0.20) [k]
White matter	0.02 (−0.06, 0.10) [b]	0.01 (−0.07, 0.09) [c]	0.15 (−0.04, 0.34)	0.16 (−0.04, 0.35)	0.06 (−0.07, 0.19) [j]	0.05 (−0.09, 0.18) [k]
Total brain volume	0.02 (−0.07, 0.11) [b]	0.02 (−0.07, 0.10) [c]	0.16 (−0.06, 0.37)	0.12 (−0.10, 0.34)	0.10 (−0.03, 0.24) [j]	0.11 (−0.03, 0..24) [k]
Serum methylmalonic acid, µmol/L						
Grey matter	−13.2 (−39.3, 12.9)	−14.5 (−40.6, 11.5) [a]	−8.9 (−38.7, 20.8)	−10.5 (−40.5, 19.5)	−136.6 (−237.2, −36.0) *	−136.2 (−241.6, −30.8) *
White matter	−7.7 (−32.2, 16.8)	−7.2 (−32.3, 17.9) [a]	−19.9 (−46.8, 6.0)	−16.7 (−44.3, 10.9)	29.9 (−70.1, 130.2)	9.4 (−98.0, 116.9)
Total brain volume	−20.9 (−47.4, 5.6)	−21.6 (−48.1, 4.8) [a]	−28.9 (−58.7, 0.90)	−27.1 (−57.7, 3.5)	−106.7 (−208.8, −4.5) *	−127.1 (−232.2, −21.9) *
Plasma homocysteine, µmol/L						
Grey matter	−0.33 (−1.26, 0.60) [d]	−0.53 (−1.47, 0.41) [e]	−0.67 (−1.98, 0.64) [g]	−0.78 (−2.09, 0.53) [h]	−1.88 (−4.47, 0.70) [i]	−2.53 (−5.24, 0.19) [L]
White matter	−0.36 (−1.22, 0.51) [d]	−0.43 (−1.31, 0.50) [e]	−1.08 (−2.23, 0.07) [g]	−1.30 (−2.50, −0.10) [h],*	−2.10 (−4.58, 0.39) [i]	−1.19 (−3.87, 1.49) [L]
Total brain volume	−0.69(−1.62, 0.25) [d]	−0.91 (−1.85, 0.03) [e]	−1.75 (−3.02, −0.48) [g],*	−1.88 (−3.21, −0.55) [h],**	−3.98 (−6.47, −1.49) [i],**	−4.09 (−6.73, −1.44) [L],**

Crude: Adjusted for intracranial volume, age, sex; Model 1: Adjusted for model 1 and BMI, alcohol, smoking, education, physical activity; [a] n = 214; [b] n = 208; [c] n = 205; [d] n = 212; [e] n = 208; [f] n = 111; [g] n = 109; [h] n = 108; [i] n = 103; [j] n = 96; [k] n = 94; [L] n = 100. * $p < 0.05$; ** $p < 0.01$.

Table 3. Differences in brain volumes (mL) between participants who received two-year B-vitamin supplementation (n = 106) and participants who did not receive this supplementation (n = 112) (adjusted means (95% confidence interval).

	Crude Model			Model 1 [a]		
	Without B-Vitamin Supplementation	**With B-Vitamin Supplementation**	***p*-Value**	**Without B-Vitamin Supplementation**	**With B-Vitamin Supplementation**	***p*-Value**
Grey matter	575.7 (570.6, 580.8)	572.5 (567.3, 577.8)	0.40	575.6 (570.4, 580.8)	573.4 (568.0, 578.7)	0.57
White matter	495.6 (490.8, 500,4)	490.3 (485.4, 495.2)	0.13	496.7 (491.7, 501.6)	490.2 (485.2, 495.3)	0.07
Total brain volume	1071.3 (1066.1, 1076.5)	1062.8 (1057.5, 1068.1)	0.03	1072.4 (1067.2, 1077.5)	1063.6 (1058.2, 1069.0)	0.03

Differences between groups were tested with ANCOVA, adjusted for intracranial volume, age, sex (crude model), BMI, alcohol, smoking, education, and physical activity (model 1);
[a] n = 111 for the group without B-vitamin supplementation, n = 103 for the group with B-vitamin supplementation.

4. Discussion

Our cross-sectional analyses showed that higher levels of Hcy and MMA were associated with lower total brain volumes, with stronger associations in the B-vitamin group than in the non-supplemented group. No associations were observed for serum vitamin B_{12} or holoTC with brain volumetric measures. Higher levels of folate, however, were also associated with lower brain volume. Furthermore, directly comparing the supplementation groups with respect to brain volumes does not point towards a beneficial effect of B-vitamin treatment; after adjustment for important covariates the group that received the B-vitamin supplementation had a lower brain volume compared to the placebo group.

Our data add to two other findings on B-vitamins and brain health within the B-PROOF study. Cross-sectionally, we observed baseline associations between MMA, Hcy and folate, indicating a better vitamin B_{12} and folate status, with episodic memory and information processing speed [20]. Two-year supplementation with folic acid and vitamin B_{12}, however, did not show beneficial effects on specific cognitive function domains [21]. Most other randomized controlled trials (RCTs) investigating the effects of B-vitamin supplementation with at least one-year follow-up also failed to show a beneficial effect on cognitive performance [22,23]. Potentially, study populations were not sensitive enough to induce an effect, study durations were too short to significantly slow down the development of cognitive decline, or the effects were too subtle to be detected by the neuropsychological testing. Neuropsychological tests may be susceptible to practice effects or short-term fluctuations in test performance [24], while structural MRI may be less susceptible to these fluctuations. Brain volume can act as a predictor for brain health [24–26]; rate of brain volume loss is correlated with performance on cognitive tasks and it may predict the conversion of mild cognitive impairment (MCI) into dementia [5,26,27]. MRI measures can thus be of added value to neuropsychological tests. For instance, within the B-PROOF study, significant positive associations were observed for vitamin D status, glucose levels and grey matter volume [28]. This added to earlier seen significant associations of vitamin D status and cognitive functioning [29].

We observed a negative association between folate and brain volume in the total population. After stratification, the cross-sectional association was not present in the group that did not receive B-vitamin supplementation, whereas a trend was still seen in the supplemented group. The non-supplemented group may reflect a sample with blood levels closer to normal instead of being intervened by folic acid supplementation, and thus it might be that these associations come closer to normal values. Folic acid, the synthetic form used in supplements, has a higher bioavailability than folate naturally present in food. The conversion of folic acid to metabolized folate is low because of low activity of the enzyme dihydrofolate reductase, and therefore high intake of synthetic folic acid may cause an accumulation of unmetabolized folic acid. Studies have suggested that high folate levels, in combination with and without vitamin B_{12} deficiency can be detrimental for cognitive health [30–32]. Especially older adults who took folic acid supplements containing >400 μg folic acid supplements, had a higher risk for cognitive decline [32]. Hence, it is possible that folate levels follow an inverted U-shape regarding optimal cognitive performance and brain health; cross-sectional studies and intervention studies, however, are inconclusive in their findings [11,33,34]. Another point to address is that serum folate, as used in the present study, reflects more recent intake, while red blood cell folate concentrations seem to be a more robust biomarker of folate status on the longer term [35]. It would be worth examining the association between this biomarker and brain volumes. Unfortunately, we do not have data on unmetabolized folic acid or on red blood cell folate concentrations.

Our cross-sectional findings regarding Hcy and vitamin B_{12} status and brain volumes are similar to other studies. Studies that investigated Hcy concentrations and brain atrophy showed clear associations, with higher levels associated with more total atrophy [2,36,37] and lower hippocampal volume [38]. In line with our findings, associations of MMA and Hcy with total brain volume measured 4.6 years later have been shown, but not with serum vitamin B_{12} [9]. A prospective study (n = 107, mean age 73 year), however, showed that lower serum concentrations of vitamin B_{12} and holoTC, but not folate, Hcy or MMA, were associated with smaller brain volumes after five years of

follow-up [10]. The only RCT currently published was performed in 168 patients with MCI, showing that B-vitamin (B_{12}, B_6 and folic acid) supplementation slowed down total brain atrophy and grey matter atrophy, but only in those with the highest Hcy levels [39]. We observed a borderline significant association between Hcy and grey matter in our healthier study population, but only in the group that received B-vitamin supplementation. Interestingly, the observed associations were thus stronger in those with lower Hcy levels as a result of the B-vitamin treatment. It might be that the stronger associations between vitamin B_{12} status, as reflected by Hcy and MMA, and brain volumes in the B-vitamin group are the results of the intervention, but because baseline MRI data are lacking, no conclusions can be drawn about the causality of the relation between the intervention and the MRI measures.

In contrast to our hypothesis, we observed that participants who received B-vitamin supplementation had lower brain volumes than the non-supplemented group. Participants who received B-vitamins were significantly younger than participants who did not receive the supplements. Age is a major predictor for brain atrophy [40] and it is possible that our findings may be the result of this two-year age difference between the two groups. This hypothesis is supported by the fact that the unadjusted model did not show differences on brain volumes between treatment groups, whereas a difference was expected due to the age difference (data not shown). When adjustments for age were made, this resulted in a significant difference in brain volume between the two groups.

The observed cross-sectional associations of higher Hcy concentrations and lower brain volume may be explained by several mechanisms. Hcy may be neurotoxic, which can induce brain atrophy and hamper neurogenesis [2,36]. Furthermore, elevated Hcy levels may lead to an increase of phosphorylated tau, which is present in neurofibrillary tangles and is associated with atrophy in specific brain regions [41,42]. Vascular pathways have also been suggested, based on negative associations between Hcy and vascular health and white matter hyperintensities [8,43]. An elevated Hcy level as indicator of low vitamin B_{12} status may have influence on myelin and consequently on the integrity of white matter, as vitamin B_{12} deficient patients show areas of demyelination on brain MRI [44,45].

To put our findings into perspective, some methodological issues need to be discussed. Limitations include the lack of baseline data on brain volumes, which makes it impossible to draw conclusions on the effects of two years B-vitamin supplementation on grey and white matter volume. Furthermore, one of the inclusion criteria of the B-PROOF study was an elevated Hcy level, which makes the generalizability to the total elderly population difficult. Last, we performed multiple statistical tests, which increased the risk on chance findings. Strengths of our study are the use of a 3T scanner to make high-precision images, our large study population for conducting MRI scans, the possibility to adjust for multiple confounders, and the available data of folate, Hcy and three markers of vitamin B_{12} status. Furthermore, previous data analyses in the total B-PROOF population showed that supplement use was very low at baseline. Vitamin B_{12} derived from diet was the main source that contributed to baseline serum vitamin B_{12} levels [46]. At follow-up, i.e., at the time MRI scans were performed, we also did not observe differences in self-initiated supplement use between treatment groups in the subpopulation of the current paper. Questionnaires to assess supplement usage, however, may not be reliable for a long-term micronutrient intakes in older adults [47].

5. Conclusions

To conclude, the results suggest that lower levels of Hcy and the vitamin B_{12}-metabolite MMA may be important in order to attenuate brain atrophy in healthy elderly people. However, the negative associations of folate and the lack of a baseline measurement withhold us from giving recommendations on whether folic acid and vitamin B_{12} supplementation will be beneficial above and beyond normal dietary intake for brain health. Furthermore, the age difference between the groups may have distorted the results. More research, especially well-designed randomized controlled trials with a sufficient follow-up time, is required to unravel the role of folate levels, in combination with and without low vitamin B_{12} levels, in brain health.

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Methionine and Choline Supply during the Periparturient Period Alter Plasma Amino Acid and One-Carbon Metabolism Profiles to Various Extents: Potential Role in Hepatic Metabolism and Antioxidant Status

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Abstract: The objective of this study was to profile plasma amino acids (AA) and derivatives of their metabolism during the periparturient period in response to supplemental rumen-protected methionine (MET) or rumen-protected choline (CHOL). Forty cows were fed from −21 through 30 days around parturition in a 2 × 2 factorial design a diet containing MET or CHOL. MET supply led to greater circulating methionine and proportion of methionine in the essential AA pool, total AA, and total sulfur-containing compounds. Lysine in total AA also was greater in these cows, indicating a better overall AA profile. Sulfur-containing compounds (cystathionine, cystine, homocystine, and taurine) were greater in MET-fed cows, indicating an enriched sulfur-containing compound pool due to enhanced transsulfuration activity. Circulating essential AA and total AA concentrations were greater in cows supplied MET due to greater lysine, arginine, tryptophan, threonine, proline, asparagine, alanine, and citrulline. In contrast, CHOL supply had no effect on essential AA or total AA, and only tryptophan and cystine were greater. Plasma 3-methylhistidine concentration was lower in response to CHOL supply, suggesting less tissue protein mobilization in these cows. Overall, the data revealed that enhanced periparturient supply of MET has positive effects on plasma AA profiles and overall antioxidant status.

Keywords: amino acids; lactation; methyl donors; periparturient dairy cow

1. Introduction

Around parturition, the increased demand for nutrients to sustain fetal growth and lactation coupled with depressed dry matter intake (DMI) impose tremendous metabolic stress on dairy cows. Consequently, health problems and compromised production performance likely occur not only due to negative energy balance-induced increases in circulating free fatty acids, but a negative amino acid (AA) balance [1–3]. For instance, increased mobilization of tissue protein is often observed due to inadequate availability of AA substrates for gluconeogenesis as well as synthesis of protein in liver and mammary gland [4]. In fact, a moderate net loss of carcass protein was observed even in animals fed to predicted metabolizable protein requirements around parturition [1,5], indicating suboptimal profiles of AA may be the limiting factor for their utilization during this period. In line with this assumption, supplementing rumen-protected limiting AA has achieved various benefits in terms of lactation performance and health status of periparturient dairy cows [6–9].

Methionine (Met) is an essential sulfur-containing AA associated with various key physiologic events. Previous research has underscored the importance of Met as a limiting AA for milk protein synthesis in many diets [10,11]. Apart from its apparent key role in mammary gland and liver protein synthesis, Met also serves as substrate for sulfur-containing antioxidants, namely glutathione (GSH) and taurine [12]. In addition, as a key component of one-carbon metabolism, hundreds of methylation reactions acquire methyl groups from Met via *S*-adenosyl methionine (SAM) [13]. Furthermore, as a gluconeogenic AA, a portion of Met may be taken up by liver to sustain the abrupt increase in demand for glucose at the onset of lactation. In line with the various biologic processes relying on Met, its deficiency has often been reported in cows around parturition [3]. In fact, circulating Met concentration decreased markedly through parturition and were not restored to prepartum levels until 28 days postpartum [3].

An unfavorable circulating AA profile around parturition likely occurs due to (1) increased production of positive acute-phase proteins (APP) and immune-related proteins induced by oxidative stress and inflammation; (2) enhanced carcass protein mobilization to provide AA for gluconeogenesis; and/or (3) limited uptake of other AA and increased N excretion due to lack of Met. Recent research with periparturient dairy cows has demonstrated benefits to overall health and production performance in response to MET supplementation [7,9,14]. However, knowledge about how plasma AA and downstream products of their metabolism respond to periparturient MET supplementation is lacking.

Although choline (CHOL) is not an AA, it may regulate AA metabolism by altering AA requirements, especially Met, around parturition. For instance, Met can be regenerated when homocysteine receives a methyl group from CHOL through betaine [15–17], suggesting that CHOL supplementation can potentially reduce Met requirements around parturition. In addition, accumulation of fat in liver has been speculated to induce inflammation and oxidative stress in ruminant liver, which almost certainly would lead to increased AA requirements for production of positive APP and other immune function-related proteins [17]. As a precursor for hepatic very low-density lipoprotein (VLDL) assembly, CHOL has a crucial role in the export of triacylglycerol to prevent fatty liver by promoting phosphatidylcholine synthesis via the Kennedy pathway instead of sequential methylation using Met derived SAM, which may also spare a portion of Met around parturition.

Few studies have attempted to characterize the profile of circulating AA and their derivatives around parturition in dairy cows [3,18–20]. To our knowledge, this is the first study profiling AA and their derivatives in response to MET or CHOL supplementation around parturition. Considering that periparturient MET supplementation resulted in greater feed intake, increased milk yield, and better overall cow health while CHOL cows did not achieve similar benefits [9] (although others have reported increased milk production with CHOL [21,22]), our hypothesis was that MET and CHOL supplementation results in different alterations in AA metabolism-associated events which ultimately contribute to their distinct roles in the overall health and production efficiency of the animal.

2. Materials and Methods

2.1. Experimental Design and Treatments

All procedures for this study (protocol no. 13023) were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Illinois. Details of the experimental design have been described previously [9,23]. Briefly, the experiment was conducted as a randomized, complete, unbalanced, block design with 2 × 2 factorial arrangement of MET (Smartamine M, Adisseo NA, Alpharetta, GA, USA) and CHOL (ReaShure, Balchem Inc., New Hampton, NY, USA) level (with or without). Cows within each block were balanced for parity, previous lactation milk yield, and BCS before the close-up diet groups were assigned. A total of 81 cows were used. Treatments were control (CON, n = 20), with no MET or CHOL supplementation; Smartamine (SMA, n = 21), CON plus MET at a rate of 0.08% of DM; Reashure (REA, n = 20), CON + CHOL at 60 g/days; or Smartamine

and Reashure (MIX, *n* = 20), CON + MET + CHOL. Dosage of MET was based on Osorio et al. [6], whereas CHOL was supplemented following the manufacturer's recommendations. Met as a % of metabolizable protein (MP) and the Lys:Met ratio for close-up diets were estimated to be 1.9% and 3.6:1 Lys:Met for CON, 2.4% and 2.8:1 Lys:Met for SMA, 1.9% and 3.6:1 Lys:Met for REA, and 2.4% and 2.8:1 Lys:Met for MIX. Met as a % of MP and the Lys:Met ratio for lactation diets were estimated to be 1.8% and 3.5:1 Lys:Met for CON, 2.3% and 2.7:1 Lys:Met for SMA, 1.8% and 3.5:1 Lys:Met for REA, and 2.3% and 2.7:1 Lys:Met for MIX. Per IACUC conclusions, a subset of 40 multiparous cows (10 cows/treatment) was deemed sufficient to achieve statistical power. Thus, these cows were selected randomly and used for this portion of the study. All cows received the same far-off diet from −50 to −22 days before expected parturition, the close-up diet from −21 days to expected parturition, and the lactation diet from parturition through 30 days in milk (DIM). The MET and CHOL supplements were both top-dressed from −21 ± 2 to 30 DIM once daily at the AM feeding using approximately 50 g of ground corn as carrier for all treatments. On average, cows received MET and/or CHOL supplementation for 23.1 ± 1.0 days prepartum. Supplementation of SMA (0.08% DM of TMR offered) was calculated daily for each cow. Smartamine M was supplied as small beads containing a minimum of 75% DL-Methionine (DL-Met), physically protected by a pH sensitive coating, which is considered to have a Met bioavailability of 80% [24]; therefore, per 10 g of SMA, the cows received 6 g of metabolizable Met. The REA supplement is reported to contain 28.8% choline chloride and is protected by microencapsulation. In terms of bioavailability, work from a graduate thesis using (methyl,^{2}H3)-CHOL and (methyl,^{2}H3)-Met indicated, based on differences in Met methyl flux rates, that the product has CHOL bioavailability of 72% [25]; therefore, per 60 g of REA, cows in our study would have received 12.4 g of metabolizable choline chloride. In contrast, a recent in vivo study evaluating bioavailability of REA detected low portal flux of free choline (13%) relative to abomasal delivery of choline [26], thus, indicating that only 2.3 g of free choline would have been available post-ruminally. Although assessing bioavailability of SMA and REA was beyond the scope of our study, it is important to note that the approaches for estimating "bioavailability" of CHOL in these studies was different. For instance, the work of de Veth et al. [26] did not consider the portion of CHOL partly oxidized to betaine, partly phosphorylated, and partly incorporated into lyso- and phosphatidylcholine within the enterocyte and prior to entering portal circulation. Thus, despite the low concentration of free choline any betaine generated during metabolism within enterocytes could have contributed methyl groups for remethylation of homocysteine to Met in the liver. Clearly, further studies would have to be conducted to define better CHOL needs of periparturient cows and the efficacy of available CHOL products in delivering metabolizable CHOL and its derivatives. To our knowledge, neither SMA nor REA have specific characteristics that may affect palatability of diets.

2.2. Animal Management

Dry cows were housed in a ventilated enclosed barn during the dry period and fed individually once daily at 06:30 am using an individual gate system (American Calan Inc., Northwood, NH, USA). After parturition, cows were housed in a tie-stall barn and were fed a common lactation diet once daily. Feed offered was adjusted daily to achieve ~10% refusals.

2.3. Blood Sample Collection and Analyses of Plasma AA and Their Derivatives

Blood was sampled from the coccygeal vein at −30 and −10 days relative to expected parturition date and at 4, 14 and 28 days relative to actual parturition date before the AM feeding. On average, prepartum samples were harvested at −30.8 ± 1.4 days and −10.9 ± 1.3 days relative to actual calving date. Samples were collected into evacuated tubes (BD Vacutainer; BD and Co., Franklin Lakes, NJ, USA) containing lithium heparin for isolation of plasma.

Plasma was used to analyze the concentrations of free arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), valine (Val), asparagine (Asn), aspartate (Asp), alanine (Ala), glutamate (Glu),

glutamine (Gln), glycine (Gly), proline (Pro), serine (Ser), tyrosine (Tyr), citrulline (Cit), carnosine, ornithine (Orn), sarcosine (Sar), cystathionine, cystine, homocystine, taurine, α-aminoadipic acid, α-aminobutyric acid, β-alanine, γ-aminobutyric acid (GABA), hydroxylysine, hydroxyproline, phosphoserine, 1-methyl histidine, and 3-methyl histidine at the University of Missouri Agriculture Experiment Station Chemical Laboratories (Columbia, MO, USA) using high performance liquid chromatography [27,28]. Plasma total GSH was measured using a commercial kit (Cat. No. NWH-GSH01; Northwest Life Science Specialties LLC, Vancouver, WA, USA). The essential amino acid (EAA) pool included Arg + His + Ile + Leu + Lys + Met + Phe + Thr + Trp + Val; the NEAA pool included Asn + Asp + Ala + Gln + Glu + Gly + Pro + Ser + Tyr; total AA was the sum of EAA and non-essential amino acid (NEAA); the total sulfur-containing compounds (TSC) included Met + cystine + cystathionine + homocysteine + taurine + GSH.

2.4. Liver Sample Collection and Quantitative RT-PCR (qPCR)

Liver was sampled via puncture biopsy [29] from cows under local anesthesia at approximately 08:00 am on days −10, 7, 20, and 30 days relative to parturition. Liver was frozen immediately in liquid nitrogen and stored until analysis. The qPCR was performed in liver samples as described previously [7].

2.5. Statistical Analysis

Data were analyzed using PROC MIXED of SAS (SAS Institute Inc., Cary, NC, USA) according to the following model:

$$Y_{ijklm} = \mu + b_i + M_j + C_k + MC_{jk} + T_l + TM_{jl} + TC_{kl} + TMC_{jkl} + A_{m:ijk} + \varepsilon_{ijklm}$$

where Y_{ijklm} is the dependent, continuous variable; μ is the overall mean; b_i is the random effect of the *i*th block; M_j is the fixed effect of MET (j = with or without); C_k is the fixed effect of CHOL (k = with or without); T_l is the fixed effect of time (day or week) of the experiment; A_m is the random effect of the *m*th animal (cow); ε_{ijklm} is the residual error. The covariate of parity (2nd vs. 3rd lactation and above) and concentrations obtained at −30 days for various AA and derivatives were maintained in the model for all variables when significant ($p < 0.05$). Plasma AA and derivatives and hepatic gene expression were analyzed at various time points that were not equally spaced. Therefore, the first order ante-dependence covariance structure 1 (ANTE(1)) was used for repeated measures. Variables were assessed for normality of distribution using the Shapiro-Wilk test. When the normality assumption was rejected, data were log-transformed before statistical analysis. Back transformed data are reported in tables and figures for ease of interpretation. Data reported are least square means for each time point and least square means separation between treatments and time points were performed using the PDIFF statement. Statistical differences were declared significant at $p < 0.05$ and tendencies at $p < 0.10$.

3. Results

3.1. Essential AA

Main effects of MET, CHOL, and their interactions with time for essential AA are presented in Table 1. Means for MET × CHOL are presented in Table S1. Overall, plasma concentrations of Arg, His, Lys, and Trp decreased soon after parturition (4 days). In contrast, circulating Met concentration increased at 4 days (Figure 1). As expected, MET-supplemented cows had greater plasma Met concentration at all time points ($p < 0.01$, Figure 1E) compared with cows without MET. In fact, the proportion of Met in EAA ($p < 0.01$, Figure 2A) and total AA pool (TAA) ($p < 0.01$, Figure 2C) also was greater in response to MET supplementation. In contrast, plasma Met ($p > 0.10$, Figure 1F) and Met%EAA ($p > 0.10$, Figure 2B) levels were not different in response to CHOL supplementation. A main effect of MET ($p = 0.02$) also was detected for Lys, the second most-limiting AA for milk

production, mainly due to greater plasma concentrations at −10 days and 14 days (M × D $p < 0.05$, Figure 1C). Similarly, plasma Arg concentration also was greater ($p = 0.02$) in MET cows owing to greater concentrations at −10 days and 14 days (M × D $p < 0.05$, Figure 1A), but not at 4 days postpartum. Although greater Thr was detected in MET cows at −10 days (M × D $p < 0.05$, Figure 1G), Thr concentrations were not greater at other time points measured postpartum in MET-supplemented cows, hence, overall only a tendency ($p = 0.09$) was detected in these cows. In addition to greater ($p < 0.01$) Trp in MET cows, a main effect of CHOL also was detected for Trp ($p = 0.04$, Figure 1I,J). Despite the lack of main effects of MET for plasma His, Phe and total branched-chain amino acids (BCAA = sum of Val, Leu, and Ile) concentrations, the EAA tended to be greater ($p = 0.06$) in response to MET mainly owing to greater EAA concentration prepartum (M × D $p < 0.05$, Figure 1K).

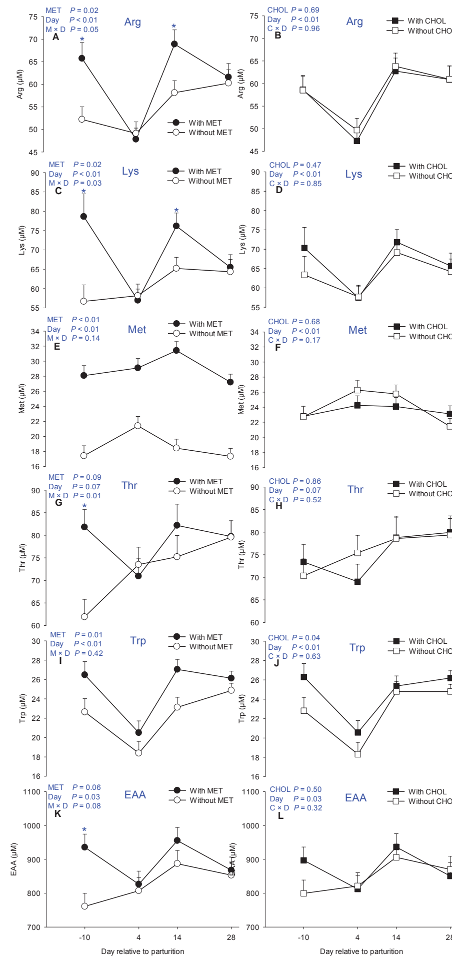

Figure 1. Effects of supplementing multiparous Holstein cows during the periparturient period (−21 through 30 days around parturition) with rumen-protected methionine (MET; Smartamine M, Adisseo NA, Alpharetta, GA, USA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc., New Hampton, NY, USA) on circulating concentrations of arginine, lysing, methionine, threonine, tryptophan, and total essential AA (EAA). Values are means, with standard errors represented by vertical bars.

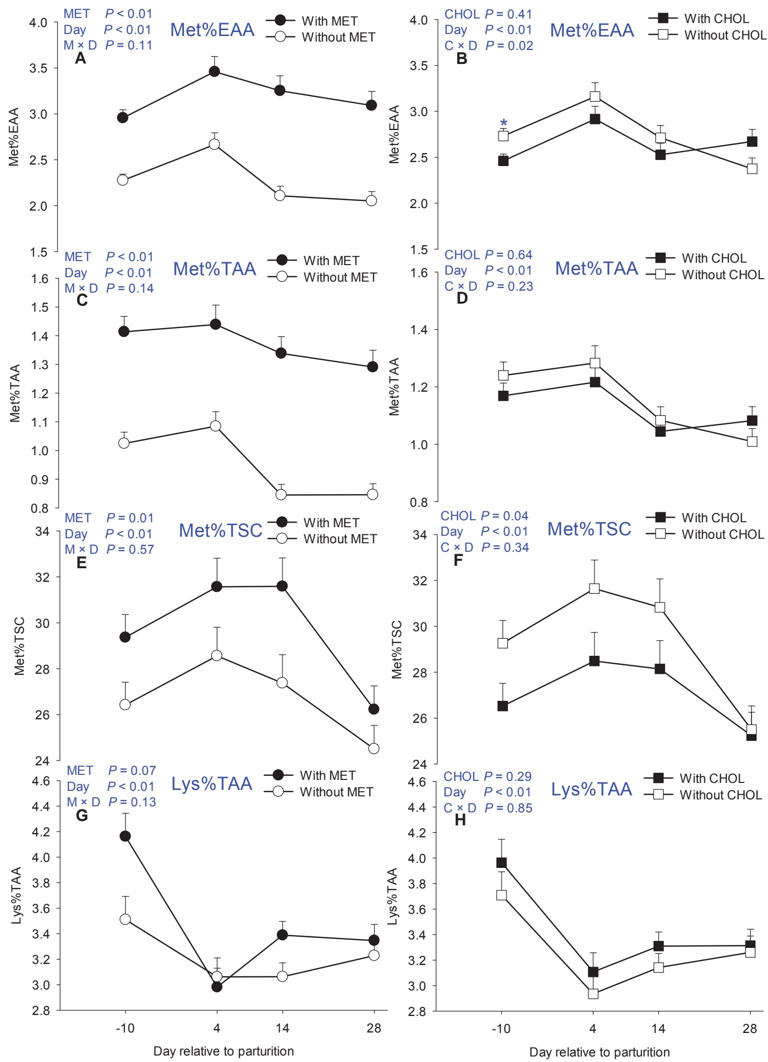

Figure 2. Effects of supplementing multiparous Holstein cows during the periparturient period (−21 through 30 days around parturition) with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on proportions of circulating Met in essential amino acids (Met%EAA), total amino acids (Met%TAA), total sulfur-containing compounds (Met%TSC), and proportion of Lys in TAA (Lys%TAA). Values are means, with standard errors represented by vertical bars.

Table 1. Plasma proteinogenic AA concentrations during the periparturient period in cows supplemented with or without rumen-protected MET and CHOL.

	MET		CHOL			Day				p-Value [2]				
AA (μM)	Without	With	Without	With	SEM [1]	−10	4	14	28	MET [3]	CHOL [4]	Time	M × T [5]	C × T [6]
Essential AA														
Arginine	54.75	60.43	57.99	57.05	1.75	58.58 ^{ab}	48.47 ^{c}	63.29 ^{a}	60.92 ^{ab}	0.02	0.69	<0.01	0.05	0.96
Histidine	52.38	54.55	54.04	52.87	1.18	57.28 ^{a}	53.53 ^{b}	52.30 ^{b}	50.92 ^{b}	0.19	0.49	<0.01	0.13	0.17
Isoleucine	95.58	103.61	100.64	98.40	4.42	98.18	95.54	106.87	97.83	0.19	0.72	0.18	0.07	0.36
Leucine	153.31	161.79	152.70	162.43	6.69	151.35 ^{b}	153.51 ^{b}	173.18 ^{a}	152.92 ^{b}	0.35	0.31	0.03	0.27	0.31
Lysine	60.99	68.74	63.47	66.06	2.45	66.75 ^{ab}	57.56 ^{c}	70.47 ^{a}	64.91 ^{b}	0.02	0.47	<0.01	0.03	0.85
Methionine	18.65	28.95	24.04	23.55	0.83	22.75 ^{c}	25.25 ^{a}	24.92 ^{ab}	22.27 ^{c}	<0.01	0.68	<0.01	0.14	0.17
Phenylalanine	46.64	47.09	45.88	47.86	1.02	45.00 ^{b}	47.64 ^{b}	51.16 ^{a}	43.97 ^{b}	0.75	0.17	<0.01	0.26	0.92
Threonine	72.55	78.67	75.93	75.30	2.50	71.87	72.22	78.70	79.66	0.09	0.86	0.07	0.01	0.52
Tryptophan	22.26	25.04	22.68	24.61	0.64	24.56 ^{ab}	19.44 ^{c}	25.08 ^{a}	25.50 ^{a}	<0.01	0.04	<0.01	0.42	0.63
Valine	226.77	244.54	229.16	241.99	9.32	230.11	223.02	249.41	240.25	0.17	0.33	0.08	0.26	0.31
BCAA [7]	476.69	510.97	483.71	503.55	19.87	480.67	473.17	530.28	491.93	0.21	0.48	0.08	0.19	0.27
EAA	827.12	896.20	849.22	874.1	25.54	848.26 ^{bc}	816.78 ^{bc}	921.2 ^{a}	860.39 ^{ab}	0.06	0.50	0.03	0.08	0.32
Met%EAA	2.26	3.18	2.73	2.64	0.09	2.59 ^{b}	3.04 ^{a}	2.62 ^{b}	2.52 ^{b}	<0.01	0.41	<0.01	0.11	0.23
Non-essential AA														
Alanine	187.39	206.37	198.37	195.39	5.55	188.83 ^{b}	175.45 ^{c}	211.30 ^{a}	211.95 ^{a}	0.02	0.71	<0.01	0.05	0.32
Asparagine	34.90	39.63	37.78	36.61	1.48	27.82 ^{c}	34.08 ^{b}	45.67 ^{a}	44.19 ^{a}	0.02	0.56	<0.01	0.22	0.42
Aspartate	4.21	4.79	4.24	4.76	0.21	4.91 ^{a}	3.78 ^{b}	4.80 ^{a}	4.57 ^{a}	0.04	0.07	<0.01	0.59	0.14
Glutamate	35.93	38.34	36.44	37.81	1.04	44.24 ^{a}	32.17 ^{d}	37.84 ^{b}	35.24 ^{c}	0.10	0.36	<0.01	0.54	0.38
Glutamine	246.22	259.53	257.92	247.82	6.18	278.17 ^{a}	252.84 ^{b}	242.52 ^{b}	237.96 ^{b}	0.15	0.25	<0.01	0.21	0.29
Glycine *	426.83	406.23	427.39	405.67	15.10	236.99 ^{c}	456.97 ^{b}	536.19 ^{a}	435.98 ^{b}	0.35	0.31	<0.01	0.92	0.22
Proline	72.30	78.24	74.03	76.50	2.02	59.19 ^{c}	67.52 ^{b}	90.21 ^{a}	84.15 ^{a}	0.04	0.39	<0.01	0.35	0.74
Serine	86.62	88.92	88.67	86.87	2.28	73.88 ^{c}	90.85 ^{b}	100.78 ^{a}	85.57 ^{b}	0.48	0.58	<0.01	0.16	0.49
Tyrosine	40.82	43.39	40.78	43.43	1.53	42.03 ^{a}	36.26 ^{b}	46.32 ^{a}	43.80 ^{a}	0.24	0.24	<0.01	0.08	0.94
NEAA [8]	1114.59	1151.58	1153.58	1112.59	24.79	946.13 ^{c}	1136.83 ^{b}	1301.27 ^{a}	1177.00 ^{b}	0.29	0.24	<0.01	0.36	0.23
TAA [9]	1926.95	2052.97	1995.87	1982.22	41.32	1781.41 ^{d}	1947.89 ^{bc}	2215.47 ^{a}	2035.97 ^{b}	0.03	0.82	<0.01	0.10	0.53
Met%TAA	0.94	1.37	1.15	1.13	0.04	1.20 ^{a}	1.25 ^{a}	1.06 ^{b}	1.05 ^{b}	<0.01	0.64	<0.01	0.14	0.23
Lys%TAA	3.22	3.47	3.26	3.42	0.10	3.84 ^{a}	3.02 ^{c}	3.23 ^{bc}	3.29 ^{b}	0.08	0.28	<0.01	0.13	0.85

* Significant ($p < 0.05$) parity effect observed. Labeled means in a row without a common superscript letter differ ($p < 0.05$). [1] Greatest SEM; [2] MET × CHOL interaction was included in the supplemental file; [3] Overall effect of MET supplementation; [4] Overall effect of CHOL supplementation; [5] Interaction of MET × time; [6] Interaction of CHOL × time; [7] Branched-chain AA; [8] Non-essential AA; [9] Total AA.

3.2. Non-Essential AA

Main effects of MET, CHOL, and their interactions with time for non-essential proteinogenic AA are presented in Table 1. Means for MET × CHOL are presented in Table S1. Similar to EAA, plasma concentrations of Asp, Glu, Gln, and Tyr decreased soon after parturition (Figure 3). In contrast, plasma concentrations of Asn, Gly, Pro, and Ser increased at 4 days compared with −10 days. Although total NEAA did not change ($p > 0.10$) in response to MET or CHOL, TAA in MET-supplemented cows was greater ($p = 0.03$, Figure 3K) as a result of an overall tendency for greater EAA together with greater Asn, Asp, Ala, and Pro ($p < 0.05$, Figure 3A,C,E,I) as well as a tendency for greater Glu ($p = 0.10$, Figure 3G).

It is noteworthy that similarly to Arg and Lys (M × D $p < 0.05$, Figure 1A,C), plasma Ala concentrations were already greater (M × D $p < 0.05$, Figure 3A) at −10 days prepartum in MET cows. After parturition, plasma Arg, Lys, and Ala were not different regardless of MET supplementation at 4 days, but were again greater at 14 days (M × D $p < 0.05$, Figure 1A,C and Figure 3A) in MET cows. Unlike Met, only a tendency for greater ($p = 0.06$, Figure 3D) Asp was detected in response to CHOL.

3.3. Sulfur-Containing Compounds

Main effects of MET, CHOL, and their interactions with time for sulfur-containing compounds are presented in Table 2. Means for MET × CHOL are presented in Table S2. As expected, MET supplementation increased plasma concentrations of cystathionine, cystine, homocysteine, and taurine ($p < 0.05$, Figure 4A,C,E,G), which account for the greater overall plasma TSC level at all time points evaluated (M × D $p < 0.05$, Figure 4I). Similar to Ala, Arg, and Lys, greater ($p < 0.01$) cystine and cystathionine concentrations were also detected in response to MET due to greater plasma level at −10, 14, and 28 days (M × D $p < 0.05$, Figure 4A,C). In addition to increased circulating sulfur-containing compounds, MET also increased the proportion of Met in TSC ($p = 0.01$, Figure 2E). In contrast, although plasma cystine concentration was greater ($p < 0.01$, Figure 4D) in response to CHOL supplementation, a decreased proportion of Met in TSC was detected in CHOL cows around parturition ($p = 0.03$, Figure 2F).

3.4. Non-Proteinogenic AA and Derivatives

Main effects of MET, CHOL, and their interactions with time for non-proteinogenic AA and derivatives are presented in Table 2. Means for MET × CHOL are presented in Table S2. Although no main effect of MET or CHOL was detected for ornithine, greater Cit ($p < 0.01$, Figure 5I) and a tendency ($p = 0.07$, Figure 5K) for greater urea were observed in MET-supplemented cows in addition to greater Arg. Besides taurine, plasma concentrations of carnosine, another antioxidant, was also greater ($p = 0.02$, Figure 5G) in MET cows. Although no main effect of CHOL was detected for carnosine, greater (C × D $p < 0.05$, Figure 5H) concentrations were detected at −10 days and 28 days. γ-aminobutyric acid was greater both in MET- and CHOL-supplemented cows ($p < 0.05$, Figure 5E,F). The indicator of protein mobilization, 3-methylhistidine, was lower in CHOL cows ($p = 0.02$, Figure 5D). Similarly, plasma 1-methylhistidine concentration also was lower in these cows ($p = 0.04$).

3.5. Pyruvate Carboxylase and Phosphoenolpyruvate Carboxykinase 1 Expression

Main effects of MET, CHOL, and their interactions with time for expression of *pyruvate carboxylase* (*PC*) and *phosphoenolpyruvate carboxykinase 1* (*PCK1*) are presented in Table 3. Means for MET × CHOL are presented in Table S3. Main effects of MET and CHOL or interactions were not detected ($p > 0.05$) for the mRNA expression of *PC* and *PCK1.*

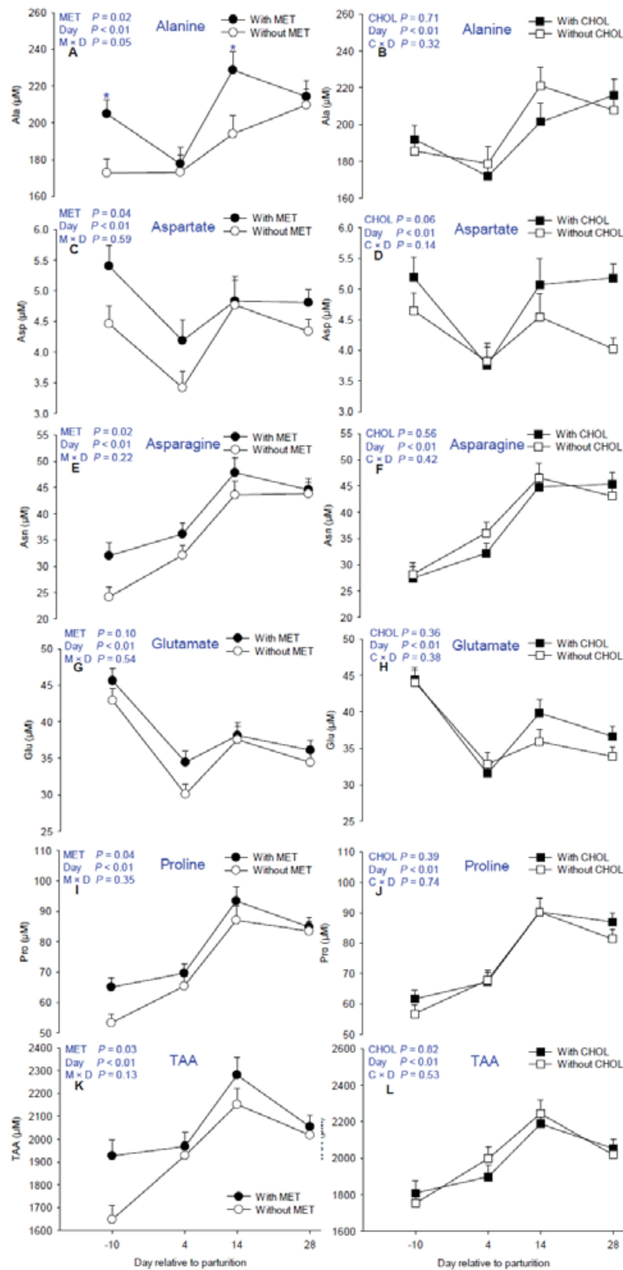

Figure 3. Effects of supplementing multiparous Holstein cows during the periparturient period (−21 through 30 days around parturition) with rumen-protected methionine (MET; Smartamine M, Adisseo NA) on circulating concentrations of free alanine, aspartate, asparagine, glutamate, proline, and total AA (TAA). Values are means, with standard errors represented by vertical bars.

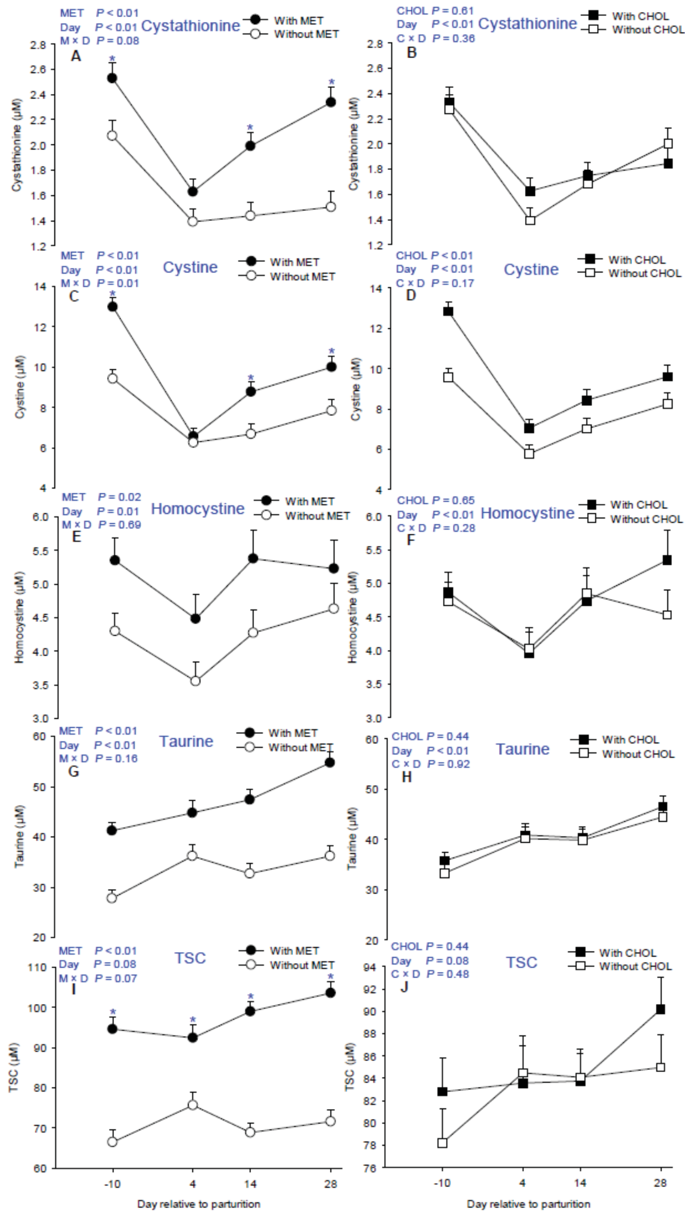

Figure 4. Effects of supplementing multiparous Holstein cows during the periparturient period (−21 through 30 days around parturition) with rumen-protected methionine (MET; Smartamine M, Adisseo NA) on circulating concentrations of cystathionine, cystine, homocysteine, taurine, and total sulfur-containing compounds (TSC). Values are means, with standard errors represented by vertical bars.

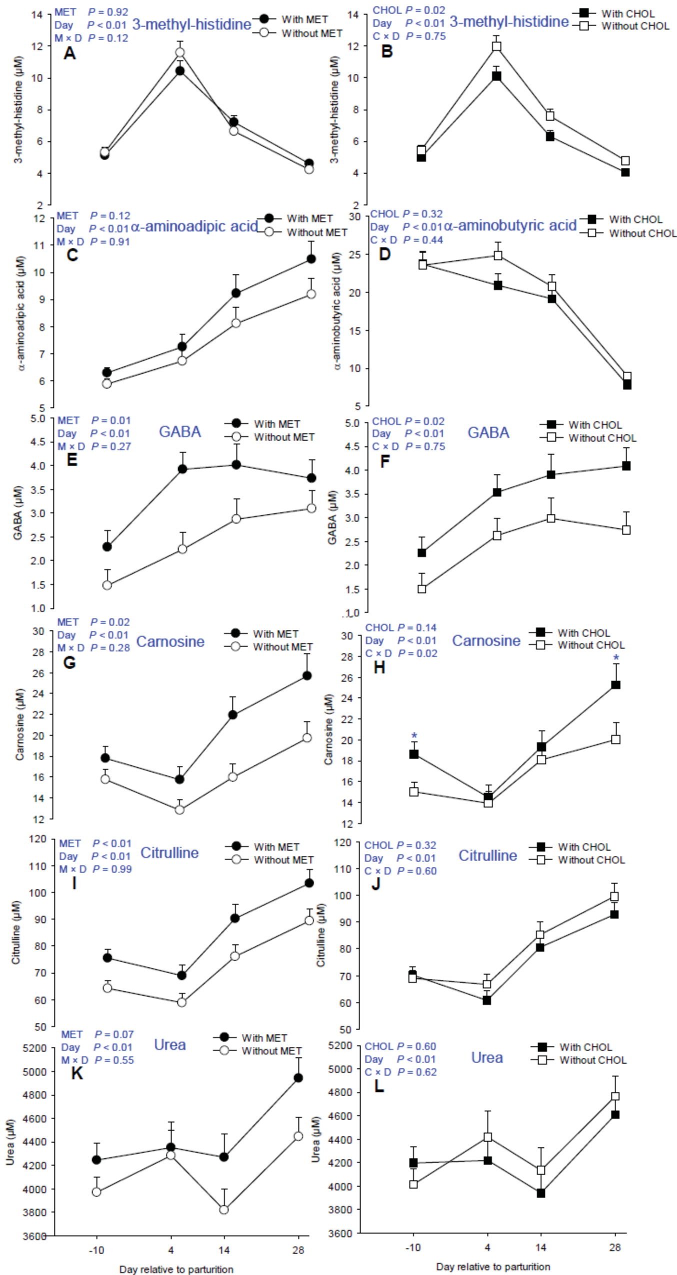

Figure 5. Effects of supplementing multiparous Holstein cows during the periparturient period (−21 through 30 days around parturition) with rumen-protected methionine (MET; Smartamine M, Adisseo NA) or rumen-protected choline (CHOL; ReaShure, Balchem Inc.) on proportions of circulating 1-methyl histidine, 3-methyl histidine, γ-amino butyric acid, carnosine, citrulline, and urea. Values are means, with standard errors represented by vertical bars.

Table 2. Plasma non-proteinogenic AA and AA derivatives concentrations during the periparturient period in cows supplemented with or without rumen-protected MET and CHOL.

	MET		CHOL			Day				p-Value [2]				
Parameter (μM)	Without	With	Without	With	SEM [1]	−10	4	14	28	MET [3]	CHOL [4]	Time	M × T [5]	C × T [6]
AA and derivatives #														
1-methyl histidine	15.36	16.56	16.79	15.15	0.57	12.82 c	12.58 c	18.38 b	21.85 a	0.12	0.04	<0.01	0.36	0.50
3-methyl histidine	6.44	6.48	6.97	5.99	0.29	5.21 c	11.00 a	6.91 b	4.40 d	0.92	0.02	<0.01	0.12	0.75
α-aminoadipic acid	7.37	8.15	8.03	7.48	0.36	6.09 d	6.99 c	8.65 b	9.81 a	0.12	0.27	<0.01	0.91	0.30
α-aminobutyric acid	15.64	19.78	18.13	17.07	0.81	8.94 c	23.60 a	22.77 a	19.93 b	<0.01	0.32	<0.01	0.61	0.44
β-alanine	9.06	8.87	8.81	9.11	0.32	8.97	8.69	9.24	8.94	0.68	0.50	0.54	0.96	0.92
γ-aminobutyric acid	2.42	3.49	2.46	3.45	0.29	1.88 b	3.08 a	3.44 a	3.41 a	0.01	0.02	<0.01	0.27	0.75
Carnosine *	15.88	19.92	16.60	19.07	1.29	16.74 c	14.20 d	18.71 b	22.51 a	0.02	0.14	<0.01	0.30	0.02
Citrulline	71.19	83.47	79.08	75.14	2.92	69.61 c	63.69 c	82.86 b	96.11 a	<0.01	0.30	<0.01	0.99	0.60
Glutathione	4.68	4.73	4.84	4.57	0.27	4.60 b	6.11 a	4.35 bc	3.76 c	0.89	0.50	<0.01	0.50	0.74
Hydroxylysine	0.32	0.28	0.30	0.29	0.04	0.33	0.29	0.33	0.25	0.37	0.79	0.21	0.61	0.70
Hydroxyproline	15.19	15.36	15.42	15.13	0.48	12.80 c	18.10 a	16.31 b	13.89 c	0.81	0.67	<0.01	0.94	0.12
Ornithine	29.14	32.00	30.48	30.60	1.35	39.37 a	22.11 d	30.04 c	33.25 b	0.13	0.95	<0.01	0.12	0.45
Phosphoserine	6.22	6.18	6.07	6.33	0.18	5.53 c	5.91 bc	6.72 ab	6.74 a	0.88	0.30	<0.01	0.71	0.91
Sarcosine	10.65	11.43	11.50	10.58	0.56	8.32 b	11.31 a	12.03 a	12.50 a	0.33	0.27	<0.01	0.76	0.37
Urea	4121.63	4442.94	4323.55	4235.16	123.89	4104.53 bc	4317.26 b	4036.81 c	4687.49 a	0.07	0.60	<0.01	0.55	0.62
Sulfur-containing compounds														
Cystathionine	1.60	2.12	1.84	1.89	0.07	2.30	1.51	1.71	1.92	<0.01	0.61	<0.01	0.08	0.36
Cystine	7.56	9.58	7.65	9.48	34.5	11.20 a	6.41 d	7.73 c	8.92 b	<0.01	0.02	<0.01	0.01	0.17
Homocystine	4.17	5.10	4.52	4.70	0.30	4.80 a	3.99 b	4.80 a	4.92 a	0.02	0.65	0.01	0.69	0.28
Taurine	33.23	47.04	39.43	40.84	1.28	34.51	40.49	40.06	45.48	<0.01	0.44	<0.01	0.16	0.92
TSC [7]	70.65	97.37	82.47	85.95	1.93	80.52	84.03	83.93	87.57	<0.01	0.44	0.08	0.07	0.48
Met%TSC	28.44	31.4	31.09	28.74	0.72	29.65 b	32.35 ab	31.15 b	26.51 c	0.01	0.03	<0.01	0.74	0.24

* Significant ($p < 0.05$) parity effect observed. Labeled means in a row without a common superscript letter differ ($p < 0.05$). # β-aminobutyric acid undetectable; [1] Greatest SEM; [2] MET × CHOL interaction was included in the supplemental file; [3] Overall effect of MET supplementation; [4] Overall effect of CHOL supplementation; [5] Interaction of MET × time; [6] Interaction of CHOL × time; [7] Total sulfur-containing AAs and derivatives (methionine, cystathionine, cystine, homocysteine, and taurine).

Table 3. Hepatic relative *PC*# and *PCK1* mRNA expression during the periparturient period in cows supplemented with or without rumen-protected MET and CHOL.

	MET		CHOL			Day				p-Value [2]				
Genes	Without	With	Without	With	SEM [1]	−10	7	20	30	MET [3]	CHOL [4]	Time	M × T [5]	C × T [6]
PC	2.12	2.02	2.09	2.05	0.07	1.49 c	2.88 a	2.23 b	1.93 d	0.33	0.70	<0.01	0.28	0.34
PCK1	2.27	2.43	2.39	2.31	0.10	1.57 c	2.62 ab	2.74 a	2.69 ab	0.27	0.55	<0.01	0.48	0.46

* Significant ($p < 0.05$) parity effect observed. Labeled means in a row without a common superscript letter differ ($p < 0.05$). # *PC* = pyruvate carboxylase; *PCK1* = Phosphoenolpyruvate carboxykinase 1; [1] Greatest SEM; [2] MET × CHOL interaction was included in the supplemental file; [3] Overall effect of MET supplementation; [4] Overall effect of CHOL supplementation; [5] Interaction of MET × time; [6] Interaction of CHOL × time.

4. Discussion

A detailed discussion of production performance and immunometabolic status of the cows has been published elsewhere [8,9,23].

4.1. Enhancing the Supply of MET Improved Plasma AA Profiles

MET and Lys in the MP are the most-limiting AA in a wide-range of diets for dairy cows due to their low concentrations in feed protein relative to their "apparent required amounts in digestible protein" [30]. During the periparturient period, the decreased feed intake coupled with increased AA requirements to sustain fetal growth and lactation lead to greater tissue protein mobilization evidenced in the present study by the highest plasma 3-methyl histidine at 4 days regardless of treatments. Although AA released from tissue mobilization can partly mitigate the demand for AA, cow body tissue protein is relatively low in Met and Lys [31]. Consequently, this physiological stage is characterized by an overall decrease in circulating AA, especially the most-limiting AA (e.g., Met and Lys). Apart from limiting milk production, according to von Liebig's hypothesis commonly described with the analogy of a water barrel with broken staves, inadequate Met availability also could potentially limit the utilization of other circulating AA [32]. Strategies to increase circulating Met are, thus, expected to improve overall circulating AA profiles and utilization during the periparturient period.

Apart from greater plasma Met, the fact that various proteinogenic circulating EAA (Arg, Lys, Met, Thr, and Trp), NEAA (Ala, Asn, Asp, Glu, Gln, and Pro) and non-proteinogenic AA (Cit) were greater in response to rumen-protected MET supplementation is an indication of a better circulating AA profile during the periparturient period. However, it cannot be ignored that MET cows had greater DMI both prepartum and postpartum [9,23]. Therefore, the DMI increase in MET-supplemented cows might have contributed to the observed increase in circulating EAA and TAA. It is noteworthy that despite concomitant increases in circulating Met, EAA and TAA, the proportion of Met in EAA and TAA (Met%EAA and Met%TAA) as well as the proportion of Lys in TAA were increased in response to MET supplementation, indicating a better AA profile at least in regards to limiting AA, i.e., Met and Lys.

Considering the overall greater milk yield, milk protein %, and milk protein yield [9,23], it is reasonable to speculate that MET supplementation contributes to better lactation performance both by increasing intake and proportion of Met and Lys in the circulating AA pool. Although CHOL can, in theory, promote in vivo synthesis of Met or indirectly spare Met by reducing the use of Met for CHOL synthesis, the lack of change in circulating AA in the present study and the fact that flux from CHOL to MET was not increased in response to CHOL supplementation in lactating goats [33] and transition dairy cows [34] do not support such hypothesis.

4.2. Utilization of Circulating AA Close to Parturition

Around parturition, the demand for AA for glucose and protein synthesis increases abruptly and results in greatly increased AA uptake from the circulation [35]. The few published reports have revealed that circulating AA concentrations in dairy cows generally reach a nadir close to parturition mainly as a result of increased demand for milk protein synthesis and gluconeogenesis coupled with insufficient dietary intake [3,18,20,36]. In agreement with these reports, regardless of treatment, we detected the lowest circulating concentrations of most AA at 4 days relative to parturition.

Although circulating concentrations of multiple AA (Ala, Arg, Lys) and derivatives (cystine, cystathionine) in MET-supplemented cows were already greater at −10 days compared with cows without MET supplementation and regained greater levels at 14 days postpartum, the circulating concentrations of all AA and derivatives at 4 days were similar regardless of MET supplementation. Whether the lack of difference in circulating concentrations of these AA at 4 days in MET cows was due to inadequate supply in the circulation (e.g., AA from intake and tissue mobilization) [37] or enhanced utilization (e.g., greater liver and mammary uptake) remains unknown. However, the fact that average DMI at day 4 remained greater (+3.33 kg/days more) in MET-supplemented cows [9,23], together with

similar 3-methylhistidine in cows with or without MET supplementation, seems to support an overall greater, rather than lower, AA supply at 4 days in response to MET supplementation. In line with the increase in AA supply, MET-supplemented cows regained greater concentrations of these AA at 14 days.

In terms of AA utilization, a previous study detected the greatest liver uptake of EAA at 4 days postpartum in dairy cows [35]. Similarly, hepatic uptake of total NEAA, especially Ala, also was substantially greater in the periparturient period, underscoring that this AA acts as a precursor for glucose synthesis [35,38]. The greater (+4.10 kg/day more) milk yield in MET-supplemented cows at 4 days [9,23] could have caused an increase in uptake of these AA by mammary gland soon after parturition, hence, resulting in lower plasma AA concentration relative to −10 days. Presumably, greater mammary availability of AA accounted for the greater milk protein % [9,23].

It is also noteworthy that a reduction in net flux of carbon from volatile fatty acids across the liver and an increase in glucose release was detected early postpartum in a previous report [35], indicating that the greatest flux through citric acid cycle occurs soon after parturition. Considering the severe negative energy balance and depressed DMI soon after parturition, the increased demand for energy and glucose synthesis by the liver likely accounted for the increase in citric acid flux. Whether periparturient MET supplementation in the present study enhanced gluconeogenesis by promoting AA flux through the citric acid cycle is unknown, but does not seem to be related to regulation at the transcriptional level as evidenced by unchanged mRNA abundance of the key gluconeogenic genes *PC* and *PCK1.*

4.3. Sulfur-Containing Compound Pool and Metabolism

Met and cysteine are the two sulfur-containing AA that are incorporated into proteins. Apart from their well-known role in contributing sulfur bonds during protein synthesis, Met and cysteine are precursors for downstream functional compounds (homocysteine, cystathionine, and taurine) and, thus, are considered the principal components in vivo of the sulfur-containing compound pool [12]. The fact that circulating concentrations of all sulfur-containing compounds measured (except GSH) were greater in MET-supplemented cows during the periparturient period indicate an enriched sulfur-containing compound pool.

It is noteworthy that other than Met, all other plasma sulfur-containing compounds measured are components of the transsulfuration pathway. Considering plasma homocysteine concentrations are highly-dependent on intracellular homocysteine metabolism in liver [39], and cystathionine is a sensitive marker of changes in flux through the transsulfuration pathway [40], the greater homocysteine and cystathionine concentrations indicate increased hepatic flux through this pathway in response to MET supplementation. In addition to the involvement of sulfur-containing compounds, the fact that circulating concentrations of α-aminobutyric acid also were greater in MET-supplemented cows indicates increased flux through the transsulfuration pathway [41]. Considering the key role of Met as the most-limiting AA for milk protein synthesis, the fact that it is the major precursor for sulfur-containing compounds in the transsulfuration pathway indicates that such increase may result in depletion of Met which could potentially give rise to unfavorable lactation performance. However, despite the increased flux through the transsulfuration pathway, the sustained greater circulating concentration of Met and proportion of Met in TSC during the periparturient period indicates that sufficient Met was available in MET-supplemented cows even at 4 days. In contrast, although greater circulating cystine was detected in response to CHOL supplementation, the proportion of Met in TSC was decreased, indicating enhanced flux through the transsulfuration pathway at the expense of Met. Whether the decrease of plasma Met proportion in TSC in the CHOL-supplemented cows contributed to the lack of benefit in performance is unknown; however, it does not seem to support the hypothesis that CHOL can promote Met synthesis in vivo in periparturient dairy cows.

Around parturition, the increased demand for nutrients and energy leads to an increase in the production reactive oxygen metabolites (ROM), the accumulation of which could deplete antioxidants

and give rise to oxidative stress that may cause substantial tissue damage and render cows more susceptible to various health disorders [42,43]. Because of their marked ability to scavenge ROM and free radicals, among the sulfur-containing compounds measured, taurine and GSH are considered potent intracellular antioxidants [44]. Hence, in non-ruminants, hepatic concentrations of GSH and taurine have been widely-used as oxidative stress biomarkers [45–47]. Because the liver is the main site of taurine synthesis and releases it into plasma [48], its concentration in the circulation reflects hepatic synthesis [49]. Therefore, the greater overall circulating taurine in response to MET supplementation indicates greater hepatic and extra-hepatic taurine availability and, hence, potentially less oxidative stress in these cows. However, although previous results from our group revealed greater total and reduced hepatic GSH in MET-supplemented cows [14,50], plasma GSH was barely detectable and did not respond to MET. Considering that concentration of GSH in whole blood was 200-fold higher than in plasma due to high concentration in erythrocytes, small amounts of hemolysis may lead to great variations in plasma GSH concentration [51]. Therefore, plasma GSH may not be a reliable oxidative stress biomarker for periparturient dairy cows.

4.4. AA Derivatives

AA derivatives have unique metabolic properties and, thus, many have been adopted as biomarkers for metabolic status. For instance, because 3-methylhistidine is released from the catabolism of actin and myosin in skeletal muscle and is not further metabolized in the body, it has been regarded as a reliable marker for tissue protein mobilization [52]. Assuming that renal blood flow was not altered by CHOL supplementation, the overall lower plasma 3-methylhistidine in response to CHOL supplementation during the periparturient period indicates a lower degree of muscle catabolism. Considering that CHOL supplementation did not result in greater milk yield, the lower degree of tissue mobilization could denote better production efficiency. However, the fact that CHOL supplementation did not alter energy corrected milk (ECM):DMI and fat corrected milk (FCM):DMI does not support such hypothesis. Although lower circulating 1-methylhistidine concentration also was observed in response to CHOL, the postpartum pattern of a gradual increase with time indicates that it is not a suitable indicator of muscle catabolism considering that it was at its greatest right after parturition.

The antioxidant activity of carnosine in non-ruminants has been demonstrated both in terms of reducing oxidative damage and improving the enzymatic and non-enzymatic activity of other antioxidants [53]. The fact that supplementation of carnosine in non-ruminants was able to rescue the prooxidant-antioxidant balance by restoring depleted levels of blood GSH and activities of antioxidant enzymes [54] indicates that circulating carnosine concentrations could decrease in events of oxidative stress. Although the contribution of carnosine to restoring prooxidant–antioxidant balance under physiologic (without carnosine supplementation) conditions remains unknown, the overall greater circulating carnosine together with greater circulating taurine as well as hepatic GSH reported previously [8] indicate a less pronounced oxidative stress status in response to periparturient MET supplementation.

As a neurotransmitter distributed in both neural and non-neural tissue, GABA has various physiologic functions including feed intake regulation [55]. In rats, GABA-B agonist administration increased intake by attenuating satiety signals [56]. In lactating dairy cows, increased DMI in response to rumen-protected GABA supplementation during mid-lactation has been reported [55,57]. The greater circulating GABA in response to periparturient MET supplementation along with the greater DMI [9,23] seem to suggest a role of GABA in mediating DMI regulation by MET. However, considering that CHOL supplementation failed to increase periparturient DMI, yet resulted in a similar increase in circulating GABA, seems to argue against a regulatory role of this molecule in the control of intake at least around parturition.

5. Conclusions

The greater Ala, Arg, and Lys together with cystine and cystathionine concentrations at −10 days and 14 days, but not at 4 days, in cows fed MET indicated greater utilization of these AA and derivatives at the time of most-severe negative AA balance. The enriched circulating sulfur-containing compound pool together with greater α-aminobutyric acid revealed enhanced transsulfuration pathway activity in response to MET supplementation. Despite the greater circulating cystine in CHOL cows, the lower proportion of Met in TSC indicates enhanced flux through the transsulfuration pathway at the expense of Met. Whether the decrease in the proportion of Met was due to insufficient choline supplementation remains unknown. As a precursor for Met synthesis in vivo, it is possible that increasing periparturient choline supplementation could mitigate a decrease in Met. Although plasma GSH did not differ in response to MET due to barely detectable concentrations in plasma, the greater circulating taurine and carnosine indicate less oxidative stress in MET-supplemented cows. In conclusion, the overall better health and production performance reported previously in MET cows was due, at least in part, to a better plasma AA profile and overall lower oxidative stress status. Hence, the lack of change in AA profiles could be one of the reasons preventing CHOL-fed cows from achieving comparable performance and health benefits during the periparturient period.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/9/1/10/s1, RNA Extraction and mRNA Expression Calculation, Table S1: Plasma proteinogenic AA concentrations during the transition period in cows supplemented with or without rumen-protected methionine (MET) and choline (CHOL), Table S2: Plasma non-proteinogenic AA and AA derivatives concentrations during the transition period in cows supplemented with or without rumen-protected MET and CHOL, Table S3: Hepatic relative *PC* and *PCK1* mRNA expression during the transition period in cows with or without rumen-protected MET and CHOL.

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Conflicts of Interest: The authors have read the journal's policy and one of the authors of this manuscript has the following competing interests: Daniel N. Luchini is an employee of Adisseo USA Inc. (Alpharetta, GA, USA). Adisseo USA Inc. had a role in the study design and provided financial support to cover costs of animal use, data collection, and sample analyses.

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B-Vitamin Intake and Biomarker Status in Relation to Cognitive Decline in Healthy Older Adults in a 4-Year Follow-Up Study

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Abstract: Advancing age can be associated with an increase in cognitive dysfunction, a spectrum of disability that ranges in severity from mild cognitive impairment to dementia. Folate and the other B-vitamins involved in one-carbon metabolism are associated with cognition in ageing but the evidence is not entirely clear. The hypothesis addressed in this study was that lower dietary intake or biomarker status of folate and/or the metabolically related B-vitamins would be associated with a greater than expected rate of cognitive decline over a 4-year follow-up period in healthy older adults. Participants (aged 60–88 years; $n = 155$) who had been previously screened for cognitive function were reassessed four years after initial investigation using the Mini-Mental State Examination (MMSE). At the 4-year follow-up assessment when participants were aged 73.4 ± 7.1 years, mean cognitive MMSE scores had declined from 29.1 ± 1.3 at baseline to 27.5 ± 2.4 ($p < 0.001$), but some 27% of participants showed a greater than expected rate of cognitive decline (i.e., decrease in MMSE > 0.56 points per year). Lower vitamin B6 status, as measured using pyridoxal-5-phosphate (PLP; <43 nmol/L) was associated with a 3.5 times higher risk of accelerated cognitive decline, after adjustment for age and baseline MMSE score (OR, 3.48; 95% CI, 1.58 to 7.63; $p < 0.05$). Correspondingly, lower dietary intake (0.9–1.4 mg/day) of vitamin B6 was also associated with a greater rate of cognitive decline (OR, 4.22; 95% CI, 1.28–13.90; $p < 0.05$). No significant relationships of dietary intake or biomarker status with cognitive decline were observed for the other B-vitamins. In conclusion, lower dietary and biomarker status of vitamin B6 at baseline predicted a greater than expected rate of cognitive decline over a 4-year period in healthy older adults. Vitamin B6 may be an important protective factor in helping maintain cognitive health in ageing.

Keywords: one-carbon metabolism; B-vitamin biomarkers; dietary intakes; vitamin B6; pyridoxal-5-phosphate (PLP); cognition; ageing

1. Introduction

Advancing age can be associated with an increase in cognitive dysfunction, a spectrum of disability that ranges in severity from normal age-related changes through mild cognitive impairment (MCI) to dementia; with the latter defined as a progressive decline in memory, thinking, language and judgment that is sufficient to impair activities of daily living [1]. An estimated 50% of those diagnosed with MCI will go onto develop dementia within 5 years of diagnosis [2]. Globally, it is estimated that

48 million people are currently suffering from dementia and the figures are predicted to triple by 2050 [3]. Dementia is a leading cause of disability, dependency and decreased quality of life among older people [3] and presents many social, economic and health care challenges that will continue to increase with an ageing population. Therefore, the identification of strategies to prevent or delay the onset of dementia has become a major global public health priority.

A number of nutritional and lifestyle factors have emerged as potential modifiable risk factors for cognitive decline in ageing [4]. In particular, there is considerable epidemiological evidence to suggest that sub-optimal status of folate, the related B-vitamins, and/or elevated concentrations of the metabolite homocysteine, contribute to cognitive dysfunction [5–10] and to a greater rate of cognitive decline in ageing [11–14]. Elevated plasma homocysteine and lower folate have been most consistently associated with cognitive dysfunction in ageing [6,8]. There is also evidence to support a role for vitamin B12 [15,16] and to a lesser extent vitamin B6, although the latter has been far less extensively investigated [9,17]. There is also some evidence in the form of randomised controlled trials to show beneficial effects of B-vitamin supplementation on cognition in ageing [18–20]. A number of other trials have failed to detect significant benefits [21–23] with recent meta-analyses concluding that there was no beneficial effect of B-vitamin supplementation on cognition [24,25]. However, a number of these trials may have been too short in duration; conducted in healthy individuals, patients with severe dementia; or in those with optimal B-vitamin status and so unlikely to benefit from vitamin supplementation [26]. The strongest evidence to date of a causal relationship between B-vitamins and cognition comes from the Homocysteine and B-vitamin in Cognitive Impairment (VITACOG) study. This study showed that combined B-vitamin supplementation for two years had beneficial effects on cognitive performance in participants with MCI and elevated plasma homocysteine concentrations [20]. More importantly, it also demonstrated that B-vitamin supplementation reduced the rate of brain atrophy by 30% as measured using MRI [27]. A subsequent report from the VITACOG investigators reported that the atrophy occurred in grey matter areas of the brain which are particularly vulnerable to Alzheimer's disease [28].

The intervention doses administered in VITACOG were well in excess of recommended dietary intakes and therefore whilst the VITACOG papers provide powerful evidence of a role for folate, vitamin B12, and/or vitamin B6 in cognition, the relevance of these results to nutrition, and thus prevention of cognitive dysfunction in ageing is unclear. Furthermore, epidemiological research generally in this area has predominantly focused on plasma homocysteine, folate and vitamin B12; most studies have overlooked vitamin B6 and all have ignored the role of vitamin B2. Consequently, the influence of all the relevant B-vitamins involved in one-carbon metabolism on cognition is not fully understood. Therefore, the aim of this study was to investigate whether lower dietary intake or biomarker status of B-vitamins (folate, vitamin B12, vitamin B6 or riboflavin) at baseline was associated with a greater rate of cognitive decline over a 4-year follow-up period in healthy older adults.

2. Materials and Methods

2.1. Participant Recruitment and Study Design

Potential participants were identified from our records of a previous cross sectional study funded by the UK Food Standards Agency investigating B-vitamin dietary intakes and biomarker status in the healthy younger and older adults in Northern Ireland as previously described [29]. Healthy participants (n = 662; aged $\geq$18 years) were recruited to the original study and as part of the protocol, those aged $\geq$60 years completed a cognitive function test (Folstein's Mini Mental State Examination MMSE; [30], the purpose of the original assessment was to ensure that the ability of participants to accurately recall food intake was not compromised. The current study involved the re-examination (4 years after initial screening) of those aged $\geq$60 years (n = 255). The exclusion criteria for the original study were: those with vitamin B12 deficiency (serum vitamin B12 < 111 pmol/L); self-reported history of cardiovascular, gastrointestinal, hepatic, renal, or haematological disease; use

of medications that interfere with B-vitamin metabolism; taking supplements containing B-vitamins; having visited a country with a mandatory fortification policy for a period ≥2 weeks in the previous 6 months; plasma creatinine concentrations >130 μmol/L (generally indicative of renal impairment); and a score of <25 on the MMSE (indicative of cognitive impairment). Ethical approval was granted by the University of Ulster Research Ethics Committee (UUREC; Ref UUREC/07/005) and all participants provided written informed consent.

2.2. Cognitive Assessment

Cognitive function was assessed at baseline and at follow-up (between 3.5 to 4 years from initial screening for each participant) using the MMSE [30], one of the most widely used cognitive screening tools in a clinical setting. It is a global test of cognitive function and assesses the domains of orientation, registration, attention and concentration, recall and language. Overall the maximum score achievable is 30, with a score <25 indicating a possibility of cognitive impairment and a score <20 dementia [30].

2.3. Dietary and Lifestyle Assessment

Dietary intake was assessed using a 4-day food diary (for 4 consecutive days, including Saturday and Sunday, to account for the known variation in day-to-day intake) in combination with a food frequency questionnaire. This combined dietary method as described previously has been validated at this centre for the assessment of the four relevant B-vitamins against each of their blood biomarkers [29]. The food frequency questionnaire requested participants to state the frequency of consumption for food groups or specific branded products fortified with B-vitamins (e.g., ready-to-eat breakfast cereals, bars, breads and margarines). Participants provided details on brand names of the products consumed so that the fortification profile of any new foods could be established. By combining the 2 dietary collection methods, we were able to estimate dietary intakes of the relevant B-vitamins from both natural food sources and from fortified foods. Each participant received oral and written instructions on how to complete the food diary and food-frequency questionnaire. Any queries or discrepancies between the 2 dietary records were discussed with the participant and were clarified within 1 week of collection to enhance the accuracy of information on usual food intakes. Food portion sizes were estimated by the participant by using household measures and were later quantified by using published food portion size data [31]. The food-composition database WEIGHED INTAKE SOFTWARE PACKAGE (WISP, version 3; Tinuviel Software, Anglesey, UK) was used to calculate mean daily energy and B-vitamin intakes. This database has been customised at our centre to enable natural food folate to be distinguished from folic acid added to foods by manufacturers, and this allows the estimation of dietary folate equivalents (DFE; [29]).

A health and lifestyle questionnaire was used to obtain information on medical history including depression, smoking, use of alcohol and medication, and educational attainment. Height (m) and weight (kg) were measured at baseline and body mass index (kg/m^2) was calculated.

2.4. Laboratory Analysis

All participants provided a fasting 30 mL blood sample at baseline. Sample preparation and fractionation were performed within 4 h of blood collection, and blood aliquots were stored at −80 °C until batch analysis. Plasma homocysteine was measured by fluorescence polarization immunoassay using the Abbot Imx analyser [32]. Red blood cell folate was measured by microbiological assay using *Lactobacillus casei* [33]. Vitamin B12 status was determined using a number of biomarkers; the direct measures were serum total vitamin B12 by microbiological assay using *Lactobacillus leichmanni* [34] and serum holoTC (the metabolically active fraction of vitamin B12) by microparticle enzyme immunoassay (AxSym Active-B12; Axis-Shield, Heidelberg, Germany); the functional biomarker serum methylmalonic acid (MMA) by gas chromatography mass spectrometry using methylchloroformate derivatization at University of Bergen, Norway. Plasma vitamin B6 (PLP) was measured by reversed phase, high performance liquid chromatography with fluorescence detection [35]. Riboflavin status

was assessed using the erythrocyte glutathione reductase activation (EGRAC) where the ratio of FAD stimulated to unstimulated enzyme activity is calculated; higher EGRAC values indicate lower riboflavin status, and sub-optimal riboflavin status is generally recognised as a coefficient $\geq$1.3 [36]. The methylenetetrahydrofolate reductase (MTHFR) 677C$\rightarrow$T polymorphism was identified by polymerase chain reaction amplification followed by *HinF1* restriction digestion [37]. Plasma creatinine was measured using a standard spectrophotometric assay with use of a chemistry analyzer (Hitachi; Roche Diagnostics Corporation, Indianapolis, IN, USA). Additionally, pepsinogen I and pepsinogen II were measured as markers of gastric atrophy by enzyme-linked immunosorbent assay (Biohit, Helsinki, Finland); a ratio of pepsinogen I:II < 3 is indicative of atrophic gastritis. All samples were analysed blind and duplicated. Quality controls were provided by repeated analysis of pooled samples.

2.5. Statistical Analysis

All statistical analysis was performed using SPSS software (version 22; SPSS UK Ltd., Chersey, UK). Prior to analysis, tests for normality were conducted and data were log transformed where appropriate. Differences at baseline and follow-up were assessed using a paired t-test for continuous data and chi-squared test for categorical data. Correlations between dietary intakes and corresponding blood biomarkers were calculated using Pearson's correlation coefficient (r). Annual cognitive decline was calculated (baseline MMSE$-$follow-up MMSE)/(duration of follow-up) on an individual basis for each participant, accelerated cognitive decline was defined as a decrease in MMSE > 0.56 points per year [38]. Binary logistic regression analysis was used to assess health and lifestyle predictors of cognitive decline. The impact of B-vitamin dietary intake and blood biomarker status as predictors of cognitive decline was assessed using binary logistic regression after controlling for significant predictors of cognitive decline (age and baseline MMSE).

3. Results

Of the 662 healthy volunteers, 255 $\geq$ 60 years were identified as potential participants and of these 155 were available to participate in the follow-up assessment (Figure 1). Only those that participated at both timepoints are presented in this paper; those lost to follow-up were older and had significantly lower vitamin B12 status (Appendix A).

The characteristics of participants at initial screening are shown in Table 1. Participants had a mean age of 70 years, were predominantly female, well-educated and had a low rate of depression. The majority of participants were regular consumers of foods fortified with B-vitamins (75%). Dietary intakes compared favourably with current UK dietary recommendations [39] as reflected in good overall B-vitamin biomarker status. As a result of the exclusion criteria, no participant was deficient in vitamin B12, however some 3% were identified as deficient in folate and 11% deficient in vitamin B6. Gastric function was assessed by using pepsinogen I:II ratio, 12% had evidence of atrophic gastritis (pepsinogen I:II ratio < 3; data not shown).

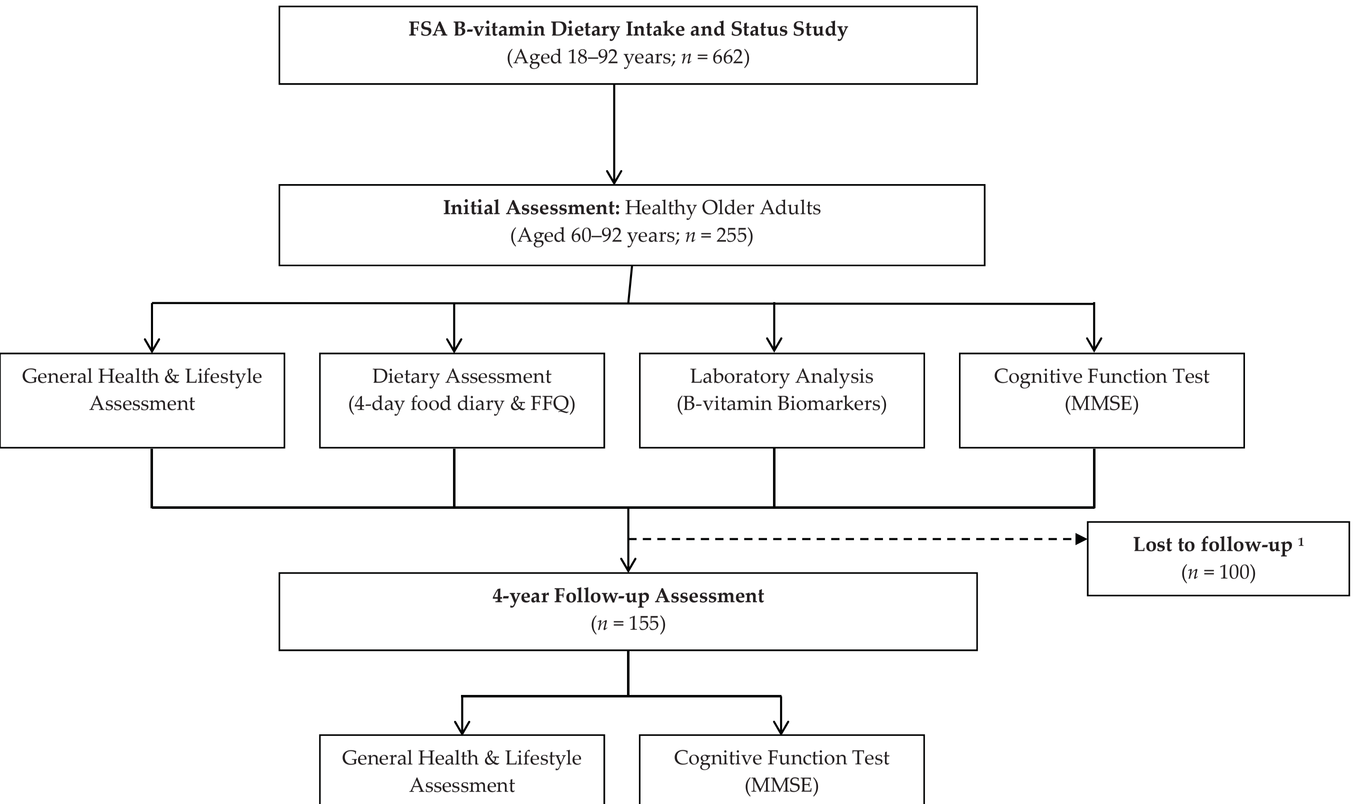

Figure 1. Study Design and flow of participants through the study. Abbreviations: FSA, Food Standards Agency; FFQ, Food Frequency Questionnaire; MMSE, Mini Mental State Examination; [1] Failed to meet inclusion criteria at follow-up assessment $n = 26$; declined to participate $n = 43$; deceased $n = 4$; non contactable $n = 21$; participation in other research $n = 6$.

Table 1. General characteristics of healthy older adults at initial investigation (n = 155).

	Participants	**Reference Range**
Age (years)	69.5 (7.3)	
Male *n* (%)	60 (39)	
BMI (kg/m^2)	27.5 (4.2)	20–25
Smokers *n* (%)	6 (4)	
3rd Level Education *n* (%)	48 (31)	
Depression *n* (%) *	11 (7)	
Cognitive Function (MMSE)	29.1 (1.3)	≤25
B-Vitamin Dietary Intakes †		
Energy (MJ/day)	7.621 (1.789)	9.71 (M); 7.96 (F)
Total Folate (μg/day)	303 (141)	200
Vitamin B12 (μg/day)	4.0 (2.4)	1.5
Vitamin B6 (mg/day)	2.3 (0.7)	1.4 (M); 1.2 (F)
Riboflavin (mg/day)	1.6 (0.4)	1.3 (M); 1.1 (F)
Fortified Food Consumer *n* (%) ‡	116 (75)	
B-Vitamin Biomarker Status §		
Red Blood Cell Folate (nmol/L)	954 (410)	340–2270
Serum total B12 (pmol/L)	282 (106)	111–740
Serum HoloTC (pmol/L)	50.8 (24.3)	40–200
Serum MMA (μmol/L)	0.24 (0.19	≤0.36
Vitamin B6 (Plasma PLP; nmol/L)	58.4 (25.8)	20–121
Riboflavin (EGRAC)	1.33 (0.14)	≤1.3
Plasma total homocysteine (μmol/L)	12.0 (3.7)	<10

Data presented as mean (SD) unless otherwise indicated. * History of depression was self-reported. † Reference ranges for dietary intakes based on reference nutrient intake values (RNIs) for 50+ years except for energy where the estimated energy requirements (EARs) for 65–74 years were used [39]; ‡ Consumers of fortified foods were defined as those who consumed foods fortified with B-vitamins at least once per week; § Reference ranges based on analytical laboratory where assay was performed. Abbreviations: BMI, body mass index; MMSE, mini mental state examination; HoloTC, Holotranscobalamin—functional indicator of metabolically active fraction of vitamin B12; MMA, methylmalonic acid—an indicator of vitamin B12 status, a higher MMA status indicates a lower vitamin B12 status; PLP, Pyridoxal-5-phosphate—a measure of vitamin B6 status; EGRAC, Erythrocyte glutathione reductase activation coefficient—a functional indicator of riboflavin status, a higher ratio indicates a lower riboflavin status

The relationship between dietary intakes and corresponding blood biomarker concentrations were examined for each B-vitamin of interest (Figure 2). Dietary intakes for total folate, vitamin B6 and riboflavin were each significantly correlated with the corresponding blood biomarker concentration. Of note, vitamin B12 intake was significantly correlated with serum holoTC but not serum total vitamin B12, the more typically measured biomarker (r = 0.134, p = 0.104; data not shown).

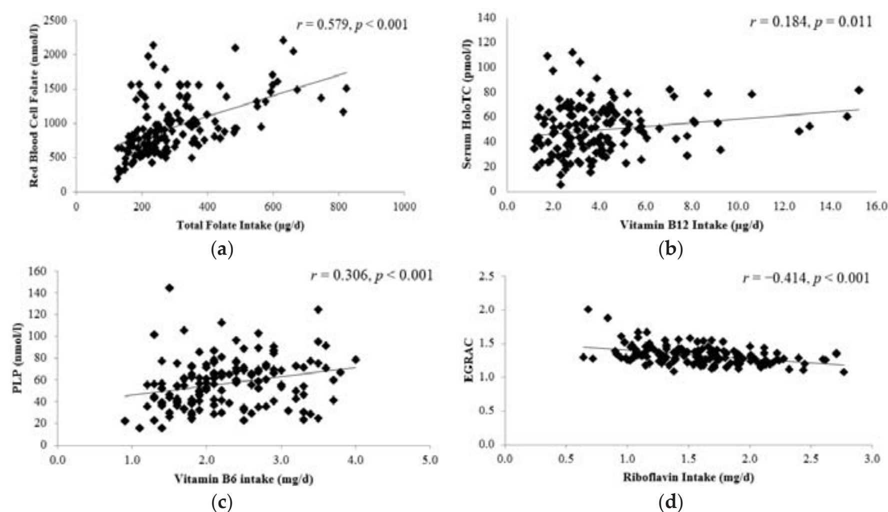

Figure 2. Relationship between dietary intake and biomarker status of B-vitamins at baseline (n = 148): (**a**) association between red blood cell folate and total folate intake; (**b**) association between holoTC and vitamin B12 intake; (**c**) association between pyridoxal-5-phosphate and vitamin B6 (**d**) association between EGRAC and riboflavin intake. Correlations were calculated using Pearson's correlation coefficients (r). $p < 0.05$ was considered significant. HoloTC, holo-transcobalamin; PLP, Pyridoxal-5-phosphate—a measure of vitamin B6 status; EGRAC: erythrocyte glutathione reductase activation coefficient, a functional indicator of riboflavin status. The change in cognitive function score, as measured using MMSE is shown in Table 2. Over the 4-year follow-up period, a significant decline in the mean MMSE by almost 2 points was observed; the scores for each component of the MMSE (i.e., orientation, attention, recall, total verbal and language) also declined significantly, with the exception of registration. Whilst all participants had a MMSE score within the normal range at baseline (i.e., according to the inclusion criteria), 12% had a score indicative of mild cognitive impairment (MMSE range 18–24) at the time of follow-up. Overall, the average decrease in MMSE score per year was 0.42 ± 0.56; but some participants 42 (27%) had a greater than expected rate of cognitive decline (i.e., decrease in MMSE score > 0.56 points per year; [38].

Table 2. Cognitive Characteristics of healthy older adults at initial examination and after 4-year follow-up (n = 155).

	Initial Assessment	**Follow-Up Assessment**	***p*-Value**
Age	69.5 (7.2)	73.4 (7.1)	<0.001
Cognitive Function Score			
MMSE Total Score	29.1 (1.3)	27.5 (2.4)	<0.001
Orientation	9.9 (0.3)	9.8 (0.7)	0.014
Registration	3.0 (0.1)	3.0 (0.1)	0.565
Attention	4.7 (0.7)	4.4 (1.1)	0.004
Recall	2.7 (0.6)	1.8 (1.0)	<0.001
Total Verbal	20.3 (1.1)	19.0 (2.0)	<0.001
Language	8.8 (0.5)	8.5 (0.8)	<0.001
Impaired Cognition n (%) *	0 (0)	19 (12)	

Data presented as mean (standard deviation) unless otherwise indicated. * Impaired cognition defined as an MMSE score <25 [30]. Differences between the two time points were assessed using a paired t-test. $p \leq 0.05$ considered statistically significant. Abbreviations: MMSE, Mini mental state examination.

The influence of several lifestyle factors, B-vitamin dietary intake and B-vitamin biomarker status, as determinants of rate of cognitive decline are shown in Table 3. Of the general health and lifestyle factors examined only age and baseline MMSE score were predictive of cognitive decline. In addition, after adjustment for age and baseline MMSE score, no associations were observed between disease history (CVD, diabetes and gastrointestinal; data not shown) or medication use with the exception of use of analgesic medication ($p = 0.035$; data not shown). Vitamin B6 was found to be the only B-vitamin that was predictive of cognitive decline. After adjustment for age and baseline MMSE score, individuals with lower vitamin B6 biomarker status (PLP range $\leq$ 43.3 nmol/L; $p = 0.002$) or lower dietary B6 intakes (0.9–1.4 mg/day; $p = 0.018$) were at a 3.5–4 fold greater risk of cognitive decline. None of the other B vitamins or plasma homocysteine concentrations were associated with the risk of cognitive decline in this cohort.

Table 3. Lifestyle factors, B-vitamin dietary intake and B-vitamin biomarker status as predictors of cognitive decline in older adults.

	Range	Odds Ratio	95% CI	*p*-Value
Age		1.11	(1.05–1.16)	<0.001
Female Gender		0.69	(0.34–1.41)	0.310
BMI		1.04	(0.95–1.13)	0.410
Smoking		2.82	(0.55–14.56)	0.216
MTHFR TT genotype		1.82	(0.56–5.93)	0.318
Secondary level education		1.37	(0.62–3.03)	0.434
Depression		0.40	(0.08–2.18)	0.293
B-Vitamin Biomarker Status				
Low folate status (RBC Folate) *	(191–719 nmol/L)	1.81	(0.83–3.91)	0.134
Low vitamin B12 (serum total B12) *	(118–231 pmol/L)	1.14	(0.52–2.49)	0.750
Low vitamin B6 (PLP) *	(15.4–42.9 nmol/L)	3.49	(1.60–7.62)	0.002
Low riboflavin status (EGRAC) †	$\geq$1.3	1.01	(0.48–2.15)	0.972
High homocysteine	(12.6–25.4 μmol/L)	1.50	(0.58–3.85)	0.402
B-Vitamin Dietary Intake ‡				
Low Folate intake	(124–166 μg/day)	2.55	(0.78–8.41)	0.123
Low vitamin B12 intake	(1.2–1.8 μg/day)	1.04	(0.29–3.78)	0.949
Low vitamin B6 intake	(0.9–1.4 mg/day)	4.08	(1.24–13.50)	0.021
Low riboflavin intake	0.6–1.0 mg/day)	0.41	(0.13–1.32)	0.136

Logistic regression was performed to determine predictors of cognitive decline (defined as a decrease in MMSE $\geq$ 0.56 points/year). The reference category for the lifestyle variables were as follows; sex, male gender; education, 3rd level; depression, no history; MTHFR 677 genotype, MTHFR 677 CC and CT genotype combined. * 'Low' B-vitamin status (with the exception of riboflavin) was defined as the bottom tertile of biomarkers; the reference category was the top two tertiles. † 'Low' riboflavin was defined by established cut-off values for EGRAC (low $\geq$1.3), the reference category was EGRAC < 1.3. ‡ 'Low' dietary intakes were identified by the bottom 10% of intake for each nutrient, the reference category was the remaining intake. Abbreviations: BMI, body mass index; MTHFR, methylenetetrahydrofolate; RCF, red cell folate; PLP, Pyridoxal-5-phosphate; EGRAC, Erythrocyte glutathione reductase activation coefficient.

4. Discussion

This study in healthy older adults, initially with normal cognitive performance, indicates that vitamin B6 is an important predictor of cognitive decline in ageing. Lower dietary intake and biomarker status of vitamin B6 were associated with a greater rate of cognitive decline over a subsequent 4 years follow-up period. No significant association of dietary intake or biomarker status with cognitive decline were observed for the other B-vitamins (folate, vitamin B12 and riboflavin). To our knowledge, this is the first longitudinal study to consider the impact of both dietary intake and biomarker status of all four relevant B-vitamins involved in one-carbon metabolism on cognitive health in ageing.

Whilst the influence of vitamin B6 on cognition has not been as fully investigated as folate and vitamin B12 a number of studies have reported observations consistent with the current study. Our results showed that participants with lower status of vitamin B6 (PLP; the measure of active vitamin B6) at baseline were 3.5 times more likely to have a greater rate of cognitive decline over a 4 years follow-up period. Furthermore, the association between vitamin B6 and cognitive decline was not confined to those with clinical deficiency, lower status included individuals in both the deficient (PLP < 30 nmol/L) and sufficient range (PLP 30–43 nmol/L) which would suggest that optimal vitamin B6 may be important for cognitive health in ageing. Consistent with the biomarker data, those with lower dietary intakes of vitamin B6 at baseline were 4 times more likely to have a greater rate of cognitive decline over the 4 years time period. Our results are in good agreement with findings from other studies, low vitamin B6 status (PLP < 46 nmol/L) and corresponding dietary intakes were previously associated with cognitive decline over a 3 years period in the Veteran Affairs Normative Ageing Study [40]. There is also evidence from several cross-sectional studies to support an association between low vitamin B6 and cognitive dysfunction [9,17,41,42] and Alzheimer's disease [43,44]. Furthermore, vitamin B6 status was associated with cognitive performance in high functioning older adults at baseline, though not with cognitive decline over the 7 years follow-up period in the MacArthur study of Successful Ageing [8]. Certain other studies have failed to detect any significant association between vitamin B6 and cognitive function, however, these studies have relied on dietary intake measures alone with no corresponding measurement of blood biomarker status [45–47]. Few RCTs have investigated the independent effect of vitamin B6 on cognitive function and only one very early study reported beneficial effects of vitamin B6 supplementation on memory [19]. Subsequent RCTs have investigated the effect of vitamin B6 in combination with folate and vitamin B12, with some studies reporting beneficial effects on cognitive function however the independent effect of vitamin B6 cannot be determined [18,20].

Whilst elevated plasma homocysteine, low folate and, to a lesser extent, vitamin B12 status have been frequently associated with cognitive decline [11–15,48] there was no evidence of significant associations for these biomarkers in the current study. A number of other studies have reported similar findings [49–52]. The findings in the current study may be explained to some degree by the fact that vitamin B6 seemed to be the limiting nutrient within the cohort. There was a greater incidence of deficiency of vitamin B6 (11% clinical deficiency) compared with folate (3%) or vitamin B12 (0%). Also, the lack of a significant association between cognitive decline and plasma homocysteine concentration is almost certainly is a reflection of the low prevalence of folate deficiency. Furthermore, the concept that the association between B-vitamin status and cognition is determined by the limiting nutrient within that population group is further supported by evidence from published RCTs. One trial of healthy older adults in New Zealand reported no benefit of combined B-vitamin supplementation on cognitive function [21], whereas another similar study from the Netherlands showed that supplementation with folic acid significantly improved cognitive performance [18]. A notable difference between these two studies was that baseline folate status tended to be far lower in the Dutch trial, suggesting that the cognitive benefit related to the correction of sub-optimal B-vitamin status whereas additional B-vitamins to an already optimal population may have no beneficial effect.

The mechanism linking vitamin B6 with cognitive health in this and other studies in ageing is not clear however, it is biologically plausible given the widespread functions of vitamin B6 within the brain and nervous system [53,54]. Vitamin B6 has a crucial role in the synthesis of a variety of neurotransmitters including dopamine and serotonin [55] and can act as a potent antioxidant [56,57]. In addition, higher vitamin B6 intakes have been associated with greater grey matter volume [58] and combined B-vitamin supplementation (including vitamin B6) has been shown to slow brain atrophy, an important feature cognitive dysfunction [27].

The current study has a number of strengths and limitations that merit comment. To our knowledge, this is the first longitudinal study to investigate the association between cognitive decline and all relevant B-vitamins along with their corresponding dietary intakes. The MMSE is the most

widely used screening tools for cognitive dysfunction and although it has been criticised for lacking sensitivity, few previous studies have used it to measure cognitive change in a healthy older population. However, a meta-analysis reported a mean decline in MMSE of between 0.16 and 0.56 points per year in cognitively healthy people which compares favourably to the overall rate of decline observed in this study (mean 0.39 points per year) [38]. In addition, the rate of decline observed in this study was identical to that observed in the Rotterdam Study of community dwelling older adults free from cognitive impairment [59]. While the use of the MMSE may be perceived as a limiting factor in the current study, it could be argued that its use would only attenuate the associations observed and that the use of more sensitive tools would have, if anything, detected more subtle differences thus strengthening the results. Another well-recognised limitation of longitudinal studies of this kind is that individuals with the greatest decline in cognitive function are more likely to be lost to follow-up [60]. Indeed, in this study the non-participants were more likely to be older but any non-response bias would ultimately underestimate the associations between baseline B-vitamin status and cognitive decline and this could not have influenced the current findings.

5. Conclusions

In conclusion, vitamin B6 may be an important (often overlooked) protective factor in helping maintain cognitive health in ageing, especially in a folate and vitamin B12 replete population. Lower vitamin B6 status (as assessed by both dietary intake and biomarker status) at baseline predicted a greater than expected rate of cognitive decline over a 4-year period in healthy free living older adults. These findings are important because optimising vitamin B6 status in older people, through the use of fortified foods or supplements, may have a positive impact on cognition in ageing. Further research in this area in the form of well-designed randomised controlled trials targeted at populations with sub-optimal status are required in order to confirm a cause and effect relationship between B-vitamin status and cognitive health in ageing.

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Abbreviations

MMSE	Mini-mental state examination
HoloTC	Holotranscobalamin—functional indicator of metabolically active fraction of vitamin B12
MMA	methymalonic acid—an indicator of vitamin B12 status, a higher MMA status indicates a lower vitamin B12 status
PLP	Pyridoxal-5-phosphate—a measure of vitamin B6 status
EGRAC	Erythrocyte glutathione reductase activation coefficient—a functional indicator of riboflavin status, a higher ratio indicates a lower riboflavin status

Appendix A

Table A1. A comparison of baseline characteristics between participants and non-participants (i.e., those lost to follow-up).

	Participants (n = 155)	Non-Participants (n = 100)	p-Value
General Characteristics			
Age (years)	69.5 (7.3)	72.2 (8.1)	0.007
Male n (%)	60 (39)	34 (34)	0.530
BMI (kg/m^2)	27.5 (4.2)	27.3 (3.5)	0.981
Smokers n (%)	6 (4)	5 (5)	
Cognitive Function Score			
MMSE Total Score	29.1 (1.3)	28.7 (1.4)	0.093
Orientation	9.9 (0.3)	9.8 (0.5)	0.140
Registration	3.0 (0.1)	3.0 (0.1)	0.273
Attention	4.7 (0.7)	4.6 (0.9)	0.243
Recall	2.7 (0.6)	2.6 (0.6)	0.825
Total Verbal	20.0 (1.1)	20.0 (1.2)	0.263
Language	8.8 (0.5)	8.6 (0.6)	0.094
B-vitamin Biomarker Status			
Red blood cell folate (nmol/L)	954 (410)	851 (359)	0.080
Serum total vitamin B12 (pmol/L)	282 (106)	257 (127)	0.013
Serum HoloTC (pmol/L)	50.8 (24.3)	47.1 (28.8)	0.381
Serum MMA (μmol/L)	0.24 (0.19)	0.36 (0.56)	0.035
Vitamin B6 (Plasma PLP; nmol/L)	58.4 (25.8)	54.3 (22.9)	0.314
Riboflavin (EGRAC)	1.33 (0.14)	1.34 (0.15)	0.387
Plasma total homocysteine (μmol/L)	12.0 (3.7)	13.1 (4.4)	0.117
Gastric Function			
Pepsinogen I (μg/L)	126.8 (70.8)	135.3 (78.2)	0.515
Pepsinogen Ratio[2]	8.4 (6.7)	8.0 (6.6)	0.713

Values represented as mean (SD). Differences in baseline characteristics between those that participated in the 4-year follow-up and those that did not participate in the follow-up were compared using one-way ANCOVA with adjustment for age for continuous variables (on log transformed data were appropriate). Differences in categorical variables were assessed using Chi-squared analysis. $p < 0.05$ was significant.

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Sex-Specific Muscular Maturation Responses Following Prenatal Exposure to Methylation-Related Micronutrients in Pigs

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Abstract: Supplementation of micronutrients involved in DNA methylation, particularly during pregnancy, is recommended because of its impacts on human health, but further evidence is needed regarding the effects of over-supplementation and differences between sexes. Here, a porcine model was used to assess effects of maternal supplementation with one-carbon-cycle compounds during prenatal and postnatal stages on offspring muscle development. Sows received either a standard diet (CON) or a standard diet supplemented with folate, B6, B12, methionine, choline, and zinc (MET) throughout gestation. Myogenesis-, growth-, and nutrient utilization-related transcript expression was assessed using quantitative PCR. Organismal phenotype and gene expression effects differed significantly between males and females. Male MET-offspring showed increased fetal weight during late pregnancy but decreased live weight postnatally, with compensatory transcriptional responses comprising myogenic key drivers (*Pax7*, *MyoD1*, *myogenin*). In contrast, female weights were unaffected by diet, and mRNA abundances corresponded to a phenotype of cellular reorganization via *FABP3*, *FABP4*, *SPP1* and Insulin-like Growth Factor-signaling. These findings in an animal model suggest that supplementation during pregnancy with methylation-related micronutrients can promote sex-specific myogenic maturation processes related to organismal growth and muscle metabolism. The usage of maternal dietary supplements should be more carefully considered regarding its ability to promote fetal and postnatal health.

Keywords: fetal programming; maternal diet; methyl donors; myogenesis; one-carbon cycle; pigs

1. Introduction

Micronutrients involved in processes related to DNA methylation (e.g., folate, vitamins B6 and B12, methionine, choline, zinc) are commonly provided as supplements during pregnancy to benefit offspring development. However, an excessive maternal intake of methylation-related micronutrients acts as a nutritional insult, inducing hypermethylation at epi-labile loci and culminating in phenotypic [1,2] and metabolic variation [3,4]. In particular, some maternal dietary methylation-related supplementation regimens have been shown to adversely affect skeletal muscle tissue development in murine fetuses [5–7]. Since rodents require a higher relative dose of such micronutrients than humans or pigs [8,9], porcine models have been used to study paternal or maternal dietary supplementation with methylation-related micronutrients [8,10,11]. In pigs, high maternal intake

of these micronutrients is associated with increased fetal mass during late gestation, i.e., during the period when secondary muscle fibers are formed and hyperplasia occurs [12]. Extracts obtained from fetal skeletal muscle implicate insulin-like growth factor (IGF)-related compounds in the observed weight differences [8]. Indeed, in vitro exposure to the methylation-related micronutrient folate affects muscle myoblast differentiation via the IGF downstream target Akt and transcription factors of the myogenic lineage, such as MyoD and myogenin [13]. Furthermore, the phenotypic and transcriptomic effects of dietary supplementation with methylation-related micronutrients have been described to be sex-specific [14–17]. Thus, important gaps exist in our knowledge regarding the beneficial effects of dietary methylation-related micronutrient supplementation.

Shifts in body weight, muscle fiber distribution, myogenesis, and nutrient utilization could be detrimental for health [18]. The goal of this study was to determine whether a maternal diet enriched with methylation-related micronutrients affects transcripts involved in myogenesis, growth, and nutrient utilization. Potential sex effects were considered. The composition of muscle by fiber type can be assessed by detecting gene expression patterns of Myosin heavy chain isoforms (MyHC) [19]. In porcine fetuses and juvenile pigs, expression of key factors was analyzed in *M. longissimus dorsi* to approximate proliferative and metabolic features, at the transcriptional level, of cells originating from the myogenic lineage.

2. Materials and Methods

2.1. Animals, Diets, Sample Collection

The experimental setup was in accordance with the Animal Protection legislation of the federal state and of the country of Mecklenburg-Western Pomerania, Schwerin, Germany. Animal care and tissue collection procedures were approved by the Scientific Committee of the Leibniz-FBN (70.1.2.03.201). The animal experiment was performed as described previously [8]. Briefly, Piétrain gilts were randomly assigned to receive either a standard diet (CON) or a standard diet supplemented with one-carbon-cycle substrates and associated cofactors (MET), starting from 10 days before artificial insemination throughout pregnancy (Figure 1). Specifically, methionine, choline, folic acid, vitamin B6, vitamin B12, and zinc were supplemented (Table 1). The doses of the altered micronutrients match about 80% of their known tolerable upper intake levels. For dietary vitamin B12 levels, no overdosing is known [20]. Hence, the chosen supplementation was adapted to high doses used in human studies [21,22] to ensure maximal uptake of vitamin B12. Gilts were individually reared in cages on flat decks in environmentally controlled rooms. Access to pelleted feed has been restricted to 2.8 kg/day. The body weight and feed intake during gestation were not significantly different between gilts fed the CON or MET diets. The number of fetuses/offspring per sow did not differ significantly due to the maternal diets.

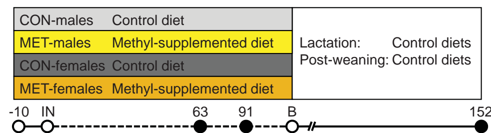

Figure 1. Experimental design. Piétrain gilts were fed either a CON or a MET diet from 10 days before insemination (IN) until tissue sampling at 63 dpc, 91 dpc, and 152 dpn, respectively, when *M. longissimus dorsi* (n = 40 per stage) was collected. IN—insemination; B—birth; dpc—days post conception; dpn—days post natum.

At 63 dpc and 91 dpc, a subset of gilts were exsanguinated and uteri were quickly removed and dissected (n = 3 per stage and diet). Fetal muscle tissue (*M. longissimus dorsi*) was immediately collected, frozen in liquid nitrogen, and stored at −80 °C until further analyses (63 dpc: n = 40; 91 dpc: n = 40). Another subset of gilts (n = 3 per diet) was kept until delivering live-born progeny (114 dpc).

During lactation, sows received a standard lactation diet. Male piglets were castrated at 4 dpn by authorized qualified personnel in approved user's establishments. Post-weaning (28 dpn, week 4), the progeny was fed control diets ad libitum. The progeny's muscle tissue (*M. longissimus dorsi*) was collected at 152.3 ± 2.6 dpn, frozen in liquid nitrogen, and stored at −80 °C until further analyses (n = 40). In total, the study comprises 120 individual samples (n = 9–11 per sex per stage per dietary group). Notably, pigs attain sexual and reproductive maturity not before six months of age [23,24].

Table 1. Dietary amount of altered micronutrients fed to gilts during gestation (per kg diet).

Methylating Micronutrient	Sow CON Diet	Sow MET Diet
Methionine, mg	2050	4700
Choline, mg	500	2230
Folic acid, mg	3	92.2
Vitamin B6, mg	3	1180
Vitamin B12, µg	31	5930
Zinc, mg	21.8	149

CON—Standard diet; MET—Standard diet supplemented with methylating micronutrients.

2.2. Phenotype Data Analyses

Weights and carcass characteristics were analyzed via variance analysis (version 9.4., SAS Institute, Cary, NC, USA), and effects represented by diet, sex, and gilt were included. A repeated measurement statement was applied to compute postnatal live weights. The carcass characteristics were corrected for live weight. Differences were considered significant at $p \leq 0.05$.

2.3. RNA Isolation and cDNA Synthesis

In total, 120 muscle tissue samples (n = 40 per stage) were used to isolate total RNA using Tri-Reagent per manufacturer's directions (Sigma-Aldrich, Taufkirchen, Germany). Quantification and purification were performed as previously described [8]. All RNA samples were stored at −80 °C until downstream analyses were performed. Samples were checked for contamination by genomic DNA. First-strand cDNA was synthesized from 2 µg of total RNA using random primers and oligo d(T) 13VN in the presence of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). The cDNA samples were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany), eluted in 20 µL Aqua dest. and stored at −20 °C until further analyses.

2.4. Primer Sequences and Primer Validation

A number of transcripts associated with myogenesis, growth, myosin heavy chains, lipid metabolism, and energy metabolism were analyzed. mRNA sequences of both target and reference genes were obtained from the National Center for Biotechnology Information (NCBI) Gene database (NCBI, Bethesda, MD, USA). Primer sequences are listed in Table S2. The specificity of primer sequences was checked using tissue-specific test samples. Transcript levels of selected target and reference genes were quantified by qPCR performed on a LightCycler 480 system (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, reactions were performed in a final volume of 10 µL using 5.0 µL of LightCycler 480 SYBR Green I Master (Roche), 0.5 µL (10 µM) of each primer, 2 µL (40 ng) cDNA, and 2.0 µL of Aqua dest. The temperature profiles were comprised of an initial denaturation step at 95 °C for 10 min followed by 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 10 s and extension/fluorescence acquisition at 72 °C for 15 s. Amplified products were subjected to melting curve analyses and gel electrophoresis to verify the absence of non-specific products.

2.5. Microfluidic High-Throughput qPCR

Selected target and reference genes were analyzed in duplicate on a BioMarkTM HD system (Fluidigm, San Francisco, CA, USA) following the manufacturer's instructions (n = 40 per stage;

balanced by diet and sex). In brief, a pre-amplification step was performed in a final volume of 5 µL using 2.5 µL of TaqMan PreAmp Master Mix (Applied Biosystems, Waltham, MA, USA), 0.75 µL of Aqua dest., 0.5 µL (500 nM) of pooled primer mixture (comprising aliquots of all primers set to be included), and 1.25 µL purified cDNA. The temperature profile was comprised of an initial denaturation step at 95 °C for 10 min followed by 10 cycles consisting of 95 °C for 15 s and 60 °C for 4 min. Subsequently, a clean-up was performed by adding 0.2 µL exonuclease I reaction buffer, 0.4 µL exonuclease I (20 U/µL), and 1.4 µL of Aqua dest. The temperature profile consisted of 37 °C for 30 min and 80 °C for 15 min. Furthermore, the pre-amplified and cleaned-up cDNA was diluted (1:10) using Tris-EDTA (Ethylenediaminetetraacetic acid) buffer (10mM Tris/HCl; 1.0 mM EDTA).

Using the 96.96 dynamic array, PCRs were performed in a final volume of 10 µL. Specifically, 5 µL sample premix (2.5 µL SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Munich, Germany) with low 6-carboxy-X-rhodamine (ROX), 0.25 µL DNA Binding Dye Sample Loading Reagent, 2.25 µL pre-amplified and diluted cDNA) and 5 µL assay premix (2.5 µL Assay Loading Reagent, 2.25 µL DNA Suspension Buffer, 0.25 µL primer mixture) were prepared and transferred to the primed array. Subsequently, the final PCR mix was formed due to the integrated fluidic circuits and physical loading protocols finally generating 22,080 data points (92 transcripts measured in duplicate in 40 samples at 3 time points). The final primer concentration per individually performed PCR was 500 nM. The temperature profile was comprised of an initial denaturation step at 95 °C for 60 s followed by 30 cycles consisting of 96 °C for 5 s and 60 °C for 20 s. Raw data (quantification cycle) was obtained using BioMarkTM Data Collection software (version 4.1.3, Fluidigm, San Francisco, CA, USA).

2.6. Transcript Data Preprocessing and Analysis

Expression data were compared to quality control criteria and predicted outliers were removed (melting curve of amplified products, lower and upper bound of quantification cycle) as proposed by the manufacturer (Real-time PCR analysis software, version 4.1.3, Fluidigm, San Francisco, CA, USA). To account for variation in RNA input and efficiency of reverse transcription, values were normalized by geometric averaging of reference genes as described previously [25,26]. Transcriptional alterations due to diet and sex were analyzed stage-specifically via variance analysis (SAS, version 9.4., SAS Institute, Cary, NC, USA), and effects represented by diet, sex, and gilt confounded with diet were included. To account for multiple testing, p-values were converted to a set of q-values [27]. Unless specified, differences were considered significant at $p \leq 0.05$ and $q \leq 0.30$. Fold changes displaying differences in mRNA abundances were calculated from least square means (positive FC: CON < MET; negative FC: CON > MET).

To model the diversity of muscle fiber types, data of transcripts associated with myosin heavy chain isoforms (*MYH1*, *MYH2*, *MYH3*, *MYH4*, *MYH7*) were used to calculate a principal component analysis. The first two dimensions were used to plot diet- and sex- dependent distribution (eigenvalue > 1 for each principal component).

2.7. Validation of Microfluidic High-Throughput qPCRs

To verify microfluidic high-throughput qPCRs, selected target (*FABP4*, *HSD11B1*, *MYH4*, *SPP1*) and reference genes (*RPL32*, *RPL10*) were quantified by qPCR performed on a LightCycler 480 system (Roche, Mannheim, Germany) as described above (n = 40 per stage). Data was factorial normalized and analyzed as described above (SAS). The level of significance was set at $p \leq 0.05$. Correlation of normalized expression values was calculated by Spearman's Rho.

3. Results

The aim of the current study was to investigate whether prenatal exposure to methylation-related micronutrients ("methyl-supplemented", or MET, versus standard dosing, or CON) affects muscle development of the offspring, and its implication for organismal growth. Responsiveness to the dietary

challenge was analyzed in male and female pigs via weight recordings and expression profiling at different ontogenetic stages.

3.1. Maternal Supplementation with Methylation-Related Micronutrients Affected Fetal Weight and Live Weight in Males

Weights at fetal stages (63 dpc; 91 dpc) and live weights (birth weight until 152 dpn) are displayed in Figure 2. Litter size was unaffected between CON and MET sows. At 63 dpc, fetal weight did not differ by diet between groups of males (CON-males: 127.8 ± 5.6 g; MET-males: 141.5 ± 5.6 g; $p = 0.094$) or females (CON-females: 151.0 ± 5.4 g; MET-females: 142.4 ± 4.9 g; $p = 0.243$). However, at 91 dpc, fetal weight was significantly higher in methyl-supplemented males (CON-males: 677.8 ± 26.6 g; MET-males: 790.4 ± 34.5 g; $p = 0.015$) and tended to be higher in methyl-supplemented females (CON-females: 656.8 ± 27.3 g; MET-females: 728.6 ± 23.8 g; $p = 0.056$) compared with sex-matched controls. Birth weight was unaffected by maternal diet in both males and females. Postnatally, MET-males had a significantly lower live weight than CON-males at 77 dpn (week 11), but females exhibited no difference in live weights by diet. Accordingly, at 152 dpn, live weight was significantly different by diet between males (CON-males: 105.0 ± 1.6 kg; MET-males: 95.1 ± 1.7 kg; $p < 0.001$) but not different between females (CON-females: 94.2 ± 1.7 kg; MET-females: 92.7 ± 2.1 kg; $p = 0.564$).

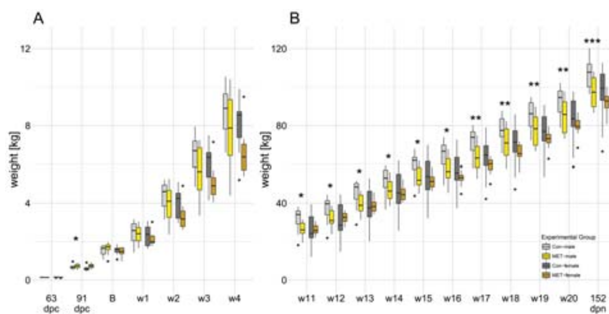

Figure 2. Box plots representing fetal weights and live weights in progeny exposed to maternal CON and MET diets. Data recorded (**A**) at fetal stages display individual experiments. Data recorded from (**A**) birth to week 4 (28 dpn), (**B**) from week 11 (77 dpn) to week 20 (140 dpn), and at 152 dpn represents a time course. Fetal weight was increased in MET-males at 91 dpc. Live weight was lowered in MET-males from week 11 (77 dpn) until 152 dpn. CON—Standard diet; MET—Standard diet supplemented with methylating micronutrients; CON-males: **light gray**; CON-females: **dark gray**; MET-males: **yellow**; MET-females: **orange**; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; dpc—days post conception; dpn—days post natum.

3.2. Increased Percent Lean Mass in MET-Males

Postnatal body characteristics are displayed in Table S1. Specifically, percent lean mass was higher in MET-males compared to CON-males (CON-males: 59.02% ± 0.40%; MET-males: 60.57% ± 0.53%; $p = 0.030$), but similar between females (CON-females: 61.67% ± 0.41%; MET-females: 61.62% ± 0.52%; $p = 0.935$). Body characteristics related to back fat, pH, and meat color were not affected by diet or sex.

3.3. Gene Expression Pattern Specific for Maternal Diet and Sex

In MET-males (63 dpc), transcripts of *Akt1*, *FLT4*, and *VEGFB* were more abundant compared to CON-males ($p \leq 0.05$ but corresponding q-value of 0.68) (Table 2 and Table S2). At 91 dpc, transcripts associated with growth (*PDGFA*, *VEGFC*), myogenesis (*MYF6*), and lipid metabolism (*PPARD*) were less abundant in MET-males compared to CON-males (corresponding q-values < 0.30). At 152 dpn, MET-males showed greater mRNA abundances of *MyoD1*, *Pax7*, and *myogenin* (corresponding q-values < 0.30).

Table 2. Transcripts affected by diet and sex at prenatal and postnatal time points (excerpt from Table S2).

Gene	Males 63 dpc		Females 63 dpc		Males 91 dpc		Females 91 dpc		Males 150 dpn		Females 150 dpn	
	p	FC [1]	*p*	FC [1]	*p*	FC [1]	*p*	FC [1]	*p*	FC [1]	*p*	FC [1]
Akt1	0.026 *	+1.40	0.002	+1.51	ns	ns	0.046	+1.29	ns	ns	ns	ns
FABP3	ns	ns	ns	ns	ns	ns	0.001	+1.38	ns	ns	ns	ns
FABP4	ns	ns	ns	ns	ns	ns	0.031	+1.39	ns	ns	ns	ns
FBXO32	ns	ns	0.038	+1.33	ns	ns	ns	ns	ns	ns	ns	ns
FLT1	ns	ns	ns	ns	ns	ns	0.017	+1.41	ns	ns	ns	ns
FLT4	0.029 *	+1.36	ns	ns	ns	ns	0.018	+1.55	ns	ns	ns	ns
FST	ns	ns	ns	ns	ns	ns	0.026	−2.65	ns	ns	ns	ns
GALK1	ns	ns	ns	ns	ns	ns	0.035	+1.25	ns	ns	ns	ns
GHR	ns	ns	0.023	+1.54	ns	ns	ns	ns	ns	ns	ns	ns
GLUT1	ns	ns	ns	ns	ns	ns	0.024	+1.37	ns	ns	ns	ns
GLUT4	ns	ns	ns	ns	ns	ns	0.025	+1.30	ns	ns	ns	ns
GSK3b	ns	ns	ns	ns	ns	ns	0.023	+1.38	0.029 *	+1.41	ns	ns
HGF	ns	ns	0.006	+1.61	ns	ns	ns	ns	ns	ns	ns	ns
HSD11B1	ns	ns	ns	ns	ns	ns	0.013	+1.40	ns	ns	ns	ns
IGFBP5	ns	ns	ns	ns	ns	ns	0.027	+1.26	ns	ns	ns	ns
KDR	ns	ns	ns	ns	ns	ns	0.018	+1.32	ns	ns	ns	ns
MAT2A	ns	ns	0.023	+1.22	ns	ns	ns	ns	ns	ns	ns	ns
MAT2B	ns	ns	ns	ns	ns	ns	0.025	+1.25	ns	ns	ns	ns
MET	ns	ns	ns	ns	ns	ns	ns	ns	0.023 *	+1.54	0.026 *	−1.50
MSTN	ns	ns	0.029	+1.56	ns	ns	ns	ns	ns	ns	ns	ns
MYF5	ns	ns	0.035	+1.29	ns	ns	ns	ns	ns	ns	ns	ns
MYF6	ns	ns	ns	ns	0.043	−1.38	ns	ns	ns	ns	ns	ns
MYH2	ns	ns	ns	ns	ns	ns	0.044	+1.69	ns	ns	ns	ns
MyoD1	nd	nd	nd	nd	ns	ns	ns	ns	0.009	+2.03	ns	ns
Myogenin	ns	ns	ns	ns	ns	ns	ns	ns	0.003	+1.86	ns	ns
NCAPD2	ns	ns	0.032	+1.29	ns	ns	ns	ns	0.030 *	+1.43	ns	ns
Pax7	ns	ns	ns	ns	ns	ns	ns	ns	0.003	+1.84	ns	ns
PC	ns	ns	ns	ns	ns	ns	0.011	+1.59	ns	ns	ns	ns
PDGFA	ns	ns	ns	ns	0.047	−1.29	ns	ns	0.028 *	+1.48	ns	ns
PDPK1	ns	ns	0.013	+1.28	ns	ns	ns	ns	ns	ns	ns	ns
PFKM	ns	ns	0.008	+1.31	ns	ns	ns	ns	ns	ns	ns	ns
PIK3CA	ns	ns	ns	ns	ns	ns	ns	ns	0.018 *	+1.54	ns	ns
PIK3CD	ns	ns	ns	ns	ns	ns	0.041	+1.47	ns	ns	ns	ns
PIK3CG	ns	ns	ns	ns	ns	ns	0.022	+1.42	0.039 *	+1.60	ns	ns
PPARA	ns	ns	0.032	+1.28	ns	ns	0.023	+1.39	ns	ns	ns	ns
PPARD	ns	ns	ns	ns	0.013	−1.50	ns	ns	ns	ns	ns	ns
PPARGC1A	ns	ns	0.040	+1.46	ns	ns	ns	ns	ns	ns	ns	ns
PRKAA2	ns	ns	0.023	+1.37	ns	ns	ns	ns	ns	ns	ns	ns
SPP1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	0.043 *	+4.34
VEGFB	0.025 *	+1.14	0.012	+1.12	ns	ns	ns	ns	ns	ns	ns	ns
VEGFC	ns	ns	ns	ns	0.032	−1.51	ns	ns	ns	ns	ns	ns

CON—Standard diet; MET—Standard diet supplemented with methylating micronutrients; FC—fold change; ns—not significant; nd—not detectable; dpc—days post conception; dpn—days post natum; [1] Positive FC: CON < MET; negative FC: CON > MET; * corresponding $q > 0.30$.

In MET-females, transcripts associated with fiber formation (*FBXO32*, *MSTN*, *MYF5*), lipid metabolism (*PPARA*), and energy metabolism (*PFKM*, *PPARGC1A*, *PRKAA2*) were more abundant compared to CON-females at 63 dpc. At 91 dpc, transcripts related to IGF signaling (*Akt1*, *GSK3β*, *PIK3CD*, *PIK3CG*), myogenic control (*FST*), growth (*FLT1*, *FLT4*, *KDR*), lipid metabolism (*FABP3*, *FABP4*, *PPARA*), and energy metabolism (*GALK1*, *GLUT1*, *GLUT4*, *PC*) were more abundant in MET-females compared to CON-females. At 152 dpn, differences for *SPP1* and *MET* reached $p \leq 0.05$; however, the corresponding q-value was 0.99.

3.4. Myosin Heavy Chain Isoforms

At 63 dpc and 152 dpn, mRNA abundances of myosin heavy chain isoforms (*MYH1*, *MYH2*, *MYH3*, *MYH4*, *MYH7*) were unaffected by diet or sex. At 91 dpc, *MYH2* and *MYH4* were upregulated in MET-females (Table 2, Tables S2 and S3). Accordingly, a principle component analysis (PCA) calculated for expression values of myosin heavy chain isoforms revealed that samples were unsystematically distributed at 63 dpc and 152 dpn (Figure 3). At 91 dpc, the cluster of MET-females might follow the shifts of the variance components represented by both *MYH2* and *MYH4*.

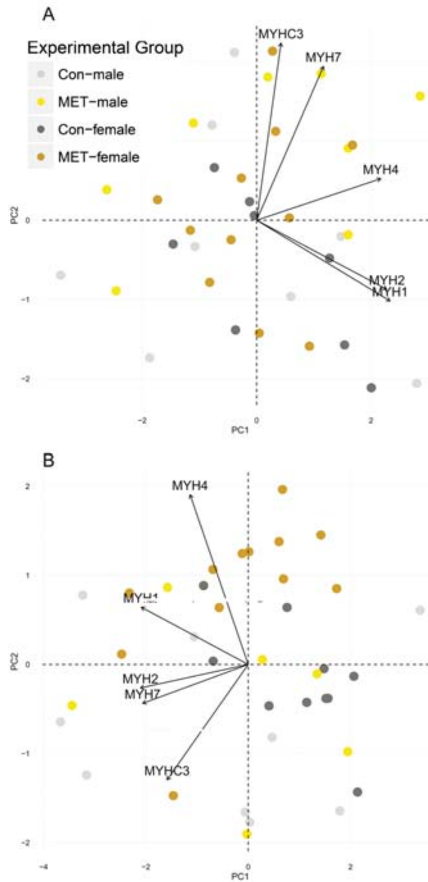

Figure 3. *Cont.*

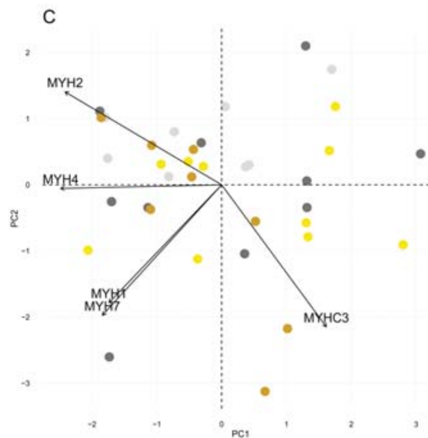

Figure 3. Principal component analysis-biplot of individual pigs and myosin heavy chain isoforms. Expression data of *MYH1*, *MYH2*, *MYH3*, *MYH4*, and *MYH7* was used to describe differences among individual pigs affiliated with CON-males (**light gray**), CON-females (**dark gray**), MET-males (**yellow**), and MET-females (**orange**) at (**A**) 63 dpc, (**B**) 91 dpc, and (**C**) 152 dpn, respectively. CON—Standard diet; MET—Standard diet supplemented with methylating micronutrients; dpc—days post conception; dpn—days post natum.

3.5. Verification of qPCR Results

The qPCR results obtained via Fluidigm and LightCycler 480 systems were correlated to verify differences in mRNA abundance of selected transcripts, specifically for *FABP4*, *HSD11B1*, *MYH4*, and *SPP1* (Table S3). Significant correlations between expression values ranged between 0.67 and 1.00, which suggest reliable results. The fold change (FC) was reproducible on both systems. Although results of *MYH4* measured via high-throughput qPCR did not differ (p = 0.08, Table S3), expression values retrieved from the LightCycler 480 system were increased in MET-females at 91 dpc.

4. Discussion

Skeletal muscle is a dynamic tissue that adapts to external stimuli through transcriptional, biochemical, and morphological variations. This study focused on the time during which primary and secondary fiber hyperplasia are completed, measuring growth performance and mRNA abundances of key factors involved in myogenesis, proliferation, and energy utilization. Male pigs exposed to methylation-related micronutrients exhibited higher fetal weight during late pregnancy but lower live weight during the postnatal period. Effects on organismal weight following excessive intrauterine exposure to methylation-related micronutrients have been reported in studies on mixed sexes, but findings were inconsistent, apparently due to either single or combined effects of such nutrients [4,28–30]. Thus, our study supports previous work indicating that methylation-related micronutrients can affect offspring growth (“methyl-supplemented”, or MET, versus standard dosing, or CON).

The IGF system may have a prominent role in mediating the observed weight effects [8,31,32]. In our study, phenotypic variations were reflected by compensatory transcriptional variations: the prenatal expression of growth-associated transcripts (*MYH6*, *PDGFA*, *VEGFC*) was lower, but the postnatal expression of myogenic key drivers (*Pax7*, *MyoD1*, *myogenin*) was higher following MET exposure. The emphasis on the expression of transcripts involved in driving myogenic precursor proliferation, such as satellite cells, might fit to the observation that MET-males exhibited greater lean mass percentage at 152 dpn. Indeed, postnatal muscle growth takes place primarily via myofiber hypertrophy [33]. However, it has been shown previously that muscle regulatory factors (MRFs)

involving *Pax7*, *MyoD1*, and *myogenin* implied the occurrence of activated satellite cells [34], which differentiate to myoblasts, fuse to myotubes, and finally mature into myofibers [35–37]. Since the mitotically quiescent satellite cells are activated via trauma, extrinsic mechanical stretch, or growth factors, the data might suggest that the response to supplemented methylation-related micronutrients changed the program of myogenic differentiation. At 152 dpn, when animals were sampled, the line between adolescence and adulthood is fading; thus, it is conceivable that MET-exposed males exhibit prolonged growth and maturation processes by exploiting the high plasticity of satellite cells during the postnatal period.

In female pigs, fetal and postnatal live weights were unaffected by the dietary challenge. However, a previous study found that female offspring exhibited decreased weight following methylation-related micronutrient supplementation during pregnancy [38]. In our study, MET-exposed females exhibited transcriptional alterations in *FABP3* and *FABP4*, which are known to act as biomarkers for muscle fiber types and lipid accumulation and are implicated in adverse metabolic states [39,40]. Moreover, transcripts associated with IGF signaling were more abundant. Since members of the GLUT family orchestrate glucose uptake and are regulated by Akt, which is, in turn, phosphorylated by PI3K, the maternal supplementation with methylation-related micronutrients might act on myogenesis and muscle differentiation during fetal development [41,42]. Furthermore, the analyses revealed increased expression of *MYH2* and *MYH4*, for which the variance components derived from a PCA of myosin heavy chain isoforms might parallel a shift from CON-females to MET-females. Whether this indicates a cellular shift towards MyHC2a and MyHC2b fibers remains unclear since no fiber typing was performed. However, maternal diets varying in micro- and macronutrient supply during gestation have been shown to affect the formation of myofibers [43–45]. Postnatally, *SPP1* had greater abundance, which may highlight a dietary impact on myogenesis [46,47] since *SPP1* is known to be regulated by the muscle regulatory factors *MyoD1* and *MYF5* [48]. Increased expression levels of *SPP1* are implicated in pathophysiological states associated with obesity and macrophage recruitment, as reviewed elsewhere [49,50].

Taken together, males might have responded via systemic mechanisms culminating in growth alterations with compensatory transcriptional responses. However, female responses comprised cellular features, i.e., metabolic demands might have pushed females toward a status of cellular reorganization (by using glycolytic features) with an emphasis on muscle fibers and adipocytes. The results suggest that male and female animals favored different strategies to adapt to the dietary challenge. The data suggest that the maternal diet might impact myogenesis. An implication for organismal growth and muscle cell reorganization is conceivable, which might account for sex-specific maturation processes. Thus, supplementing methylation-related micronutrients like folate, B6, B12, methionine and choline during pregnancy at higher-than-recommended doses may alter organismal processes beyond preventing birth defects.

In humans, maternal supplementation with methylation-related micronutrients may benefit individuals exposed to prenatal dietary undersupply or even famine, which still burdens a considerable proportion of the global population [51]. However, our findings in a porcine model indicate that it might be useful to consider diversified nutritional recommendations by offspring sex. To maximize the therapeutic potential of methylation-related micronutrients, further investigations (e.g., histological or metabolic studies) are warranted because potential metabolic risks are of scientific interest and public health importance.

5. Conclusions

In summary, the study demonstrated that there are effects due to the supplementation of methylation-related micronutrients at the phenotypic and transcriptomic level. The study revealed sex-dependent strategies to adapt to a prenatal exposure to methylation-related micronutrients. Potentially, growth and maturation processes may be prolonged in MET-exposed males, whereas the programming of female muscle tissue might result in a cellular reorganization. Hence, known

advantageous phenotypes mediated by methylation-related micronutrient intake during pregnancy should be further discussed with regard to their acute and persistent impact on muscle plasticity with a special emphasis on sexual dimorphism.

Supplementary Materials: The following files are available online at www.mdpi.com/2072-6643/9/1/74/s1, Table S1: Postnatal carcass characteristics related to percent lean mass, back fat, pH, and meat color, Table S2: Gene symbols, accession numbers, primer sequences, and statistics (variance components mediated by diet and diet*sex) of selected genes; see Material and Methods section for details, Table S3: Comparison of Fluidigm and LightCycler 480 results for selected transcripts to verify Fluidigm data.

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Author Contributions: K.W. and A.Z. conceived and designed the experiments; M.O., N.T., H.R. and E.M. performed the experiments; M.O., N.T., H.R. and E.M. analyzed the data; E.M. and S.P. contributed reagents/materials/analysis tools; M.O. and K.W. wrote the paper.

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Abbreviations

The following abbreviations are used in this manuscript:

B	Birth
CON	Standard diet
dpc	days post conception
dpn	days post natum
FC	Fold change
IN	Insemination
MET	Standard diet supplemented with methylating micronutrients
nd	not detected
ns	not significant
PCA	Principle component analysis
qPCR	Quantitative polymerase chain reaction
w	week

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nutrients

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Review

Nutrients in Energy and One-Carbon Metabolism: Learning from Metformin Users

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Abstract: Metabolic vulnerability is associated with age-related diseases and concomitant co-morbidities, which include obesity, diabetes, atherosclerosis and cancer. Most of the health problems we face today come from excessive intake of nutrients and drugs mimicking dietary effects and dietary restriction are the most successful manipulations targeting age-related pathways. Phenotypic heterogeneity and individual response to metabolic stressors are closely related food intake. Understanding the complexity of the relationship between dietary provision and metabolic consequences in the long term might provide clinical strategies to improve healthspan. New aspects of metformin activity provide a link to many of the overlapping factors, especially the way in which organismal bioenergetics remodel one-carbon metabolism. Metformin not only inhibits mitochondrial complex 1, modulating the metabolic response to nutrient intake, but also alters one-carbon metabolic pathways. Here, we discuss findings on the mechanism(s) of action of metformin with the potential for therapeutic interpretations.

Keywords: diabetes mellitus; energy intake; epigenetics; folic acid; food-drug interactions; food source; obesity; vitamins B

1. Introduction

Food restriction extends health and lifespan in some models [1], but what nutrients should be restricted and to what extent? Qualitative changes in the provision of dietary macronutrients induce metabolic and endocrine adaptations that are clinically relevant to the nutritional status of both, patients with low food availability and those with a persistent intake of excessive amounts of food [2]. In particular, the relationship between energy and one-carbon (1C) metabolism is extremely sensitive to food intake [3–6]. We envision that the inclusion of metformin in current strategies promoting metabolic fitness [7] is in accordance with the clinical response to dietary environment in disease states and the beneficial effects in metabolically vulnerable cells [8–11]. That is, it is important to revise what we can learn from metformin users and which are the potential implications. Metformin, as a

calorie-restriction mimetic drug affecting mitochondrial function, integrates metabolic signals and the direct effect on folate metabolism may provide therapeutic clues [12–15]. We highlight the effects of metformin on signaling pathways associated with some of the hallmarks of aging and the likely beneficial effects in the pathogenesis of comorbidities associated with metabolic diseases (Figure 1). Whether metformin can be safely used with these new indications remains to be established.

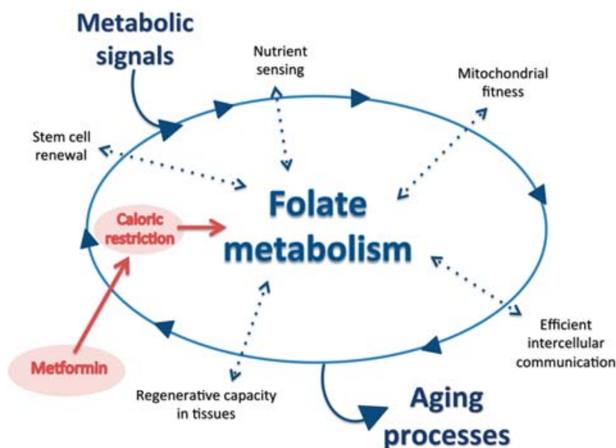

Figure 1. The effect of metformin on signaling pathways associated with nutrient excess. These effects are likely regulated by folate metabolism and represent direct effects on some of the hallmarks of tissue aging. It remains to be determined whether metformin can safely slow the development of age-related comorbidities in metabolic diseases.

Metformin is currently used exclusively in the treatment of type 2 diabetes mellitus (T2DM), but there is potential for additional indications in obesity, inflammatory disorders, cardiovascular diseases and cancer. For instance, in obese patients without diabetes, the weight loss effects of metformin are not inferior to those of a recently approved drug for obesity [16] and the antenatal administration of metformin during pregnancy reduces maternal weight gain without effects on neonatal outcomes [17–19]. Moreover, the Diabetes Prevention Program has found beneficial effects in diabetes prevention and a durable weight loss attributable to metformin [20–22]. Cardiovascular benefits are also likely, and, when compared to sulfonylurea or insulin therapy, metformin monotherapy is associated with a higher reduction in cardiovascular events [23,24]. In addition, the anti-inflammatory actions of metformin can be separated from its metabolic effects, and there is ongoing research assessing the role of metformin in the prevention and recurrence of breast cancer [25,26].

All these effects are crucial in preventing disease and promoting health. We discuss here the metabolic alterations connecting the metformin response and the function of essential nutrients, which emphasize that careful attention to diet might shape clinical strategies [27]. Our arguments are framed in the relationship between energy and one-carbon metabolism, reinforced by the mechanisms of action proposed for metformin.

2. One-Carbon Metabolism: Inputs and Outputs

Folate coenzymes play a crucial role in health and disease and are present in virtually all organisms and cell types. Moreover, although controversial, some dietary arguments support the addition of folic acid or related compounds to common foods [28–30]. In humans, dietary folic acid is reduced to 7,8-dihydrofolate (DHF) and then to 5,6,7,8-tetrahydrofolate (THF) by dihydrofolate reductase (DHFR), initiating the folate cycle. Biochemical reactions converge here, using the ability of THF

to carry 1C units in different oxidation states. Folate coenzymes contain poly-γ-glutamate tails attached to a p-aminobenzoic acid moiety by the activity of folylpolyglutamate synthetase (FPGS), an essential enzyme that regulates the distribution of different folate forms in cellular compartments and specific actions in cell proliferation pathways [31,32]. Serine, glycine and methionine are readily provided in the diet. Serine is oxidized in the mitochondria and transferred to THF by serine hydroxyl-methyl-transferase (SHMT), resulting in glycine and 5,10-methylene-THF (CH2-THF). Glycine may be incorporated directly into purine nucleotide bases or glutathione (GSH) and CH2-THF can be converted to 5-methyl-THF in a reaction catalyzed by methylenetetrahydrofolate reductase (MTHFR) or 10-formyl-THF according to cellular needs. Of note, serine and glycine may be synthesized de novo through glycolysis, aldol cleavage (from threonine, in some cells) or reactions involving demethylation (from choline, betaine, dimethylglycine and sarcosine) [31,32].

The folate cycle, closely linked to the methionine cycle, regulates the availability of methyl groups (CH3) through the sequential conversion of methionine to S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine. Conversely, in the presence of 5-methyl-THF (5-mTHF) and methionine synthase, which requires vitamin B12 (cobalamin), methionine can be regenerated. Transmethylation reactions and intermediate metabolites are crucial to the synthesis of high-energy molecules, structural macromolecules, thymidine, and purines and to the maintenance of the cellular redox state and the transsulfuration pathway [33,34]. Dietary vitamins also communicate bioenergetics and 1C metabolism. For instance, the conversion of homocysteine to cysteine (transsulfuration pathway) and the synthesis of glutathione require vitamin B6 (pyridoxine) and vitamin B12 is essential (via the mitochondrial enzyme methylmalonyl-CoA mutase) to form succinyl-CoA, a key substrate of the citric acid cycle (Figure 2).

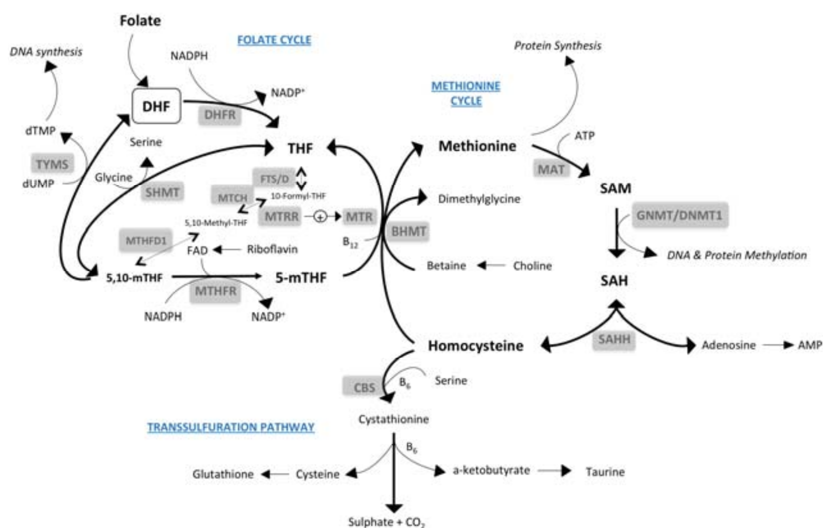

Figure 2. Metabolic pathways indicating the close dietary dependence in the folate cycle, methionine cycle and transsulfuration pathways. The role of B vitamins is paramount in regulating the expected complexities in relevant enzymes and circulating levels of metabolites. BHMT, betaine-homocysteine *S*-methyltransferase; CBS, cystathionine beta-synthase; DHFR, dihydrofolate reductase; DNMT, DNA methyltransferase; FAD, flavin adenine dinucleotide; GNMT, glycine *N*-methyltransferase; MAT, aminomethyltransferase; MTHFR, methylenetetrahydrofolate reductase; MTR, methyltransferase; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate; TYMS, thymidylate synthase; UDP, uridine diphosphate.

The cellular functions that depend on these micronutrients illustrate how important careful dietary intake is to avoid deficiencies and how difficult it is to establish clinically based data to be used in both the management of disease states and drug treatments. It may appear paradoxical, but diseases associated with excessive food intake may present deleterious effects that could result from excess in micronutrients [30]. The challenge is to achieve an adequate nutrient balance in the different groups within the general population. In this context, several complexities at the organismal level may be illustrative. For instance, the hepatic effects of diets deficient in choline and methionine are practically indistinguishable from those observed with methionine rich, high-fat, high-energy diets. In contrast, the balanced provision of methionine increases healthy lifespan in experimental models [35–37]. These nutritional observations exemplify the close dependence among regulatory pathways in energy and one-carbon metabolism and the critical importance of the equilibrium between inputs and outputs (Figures 2 and 3).

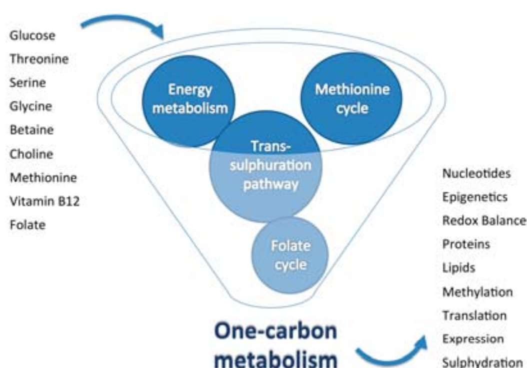

Figure 3. Schematic representation summarizing the importance of appropriate equilibrium in inputs and outputs of one-carbon metabolism. Excessive intake of a given nutrient influences the availability of other nutrients and can perturb metabolism with deleterious consequences. Understanding the events linking metabolism and diet-dependent pathways may provide crucial insight into their role in health and disease.

3. The Lack of Redundancy between Cytoplasmatic and Mitochondrial One-Carbon Metabolism Affects Tissue-Specific Responses to Nutrients

Deficient or excessive intake of nutrients causes changes in the mitochondrial electron transport chain that model the organismal response to the environment. In human cells, the specific forms of folate cofactors and the extent of polyglutamylation differ in cytosolic and mitochondrial pools. Curiously, 1C metabolism is compartmentalized, and mechanisms controlling the flux of components through these compartments are strictly regulated ([38] and references therein). Whether mitochondrial dysfunction is a cause or a consequence of disease remains debatable, but it appears that mitochondria receive monoglutamate THF, which is polyglutamylated, charged with 1C units, and later released to the cytosol [39,40], ensuring the correct function of cellular 1C metabolism.

Energy and one-carbon metabolism jointly integrate the cellular nutrient status. Reactions of glycine and serine are particularly important connecting nutrition, 1C metabolism and the effects of biguanides, as demonstrated in experimental models of glycine auxotrophy. Data in these models indicate that the cytoplasmic serine hydroxymethyltransferase (SHMT) isozyme is not essential, but glycine biosynthesis requires a different mitochondrial isozyme, which is not reversed by external addition of nutrients [41]. Moreover, in the absence of cytoplasmic SHMT, serine donates 1C units and formate flows normally to the cytoplasmic THF pool through the activity of mitochondrial methylene tetrahydrofolate dehydrogenase (MTHFD), a trifunctional folate-dependent enzyme with

activity as a CH2-THF dehydrogenase, CH+-THF cyclohydrolase and 10-formyl-THF synthetase. Mice without these mitochondrial isozymes (MTHFD2/MTHFD2L) are not viable [42–44]. In addition, glycine is broken down by the exclusively mitochondrial glycine cleavage system (GCS) and in this reaction direction, the electrons are delivered to complex I of the mitochondrial respiratory chain [45]. Metformin affects mitochondrial biology at this point, but information on compensatory pathways is limited [8,31].

Understanding the transport processes between compartments and how these differences in cytoplasm and mitochondria control metabolic processes might provide a rationale for treating metabolic abnormalities. The strictly mitochondrial GCS activities support a model of multiple carrier-facilitated diffusion of metabolites between cytosol and mitochondria [30]. Because there is no methionine adenosyltransferase (MAT) activity in mitochondria, putative carriers are also necessary for the constant transport of SAM from the cytosol to the mitochondria [46]. The relationship between compartments might also explain the metabolism of choline, which is obtained primarily as phosphatidylcholine in the diet. In the liver, choline is a major source of methyl groups through conversion to betaine (*N*,*N*,*N*-trimethylglycine). The cytosolic betaine hydroxymethyltransferase (BHMT) generates methionine and *N*,*N*-dimethylglycine (DMG), but DMG absolutely requires the activity of mitochondrial dehydrogenase (DMGDH) to act as a 1C donor in mitochondria. Similarly, sarcosine (*N*-methylglycine) can be a 1C donor in the cytoplasm, but in the mitochondria, it requires sarcosine dehydrogenase (SDH) [47].

Collectively, these multiple regulatory steps indicate essential mechanisms for preventing disease. Experiments with genetically engineered mice, examining each step, are of limited value to understand human disease [48], but in humans there are associations between genetic variants and several diseases [49–54]. Similarly, available data suggest that mitochondrial 1C metabolism is critical for metabolic adaptations and cell survival in cancer and T cell-mediated (immune) pathologies [55–57]. T cell activation does not increase the expression of proteins related to glycolysis, pentose phosphate pathway or oxidative phosphorylation. In contrast, T cell activation produces new and specialized mitochondria characterized by the massive induction of enzymes involved in folate-mediated 1C metabolism [55], indicating that the mitochondrial and cytosolic pathways for generating and processing 1C units are not redundant. That is, cytosolic 1C metabolism is insufficient to support T cell proliferation, cancer cell immortality and excessive metabolic impact when mitochondrial 1C metabolism is impaired [31,58–62] (Figure 4).

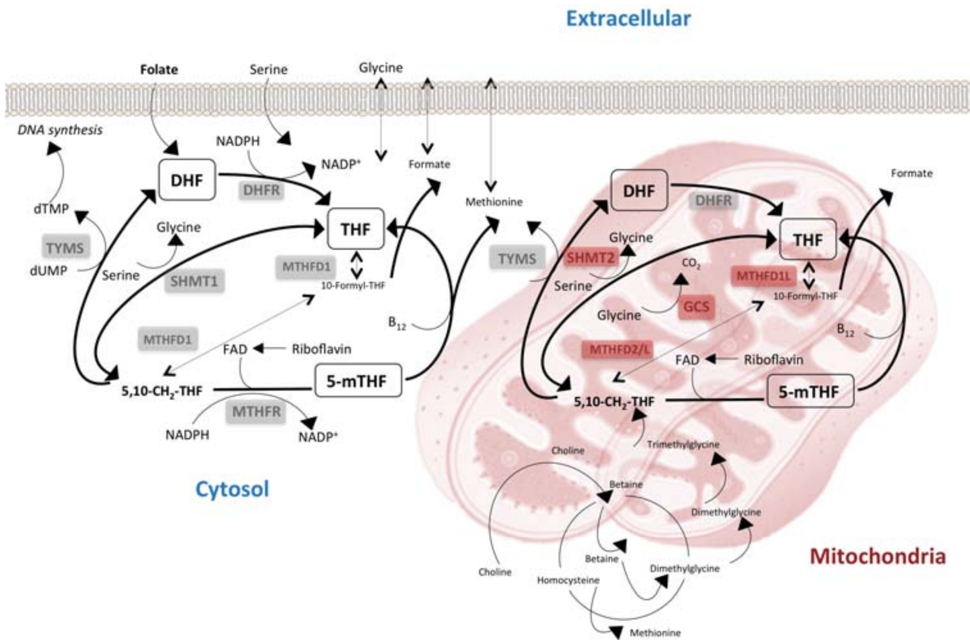

Figure 4. Mitochondrial and cytosolic pathways for generating and processing 1C units are separated but not redundant and depend on extracellular provision. Metabolically active cells may survive deficiencies in cytosolic isozymes but require the correct function of mitochondrial isozymes (marked in red). GCS, glycine cleavage system.

Mechanisms affecting energy and 1C metabolism are likely linked to cellular biosynthesis, redox maintenance and epigenetic status. Accurate information on the activities of the aforementioned enzymes in complex diseases might provide clues regarding how nutritional status or dietary and pharmacologic manipulations affect physiological regulation with evident therapeutic opportunities.

4. The Importance of Drugs and Diets That Modulate Mitochondrial Activity

Mitochondrial function, epigenetic signals and nutrient-sensing pathways are likely combined to promote health at both the cellular and the organismal levels [63,64]. Drugs and dietary regimens that directly modulate mitochondrial activity are promising [65].

Mitochondria are not only providers of energy but also of signaling units, and the existence of mitochondrial-derived peptides (MDPs) suggest the new concept of the existence of mitochondrial hormones and metabolic regulators [66–68]. Similar to metformin [12,69], targets of these MDPs include one-carbon metabolism, thiosulfate sulfurtransferase and AMP-activated protein kinase (AMPK), critical links between nutrients and health. Nutrient sensing is important for the distribution of energy, and changes in food intake alter metabolic strategies [70,71]. Notably, metformin activates AMPK, inducing changes in bioactive metabolites connected to transcriptional regulators through as yet undefined mechanisms [72,73] but probably including an interplay among food intake, metabolism and mTOR signaling in clinically relevant settings [74,75].

In particular, metabolic aspects in cancer are now a renewed source of potential therapeutic targets. Cells depleted of mitochondrial DNA identify metabolic vulnerabilities and illustrate that 1C metabolism, serine biosynthesis and transsulfuration are sensitive to mitochondrial dysfunction and stress [76]. Similarly, cancer cells without the ability to catalyze the conversion of isocitrate to α-ketoglutarate reveal that perturbing mitochondrial metabolism with metformin may help to

kill cancer cells [77]. The challenge is to learn how to use cell- and tissue-specific variations in the mitochondrial management of 1C donors and how to interpret the clinical response to manipulations in nutrient availability. Several lines of evidence indicate that crosstalk between epigenetic signals and cellular metabolism may be a clinically useful field of research because it represents a mechanism to convert dietary-induced metabolic changes into stable patterns of altered gene expression [78–81].

5. Is Gene Expression Reprogrammed in Response to Metabolic and Dietary Stimuli Affecting One-Carbon Metabolism?

Whether epigenetic mechanisms are causally coupled to changes in metabolic phenotypes is a question posed by epidemiological studies examining extreme nutritional changes during fetal development [82–85]. It is not surprising, however, to find contradictory data because exposure windows and conditions are not controlled in documented "experiments" on long-term famine [82–84]. These studies do not reveal the contribution of specific nutrients, but studies in other cohorts associate defects in energy and one-carbon metabolism in pregnant women with future risk in offspring ([85] and references therein). Taken together, these data indicate that epigenetic modulation of metabolic pathways may promote adverse phenotypes in later life [86,87].

In the current context of excessive food intake, it is urgent to understand the potential implications of modifying epigenetic marks by nutrition and whether diet-induced changes in metabolic or phenotypic traits may affect future generations. Nutritional, pharmacological and metabolic signals induce epigenetic drift (i.e., metabolic states correlated with chromatin states), and several epigenetic marks influence the expression of neighboring genes through generations. It is apparently the feedback or combination among different epigenetic mechanisms that dynamically configures the chromatin landscape throughout life [88,89] but DNA methylation alone has strong mechanistic support to explain modifications by dietary changes. DNA methylation and certain metabolites generated by mitochondrial respiration interact with transcription factors, suggesting metabolo-epigenetic links between energy and one-carbon metabolism [90–92]. It is plausible that cellular transcriptional machinery and chromatin-associated proteins integrate inputs derived from food as part of the response of living organisms to continuous fluctuations in the availability of energy substrates.

Alterations in genes that encode enzymes affecting chromatin regulation, such as kinases, acetyltransferases, demethylases and methyltransferases, are common in dietary-related diseases [93]. These enzymes use cellular metabolites as sources of phosphate, acetyl or methyl groups, and interpret the metabolic state of a specific cell. For instance, the levels of SAM, a methyl donor, SAH and threonine alter methylation status through pathways involving acetyl-coA metabolism, and specific dietary restrictions cause transcriptional and metabolic responses [94,95]. We recently provided an example of how metformin-driven reduction of acetyl-CoA is sufficient to correct histone H3 acetylation, indicating that metformin regulates mitonuclear communication and the cellular epigenetic landscape. These data may result in knowledge that can be applied to metabolo-epigenetic strategies for prevention or therapy [96]. Future research will ascertain how dietary manipulations and synthetic epigenetic modifiers contribute to DNA methylation and how plastic the genome is to dietary changes. It is also important to establish what magnitude of metabolic stimuli is required to produce appreciable changes and whether epigenetic changes can be reversed.

6. The Ability of Metformin to Target One-Carbon Metabolism: Perspectives in Clinical Practice Outcomes

The dietary inputs associated with the folate and methionine cycles support cellular integrity and health, but there is some risk in manipulating bioactive elements in the absence of well-proven associations between consumption and disease. This probably explains why nutritional manipulation is not incorporated into clinical practice against cancer, but we should not forget that the action of B vitamins led to the discovery of folate antagonists as a major class of cancer chemotherapy agents [97] and that restriction in some nutrients negatively affect tumor formation in mice [98].

The multi-faceted activity of metformin and its likely interconnected mechanisms of action are relevant to potential applications in diseases characterized by mitochondrial dysfunction, gene deregulation and failure in metabolic homeostasis [99–103]. The global metabolic impact of metformin and the generated energetic stress suggest a strong association with the overall management of food intake. Metformin stimulates glucose uptake by muscle, inhibits hepatic gluconeogenesis and stimulates AMPK through effects in NADH ubiquinone oxidoreductase, the first component of the electron transport chain. Other putative actions with dietary associations include AMPK-independent signaling through glucagon-dependent cyclic AMP and the inhibition of mitochondrial glycerophosphate dehydrogenase [103–105]. These findings on metformin action suggest an approach for treating metabolic diseases not restricted to the field of diabetes.

It could be argued that drugs reducing insulin levels may reduce cancer risk, and drugs that increase circulating insulin may increase cancer risk, but the effects of metformin in cancer cells seem specific, preventing the boost in glycolytic intermediates, decreasing citric acid cycle intermediates, and depleting the cellular glutathione content [106]. Moreover, metformin impairs one-carbon metabolism in a manner similar to drugs that target folate-related enzymes and have long been used to treat inflammatory diseases and cancer [8,12,106–109]. Efforts to clinically address this issue remain incomplete and difficult to interpret because heterogeneity in metformin response is considerable and only partially explained by genetic and nutritional factors. Metformin pharmacodynamics and pharmacokinetics have been reviewed recently ([110] and references therein). Current formulations have a bioavailability of ~50% (approximately 40% is absorbed in the duodenum and proximal jejunum, and ~10% in the ileum and colon). Unabsorbed drug is eliminated in the feces, and metformin circulates in the plasma unbound and without transformation until cleared by the kidneys. Metformin is a hydrophilic molecule requiring specific mechanisms of transport, primarily organic cation transporters (OCT). Briefly, plasma membrane monoamine transporter (PMAT) and OCT3 contribute to gastrointestinal uptake, and OCT1 may be responsible for transport into the interstitial fluid of the intestine. OCT1, OCT3 and multidrug and toxin extrusion proteins (MATE) are expressed on the basolateral membrane of hepatocytes. In the kidney, metformin is taken up into renal epithelial cells by OCT2 and excreted into the urine via MATE1 and MATE2. OCT1 and PMAT may contribute to metformin reabsorption. Genetic variants of these and other transporters may be important to explain the therapeutic action of metformin, the high inter-individual variability in gastrointestinal tolerance and drug–drug interactions [111–114]. It would be useful to ascertain whether these genetic variants could have predictive value in patients before they take the drug [115], but genetics alone frequently fail to explain phenotypes. In this context, we foresee that considering metformin for non-diabetic indications is an example of the value of applying metabolomics to useful clinical research. The next task is connecting energy and one-carbon metabolism to physiology, describing heterogeneous phenotypes and defining the role of nutrition in the response to drug treatment.

7. The One-Carbon Cycle in Metformin Users and Potential Adverse Effects

We believe that metformin is best described as an "antimetabolite" drug, and, as such, several deleterious effects might be expected among long-term metformin users. Trials to ascertain these effects are scarce, and data are frequently contradictory, but people at risk of deficiency in one-carbon-related metabolites include vegans, vegetarians, pregnant women, breastfeeding women, the elderly and patients with anemia or poor renal function [116].

There is no evidence of risk in humans from taking metformin during pregnancy, but this issue requires attention [17]. Folate deficiency in metformin users is rare, but a decrease in serum and red blood cell (RBC) folate concentration is common [117]. It is difficult to ascertain the effect of folate fortification in some foods due to discrepancies in results [118] and the lack of data regarding folate bioavailability in metformin users [34,119]. Favoring the consumption of foods that are endogenously high in folates is probably reminiscent of decades using folates with cytotoxic agents, but this empirical practice requires caution. For instance, lessons from a prospective study in rural India indicate

that normal/high folate concentrations associated with deficiencies in vitamin B12, attributable to a lacto-vegetarian diet combined with folic acid supplementation, may be associated with a higher risk of insulin resistance in the offspring [120]. This is relevant because metformin might facilitate the mechanism known as "methyl folate trap", which acts on the regulation of the methionine/folate cycle and cysteine oxidation [121]. According to this concept, although only demonstrated in pernicious anemia, the cell would mistakenly interpret vitamin B12 deficiency as a lack of methionine, and will divert the remaining folate away from DNA biosynthesis towards the methylation of homocysteine to methionine, building up 5 methyl THF that the cell will not be able to use [121].

The relationship between vitamin B12 deficiency and metformin treatment has been studied since the beginning of the 1970s with some clinical confusion likely related to the extreme heterogeneity among studies [122–124]. Among metformin users, a slight reduction in serum vitamin B12 concentrations is common, but some studies have reported contradictory results indicating that metformin has no effect or even might improve vitamin B12 metabolism [125–127]. No mechanism has been proposed and the issue requires further research but there is probably no clinical relevance in these observations as discussed below. Moreover, the possible benefits of vitamin B12 supplements in metformin users have not been assayed and the notion of causality is complicated because diabetes and obesity are also associated with vitamin B12 reductions [128].

Metformin does not significantly increase blood lactate levels, but we are probably depriving some patients from potential benefits based on the putative risk of lactic acidosis [129]. After 70 years of real-world clinical experience, the incidence of lactic acidosis is mostly based in anecdotal reports. However, caution is important in individuals with reduced metformin clearance (i.e., poor renal function), reduced lactate clearance (i.e., impaired hepatic metabolism), and/or increased lactate production (i.e., sepsis or reduced tissue perfusion). Advanced age may also increase risk because of age-related decline in renal function and increased risk for acute renal failure (i.e., dehydration), drug–drug interactions and other medical conditions. Moreover, elderly patients with type 2 diabetes are independently at greater risk for hyperlactatemia and have a reduced threshold for the development of lactic acidosis in response to a secondary event [130].

The most frequent adverse effects are gastrointestinal in nature: diarrhea, nausea, and to a lesser extent, vomiting, flatulence or heartburn. Of note, in randomized controlled trials, similar effects are found in the placebo group, indicating potential bias [131]. Those patients without preexisting gastrointestinal conditions are apparently free of these effects, but it is not uncommon that some patients decline using metformin [112,113]. Metformin response and tolerance are intrinsically associated with the gut and the intake of nutrients [132]. Metformin could increase serum glucagon-like peptide 1 concentration by increasing its secretion from L cells, distributed throughout the intestine, and/or by reducing its breakdown by dipeptidyl peptidase-4 in the intestinal mucosa and portal system [133]. Curiously, serotonin and histamine release from the intestine are associated with similar effects (nausea, vomiting, increased gut motility and diarrhea). It is possible that metformin inhibits diamine oxidase, which is highly expressed in enterocytes and responsible for the metabolism of these gut peptides [134]. Metformin might also disrupt the enterohepatic circulation of bile salts, predominantly through reduced ileal absorption and osmotic effects facilitating diarrhea [135]. Finally, the gut microbiome is considered an environmental factor contributing to the development of metabolic diseases and a possible target of metformin. A reduction in butyrate-producing bacteria and an increase in opportunistic pathogens are common in type 2 diabetes and could potentially influence gastrointestinal tolerance [136–138].

8. Measuring the Impact of Folate-Related Deficiencies in the Clinical Setting: Potential for Targeted Metabolomics

Results from immunoassays in clinical laboratories are difficult to interpret and limited by the availability of reagents and automated biochemistry platforms. Methodological constraints, confounding factors, and poor inter-laboratory reproducibility are common pitfalls [139–141].

Consequently, the picture obtained when exploring folate metabolism is partial at best. For instance, total circulating B12 in serum is unreliable in some clinical conditions and practically useless as a method to detect true, functional B12 deficiency [142]. Most (80%) of B12 is bound in serum to haptocorrin, and a variable proportion (5%–20%) is bound to transcobalamin II and ready for tissue uptake (holotranscobalamin). Measuring in the same batch serum holotranscobalamin, homocysteine and/or methylmalonic acid can mitigate limitations [125,143]. Some pitfalls were made evident in a large cohort of participants enrolled in the REasons for Geographic and Racial Differences in Stroke (REGARDS) study [144]. The proportion of participants with low serum B12 concentration was exactly the same (2%; not clinically evident) in participants without diabetes and in long-term metformin users with diabetes. However, serum B12 concentrations were lower in metformin users than in those who did not use metformin. Curiously, metformin users were less likely to have taken multivitamins (6–25 μg of vitamin B12 per dose), and multivitamin users had a significantly higher serum B12 concentration compared to those who did not take multivitamins [144]. A longitudinal study to assess the impact of metformin is warranted, but it appears that laboratory biomarkers do not add significantly to clinical decisions and that dietary advice might contribute to better management of metformin users [145].

Similarly, measurement of serum folate with standard immunoassays is accompanied by possible errors in interpretation. For instance, obesity appears to be associated with decreased serum (measuring recent folate intake) but also with increased RBC folate (measuring a long term intake) concentrations compared with lean subjects. The association is plausible but the presence of obesity-associated metabolic disturbances hampers further interpretations [146]. It was recently clarified that folate measurements in serum and in RBC display similar performance in assessing folate status [147]. The use of both measures generates higher and unnecessary costs, but the RBC folate assay is less likely to provide falsely normal levels attributable to dietary behavior or recent supplements [147]. The observation that metformin is associated with a slight, but sometimes significant, raise in homocysteine and/or decrease in folate needs careful consideration, but there is no sufficient evidence to recommend folic acid supplements to metformin users [148,149].

It remains unknown how nutritional manipulations may affect the complex relationships among metabolites involved in the 1C cycle, but studies in women with seasonal variations in nutrient intake highlight the need to measure all metabolites and cofactors [150,151]. Analytical platforms should provide measurements of different folate species, especially 5-methyltetrahydrofolate as the active 1C donor [150,151]. Specifically, it is important to confirm data suggesting that levels of maternal one-carbon metabolites at conception influence DNA methylation in the early embryo and that offspring methylation correlates with the paternal somatic methylation pattern [152]. This effort implies better tools for measuring intermediate metabolites either side of the involved reactions [153,154]. Targeted metabolomics may help in pursuing a better interpretation. In this context, ultra-high pressure liquid chromatography coupled to an electrospray ionization source and a triple-quadrupole mass spectrometer is an affordable choice to quantitatively examine the methionine/folate bi-cyclic 1C metabolome [155]. This method has been used to explore the activation of methylogenesis in some models. This essential function of 1C metabolism provides a labile pool of methyl groups required for successfully establishing and maintaining the DNA methylation imprint [155]. A similar approach has been used to explore energy metabolism with a simple and rapid method based on gas chromatography coupled to quadrupole time-of-flight mass spectrometry and an electron impact source [156]. The accurate and simultaneous measurement of selected metabolites facilitates the understanding of metabolic responses to changing environmental factors and has the potential for searching quantitative biomarkers of disease and signals indicating the ability of drugs to restore cellular homeostasis [157]. In addition, this analytical approach may serve to assess the expected toxicity in potential applications for metformin employed in oncology at doses notably higher than those used chronically in the management of diabetes [8].

9. Conclusions

Metformin users may provide data on the effect of nutrients in health and disease. There is growing evidence demonstrating the multiple protective effects of metformin against obesity-associated diseases, a major challenge to global public health. Some findings support the idea that metformin mediates the mitochondrial response to excessive food intake and the effect of different micronutrients. In particular, the mechanism of action of metformin involves effects on both energy and one-carbon metabolism and suggests novel strategies that involve the combination of lifestyle modification with pharmacotherapy. The concept is more important in individuals whose risk factors are not reduced by dietary interventions and dietary regimens in metformin users may provide valuable information. We envision that several analytical approaches in the field of metabolomics can provide diagnostic indicators on multiple metabolic aspects and may ascertain the effects of nutrient intake. Accordingly, it is especially relevant to assess the role of significant nutrients, such as serine, glycine, methionine, folic acid or other B vitamins, affecting one-carbon metabolism. Efforts to repurpose metformin, the first choice as an oral treatment of type 2 diabetes, as an antimetabolite drug, reinforce the interest in understanding food and drug interactions and the expected toxic effects caused by a change in dose range.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

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Review

Pyridoxine (Vitamin B_6) and the Glutathione Peroxidase System; a Link between One-Carbon Metabolism and Antioxidation

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Abstract: Vitamin B_6 (B_6) has a central role in the metabolism of amino acids, which includes important interactions with endogenous redox reactions through its effects on the glutathione peroxidase (GPX) system. In fact, B_6-dependent enzymes catalyse most reactions of the transsulfuration pathway, driving homocysteine to cysteine and further into GPX proteins. Considering that mammals metabolize sulfur- and seleno-amino acids similarly, B_6 plays an important role in the fate of sulfur-homocysteine and its seleno counterpart between transsulfuration and one-carbon metabolism, especially under oxidative stress conditions. This is particularly important in reproduction because ovarian metabolism may generate an excess of reactive oxygen species (ROS) during the peri-estrus period, which may impair ovulatory functions and early embryo development. Later in gestation, placentation raises embryo oxygen tension and may induce a higher expression of ROS markers and eventually embryo losses. Interestingly, the metabolic accumulation of ROS up-regulates the flow of one-carbon units to transsulfuration and down-regulates remethylation. However, in embryos, the transsulfuration pathway is not functional, making the understanding of the interplay between these two pathways particularly crucial. In this review, the importance of the maternal metabolic status of B_6 for the flow of one-carbon units towards both maternal and embryonic GPX systems is discussed. Additionally, B_6 effects on GPX activity and gene expression in dams, as well as embryo development, are presented in a pig model under different oxidative stress conditions.

Keywords: glutathione peroxidase; one-carbon; pig; pyridoxine; remethylation; transsulfuration

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1. Introduction

Among various metabolic pathways present in the organism, the metabolism of sulfur- (S) methionine and its seleno- (Se) analogous (Se-methionine) are particularly important because they not only contribute to protein mass but also produce (Se) homocysteine, a key metabolite connecting two fundamental metabolic functions, the one-carbon metabolism and the antioxidative system.

The transfer of one-carbon groups, represented by methyl (–CH3), methylene (–CH2–), formyl (–CHO), formimino (–CHNH), and methenyl (–CH=), is involved in the remethylation of (Se) homocysteine to (Se) methionine, as well as related pathways such as the folate cycle and the choline oxidation pathway [1]. Additionally, during the demethylation of (Se) methionine to (Se) homocysteine, the universal bioactive methyl donor S-adenosylmethionine (SAM) is synthesized and donates its methyl group to a large number of methyl acceptors catalyzed by methyltransferases [1,2] with profound impacts on DNA synthesis, protection and repair, cellular metabolism, and cell proliferation and, consequently, with direct effects on embryo/fetal growth [3,4].

During redox challenges, however, the high level of reactive oxygen species (ROS) induces a negative feedback on methionine synthase (MTR) [5] and betaine-homocysteine S-methyltransferase (BHMT) [6], which are enzymes that catalyze the regeneration of methionine from homocysteine [7]. Concomitantly, a positive feedback on cystathionine β-synthase (CBS), the first enzyme of the transsulfuration pathway, increases the flow of carbon towards transsulfuration reactions [8].

The transsulfuration of homocysteine, catalyzed by two vitamin B_6- (B_6) dependent reactions, consumes one-carbon units and is a major source of cysteine, contributing with about 50% of the cysteine used for glutathione (GSH) synthesis [8], the major redox buffer in mammalian cells. Se-homocysteine follows these same B_6-dependent steps of the transsulfuration pathway but syntheses Se-cysteine (SeCys). Due to the action of the B_6-dependent and Se-specific enzyme selenocysteine lyase (SCLY), SeCys loses its organic part and releases selenide. Selenide is also formed after dietary selenite (mineral Se) supplementation [9]. Considering the high cytotoxicity of selenide, it has to be immediately metabolized through either methylation reactions consuming SAM or phosphorylation to merge Se into an organic moiety in a B_6-dependent reaction (Figure 1). After another B_6-dependent reaction, phosphorylated Se is incorporated into tRNA Se-cysteinyl with further translation into Se-proteins such as Se-dependent glutathione peroxidases (SeGPX), the enzyme responsible for the oxidation of GSH during the important detoxification of ROS [10].

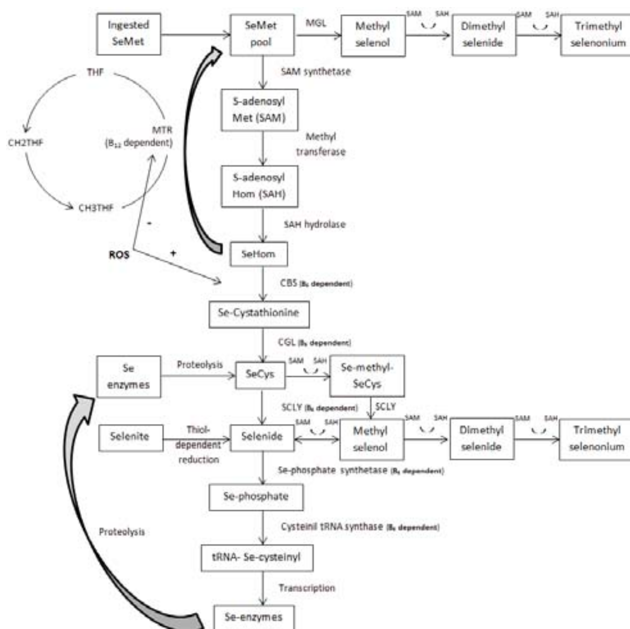

Figure 1. Transmethylation and transsulfuration pathways of selenium (similar for sulfur counterparts) in adults. Selenomethionine (SeMet) is methylated to methyl selenol by methionine γ lyase (MGL) or demethylated to selenohomocysteine (SeHom). SeHom may follow remethylation back to SeMet or transsulfuration to form selenocysteine (SeCys). The substrate-specific enzyme selenocysteine lyase (SCLY), which represents the landmark between sulfur and selenium metabolisms, catalyses the synthesis of selenide from SeCys. Selenide may also be formed from dietary selenite after thiol-dependent reactions. Selenide may be methylated and excreted or phosphorylated and its selenium (Se) incorporated into tRNA Se-cysteinyl with further translation to Se-enzymes. THF = tetrahydrofolate; CH2THF = 5,10-methylene-THF; CH3THF = 5-methyl-THF; MTR = methionine synthase; CBS = cystathionine β synthase; CGL = cystathionine γ lyase; ROS = reactive oxygen species.

Although the transmethylation of (Se) methionine is not dependent on B_6 [11], the direct impact of this vitamin on the transsulfuration of (Se) homocysteine to (Se) cysteine and the intrinsic relation between these two metabolic pathways make the B_6 status of an individual important not only for the antioxidant system but for the one-carbon pool as well. Considering that the transsulfuration of homocysteine to the synthesis of GSH requires two B_6-dependent reactions, whereas Se-homocysteine requires five B_6-dependent reactions to synthesize SeGPX, the impact of B_6 is expected to be more evident for Se metabolism. Additionally, the dissimilar metabolisms between organic and mineral Se, including different mechanisms of regulation for the synthesis of selenoprotein, indicate possible different fates for the one-carbon metabolism.

All the above concepts are even more fundamental in developing embryos because of the singular transfer of Se to pre-implantation embryos [12], the redox alterations brought by the placentation process [13], and the incomplete transsulfuration pathway [14].

The present review discusses the impact of B_6 on the equilibrium between the synthesis and the consumption of one-carbon units under different oxidative stress conditions, focusing not only sulfur-related metabolism but also Se metabolism and the differences between Se sources both in adults and embryos at 5- and 30-days of gestation using a pig model.

2. Vitamin B_6 Metabolism

Vitamers and Their Metabolism

Vitamin B_6 is a general description of six interconvertible metabolites, pyridoxine (alcohol), pyridoxamine (amine), pyridoxal (aldehyde), and their respective phosphates. Its main metabolic form differs between plant (pyridoxine) and animal (pyridoxal and/or pyridoxamine) sources, but, independently of origin, they are mainly found in the phosphorylated state or bound to proteins [15].

The enteric absorption of the phosphorylated protein-bound B_6 vitamers, mostly at jejunum and ileum, is dependent on their dephosphorylation by membrane-bound alkaline phosphatases present in the intestinal mucosa [16]. All three dephosphorylated vitamers are absorbed by passive diffusion, and, within the enterocytes, these metabolites are re-phosphorylated, generating a metabolic trapping of the vitamin with further oxidation to pyridoxal-5-phosphate (P-5-P), its metabolically active form [17]. This latter metabolite must be dephosphorylated at the intestinal serosal surface before its release into the portal circulation. Dephosphorylated forms of B_6 are readily taken up by membranes, whereas the phosphorylated analogs are not; therefore, phosphorylation may be considered a mechanism for the intracellular retention of this vitamin [15].

Pyridoxal released into the portal circulation is absorbed in the liver by passive diffusion, followed by re-phosphorylation within the cells. In order to cross liver cell membranes, P-5-P is hydrolyzed to pyridoxal and released into the general circulation bound to albumin and/or hemoglobin [18]. Any P-5-P that is not bound to proteins is readily hydrolyzed and the free pyridoxal remaining in the liver is oxidized to 4-pyridoxic acid and excreted.

Considering that no specific tissular storage of B_6 is present in the organism, both short- and long-term whole body pools of B_6 (about 12 h and 1 month, respectively) are present as P-5-P bound to enzymes/proteins [15]. Blood plasma is the major source of extrahepatic B_6, which occurs mainly as P-5-P bound to albumin [16]. Circulatory P-5-P must be hydrolyzed (by extracellular alkaline phosphatases) to pyridoxal that can cross cell membranes and then is trapped intracellularly by phosphorylation. Pyridoxal-5-P is over six times more concentrated in erythrocytes than in plasma possibly because the Schiff base with hemoglobin is stronger than the one with albumin, driving the uptake of the vitamin to erythrocytes; however, its storage in these cells is saturable [16,19].

3. Vitamin B_6 Metabolic Functions

In addition to the role of B_6 as a cofactor for the degradation of stored carbohydrates [20] and its function as a protective agent against ROS generated in vitro [21], P-5-P was shown to interact with

steroid hormone-receptors, affecting their genes expression [22]. Pyridoxal-5-P also acts as a cofactor in the biosynthesis of many neurotransmitters such as dopamine, epinephrine, gamma-aminobutyric acid, histamine, norepinephrine, and serotonin, as well as the neuromodulator serine [17]. However, the most recognized role of P-5-P is the catalysis of many important steps in the metabolism of amino acids, such as transamination, racemization, decarboxylation, and α,β-elimination reactions [23,24]. The various reactions of P-5-P in the metabolism of amino acids depend on its ability to stabilize amino acid carbanions. In the absence of a substrate, P-5-P forms an internal aldimine (Schiff base) with the lysyl residue (ε-amino group) of the enzyme. Once a substrate amino acid displaces its lysyl residue (α-amino group), P-5-P transfers the aldimine linkage from the ε-amino group to the α-amino group.

4. Transmethylation vs. Transsulfuration

4.1. Transmethylation and the One-Carbon Metabolism

Methylation is an essential metabolic function that controls the addition of –CH3 groups to a variety of organic compounds in every cell of the body. Transmethylation represents metabolic reactions in which –CH3 groups are transferred from one compound to another, comprising both demethylation and remethylation reactions. The methionine cycle contains good examples of transmethylation reactions, in which methionine is demethylated to homocysteine, *S*-adenosylmethionine acts as a methyl donor, with a further possible remethylation to methionine with either 5-methyl tetrahydrofolate or betaine as the methyl donor.

During the methionine cycle, the –CH3 group of methionine, which contains a sulfur atom, is activated by adenosine triphosphate (ATP) through the addition of adenosine to its sulfur. This reaction, which is catalyzed by methionine adenosyltransferase (MAT), forms *S*-adenosylmethionine (SAM) [25], which is an important donor of –CH3 groups to nucleic acids, proteins, and neurotransmitters.

Upon transfer of its –CH3 group, SAM is rapidly converted to S-adenosylhomocysteine (SAH) that, by removal of the adenosine molecule catalyzed by SAH hydrolase (SAHH), is immediately hydrolyzed to homocysteine [26]. Homocysteine can then follow two major pathways; transsulfuration (see next sub-item) or remethylation (Figure 1). Two remethylation pathways regenerate methionine; one is independent of cobalamin (Cbl) but depends on betaine as the one-carbon donor, and the other is Cbl-dependent and requires folate (5-methyltetrahydrofolate) as the one-carbon donor [27].

Folate has two carbon-carbon double bonds that yield dihydrofolate (DHF) and tetrahydrofolate (THF) after saturation of its first and second carbon, respectively, by hydrogen. Folates serve as donors of single carbons in their reduced (5-methyl-THF; CH3THF), intermediate (5,10-methylene-THF; CH2THF), and oxidized (10-formyl-THF; CHOTHF) states [28]. Although not clearly shown in the literature [11,29,30], B_6 status may influence the flux –CH3 groups through remethylation because 5,10-methylene-THF is formed by the methylene group of the side-chain of serine (after its conversion into glycine) along with THF in a reaction catalyzed by the B_6-dependent enzyme serine hydroxylmethyltransferase. Although THF and serine, respectively, are the most important metabolic carrier and source of one-carbon groups, they do not promote the most energetically favorable reactions. The interaction of methionine with ATP produces SAM that easily donates its –CH3 group; SAM is considered the most important one-carbon donor [3]. Folate and SAM transfer of single carbons, which are volatile and bind easily to other molecules, generates the one-carbon metabolism. The one-carbon metabolism creates interplay between amino acid and nucleotide metabolisms, playing a fundamental role in DNA synthesis, repair, and replication [3]. The one-carbon donor 5-methyl-THF is used to convert homocysteine into methionine. The overall reaction transforms 5-methyl-THF into THF while transferring a –CH3 group to homocysteine to form methionine [4].

Methionine synthase (MTR), a vitamin B_{12} (Cbl)-dependent enzyme, catalyzes the final step in the regeneration of methionine from homocysteine [7]. The complex Cbl(I)MTR binds the –CH3 group of 5-methyl-THF to form methylCbl(III)MTR, activating the enzyme. The activated –CH3 group is transferred from methylCbl(III)MTR (regenerating Cbl(I)MTR) to homocysteine synthesizing

methionine, which is released from the enzyme [31]. Under folate and/or vitamin B_{12} deficiency, MTR reactions are severely impaired.

4.2. Transsulfuration and the GPX System

As described above, after its synthesis from SAH, homocysteine can follow the remethylation or the transsulfuration pathway (Figure 1). Transsulfuration is a metabolic pathway involving the conversion of homocysteine into cysteine through the intermediate metabolite cystathionine. Briefly, in a reaction catalyzed by cystathionine β-synthase (CBS; B_6-dependent enzyme), a β-replacement of the thiol group of homocysteine by the acetyl or succinyl group of homoserine forms cystathionine [32]. By acting at the homocysteine junction, CBS represents a critical step, regulating both the maintenance of the methionine pool and the synthesis of cysteine. Therefore, this enzyme would be expected to be strictly regulated. In fact, CBS is allosterically regulated by SAM, in which low SAM concentrations direct homocysteine into remethylation, whereas at high SAM concentrations, transsulfuration is favored [33]. Following cystathionine formation, the B_6-dependent enzyme cystathionine γ-lyase (CGL) cleaves the molecule by γ-elimination of its homocysteine portion. This reaction leaves an unstable amino acid that binds to water molecules to form cysteine, α-ketobutyrate, and ammonia [34]. By the action of gamma-glutamylcysteine synthetase (GCL), cysteine and glutamate synthesize gamma-glutamylcysteine, a rate-limiting step in GSH synthesis [35]. Further, glycine binds to the C-terminal of gamma-glutamylcysteine via the enzyme GSH synthetase to form GSH.

Therefore, the transsulfuration pathway is a straight connection between homocysteine and GSH, the major redox buffer in mammalian cells. Consequently, it is expected that enzymes related to this metabolism display sensitivity to redox changes. Indeed, studies on purified mammalian MTR and CBS have revealed the reciprocal sensitivity of these two major homocysteine-utilizing enzymes to oxidative conditions [5,36]. In mammals, CBS contains a heme cofactor that functions as a redox sensor, increasing CBS activity and consequently transsulfuration under oxidizing conditions [36]. In contrast, under these same conditions, remethylation is depressed because MTR activity is reduced, most likely due to the lability of the reactive cofactor intermediate Cbl(I) [5]. Additionally, the betaine-related enzyme BHMT was also shown to be inhibited by oxidizing agents [6]. Considering that mammals metabolize seleno-amino acids in the same way as their sulfur counterparts [37], recent studies by this laboratory on the effects of selenium (Se) sources and levels combined or not with B_6 provided indirect support to this effect of ROS. Dalto et al. [38] reported that, under the oxidative stress of ovulation, the gene expression of GPX1, 3, and 4 in the livers (Table 1) and kidneys (Table 2) of Se-supplemented animals were higher than in the control Se-unsupplemented group, and animals supplemented with organic Se plus B_6 had the highest gene expression of GPX1 and selenocysteine lyase (SCLY), indicating that the transsulfuration pathway was stimulated. In contrast, Dalto et al. [39] observed, under basal oxidative stress conditions, no differential expression for the same genes (Tables 1 and 2).

Table 1. Real-time mRNA abundance of liver glutathione peroxidase (GPX) and selenocysteine-lyase (SCLY) genes in gilts three days after the fourth estrus and at day 30 of gestation, according to selenium and vitamin B_6 treatments.

	Day 3 Post-Estrus [a]					30 Days Gestation [b]				
	CONT	MSe $B_6$0 [c]	MSe $B_6$10 [c]	OSe $B_6$0 [c]	OSe $B_6$10 [c]	CONT	MSe $B_6$0 [c]	MSe $B_6$10 [c]	OSe $B_6$0 [c]	OSe $B_6$10 [c]
GPX1	0.44	1.02	1.09	0.77	1.57	1.15	1.19	1.37	1.27	1.15
GPX3	0.43	1.02	1.22	0.67	1.17	0.77	1.03	0.87	0.96	0.93
GPX4	0.59	1.00	1.08	0.66	1.19	1.05	1.05	1.19	1.07	0.98
SCLY	0.72	0.82	0.84	0.89	1.45	0.82	0.92	0.84	0.71	0.82

Adapted from Dalto et al. [38,39]. Standard error means for day three post estrus and 30 days gestation respectively equal 0.06 and 0.17 for GPX1, 0.05 and 0.12 for GPX3, 0.05 and 0.10 for GPX4, and 0.07 and 0.12 for SCLY. [a] For all treatments, GPX1, GPX3, GPX4, and SCLY were higher expressed than in the control diet ($p < 0.01$); Among all treatments, OSe$B_6$10 presented the highest gene expression for GPX1 and SCLY ($p < 0.01$); [b] No statistical difference ($p \geq 0.22$); [c] CONT = basal diet; MSe = inorganic selenium; OSe = organic selenium.

Table 2. Real-time mRNA abundance of kidney glutathione peroxidase (GPX) and selenocysteine-lyase (SCLY) genes in gilts three days after the fourth estrus and at day 30 of gestation, according to selenium and vitamin B_6 treatments.

	Day 3 Post-Estrus [a]					30 Days Gestation [b]				
	CONT [c]	MSe $B_6$0 [c]	MSe $B_6$10 [c]	OSe $B_6$0 [c]	OSe $B_6$10 [c]	CONT [c]	MSe $B_6$0 [c]	MSe $B_6$10 [c]	OSe $B_6$0 [c]	OSe $B_6$10 [c]
GPX1	0.72	1.16	1.25	1.19	1.83	0.97	1.11	0.95	1.03	1.14
GPX3	0.73	1.14	1.33	1.41	1.75	1.09	1.17	1.09	1.11	1.03
GPX4	1.06	1.12	1.18	1.18	1.47	1.10	1.22	1.12	1.18	1.17
SCLY	1.20	1.18	1.20	1.48	2.05	1.28	1.15	1.17	1.07	1.25

Adapted from Dalto et al. [38,39]. Standard error means for day three post estrus and 30 days gestation respectively equal 0.14 and 0.10 for GPX1, 0.10 and 0.09 for GPX3, 0.14 and 0.07 for GPX4, and 0.15 and 0.14 for SCLY. [a] For all treatments, GPX1 and GPX3 were higher ($p < 0.01$) expressed than control diet ($p < 0.01$); Among all treatments, OSe$B_6$10 presented the highest gene expression for GPX1 and SCLY ($p < 0.05$); Only for GPX3, OSe was higher than MSe ($p < 0.01$) and $B_6$10 was higher than $B_6$0 ($p < 0.01$); [b] No statistical difference ($p \geq 0.18$); [c] CONT = basal diet; MSe = inorganic selenium; OSe = organic selenium.

5. The Role of B_6

Differential Effects on Organic and Mineral Se Metabolisms in Gilts

Selenium is an essential trace element derived from inorganic (MSe) or organic (OSe) sources. Both forms are involved in the activation of SeGPX. The metabolism of Se-methionine, the natural organic source present in food, is interchangeable with a sulfur-methionine metabolism [37], and, therefore, the influence of B_6 on the one-carbon metabolism by the regulation between remethylation and transsulfuration is predictable. Although less evident, selenide, which is the metabolized form of selenite (commonly used dietary MSe) and a key intermediate for the utilization and/or excretion of both OSe and MSe [40,41], may represent another regulatory step influencing one-carbon metabolism. In this sense, two important B_6-dependent reactions direct selenide to be incorporated into Se-enzymes in preference to its excretion through the use of one-carbon groups in methylation reactions.

For Se-methionine, it is converted to Se-homocysteine through the action of SAM synthetase and SAHH, supplying the one-carbon system with –CH3 groups (Figure 1). As discussed above for sulfur-homocysteine, Se-homocysteine may be remethylated to Se-methionine or transsulfurated to Se-cystathionine, depending on the influence of SAM levels and ROS feedback on CBS. In this context, the SAM levels may promote equilibrium between remethylation and transsulfuration depending on dietary OSe levels, whereas the positive feedback of ROS on CBS (and the negative feedback of MTR) favors the transsulfuration pathway independently of dietary Se levels. Se-methionine may also be transaminated to methylselenol and then transformed to selenide via methyltransferases [42] or methylated to excretory forms. However, considering the importance of this amino acid in protein synthesis and the vital consequences of transmethylation, the partition of its pool to transamination and methylation may have a secondary relevance.

After SeCys synthesis from Se-cystathionine via Se-cystathionine gamma lyase (CGL; B_6-dependent enzyme), this amino acid can be incorporated into proteins or metabolized by the B_6-dependent enzyme SeCys lyase (SCLY) to alanine and selenide [43]. Considering that SCLY is substrate specific, this step is the landmark between sulfur and Se metabolisms and settles their fates in relation to the metabolism of glutathione. It has to be stated that the greater volume of sulfur, rather than SeCys, molecules available in the organism makes the impact of B_6 important not only for the enzyme SeGPX but also for its substrate (glutathione) as well.

Dietary selenite is non-enzymatically reduced via thiol-dependent reactions to selenide [44]. This direct reduction of selenite, short-cutting the transsulfuration pathway and with no regulatory mechanism, allows the accumulation of toxic levels of selenide. To avoid toxicity, selenide from both OSe (synthesized from SeCys) and MSe may follow two pathways: synthesis of Se-proteins or methylation (excretion). By the action of Se-phosphate syntethase 2 (B_6-dependent enzyme), selenite is

converted into Se-phosphate [45]. This last molecule acts as a Se donor in the exchange of the phosphate moiety of serine-tRNA for Se, generating the SeCys-specific tRNA(Ser)Sec, in a B_6-dependent reaction [46]. This special tRNA, which contains the SeCys-insertion sequence (SECIS) along with a SeCys-tRNA-specific elongation factor (eEFSEC) and a specific SECIS binding protein (SECISBP2), is essential for SeCys incorporation into Se-proteins [47]. Remaining selenide must be successively methylated using –CH3 groups from SAM to generate the monomethylated intermediate methylselenol and multimethylated excretory metabolites (dimethylselenide and trimethylselenonium) [48].

Although the metabolism of OSe also generates selenide, the balance between SeCys incorporation into protein and its degradation by the saturable enzyme SCLY prevents the accumulation of excessive selenide. It is known that methylation and demethylation reactions between selenide and methylselenol promote equilibrium between the two molecules [41,42,49]; however, considering that low levels of dietary selenite supplementation provoke toxicity, whereas dietary Se-methionine is tolerated up to extreme high levels [50], one can assume that the utilisation of dietary selenite will direct the metabolism to consume greater amounts of one-carbon groups for the methylation of selenide than dietary Se-methionine. In contrast, OSe metabolism may not only preserve –CH3 molecules through the controlled synthesis of selenide, but also acts generating these one-carbon groups via demethylation/remethylation reactions between Se-methionine and Se-homocysteine. In this context, the regulatory steps (SAM levels, ROS feedback on CBS and MTR, and controlled synthesis of selenide from SCLY) in this metabolic pathway may promote a balance between the synthesis of one-carbon molecules and Se-proteins with possible beneficial effects to the organism.

Recent studies on Se and B_6 metabolisms by this laboratory, using the peri-estrus period as a model for oxidative stress in gilts [38,39,51], provided peculiar information about the control of this metabolic pathway. Although those studies did not specifically address the homeostasis of –CH3 groups, based on the above information this review makes some indirect inferences about the one-carbon metabolism from responses reported in relation to the transsulfuration pathway.

According to Dalto et al. [38,39], B_6 supplementation (10 mg/kg) does not affect the deposition of blood or tissue Se on a long-term basis (3 to 5 estrus), as well as blood Se levels during the peri-estrus period (oxidative stress condition) for both MSe and OSe. For MSe it is not surprising because, independently of the physiological state, absorbed selenite is quickly transformed into selenide and then converted into functional Se-proteins or methylated instead of being actively stored [9]. Consequently, the impact of MSe on the pool of one-carbon molecules for the methylation of selenide would be mainly related to the level of selenite supplementation rather than to B_6 availability.

For OSe supplemented animals however, the explanation is not so straightforward. Under basal oxidative stress conditions, the absence of positive feedback of ROS on CBS suggests that the remethylation of Se-homocysteine would be the preferential pathway, whereas the high SAM levels would stimulate CBS to transsulfurate Se-homocysteine. However, according to Bekaert et al. [52], in humans, high doses of daily supplementation with selenium yeast (a source of OSe) over six months did not affect the homocysteine concentrations in plasma. Also, considering that CBS is a B_6-dependent enzyme, its activity would direct more Se to SeGPX synthesis under B_6 supplementation. Davis [53] and Lima [54] reported that CBS is slightly affected by marginal vitamin B_6 status, whereas CGL is more sensitive, but neither of them affected cysteine synthesis. Although under marginal B_6 levels, these studies indicate that the B_6 status may not be a determining factor of the transsulfuration pathway flux, which is possibly mainly controlled by ROS and SAM levels. In fact, Dalto et al. [39] reported no effect of B_6 on either blood SeGPX activity or GPX and SCLY gene expression in gilts at 30-days of gestation (Tables 1 and 2), a conditions shown to be of low oxidative stress in this species. These results suggest that under basal oxidative stress conditions, in animals supplemented with Se and B_6, neither remethylation nor transsulfuration but Se deposition into proteins is the major route for this mineral.

In contrast, as also described above, Dalto et al. [38] reported that, under the oxidative stress of the peri-estrus period (3-days after estrus), GPX's and SCLY genes were highly expressed in OSe + B_6 supplemented animals in the liver and kidneys, in agreement with the positive feedback of ROS on

CBS, increasing the ratio of transsulfuration. Interestingly, however, these same authors did not find any B_6 effect on SeGPX activity in the liver and kidneys (day 3 after estus) or whole blood (day − 4 to day + 3 of the peri-estrus period). In this regard, Lubos et al. [55] reported that GPX can be regulated by transcriptional, post-transcriptional, translational, or post-translational factors, and changes in gene transcription may not be reflected on GPX protein or its related enzymatic activity.

6. Embryo Metabolism

6.1. 5-Days Porcine Embryos

Using microarray technology to evaluate the global gene expression in 5-days porcine embryos, Dalto et al. [56] provided unique and interesting information on Se and B_6 metabolisms and, consequently, on the distinct interplay between remethylation and transsulfuration during early porcine embryo development.

The first aspect to be considered in 5-days embryos is the transfer of Se from dam to embryos. Dalto et al. [56] showed that Se was undetectable in the uterine flushing, indicating that this is a negligible source of this mineral for the pre-implantation embryo. Nevertheless, embryos from OSe supplemented dams are richer in Se than those from MSe supplemented dams. It was hypothesized that the most probable source is the pre-ovulatory oocyte that might be affected by the systemic maternal blood Se concentration through follicular fluid, considering that OSe is known to be more common deposited in tissues than is MSe [39,51,57,58]. In fact, Dalto et al. [56] observed that maternal supplementation with OSe plus B_6 stimulated 28.8 times more genes than MSe plus B_6 (six and 173 genes for MSe and OSe, respectively). These findings suggest that embryos coming from OSe supplemented dams have greater Se-methionine reserves and, therefore, are more suitable to go through demethylation steps to Se-homocysteine than are embryos from MSe supplemented dams.

A crucial aspect when studying the remethylation/transsulfuration pathway in embryos, fetuses, and newborns is the absence of CGL activity, in spite of its mRNA expression [10,14] (Figure 2). This relevant finding implies that, from conception to neonatal age, individuals are not able to convert Se-methionine into SeCys via the transsulfuration pathway. Therefore, Se-methionine would be directly incorporated into protein, methylated to methylselenol (with further methylation to excretory forms or demethylation to selenide), or demethylated to Se-homocysteine (generating the –CH3 donor SAM) and thereafter remethylated to Se-methionine via folate-dependent reactions, supplying the one-carbon metabolism. Dalto et al. [56] evaluated the expression of genes exclusively related to each source of Se (common genes between sources were excluded from the analysis) in 5-days embryos from gilts supplemented with OSe or MSe combined with B_6 and did not find differentially expressed genes related to the demethylation or remethylation of Se-methionine, whereas several genes related to general elongation factors and biological processes related to translation and mitotic cell cycle were stimulated by OSe. Those authors concluded that, in 5-days porcine embryos, Se-methionine preferentially follows protein deposition. However, recent results from this laboratory (data not yet published), using the database generated by Dalto et al. [56], evaluated global gene expression in 5-days embryos (common genes between sources of Se were not excluded from the analysis), showing that OSe, but not MSe, may affect DNA methylation and epigenetic events in 5-days embryos through the higher generation of –CH3 groups (stimulation of methyltransferases and SAM carrier genes). Therefore, although Se-methionine may be preferentially incorporated into protein, the magnitude of the demethylation and remethylation reactions that compose the one-carbon metabolism cannot be disregarded.

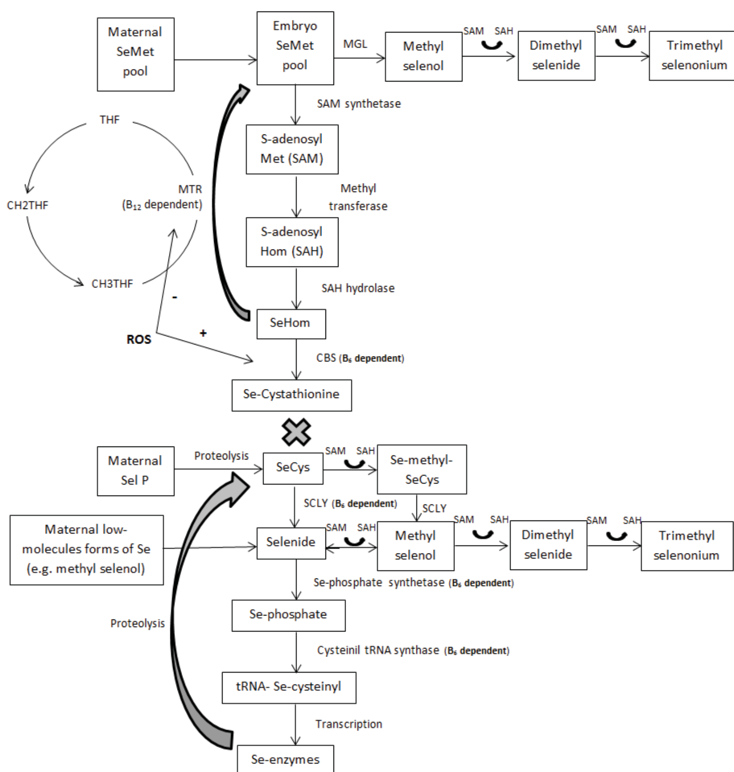

Figure 2. Embryo transmethylation and transsulfuration pathways of selenium (similar for sulfur counterparts) and possible maternal selenium transfer routes. Maternal selenomethionine (SeMet) is transferred to the embryo and may be methylated to methyl selenol by methionine γ lyase (MGL) or demethylated to selenohomocysteine (SeHom). SeHom may follow remethylation back to SeMet or transsulfuration to form selenocystathionine because the enzyme cystathionine γ lyase (CGL) that catalyzes the synthesis of selenocysteine (SeCys) from selenocystathionine is inactive in embryos. Pre-formed SeCys may be available after maternal selenoprotein P proteolysis in the placenta. Through the actions of selenocysteine lyase (SCLY), selenide is formed. Selenide may be methylated and excreted or phosphorylated and its selenium (Se) incorporated into tRNA Se-cysteinyl with further translation to Se-enzymes. THF = tetrahydrofolate; CH2THF = 5,10-methylene-THF; CH3THF = 5-methyl-THF; MTR = methionine synthase; CBS = cystathionine β synthase; ROS = reactive oxygen species.

Although the transsulfuration pathway is not complete in embryos, many genes involved in Se-enzymes synthesis were found in Dalto et al. [56] suggesting that 5-days embryos are potentially capable of synthesizing these enzymes. If so, the most probable sources of Se to the embryo are SeCys, methylselenol, and/or Se-proteins (after proteolysis in the embryo) coming from the pre-ovulatory oocyte. Furthermore, SeGPX synthesis would possibly not be under the feedback control by ROS but limited by the B_6-dependent enzyme SCLY, which controls the synthesis of selenide and, consequently, the consumption of one-carbon molecules for its methylation (Figure 2). Considering the importance of these one-carbon molecules for DNA processing and genetic stability, these routes of maternal Se transfer to early embryos may be considered a mechanism of defense to protect the pool of –CH3.

6.2. 30-Days Porcine Embryos

Similarly to 5-days embryos, the enzyme CGL is absent in 30-days embryos and, consequently, the above aspects related to remethylation/transsulfuration are expected to be analogous. However, at 30 days of gestation the presence of the placenta may change the dynamic of the interaction between these metabolic pathways.

One of the main features brought about by the presence of the placenta is the rise in the oxygen tension in the embryo, increasing the production of ROS. These levels are, in fact, higher in early gestation than thereafter [13], possibly because of the more immature embryonic antioxidant system. This suggests that, under oxidative stress conditions, the feedback of ROS stimulates CBS, directing Se-homocysteine to transsulfuration, whereas it would affect remethylation by reducing MTR activity. According to Kalhan [2], alterations to the metabolism of one-carbon may be the main cause of impaired fetal growth. Additionally, because the transsulfuration pathway is interrupted at Se-cystathionine, large amounts of this metabolite could possibly accumulate with negative effects in embryos from OSe supplemented dams. In this sense, under these conditions, B_6 supplementation levels might be carefully considered due to its effect on CBS activity. Evidences of this negative effect of B_6 on embryo development were observed in a study by Dalto et al. [39], in which maternal supplementation with 12.4 mg/kg of feed of B_6, with either MSe or OSe at 0.6 mg/kg of feed, increased the within-litter Se content variation and the within-litter weight variation, compared to animals receiving 2.4 mg/kg of B_6. Under similar experimental conditions and pig genetic lines, Fortier et al. [51] fed gilts with different sources of Se (MSe or OSe at 0.5 mg/kg of feed) but a fixed amount of B_6 at 3 mg/kg and observed improved within-litter Se content variation, embryo weight and length, and protein and DNA content, with no detrimental effect on the within-litter weight variation, compared to the control diet at similar levels of B_6 but 0.2 mg/kg of feed of Se.

As mentioned above, OSe increases body Se concentration more than MSe due to its deposition in proteins following the methionine metabolism, and B_6 does not interfere with Se deposition in the tissues of adult individuals [38,39]. Also in 30-days porcine embryos, Fortier et al. [51] and Dalto et al. [39] showed that, independently of B_6 status, gilts supplemented with OSe produced embryos with higher Se concentrations than gilts supplemented with MSe. Considering that the transsulfuration pathways is not complete at this stage of development, this higher load of Se-methionine in OSe embryos is not utilized in the synthesis of selenoenzymes but rather will be deposited in proteins and/or transmethylated, producing one-carbon groups. However, it has to be stated that even MSe supplemented gilts supply their embryos with OSe, not as Se-methionine but as SeCys, after selenoprotein P catabolism in the placenta [12]. In spite of the low activity of SCLY in vivo [59], one can assume that the majority of SeCys would be metabolized through SCLY because its concentration in tissues is enough to metabolize all SeCys available and the K_M (Michaelis constant) value of the enzyme is greater than the tissue levels of the substrate. However, Fortier et al. [51] and Dalto et al. [39] reported no effect of maternal Se levels (control vs. Se-supplemented gilts) and sources (OSe vs. MSe) on SeGPX activity in 30-days porcine embryos, indicating that at this stage of development the enzyme flux is not primarily substrate driven. Therefore, it is reasonable to hypothesize that embryo SeCys, from the catabolism of selenoprotein P in the placenta, follows primarily protein deposition and secondarily catabolism by SCLY, which is not controlled by the feedback of ROS, for the synthesis of selenide and further selenoenzymes. This mechanism would protect the embryo from wasting one-carbon groups for the methylation and elimination of excess selenide.

7. Conclusions

Both sulfur and Se methionine are important metabolic suppliers of one-carbon molecules through transmethylation reactions. The equilibrium between transmethylation and transsulfuration pathways, majorly regulated by redox conditions on CBS and MTR, influences the flow of –CH3 molecules between the one-carbon and antioxidation metabolisms.

Vitamin B_6 is important for the interplay between the synthesis (transmethylation) and the utilization (transsulfuration) of one-carbon groups by acting in most of their regulatory enzymes. However, its effects on the GPX system depend on other parameters such as the oxidative stress conditions and metabolic maturity.

Finally, whereas MSe may act by wasting one-carbon molecules to methylate the excess of selenide produced, OSe not only preserves one-carbon groups (due to the control of SCLY) but also promotes equilibrium between transsulfuration and transmethylation via the control of B_6-dependent CBS by SAM and ROS.

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Voluntary Folic Acid Fortification Levels and Nutrient Composition of Food Products from the Spanish Market: A 2011–2015 Update

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Abstract: Introduction. Folic acid (FA) is a synthetic compound commonly added for voluntary fortification of food products in many European countries. In our country, food composition databases (FCDB) lack comprehensive data on FA fortification practices and this is considered a priority research need when undergoing nutritional assessment of the population. Methods. A product inventory was collected and updated by visiting retail stores in Madrid Region, conducting online supermarket searches, and by the provision of food label information by manufacturers. Euro-FIR FCDB guidelines for data compilation and harmonization were used. Results. The FCDB, compiled between 2011 and 2015, includes FA as well as macro and micronutrient data from 338 fortified foodstuffs. As compared to previous FCDB updates (May 2010), 37 products have ceased to declare added FA in their labels, mainly yogurt and fermented milk products. The main food subgroup is 'breakfast cereals' (n = 95, 34% of total). However, the highest average FA fortification levels per recommended serving were observed in the 'milk, milk products, and milk substitutes' group at ≥35% FA Nutrient Reference Values (NRV, 200 µg, EU Regulation 1169 of 2011) (60–76.3 µg FA per 200 mL). Average contribution to the FA NRV per food group and serving ranged between 16%–35%. Conclusion. Our data show a minor decrease in the number of FA fortified products, but vitamin levels added by manufacturers are stable in most food groups and subgroups. This representative product inventory comprises the main FA food source from voluntary fortification in our country. It is therefore a unique compilation tool with valuable data for the assessment of dietary intakes for the vitamin.

Keywords: folic acid; voluntary fortification; food composition database

1. Introduction

Folic acid (FA) is the synthetic form of an essential water-soluble vitamin generically regarded as folates or B9. It is involved in one-carbon metabolism, and it has been linked to lowering Neural Tube Defect (NTD) risk when taken as a supplement around the time of conception [1]. Folates are also naturally present in foods such as green vegetables, fruits, liver, legumes, and nuts. Women of childbearing age are strongly recommended to maintain an adequate folate status through diet and supplementation, although the strategy has been proven to be somewhat ineffective in lowering the risk of NTD in Europe due to a high percentage of unplanned pregnancies and the relatively low compliance with FA pharmacological supplementation [2]. With an aim to increasing women's FA intakes because of its public health relevance, fortification policies have been implemented worldwide. At present, only voluntary fortification of food products with FA takes place in Spain and the rest of Europe, whereas more than 60 countries add FA to wheat flour and other cereal products in a mandatory fortification scheme, according to data from the Food Fortification Initiative [3]. Voluntary fortification, also known as "discretionary fortification", is the addition of vitamins or minerals to

foods at the discretion of the manufacturer in order to restore micronutrients, ensure the nutritional equivalence of substitute foods, and/or to enhance the nutritive value of a product. In this regard, FA addition is endorsed by the European Regulation 1925/2006 of the European Parliament and of the Council of 20 December 2006, on the addition of vitamins and minerals and of certain other substances to food [4], and Regulation 1169/2011 of the European Parliament and of the Council of 25 October 2011, on the provision of food information to consumers [5]. This last regulation lays down the levels of "significant" vitamin addition, the requirements for nutritional and health claims, as well as the Nutrient Reference Values (NRV) for different vitamins, including folate.

In the last few years, an increasing number of researchers have questioned whether Europe should consider implementing mandatory fortification with FA, since current strategies, such as supplementation campaigns, have not been successful in reducing NTD prevalence [2,6]. On the other hand, concerns about the effects of extra FA intakes in children and the elderly are still a major issue that delays the implementation of this population-wide measure [7,8]. Although the Mediterranean diet is naturally a good folate source, data show that the Spanish population is folate deficient [9] and, most remarkably, the Mediterranean Diet is moving towards a less healthy pattern. Traditionally, the Mediterranean diet is characterized by a high consumption of vegetable foods (fresh fruit, vegetables, legumes, wheat bread, and olive oil) and fish, and a low intake of meats (mainly poultry). The latest national data indicate that fresh fruit, legume, and vegetable intakes are decreasing [9], which is in agreement with the data from the Spanish Food Consumption Survey Panel. Approximately only 50%–58% of the adult population reach current FA recommended intakes [10].

Food Composition Tables and Databases (FCDB) are key tools for nutritional assessment of the population's diet. However, they are usually outdated in terms of inclusion of fortified products [11]. Important efforts have been made so far in developing specialized and standardized FCDB such as the one for the EPIC Project (European Prospective Investigation into Cancer and Nutrition) [12], and the Spanish FCDB BEDCA (Base Española de Datos de Composición de Alimentos) [13], both in line with the EuroFIR (European Food Information Resource Network) guidelines for harmonization and interchangeability [14]. However, the number of fortified food items included remains somewhat limited. It has been estimated that fortified foods provide only 5%–8% of the total energy intake of the European population [8], even though, market availability in the last 10 years has been consistently increasing [11]. Interestingly, data from surveys on total intakes of micronutrients (including fortified foods) in Europe and the US show that small proportions of the population, particularly children, may exceed the Upper Intake Levels (UL) for FA [15,16]. However, current fortification practices do not appear to contribute appreciably to the risk of adverse effects derived from nutrient intake [17]. Many researchers have outlined the importance of monitoring fortification practices and consumption of fortified foods in order to continuously assess the efficacy and safety of vitamin and mineral addition to foodstuffs [18–22]. At present, voluntary fortification practices are not being strictly monitored. An important number of brands commercialize fortified products in Spain [23], and continuous market evolution (new product launches and formulation changes) implies fortification level variations for specific vitamins. Food consumption surveys rarely assess fortified food products' potential impact because of the absence of updated data on these products in most commonly used food composition tables and databases [11]. Nonetheless, important efforts have been made to include them, namely the Enkid study, which assessed consumption of fortified products such as breakfast cereals in children and adolescents [24].

For all the aforementioned, since December 2007 we have been actively working on the development of a comprehensive FCDB including all available FA fortified products from the Spanish market [23]. Enhanced reliability and comprehensiveness of food composition tables has been identified as a key research need worldwide in the context of a rapidly changing food supply [25]. Therefore, in this article we present the main findings and trends from the latest database update comprising FA fortified products commercialized in Spain.

2. Materials and Methods

Database design and structure has been previously described [23]. Briefly, Microsoft Office Access® 2003 software (Microsoft Co., Redmond, Washington, DC, USA) was used for designing a tailor-made relational database. The LanguaL™ food description thesaurus and EuroFIR guidelines were adopted, including food group classification schemes [26]. The FCDB update was conducted through a market and online survey from January 2011 to June 2015 based in the Madrid Region. The consumption of fortified foodstuffs in the Madrid Region is higher as compared to the national average consumption [27], and the region includes the Capital City and a highly urbanized area (Metropolitan Madrid). Therefore, food availability in Madrid may be considered to include all fortified food products that would be available throughout different regions of the country. Retail centres such as hypermarkets, supermarkets, and convenience stores were visited over a four week period each year of the study and were selected in accordance with their sales data [28]. In addition, commercial online stores were accessed for product search, which includes food product availability nationwide. Foods that declared FA on the ingredient list by the following terms: B_9, folate, folacin, or FA were identified and listed as potential FCDB inclusions. Compiled data included macro and micronutrients per 100 g or mL, recommended serving size, nutritional and health claims, and a photograph of the product package. Once completed, quality control checks were applied to the FCDB numerical data to assess potential errors and inconsistencies in recorded data. FA fortification levels were assessed as percentage of FA NRV (200 μg, [5]) provided per recommended serving for each food group as described in our previous studies [21]. All values are expressed per 100 g of edible portion on a fresh weight basis, unless otherwise stated. Food and Agriculture Organization (FAO) Guidelines for checking food composition prior to publication of a user database were followed when applicable [29].

Results of macro- and micronutrient contents are presented as median and interquartile range for skewed variables. Variables were tested for normality using a Kolmogorov-Smirnov test. All statistical analyses were performed using SPSS Software (SPSS 20.0, SPSS Inc., Chicago, IL, USA).

3. Results

A total of 338 FA fortified products were compiled and assessed. 37 products were removed from the FCDB as they are no longer available for purchase or because FA is no longer included in their composition (Figure 1), mainly breakfast cereals, cereal bars, flours or starch, foods for weight reduction, milk, yogurts (fermented milks), cheese, juice or nectar, margarine and vegetable fats and oils, and chocolate products. A total of 25 products were new to the database, mainly foods for infants, coffee, tea, cocoa or infusions, soft drinks, pastries and cakes, and bread. Four food groups and 13 subgroups were included in the FCBD and their distribution is presented in Table 1. 'Grain and grain products' (50%, n = 169), and 'products for special nutritional uses or dietary supplements' (31%, n = 105) represented the highest proportion of available fortified products, while 'milk, milk products, or milk substitutes' (15%, n = 52) and 'beverages (non-milk)' (4%, n = 12) were minor. Total number of brands was 39, of which 31 were traditional manufacturer's brands and 8 were from distribution (supermarket own brand). Ten products declared FA content on labels (ingredients list) but did not specify the quantity per 100 g or serving in the nutritional information label. Median declared FA contents ranged from 15 μg per 100 g (30 μg per 200 mL serving) in 'nectar and juices' and 'soft drinks' to 199 μg per 100 g (39.8 μg per 20 g serving) in 'coffee, tea, cocoa, or infusions' (Table 2). 'Breakfast cereals' is the subgroup that represented the highest proportion of FA fortified products (n = 95, 28% of total), with median declared FA levels of 170 μg per 100 g (51 μg per 30 g serving). Secondly, 'foods for infants' accounted for 20% (n = 70) of fortified products, with median declared FA levels of 65 μg per 100 g (16.25 μg per 25 g serving). Median energy content provided by each FA fortified food group ranged from 22.5 kcal per 100 mL in the case of 'soft drinks', to 406 kcal per 100 g in 'cereal bars'. Carbohydrates were the main declared macronutrient in all groups, ranging from 5 to 80 g per 100 mL or g; of these, added sugars accounted for 5 to 72 g per 100 mL or g, and fibre content was 0.5 to 12 g per 100 g. Starch content, however, was not declared in most products. Total fat contents were 1.6 to

10 g per 100 g and the lipid profile (proportion of monounsaturated, polyunsaturated, and saturated fatty acids) was not declared in most food groups. Protein contents were 0.2 to 14 g per 100 mL or g.

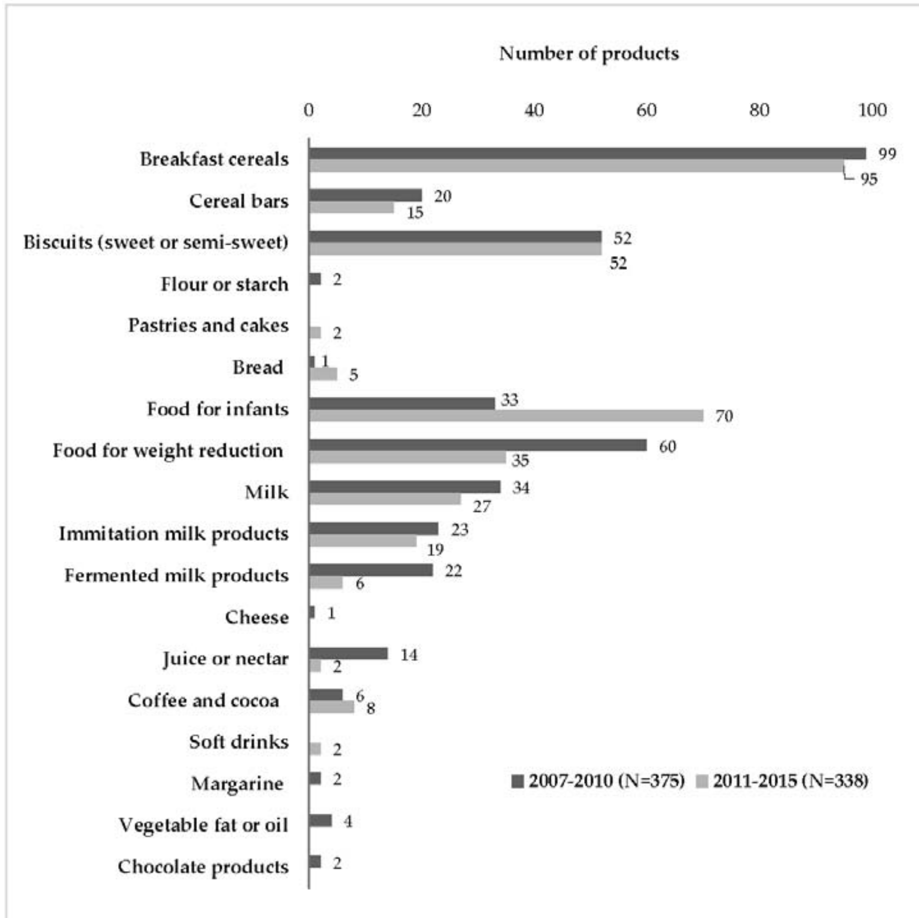

Figure 1. Food subgroup distribution in the Voluntary Folic Acid Fortification Food Composition Database: comparison between first (2007–2010) and second (2011–2015) compilation.

A general outlook of the Access® relational database, as well as an example of two database tables compiling fortified food data can be found at the Supplementary Material section. Most frequently added vitamins and minerals to FA fortified food formulations are shown in Tables 2 and 3. All data are presented as medians and interquartile ranges. 'Foods for infants' and 'foods for weight reduction' were the groups that presented the highest proportion of simultaneous addition of vitamins other than FA, containing nearly all vitamins with the exception of vitamin K in 'foods for weight reduction'. 'Milk products' declared to contain vitamins C, B1, B2, B3, B6, B12, pantothenic acid, and biotin; 'imitation milk products' contained vitamins A, D, E, and B6; 'coffee, tea, cocoa or infusions' declared vitamins A, E, D, C, B1, B2, B3, B6, B12, and pantothenic acid; and 'breakfast cereals' included mainly vitamins D, B1, B2, B3, B6, B12 and pantothenic acid, together with FA. In the case of added minerals, again 'foods for infants' and 'foods for weight reduction' were those that declared a higher number as compared to the other subgroups. All 'grain products' declared iron addition, and specifically 'cereal bars' and

'bread products' also declared the addition of calcium and sodium, respectively; some 'milk products' included calcium, phosphorus, and zinc addition. This shows that in most analysed products mineral addition was limited when compared to vitamins.

FA fortification levels from compiled products were calculated from labelled FA values and recommended servings per each product, using the FA Nutrient Reference Value (200 µg/day, NRV) as guidance (Figure 2). In the major food group of the FCDB, 'grain and grain products', 56% of items presented fortification at level 2 which accounts for 16.1% to 25.9% of FA NRV per recommended serving. Fortification at level 1 (≤16% FA NRV) was observed in 63% of 'products for special nutritional uses' ('foods for infant' and 'weight reduction') and 72% of 'milk, milk products or milk substitutes' were fortified at the highest level available (level 4, ≥35% FA NRV). Finally, the 'beverages' group showed a higher proportion of level 1 fortification, as 42% of products provided less than 16% of FA NRV per recommended serving. These data should be interpreted with care in terms of FA contribution since NRV are set at 200 µg/day of FA according to EU Regulation, while in Spain, recommended FA intakes for women of childbearing age are 400 µg/day. Accordingly, the actual contribution of these products is well below half of this group's needs.

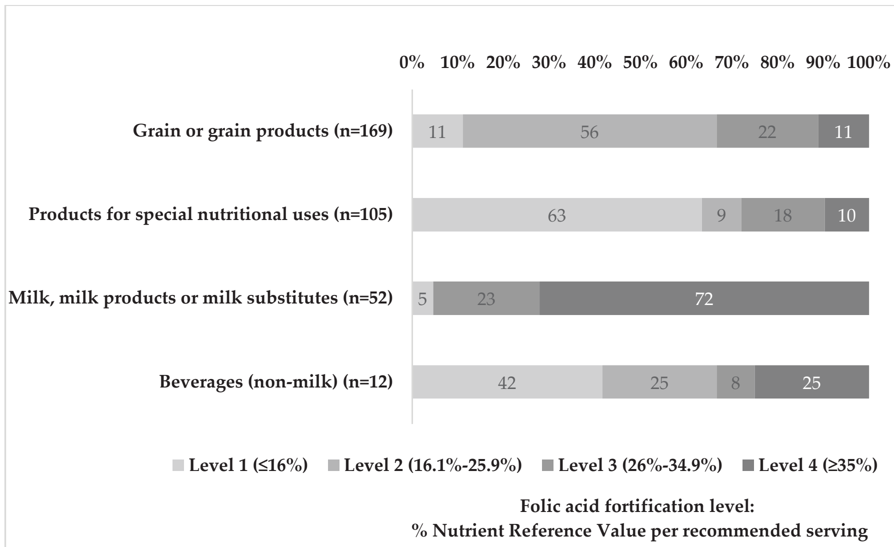

Figure 2. Folic acid fortification levels calculated per manufacturer's recommended serving in compiled food groups from the Voluntary Folic Acid Fortification Food Composition Database.

Table 1. Macronutrient distribution, fibre and salt content in folic acid-fortified food products from the Spanish market.

Food Groups and Subgroups	*N*	Energy (kcal)	Declared Serving (g or mL)	Fats (Total) (g)	SFA (g)	MUFA (g)	PUFA (g)	Carbohydrates (g)	Sugars (g)	Starch (g)	Fibre (g)	Protein (g)	Salt (g)
Grain or grain products	169												
Breakfast cereals	95	386 (378–403)	30 (25–30)	3 (1–7)	1 (0–3)	ND	ND	76 (67–81)	24 (20–30)	ND	5 (3–8.25)	8 (6–8)	0 (0–1)
Biscuits, sweet and semi-sweet	52	444 (423.2–462.7)	25 (25–29.5)	15.5 (12–17.5)	2 (1–4)	ND	ND	66.5 (63–71.5)	21 (17.5–23)	ND	4 (2–7.75)	7 (6–8)	0.41 (0.23–0.83)
Cereal bars	15	406 (380–416.5)	25 (23–30)	10 (7–13)	4 (3–9)	ND	ND	67 (61–74)	30 (23–35)	0 (0–36.5)	4 (3–6)	6 (5–7)	ND
Bread and similar products	5	375 (368–389)	ND	4 (3–5.5)	0.6 (0.45–1.65)	1.25 (0.75–2.2)	2.5 (0.87–4.05)	63 (59–70)	2 (1–4)	ND	12 (6.5–14)	14 (12–15)	1 (0.5–1)
Pastries and cakes	2	383 (383–389)	40	13 (13–13.5)	ND	ND	ND	58	34 (34–36)	ND	8 (8–8.5)	4	0.51 (0.45–0.58)
Products for special nutritional uses or dietary supplements	105												
Foods for infants	70	387 (90–483.2)	25 (20–24.75)	3 (2–21.2)	1.3 (0.47–7.72)	7.9 (1.7–9.7)	3.2 (0.5–5.1)	60 (13–78)	25 (7.7–39.2)	ND	0.5 (0–4.25)	9 (2–10)	0.1 (0.025–0.3)
Foods for weight reduction	35	383 (368–462)	45 (20–63)	12 (10–17)	5 (3–11)	ND	ND	45 (37–55)	29 (14–34)	ND	5 (2–8)	24 (6–26)	ND
Milk, milk products, or milk substitutes	52												
Milk	27	46 (38–52)	250	1.6 (0.3–1.95)	1 (0.275–1.1)	0.1 (0–0.6)	0 (0–0.3)	4 (4–5)	4 (4–5)	ND	ND	3 (3–3)	0.13 (0.13–0.2)
Imitation milk products	19	52 (49–60)	250	1.9 (1.6–2.5)	0.56 (0.41–1.05)	1.2 (0.4–1.4)	0.2 (0.2–0.3)	5 (4–6)	5 (4–6)	ND	0 (0–0.225)	3 (2–3)	0.13 (0.1–0.18)
Fermented milk products	6	49 (45–77)	65 (65–100)	1.85 (0.32–2.2)	0.2 (0.17–0.37)	1.15 (0.17–1.4)	0.6	3 (3–12.7)	3 (3–12)	ND	1.1	2 (2–2.25)	0.1 (0.1–0.12)
Beverages (non-milk)	12												
Coffee, tea, cocoa, or infusion	8	386.5 (371–548.5)	20 (15.25–30)	3.5 (2.5–16)	1 (1–8)	ND	ND	80 (73.5–81)	72 (61.5–75)	ND	6.5 (4–8.5)	5.5 (4.25–35.25)	0 (0–2.25)
Juice or nectar	2	24	200	ND	ND	ND	ND	5.5	5.2	ND	0.4	0.2	0.025
Soft drinks	2	22.5 (22–22.5)	310	ND	ND	ND	ND	5	5	ND	ND	ND	ND
TOTAL	338												

N = number of products. Values are expressed as median and interquartile range per 100 g or mL. ND = not declared; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Table 2. Vitamin content distribution in folic acid-fortified food products from the Spanish market.

Food Groups and Subgroups	*N*	A (μg)	D (μg)	E (mg)	K (μg)	C (mg)	B1 (mg)	B2 (mg)	B3 (mg)	B6 (mg)	Folic Acid (μg)	B12 (μg)	Biotin (μg)	Pantothenic Acid (mg)
Grain or grain products	169													
Breakfast cereals	95	ND	0 (0–1)	0 (0–10)	ND	ND	0 (0–1)	1 (1–1)	13 (13–14)	1 (1–1)	170 (166–200)	2 (0–2)	ND	0 (0–5)
Biscuits, sweet and semi-sweet	52	ND	ND	ND	ND	ND	ND	ND	ND	ND	100 (71.5–100)	ND	ND	0 (0–1)
Cereal bars	15	ND	0 (0–2)	0 (0–4)	ND	ND	0 (0–1)	1 (1–1)	13 (11–16)	1 (1–1)	170 (140–200)	2 (0–2)	ND	2 (0–5)
Bread and similar products	5	ND	ND	ND	ND	ND	ND	ND	0 (0–4.5)	0 (0–0.5)	100 (0–100)	ND	ND	0 (0–1.5)
Pastries and cakes	2	ND	ND	ND	ND	ND	ND	ND	10	ND	126	1	ND	ND
Products for special nutritional uses or dietary supplements	105													
Foods for infants	70	375 (101–459)	7.5 (1.7–8.9)	4 (1.3–6.2)	5.8 (0–33)	25 (14–71.5)	0.4 (0.15–0.58)	0.6 (0.11–0.87)	4.5 (1.7–6.95)	0.34 (0.1–0.6)	65 (15–70)	0.5 (0.1–1.18)	12 (1.9–15)	2.8 (0.4–3)
Foods for weight reduction	35	375 (105–467)	2 (1–3)	5 (4–7)	ND	25 (0–36)	0.7 (0.44–0.97)	0.76 (0.37–1.1)	8.4 (5.5–12)	0.9 (0.7–1.2)	120 (76–150)	0.38 (0.7–1.2)	12 (8–33)	1.9 (0.9–3)
Milk, milk products, or milk substitutes	52													
Milk	27	120 (120–120)	0.75 (0.75–0.76)	1.8 (1.5–1.8)	ND	0 (0–4.5)	0 (0–0.085)	0 (0–0.105)	0 (0–0.8)	0 (0–0.105)	30 (30–30)	ND	ND	0 (0–0.45)
Imitation milk products	19	120 (120–120)	0.75 (0.75–0.8)	1.8 (1.5–1.8)	ND	0 (0–12)	0 (0–0.21)	0 (0–0.23)	0 (0–2.4)	0.24 (0.2–0.3)	30 (30–30)	0.38 (0–0.4)	0 (0–4.9)	0 (0–0.8)
Fermented milk products	6	ND	0 (0–0.75)	ND	ND	ND	ND	ND	ND	0.85 (0.2–0.9)	90 (30–145)	0 (0–0.38)	ND	ND
Beverages (non-milk)	12													
Coffee, tea, cocoa, or infusion	8	0 (0–716)	5 (5–7)	0 (0–13.75)	ND	48 (46–78)	1 (0–1)	0 (0–1)	18 (16.25–21)	1.5 (1–2)	199 (190–283)	0 (0–1.5)	ND	3 (1–5.5)
Juice or nectar	2	60 (0–60)	ND	ND	ND	6 (0–6)	ND	ND	1 (0–1)	ND	15 (0–15)	ND	3 (0–3)	ND
Soft drinks	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	15 (0–15)	ND	ND	ND
TOTAL	338													

N = number of products. Values are expressed as median and interquartile range per 100 g or mL. ND = not declared.

Table 3. Mineral content distribution in folic acid-fortified food products from the Spanish market.

Food Groups and Subgroups	*N*	Sodium (mg)	Potassium (mg)	Calcium (mg)	Phosphorus (mg)	Magnesium (mg)	Iron (mg)	Zinc (mg)	Copper (mg)	Manganese (mg)	Selenium (µg)	Iodine (µg)
Grain or grain products	169											
Breakfast cereals	95	ND	ND	ND	ND	ND	7 (7–8)	ND	ND	ND	ND	ND
Biscuits, sweet and semi-sweet	52	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cereal bars	15	ND	ND	0 (0–760)	ND	ND	7 (0–10)	ND	ND	ND	ND	ND
Bread and similar products	5	400 (0–480)	ND	ND	ND	ND	0 (0–3.5)	ND	ND	ND	ND	ND
Pastries and cakes	2	ND	ND	ND	ND	ND	6	ND	ND	ND	ND	ND
Products for special nutritional uses or dietary supplements	105											
Foods for infants	70	0 (0–139)	0 (0–507.7)	175 (121–372)	59 (0–228)	0 (0–38.2)	6 (2.1–7.5)	0.8 (0–3.8)	0 (0–0.3)	0 (0–0.525)	0 (0–8.95)	12 (0–74.7)
Foods for weight reduction	35	29 (0–91.5)	781 (267.5–1091)	328 (0–436)	367 (0–469)	88 (0–110)	8 (0–10)	5 (0–6)	0 (0–0.65)	0.47 (0–0.8)	28 (0–31.5)	61 (0–86)
Milk, milk products, or milk substitutes	52											
Milk	27	ND	ND	120 (110–160)	0 (0–120)	ND	ND	ND	ND	ND	ND	ND
Imitation milk products	19	ND	ND	120 (105–132)	0 (0–67)	ND	ND	0 (0–1)	ND	ND	ND	ND
Fermented milk products	6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Beverages (non-milk)	12											
Coffee, tea, cocoa, or infusion	8	ND	0 (0–3132)	260 (145.2–1079)	301.5 (0–1091.5)	170 (0–236)	7.5 (0–24)	0 (0–11)	0 (0–0.75)	0 (0–0.75)	0 (0–45)	0 (0–139.5)
Juice or nectar	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Soft drinks	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TOTAL	338											

N = number of products. Values are expressed as median and interquartile range per 100 g or mL. ND = not declared.

4. Discussion

To our best knowledge, this is a unique updated food composition data compilation and assessment of FA fortified products available in the Spanish market. The Spanish fortified food supply is widespread through almost all available food groups. However, staple food products such as breads are only fortified to a limited extent when compared to other European countries such as Ireland and the UK, where breads, fat spreads, and fruit juices are commonly fortified [30]. In our country, fat spreads do not have added FA anymore, while only five years ago, up to six products containing FA were available [23]. Although it was not a main aim of our study, a limitation pertaining the use of the label-declared FA values is that we found fortification overages (higher than declared values added to products) in a number of products in previous studies [21]. The use of analytical FA contents from foodstuffs is always advisable, but the present work includes such a high number of products and types of food matrixes, that it would be a highly expensive and unaffordable task to undertake.

In order to assess the potential impact of FA-fortified products on a population's nutritional status, it is also necessary to evaluate if product market availability is in line with the most-consumed products in our country. Although data on fortified food consumption in Spain are scarce, in 2011 a study by the Spanish Nutrition Foundation showed that fortified milks were the most consumed fortified product amongst the population (49.61 g/person/day) followed by fortified yogurt (14.22 g/person/day) [27] by using data of the Food Consumption Panel. Our data indicates that voluntary FA fortification strategies do not follow this trend as fortified 'milk and milk products' are decreasing over time, whilst 'breakfast cereal' availability remains constant.

The main challenges of voluntary food fortification are reaching target populations such as women of childbearing age, which should be the main objective, and avoiding extra FA intakes in non-target groups. In Spain, women of childbearing age are at risk of insufficient folate intakes since only 50% of recommended folate intake is ingested through diet at present [10]. The daily inclusion of fortified products could provide this population group with 20%–60% of FA recommended intake, when fortification level 4 products are consumed [31]. On the other hand, there is concern that population subgroups, including children and the elderly, may be at risk of consuming usual intakes above the UL. In a previous study we assessed the potential intake of the main FA fortified products by children aged 2–13 years and the ULs were exceeded in no case [32]. In addition, the composition of these foodstuffs has also been discussed previously, as a high percentage of them contain high levels of added simple sugars, salt, and fat [33]. According to our results, fortified groups contain between 2–24 g of sugar per 100 g in 'bread and similar products' and 'breakfast cereals'. However, these quantities should be considered on the serving basis, since 'breads' may contain 1.2 g of sugar per 60 g serving and 'breakfast cereals', 7.2 g of sugar per 30 g serving (Table 1). Taking into consideration the World's Health Organization (WHO) and the European Food Safety Authority (EFSA) recommendations to keep simple sugar intake below 10% of daily Total Energy Intake [34], especially amongst the child population, specific fortified food products could be included with moderation in the context of a varied diet. The nutritional benefits of increased vitamin intakes should not be outweighed, however, by the risks according to the present results. Whether voluntary fortification is beneficial depends on which foods manufacturers fortify, which nutrients are chosen as fortificants, how much of the fortificant is added, and what portion of the population consumes the fortified products.

For research purposes, databases must be constantly updated to reflect the rapidly evolving marketplace, so that the contribution of both added and intrinsic micronutrients may provide accurate estimates of population intakes. In 2007, Irish researchers examined the effect of voluntary food fortification on dietary intake and biomarker status of folate and other homocysteine-related B vitamins in a healthy adult population (aged 18–92 years) using an updated FCDB [22]. They found that red blood cell folate concentrations were 387 nmol/L higher and plasma total homocysteine concentrations were 2 μmol/L lower in the group with the highest fortified food intake (median FA intake: 208 μg/day), as compared to the non-consumers of fortified foods, showing a substantial

increase in dietary intakes as well as biomarkers of folate status. Regardless the widespread availability of FA fortified products, this type of assessment has not been performed to date in a representative sample of the Spanish population. It would be advisable to have detailed food consumption data at a brand level from the Spanish population in order to evaluate the actual impact of the observed fortification practices with FA. Data is scarce and therefore quantification of the underestimation of FA intake due to the non-consideration of fortified products is still speculative. According to our previous studies, the non-consideration of FA fortification may underestimate as much as 40% of actual folate and folic acid intake in women [31] and children [32]. In the Irish study [22] the difference in total folate and FA intake between consumers and non-consumers of fortified products is above 50%. These observations should be taken into account if the availability of FA fortified food items prevails and may be consumed by a significant number of individuals.

5. Conclusions

This newly updated and representative database reflects the energy and nutrient composition data of FA voluntarily fortified foods from four main categories commercialized in Spain. There is an important number and variety of available products. Our data show a minor decrease in FA fortified products, but vitamin levels added by manufacturers are stable in most food groups and subgroups. It is therefore a unique compilation tool with valuable data for the monitoring and assessment of dietary intakes of this vitamin.

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Article

Correlations between Maternal, Breast Milk, and Infant Vitamin B12 Concentrations among Mother–Infant Dyads in Vancouver, Canada and Prey Veng, Cambodia: An Exploratory Analysis

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Abstract: Vitamin B12 plays an essential role in fetal and infant development. In regions where animal source food consumption is low and perinatal supplementation is uncommon, infants are at risk of vitamin B12 deficiency. In this secondary analysis, we measured total vitamin B12 concentrations in maternal and infant serum/plasma and breast milk among two samples of mother–infant dyads in Canada (assessed at 8 weeks post-partum) and in Cambodia (assessed between 3–27 weeks post-partum). Canadian mothers (n = 124) consumed a daily vitamin B12-containing multiple micronutrient supplement throughout pregnancy and lactation; Cambodian mothers (n = 69) were unsupplemented. The maternal, milk, and infant total vitamin B12 concentrations (as geometric means (95% CI) in pmol/L) were as follows: in Canada, 698 (648,747), 452 (400, 504), and 506 (459, 552); in Cambodia, 620 (552, 687), 317 (256, 378), and 357 (312, 402). The majority of participants were vitamin B12 sufficient (serum/plasma total B12 > 221 pmol/L): 99% and 97% of mothers and 94% and 84% of infants in Canada and Cambodia, respectively. Among the Canadians, maternal, milk, and infant vitamin B12 were all correlated ($p < 0.05$); only maternal and infant vitamin B12 were correlated among the Cambodians ($p < 0.001$).

Keywords: vitamin B12 (cobalamin); lactation; human milk; Canada; Cambodia

1. Introduction

Vitamin B12 is found only in animal-source or fortified foods [1], so deficiency is most common in those who consume vegan diets [2] or in populations in low- and middle-income countries where animal foods are not available or affordable [3]. Maternal vitamin B12 status in pregnancy strongly influences infant vitamin B12 status [4]; breast milk vitamin B12 content is reduced when maternal vitamin B12 status is poor [5]. Since exclusive breastfeeding is recommended for the first 6 months of life [6], infants rely solely on their stores and breast milk for vitamin B12 during this time [2]. Attaining adequate vitamin B12 status in infancy—a time of rapid growth and development—is essential for normal cognitive development in infants [7]. Deficiency, which typically presents in infants between 4 and 10 months but may appear within months of birth, can lead to failure to thrive, developmental regression [2], and severe neuropathy [8].

There is currently little published data describing blood and milk vitamin B12 concentrations of purportedly well-nourished mother–infant dyads [4]. For instance, there is currently no agreed-upon cut-off for vitamin B12 deficiency in breast milk; although a cut-off of <362 pmol/L was used to define deficiency previously, it was developed over 25 years ago using older laboratory methods that may have incorrectly measured vitamin B12 analogues, and included only 19 milk samples [9,10]. Greibe et al. measured plasma vitamin B12 concentrations among 60 Danish mother–infant dyads, and breast milk vitamin B12 among a subset of 25 mothers at 2 weeks, 4 months, and 9 months postpartum [11]. The majority of those women were consuming daily vitamin B12-containing multivitamin supplements. Moderate correlations (r = 0.27 to 0.52) were found between maternal and infant vitamin B12 concentrations at all three time-points. Milk vitamin B12 concentrations, however, fluctuated considerably throughout lactation, and were only significantly correlated with maternal and infant plasma vitamin B12 concentrations at 4 months postpartum [11]. In contrast, the authors of an earlier study reported that breast milk B12 concentrations remained relatively constant after falling from their highest levels in colostrum [12].

With the relatively limited data currently available on perinatal vitamin B12 status, we used secondary data from two recent randomized control studies [13,14] to assess the vitamin B12 status of mothers, their breast milk, and their infants in two regions with differing perinatal supplementation and dietary patterns: Canada and Cambodia. We also explored associations between vitamin B12 concentrations in maternal blood, breast milk, and infant blood samples.

2. Materials and Methods

2.1. Study Populations and Biological Sample Collection

This was a secondary analysis of two studies conducted with exclusively breastfeeding mother–infant dyads in Vancouver, British Columbia, Canada (Canada sample), and Prey Veng province, Cambodia (Cambodian sample). In both samples, a short demographic questionnaire was conducted among participants to gather information such as age, antenatal care, and socioeconomic status; the questionnaire was self-administered in Canada, and interviewer-administered in Cambodia.

2.1.1. Canadian Sample

Participants in the Canadian sample were enrolled as part of a randomized control trial from June 2010 through March 2013, investigating the efficacy of three doses of maternal perinatal vitamin D to improve infant 25-hydroxyvitamin D concentrations at 8 weeks postpartum; results are published elsewhere [13]. Briefly, healthy pregnant women 18–42 years with a low-risk singleton pregnancy in Vancouver, British Columbia were recruited using convenience sampling. Women in this study were randomized to consume an identical daily perinatal multivitamin supplement containing 12 μg vitamin B12 starting at 13 to 22 weeks gestation through to 8 weeks post-partum, and a second daily supplement that contained 10, 25, or 50 μg vitamin D3. This study was approved by the British Columbia Children's and Women's Clinical Research Ethics Board (H13-01971).

At 8 weeks postpartum, non-fasting venous blood samples were collected from mothers and infants into trace element-free evacuated tubes (Vacutainer, Becton Dickinson, Mississauga, ON, Canada). The samples were allowed to clot at room temperature for 30 min, centrifuged (2000× *g* for 10 min at 4 °C), and then the serum was removed, aliquoted, and stored at −80 °C until analysis. Breast milk samples were collected either at the clinic during the phlebotomy visit or by women themselves the morning of the phlebotomy visit. Regardless of collection site, milk from one full breast expression was collected using an electric breast pump (the woman's own, or Swing breast pump, Medela), more than two hours after the previous feeding. If women collected milk themselves at home, the sample was stored in their refrigerator until the phlebotomy visit (<10 h). At the clinic, the volume of the milk sample was recorded, and then mixed thoroughly, aliquoted, and stored at −80 °C until analysis.

2.1.2. Cambodian Sample

Participants in the Cambodian sample were recruited as part of a randomized control trial designed to test the efficacy of maternal ad libitum consumption of thiamin-fortified or control (non-thiamin fortified) fish sauce to improve maternal and infant erythrocyte thiamin diphosphate concentrations and breast milk thiamin concentrations [14]. Healthy women 18–45 years with a low-risk singleton pregnancy residing in Prey Veng province, Cambodia, were recruited between 3–8 months gestation (self-report), and consumed fish sauce for 6 months as part of this study. All women received 90 iron-folic acid supplements during pregnancy; no participants consumed vitamin B12-containing supplements. The National Ethics Committee for Health Research in Cambodia approved this study (0245 NECHR; 386 NECHR).

Between 3–27 weeks postpartum (in April 2015), trained phlebotomists collected non-fasting venous blood samples from mothers and their infants into EDTA-coated tubes (Vacutainer, Becton Dickinson). The samples were placed immediately on ice, and were transported to the National Institute of Public Health (NIPH) laboratory in Phnom Penh within 5 h of collection. Samples were centrifuged (3000 rpm for 15 min at 4 °C), and plasma removed, aliquoted, and stored at −80 °C. Breast milk samples were collected during the phlebotomy visit using a procedure identical to that in the Canadian study. Breast milk samples were placed on ice and transported to the NIPH laboratory with the blood samples. Total milk volume was recorded, then each sample was mixed thoroughly, aliquoted into amber cryovials, and stored at −80 °C. Both blood and breast milk samples were shipped on dry ice to The University of British Columbia in Vancouver, Canada, for storage at −80 °C until being shipped to other laboratories for analysis.

2.2. Biological Sample Analysis

Serum/plasma samples were shipped on dry ice to the University of Otago in Dunedin, New Zealand for analysis in the Houghton Lab. Total vitamin B12 concentration was measured using an electrochemiluminescence immunoassay (Roche Diagnostics) on an Elecsys 2010 (Roche, New Zealand). Control samples fell within the recommended manufacturer detection range of 22–1495 pmol/L; inter-assay coefficient of variance of a pooled serum sample with a known vitamin B12 concentration within the detection range was 8.3% (n = 13). Total vitamin B12 concentration was measured in serum samples from 124 Canadian mothers and 102 infants; and in plasma samples from 69 Cambodian mothers and 50 infants.

Human milk vitamin B12 concentrations were measured using chemiluminescence on an IMMULITE automated analyzer in the Allen Lab, US Department of Agriculture/ARS Western Human Nutrition Research Centre, Davis; methods reported in detail elsewhere [15]. Milk samples were boiled in dithiothreitol and potassium cyanide to release protein-bound vitamin B12 before analysis. Breast milk vitamin B12 concentration was measured in 109 Canadian and 59 Cambodian samples.

2.3. Statistical Analysis

Only those mothers who self-reported exclusive breastfeeding were included in the analysis. Demographic characteristics are expressed as mean (95% CI) and n (%) for continuous and categorical variables, respectively, unless otherwise noted. Concentrations of vitamin B12 were not normally distributed in either sample for any of the biological specimens ($p < 0.05$ for Shapiro–Wilk test of normality), and thus were transformed using the natural log for analyses and back-transformed and expressed as geometric mean (95% CI). Independent sample t-tests were used to assess differences in maternal age, parity, and maternal, milk, and infant total vitamin B12 concentrations, and chi-square tests were used to assess differences in educational attainment and household income between the Canadian and Cambodian samples. Pearson's bivariate correlations (Canada) and partial correlations (Cambodia, controlled for infant age) were used to determine correlations between maternal and infant total vitamin B12 concentrations, and between milk vitamin B12 concentrations and both maternal and infant total vitamin B12 concentrations.

All infants in the Canadian sample were evaluated at 2 months $\pm$ 1 week of age; infants in the Cambodian sample ranged from 3 to 27 weeks of age, with a mean (SD) age at sample collection of 15 (7) weeks. Therefore, linear regression models were run in the Cambodian sample to assess the effect of infant age on maternal, infant, and milk vitamin B12 concentrations.

Vitamin B12 deficiency is defined here as serum/plasma total vitamin B12 concentration < 148 pmol/L, and marginal deficiency between 148–221 pmol/L [1]. Since there are no cut-offs specifically designed for use in infants, the same cut-offs were employed for both mothers and infants. Chi-square tests were used to determine if there was a difference in the prevalence of serum/plasma vitamin B12 deficiency between groups.

All analyses were performed on SPSS for Macintosh version 23.0 (IBM Corp, Armonk, NY, USA), with a significance level of $p < 0.05$.

3. Results

All Cambodian mothers were Khmer; in Canada, the majority of mothers (n = 97; 79%) were of European descent (7% were Chinese, and 14% were of 12 other ethnicities). Canadian mothers were older than the Cambodian mothers: mean (95% CI) age was 37 (36, 38) and 26 (25, 27) years, respectively ($p < 0.001$). The Canadian mothers also had greater educational attainment and annual household income than the Cambodian participants ($p < 0.001$). Approximately half of mothers—53% in Canada and 54% in Cambodia—were pregnant for the first time ($p = 0.66$).

Biochemical vitamin B12 status is presented in Table 1. Maternal and infant serum/plasma and breast milk total vitamin B12 concentrations were all significantly higher among the Canadian as compared to the Cambodian sample ($p < 0.05$), although the distributions of mothers' and infants' B12 status did not differ between groups. Histograms showcasing the maternal, milk, and infant total vitamin B12 concentrations of the Canadian and Cambodian samples are shown in Figure 1.

Correlations between maternal, infant, and breast milk total vitamin B12 concentrations are shown in Table 2. In the Canadian sample, all three variables were significantly correlated with one another ($p < 0.05$). In the Cambodian sample, however, only maternal and infant total vitamin B12 concentrations were correlated ($p < 0.001$); breast milk total vitamin B12 concentrations were not correlated with either maternal ($p = 0.43$) or infant ($p = 0.64$) total vitamin B12 concentrations.

Unlike in Canada, where all samples were collected at 2 months post-partum, data and sample collection in Cambodia took place when infants were aged between 3 and 27 weeks, or mean (SD) of 15 (7) weeks postpartum. We used linear regression models to examine the association between infant age and plasma and milk vitamin B12 concentrations among the Cambodian sample, and found that none of these vitamin B12 measurements were significantly correlated with infant age (Table 3). While infant age was borderline inversely associated with milk vitamin B12 concentrations ($p = 0.05$), age would have accounted for <5% of the variance in milk B12 concentrations. Table 4 displays the prevalence of vitamin B12 inadequacy among the Cambodian sample by infant age; 8 weeks was used

as a cut-off to align with the Canadian sample. Again, infant age appeared to influence only milk vitamin B12 concentrations, with older infants (>8 weeks) tending to have lower mean concentrations.

Table 1. Vitamin B12 status among mothers and their infants in Vancouver, Canada, and Prey Veng province, Cambodia [1,2].

Vitamin B12 Marker	*n*	Vancouver, Canada	*n*	Prey Veng, Cambodia	*p* Value
Maternal vitamin B12	124	698 (648, 747)	69	620 (552, 687)	0.009
Deficient (<148)		-		1 (~1%)	0.37
Marginal (≥148 to 221)		1 (1%)		1 (~1%)	
Sufficient (≥221)		123 (99%)		67 (97%)	
Infant vitamin B12	102	506 (459, 552)	50	357 (312, 402)	<0.001
Deficient (<148)		2 (2%)		4 (8%)	0.10
Marginal (≥148 to 221)		4 (4%)		4 (8%)	
Sufficient (≥221)		96 (94%)		42 (84%)	
Breast milk vitamin B12 [3]	109	452 (400, 504)	59	317 (256, 378)	<0.001

[1] Serum (Canada) and plasma (Cambodia) total vitamin B12 (pmol/L), and breast milk vitamin B12 (pmol/L) expressed as geometric mean (95% CI); vitamin B12 status expressed as *n* (%); [2] Independent samples *t*-tests and chi-square tests were employed to assess differences between samples from Canadians and Cambodians of vitamin B12 concentrations and adequacy status categories, respectively; [3] Breast milk samples collected at 2 months postpartum among Canadian women, and between 3–27 weeks (mean (SD) was 15 (7) weeks) postpartum in the Cambodian sample.

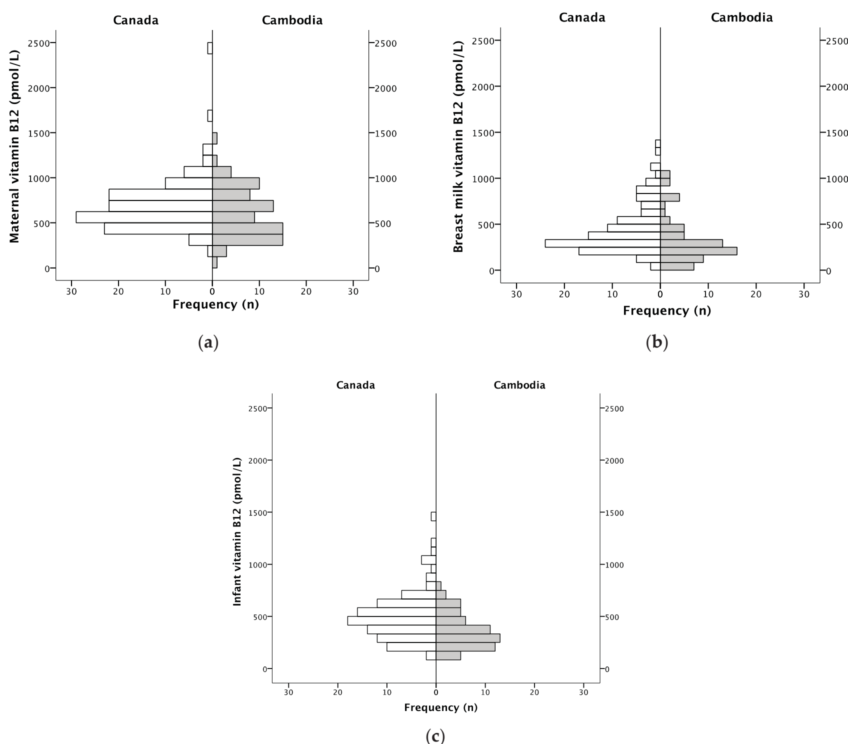

Figure 1. Histograms of (**a**) maternal total vitamin B12 concentrations (pmol/L), (**b**) human milk total vitamin B12 concentrations (pmol/L), and (**c**) infant total vitamin B12 concentrations (pmol/L) among the Canadian (white bars) and Cambodian (shaded bars) samples.

Table 2. Correlations between maternal, infant, and milk total vitamin B12 concentrations among samples of mother–infant dyads in Canada and Cambodia.

Vitamin B12 marker	*n*	Correlation [1]	*p* Value
Maternal vitamin B12 and breast milk vitamin B12			
Vancouver, Canada	109	0.498	<0.001
Prey Veng, Cambodia	59	0.105	0.43
Maternal vitamin B12 and infant vitamin B12			
Vancouver, Canada	102	0.208	0.04
Prey Veng, Cambodia	49	0.562	<0.001
Breast milk vitamin B12 and infant vitamin B12			
Vancouver, Canada	88	0.370	<0.001
Prey Veng, Cambodia	45	0.073	0.64

[1] Pearson's bivariate correlations were used for the Canadian sample; partial correlations controlling for infant age were used in the Cambodian sample.

Table 3. Linear regression models of the association between infant age and plasma and milk vitamin B12 concentrations among the Cambodian sample.

Vitamin B12 Marker	Adjusted R^2	Unstandardized		Standardized β	*p* Value
		β	95% CI		
Maternal plasma vitamin B12	0.038	−0.015	−0.031, 0.001	−0.229	0.06
Milk vitamin B12	0.049	−0.028	−0.056, 0.000	−0.256	0.05
Infant plasma vitamin B12	0.021	−0.013	−0.032, 0.005	−0.202	0.16

Table 4. Total vitamin B12 concentrations and prevalence of vitamin B12 sufficiency by infant age (older or younger than 8 weeks) among the Cambodian sample [1].

Vitamin B12 Marker	*n*	Infant ≤ 8 weeks	*n*	Infant > 8 weeks	*p* Value
Maternal plasma vitamin B12	18	634 (464, 803)	51	615 (541, 688)	0.85
Deficient (<148)		-		-	
Marginal (≥148 to 221)		-		1 (2%)	0.73
Sufficient (≥221)		18 (100%)		50 (98%)	
Infant plasma vitamin B12	12	343 (253, 433)	38	361 (307, 415)	0.93
Deficient (<148)		-		4 (10%)	
Marginal (≥148 to 221)		1 (7%)		3 (8%)	0.50
Sufficient (≥221)		11 (93%)		31 (82%)	
Breast milk vitamin B12 [2]	13	427 (266, 588)	46	286 (222, 351)	0.051

[1] Total plasma vitamin B12 (pmol/L), and breast milk vitamin B12 (pmol/L) expressed as geometric mean (95% CI); vitamin B12 status expressed as *n* (%). Independent samples *t*-tests were employed to assess differences in vitamin B12 concentrations, and chi-square tests were used to assess differences in the prevalence of vitamin B12 adequacy between infant age groups; [2] Breast milk samples were collected between 3–27 weeks (mean (SD) was 15 (7) weeks) postpartum.

4. Discussion

Milk vitamin B12 concentrations reported in the literature vary considerably. Greibe and colleagues reported median concentrations of vitamin B12 in the hindmilk of 25 well-nourished Danish mothers of 760, 290, and 440 pmol/L at 2 weeks, 4 months, and 9 months postpartum, respectively [11], indicating variability with lactation stage. In this study, we found higher total vitamin

B12 concentrations in the milk of Cambodian mothers $\leq$ 8 weeks postpartum (n = 13) compared with >8 weeks postpartum (n = 46) (427 versus 286 pmol/L, respectively), however this was not statistically significant (p = 0.051; Table 4). Regardless, these results are difficult to compare due to the low sample sizes, differing time-points for milk collection, and because we collected a full milk expression, while only hindmilk was collected in the Danish study.

As noted earlier, a milk vitamin B12 deficiency cut-off of <362 pmol/L exists [9,10], but is not commonly employed due to potential overestimation of inadequacy. Nearly all mothers in both Canada and Cambodia ($\geq$97%) had adequate vitamin B12 status (>221 pmol/L), as did their infants—94% and 84%, respectively. However, the vitamin B12 status of breast milk was below the cut-off of 362 pmol/L [9,10] among 50% and 75% of Canadian and Cambodian mothers, respectively (data not shown). The high prevalence of adequate vitamin B12 status among infants despite less than adequate breast milk vitamin B12 levels highlights the necessity for more research to better define deficient vitamin B12 breast milk levels given current laboratory methods.

Consistent with previous studies, we found significant correlations between maternal and infant serum/plasma total vitamin B12 concentrations in both the Canadian and Cambodian samples. For example, Greibe et al. identified significant correlations (p < 0.05) between maternal and infant vitamin B12 concentrations at three different time-points among Danish infants aged 2 weeks (r = 0.52), 4 months (r = 0.47), and 9 months (r = 0.29) [11]. Similarly, significant correlations between maternal and infant plasma vitamin B12 were reported among Malawian dyads (n = 521) in the Breastfeeding, Antiretroviral, and Nutrition (BAN) study—r = 0.42 and 0.32 at 2 or 6 weeks and 24 weeks, respectively [16].

In our study, we found that milk vitamin B12 concentration was not significantly correlated with infant total vitamin B12 in the Cambodian sample; however, at 2 months post-partum, milk was significantly but weakly correlated with infant serum vitamin B12 concentrations in the Canadian sample (r = 0.108, p < 0.001; see Table 2). The vitamin B12 content of milk is impacted by maternal dietary vitamin B12 intake and depletion [5]; however, the association between milk vitamin B12 and infant vitamin B12 status is not consistent. Greibe et al. found that milk was only significantly correlated with infant plasma vitamin B12 concentrations at 4 months postpartum (r = 0.58; p = 0.005) but not 2 weeks or 9 months [11]. In a Guatemalan study of 113 dyads at 3 months postpartum, the authors reported that milk vitamin B12 concentrations were inversely associated with infant vitamin B12 status (r = $-$0.22; p < 0.05) as assessed by urinary methylmalonic acid [10]. The BAN study in Malawi reported significant correlations between maternal plasma, breast milk, and infant plasma vitamin B12 concentrations at both 2 or 6 and 24 weeks, but speculated that maternal vitamin B12 status in pregnancy is likely a more important predictor of infant status than milk concentrations [16]. It may be that maternal vitamin B12 status during pregnancy is a better indicator of infant vitamin B12 adequacy in the first year of life than milk vitamin B12 concentrations, given that infants of well-nourished mothers are born with ~25 μg of vitamin B12 stores [17], which should protect the infant from inadequacy to 12 months of age regardless of milk vitamin B12 content.

Maternal and infant serum/plasma and breast milk total vitamin B12 concentrations were significantly higher among the Canadian sample compared to the Cambodian sample (p < 0.05). These differences were not unexpected, because the Canadian mothers were consuming a daily vitamin B12-containing perinatal supplement providing >400% of the recommended dietary allowance for lactation [18]. Although all Cambodian mothers were consuming fish sauce as part of the original randomized control trial [14], this is not a significant source of dietary vitamin B12; American fish sauce provides only ~0.05 μg vitamin B12 per 10 mL fish sauce [19] as per international CODEX standards [20]. The lack of vitamin B12 deficiency in Cambodia is consistent with the most recent Cambodian Demographic and Health Survey (2014), which reported only 1% of mothers (n = 731) were vitamin B12 deficient (<150 pmol/L) [21].

This study has several strengths, namely the collection of three biological samples from mother–infant dyads at the same time-point (mother and infant blood, and breast milk). In addition,

a full breast expression was collected, and samples were collected from the majority of women first thing in the morning, which helps to limit intra-participant diurnal variation, although this is not a major concern with vitamin B12 [22]. We also employed current laboratory methods to assess milk vitamin B12 [15]; older methods may have incorrectly measured vitamin B12 analogues. However, as a secondary analysis, this study has several shortfalls. It would have been ideal to measure at least one direct and one functional indicator for vitamin B12 status in addition to total vitamin B12 concentration. However, due to a lack of blood samples and funding, we were unable to measure methylmalonic acid or total homocysteine. We selected total vitamin B12 concentration as our indicator because it is the most commonly employed and inexpensive direct biomarker [23]. The lack of longitudinal data is a limitation, especially given that vitamin B12 levels appear to fluctuate throughout lactation. In addition, breast milk values from Cambodia cannot be directly compared against those from Canada because they were not collected at the same time; while early literature indicates that breast milk B12 concentrations remain relatively constant after falling from their highest levels in colostrum [12], a more recent study reported fluctuation throughout lactation [11]. Our analyses included only exclusively breastfeeding dyads, and therefore it is less relevant for comparison with children consuming breast milk substitutes or those who have started consuming complementary foods. Dietary data were not collected, limiting our ability to generalize these findings to populations with known high- or low-vitamin B12 intakes. Anthropometric measures were not assessed at the time of blood and milk collection in either group. In addition, these samples were not representative, limiting generalizability.

5. Conclusions

The vast majority of both Canadian and Cambodian mothers (≥97%) and infants (≥84%) had sufficient vitamin B12 status (>221 pmol/L), however maternal, infant, and milk total vitamin B12 concentrations were significantly higher among the Canadian sample compared to the Cambodians. In the Canadian sample, maternal, milk, and infant vitamin B12 concentrations all significantly correlated, while only maternal and infant plasma vitamin B12 concentrations were correlated in the Cambodian sample. The wider age range among Cambodian infants (3 to 27 weeks) allowed for the exploration of associations in infant age revealing a borderline significant inverse relationship with infant age and infant B12 status. The vast majority of both mothers (≥97%) and infants (≥84%) in both groups had sufficient vitamin B12 status (>221 pmol/L).

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South Asian Ethnicity Is Related to the Highest Risk of Vitamin B12 Deficiency in Pregnant Canadian Women

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Abstract: Vitamin B12 (B12) adequacy during pregnancy is crucial for maternal health and optimal fetal development; however, suboptimal B12 status has been reported in pregnant Canadian women. Methylmalonic acid (MMA) is a sensitive indicator of B12 status. Since few studies have measured MMA during pregnancy in Canadian women, the objective of this study was to evaluate B12 status in pregnant women living in Metro Vancouver, using both plasma total B12 and MMA. We recruited a convenience sample of 320 pregnant women between 20 and 35 gestational weeks from local healthcare facilities. Plasma total B12 concentrations indicative of deficiency (<148 pmol/L) and suboptimal B12 status (148–220 pmol/L) were found in 18% and 33% of the women, respectively. Normal plasma MMA concentration (<210 nmol/L) was observed in 82% of all women. Gestational age was a strong predictor of plasma total B12 and MMA concentration, and South Asian ethnicity of B-12 deficiency and MMA concentrations. Overall, there was a high discrepancy between the prevalence of B12 inadequacy depending on the biomarker used. Independently, however, South Asian women were at particular risk for B12 deficiency, likely due to lower animal source food intake. Further study of this vulnerable group and performance testing of B12 biomarkers is warranted.

Keywords: pregnancy; cobalamin; vitamin B12; low vitamin B12 status; deficiency; methylmalonic acid; MMA; ethnicity; South Asian; predictor

1. Introduction

Maternal vitamin B12 (B12) adequacy is important for fetal growth and neurodevelopment, and for maternal and infant health. Low maternal B12 status has been associated with preterm birth [1], intra-uterine growth restriction [2], congenital heart defects [3], neural tube defects (NTDs) [4], and impaired cardiometabolic health in the offspring [5,6]. Maternal B12 status is a key determinant of infant B12 status [7,8]. Poor infant B12 status can have irreversible long-term consequences, including poor intellectual and cognitive performance later in childhood [9].

In the Canadian Health Measures Survey (Cycle 1; 2007–2009), suboptimal B12 status (serum total B12 =148–220 pmol/L) and B12 deficiency (serum total B12 <148 pmol/L) were found in 20% and 6% of adult women of all ages, respectively [10]. We previously reported a 20% prevalence of suboptimal

B12 status and 14% B12 deficiency in a convenience sample of 206 reproductive-aged women of South Asian and European ethnicity living in Metro Vancouver, with a non-significant trend for a higher prevalence of B12 deficiency in South Asian women [11]. Results of two recent prospective cohort studies of pregnant Canadian women revealed a high prevalence of low B12 status in pregnancy, with deficiency as high as 38% at delivery [12,13].

B12 deficiency is also known to be particularly high in South Asian women, with 40%–90% of women of childbearing age in India having low serum total B12 concentration and/or elevated MMA concentration [6,14,15]. This high prevalence is attributed to dietary patterns associated with little intake—low frequency and small portion sizes—of animal source foods [6]. South Asian women of childbearing age who immigrate to high-income countries are at higher risk of B12 deficiency compared to those of European descent [11,16]. The prevalence of B12 deficiency in South Asian pregnant women living in Canada has not been reported.

Biochemical B12 status can be assessed using direct indicators such as total B12 and holotranscobalamin, and functional biomarkers such as methylmalonic acid (MMA) and total homocysteine. Due to problems with the sensitivity and specificity of single measurements, the combined use of one direct and one functional biomarker has been recommended [17]; however, the majority of studies investigating B12 status have only measured total B12, including a previous study of pregnant women in Vancouver [13].

Our objective was to determine B12 status and the prevalence of B12 deficiency, using combined plasma total B12 and MMA concentrations, in a sample of healthy pregnant women in Metro Vancouver. We also aimed to identify dietary, demographic, and lifestyle factors associated with B12 status.

2. Materials and Methods

2.1. Study Population

This cross-sectional study was conducted in Metro Vancouver, British Columbia, Canada, between February 2009 and February 2010 [18]. Using convenience sampling, 340 pregnant women were recruited from BC Women's Hospital and Health Centre, Douglas College prenatal programs, and various Vancouver Coastal Health Community Health Centres. Recruitment was conducted actively by health professionals such as nurses and dietitians at these locations, as well as passively by brochures left at clinics and advertisements in local newspapers. Women were eligible to participate if they were between the 20th and 35th week of gestation, and having a singleton pregnancy. Women were excluded from participating if they had any co-morbid conditions such as gestational diabetes, cardiac or renal disease, HIV/AIDS, chronic hypertension, or autoimmune disease. The study was approved by the University of British Columbia Children's and Women's Research Ethics Board (#H08-01447). Participants provided written informed consent prior to the study.

2.2. Demographic, Lifestyle, Dietary, and Anthropometric Variables

Data were collected at a healthcare centre during a one-time interview lasting approximately 30–60 min. Participants completed a questionnaire on demographics and lifestyle factors to collect the following information: age, week of gestation, supplement use, smoking status, self-identified ethnicity, income, and highest educational level attained. We categorized women's ethnicity into European, Chinese Asian, South Asian, and "Other" ethnicities including participants of Korean, Japanese, Southeast Asian, African, Latin American, West Asian, and Arabian descent. Data for highest education level were trichotomized into the following categories: less than high school—no schooling or some elementary or completed elementary or some high school; high school degree—completed high school or some trade/vocational training or some university; university or trade school—completed trade/vocational training or completed university. Information on usual consumption of meat, fish, egg, and dairy products was collected using a qualitative questionnaire (yes/no). Participants were asked to provide their pre-pregnancy weight, and height was measured by a research assistant in order to calculate BMI.

2.3. Biochemical Analysis

Participants were asked to provide a non-fasting blood sample, from which plasma was extracted and subsequently stored at −80 °C until further analysis. Plasma samples were analyzed for B12 biomarkers unless they were haemolysed (defined by visual assessment). Plasma total B12 concentrations were measured using Microparticle Enzyme Immunoassay technology (Abbott Laboratories, Abbott Park, IL, USA). Inter-assay variation (CV %) for the medium quality control was 3.7%. Plasma MMA concentrations were quantified using liquid chromatography–tandem mass spectrometry (LC–MS/MS) (LC: Agilent Technologies, Santa Clara, CA, USA; MS/MS: SCIEX, Framingham, MA, USA) [19]. The intra- and inter-assay variations (CV %) of the method were 0.8% and 3.0%, respectively. The use of non-fasting blood samples for determining B12 status does not pose challenges in results interpretation because the indicators plasma total B12 and MMA do not underlie postprandial changes [20,21].

2.4. Statistical Analysis

To date, there are no established pregnancy-specific cutoffs for B12 biomarkers. Vitamin B12 status was categorized using the non-pregnant adult cutoffs for plasma total B12: B12 deficiency: <148 pmol/L; suboptimal B12 status: 148–220 pmol/L; B12 adequacy: >220 pmol/L. Plasma MMA concentrations were categorized as follows: <210 nmol/L, B12 replete; 210–370 nmol/L, mildly elevated MMA concentration; >370 nmol/L functional B12 deficiency [22,23].

Because plasma total B12 and MMA concentrations were skewed, non-parametric tests were applied and results are presented as medians and interquartile ranges (IQR). The impact of categorical health and lifestyle variables on total B12 concentrations was assessed using the Mann-Whitney *U* test or the Kruskal-Wallis test. The distribution of B12 deficiency, suboptimal status, and adequacy in relation to qualitative characteristics was examined with chi-squared tests using Bonferroni adjustment. Spearman's Rank correlation was used to examine the association between the biomarkers. Multiple linear and logistic regression analyses were applied to identify predictors of plasma total B12 and MMA concentrations, and predictors of B12 deficiency (dichotomized; defined as plasma total B12 <148 pmol/L) and mildly elevated MMA concentration (dichotomized; defined as plasma MMA >210 nmol/L [22]), respectively. Residuals were examined for heteroskedasticity and normality. No obvious heteroskedasticity was observed, and there were only minor deviations of the residuals from normality, which was mitigated by the large sample. Therefore, no transformation of the plasma total B12 and MMA concentrations was performed. Backwards model selection based on Akaike's Information Criterion (AIC) was used to construct the best-fit model to predict plasma total B12 or MMA concentration, B12 deficiency, or elevated MMA concentration. Prior to entry into the models, collinearity of the potential predictor variables was assessed. Statistical significance was defined as $p < 0.05$. Statistical analyses were conducted using Stata 12.1 (StataCorp LLC, College Station, TX, USA) for Windows 10 (Microsoft Corp., Redmond, WA, USA).

3. Results

3.1. Participant Characteristics

Biomarker results were obtained from 320 pregnant women; the characteristics of these participants have been previously reported [18]. Briefly, the median age was 31 years (range 16–47 years) and 63% of the participants were >30 years. Median gestational age was 30 weeks and over two-thirds of the participants were in their third trimester of pregnancy (≥27 gestational weeks). Women had a median pre-pregnancy BMI of 22.5 kg/m^2; 73% were classified as normal weight, 20% as overweight and 7% as obese. Almost half of the participants were of European descent (47%), 19% Chinese, 8% South Asians, and the remaining 26% of "other" ethnicities. The majority of women (57%) had a university degree and 6% reported to have used tobacco during pregnancy.

3.2. Plasma Total B12 and MMA Concentration

The median (IQR) plasma total B12 concentration was 215 (160, 283) pmol/L (Table 1). Plasma concentrations indicative of B12 deficiency were found in 18% of women and 33% had suboptimal B12 status (Table 1). The highest prevalence of B12 deficiency, 61.5%, was observed in women of South Asian ethnicity. Over 90% of the participants reported taking B12-containing supplements. Usual consumption of fish, meat, eggs, and dairy products was reported by 74%, 83%, 95% and 99% of the women, respectively. Plasma total B12 concentrations were higher in B12 supplement users and women with higher education. Women of South Asian ethnicity had lower plasma total B12 concentration (Table 1).

Table 1. Plasma total B12 concentration (pmol/L) and prevalence of B12 deficiency, suboptimal B12 status and B12 adequacy, stratified by demographic, anthropometric, lifestyle and dietary characteristics.

	n	Median (Interquartile Range) *	Prevalence, *n* (%)			
			<148 pmol/L	148–220 pmol/L	>220 pmol/L	*p* Value $^{\$}$
All participants	320	215 (160, 283)	58 (18)	106 (33)	156 (49)	
Age (years)						
<30	120	185 ^{a} (145, 245)	31 (26)	41 (34)	48 (40)	0.001 $^{\#}$
≥30	200	230 ^{b} (182, 298)	27 (14)	53 (26)	120 (60)	
Gestational age (weeks)						
<27	106	229 (176, 298)	16 (15)	28 (26)	62 (59)	NS
≥27	214	210 (154, 275)	42 (20)	66 (31)	106 (49)	
Pre-pregnancy body mass index (kg/m^2)						
<25	235	222 (166, 291)	39 (17)	67 (28)	129 (55)	NS
25–29.9	64	204 (151, 263)	15 (23)	19 (30)	30 (47)	
≥30	21	189 (159, 243)	4 (19)	8 (38)	9 (43)	
Ethnicity						
European	150	203 ^{a} (160, 277)	24 (16)	61 (41)	65 (43)	<0.001 $^{\#}$
Chinese	60	256 ^{b} (202, 311)	4 (7)	14 (23)	42 (70)	
South Asian	26	132 ^{c} (105, 231)	16 (61.5)	3 (11.5)	7 (27)	
Other	84	220 ^{a,b} (171, 269)	14 (17)	28 (33)	42 (50)	
Education						
Less than high school	21	172 ‡ (161, 209)	3 (14)	13 (62)	5 (24)	0.004 $^{\#}$
High school degree	77	206 (147, 264)	20 (26)	21 (27)	36 (47)	
University or trade school	221	228 ‡ (171, 287)	35 (16)	60 (27)	126 (57)	
Family income per year ($)						
<40,000	36	184 (151, 230)	7 (19)	16 (44)	13 (36)	NS
40,000 to <80,000	52	223 (171, 280)	9 (17)	14 (27)	29 (56)	
80,000 to <120,000	57	231 (160, 302)	10 (17)	17 (30)	30 (53)	
≥120,000	55	211 (176, 280)	8 (14)	18 (33)	29 (53)	
Unknown	72	225 (164, 306)	13 (18)	16 (22)	43 (60)	
Not answered	48	208 (152, 271)	11 (23)	13 (27)	24 (50)	
Smoking of tobacco during pregnancy						
Yes	18	200 (160, 237)	3 (17)	9 (50)	6 (33)	NS
No	302	219 (160, 284)	55 (18)	85 (28)	162 (54)	
Use of B12-containing supplements						
Yes	297	222 ^{a} (162, 284)	50 (17)	85 (29)	162 (54)	0.002 $^{\#}$
No	23	173 ^{b} (140, 221)	8 (35)	9 (39)	6 (26)	
Fish consumption						
Yes	238	223 (160, 287)	42 (18)	73 (31)	123 (52)	NS
No	82	204 (153, 258)	16 (20)	33 (40)	33 (40)	
Meat consumption						
Yes	267	216 (160, 280)	49 (18)	80 (30)	138 (52)	NS
No	18	204 (145, 302)	5 (28)	4 (22)	9 (50)	

NS, non-significant ($p > 0.05$), * Estimates within a column subgroup not sharing a common superscript letter are significantly different; Mann-Whitney *U* test or Kruskal-Wallis test (all $p < 0.001$) with Dunn's test for pairwise comparison ($p < 0.05$); ‡ $p = 0.065$. $^{\$}$ Chi-squared test to determine if the number of participants across categories of B12 status is random; $^{\#}$ significant after Bonferroni correction, i.e., $p < 0.05/(n \text{ of tests}) = 0.05/10 = 0.005$.

The median (IQR) plasma MMA concentration was 140 (110, 187) nmol/L. The majority of pregnant women (82%) had plasma MMA concentrations indicative of B12 adequacy (<210 nmol/L; [22]), and six women (1.9%) had functional B12 deficiency (defined as having elevated MMA concentrations of >370 nmol/L; [23]). Overt B12 deficiency (defined as plasma total B12 <148 pmol/L and concurrent plasma MMA >370 nmol/L) was present in five out of the 320 women (1.6% of the study population), with three women of South Asian ethnicity. The remaining women with plasma B12 concentrations indicative of B12 deficiency had either normal (n = 31) or marginally elevated (n = 22) MMA concentrations.

There was a significant negative correlation between plasma total B12 and MMA concentrations ($\rho = -0.38$, $p < 0.0001$). Women with plasma MMA concentrations <210 nmol/L had significantly higher plasma total B12 concentrations (median 229; IQR 176, 297 pmol/L) compared to women with mildly elevated (median 157; IQR 114, 212 pmol/L) and elevated MMA concentration (median 132; IQR 107, 209 pmol/L) ($p < 0.0001$).

Plasma MMA concentrations were significantly higher in pregnant women of South Asian descent (median 223; IQR 131, 288 nmol/L) compared to European (median 144; IQR 115, 180 nmol/L), Chinese Asian (median 135; IQR 103, 190 nmol/L), and other ethnic groups (median 137; IQR 104, 174 nmol/L) ($p = 0.004$). Plasma MMA concentrations were significantly higher in third-trimester (median 146; IQR 117, 199) compared to second-trimester (median 128; IQR 96, 168) pregnant women ($p < 0.001$). There was no difference in plasma MMA concentrations with respect to pre-pregnancy BMI, supplement use, fish and meat intake, smoking, education, or income.

3.3. Predictors of B12 Status

There were complete data for multivariate analyses of 284 women after exclusion of 36 cases with missing data for meat intake and an additional case with missing data for education. The income variable was missing for 100 participants (~35%) and therefore not included in the modelling. Fish, egg, and meat intake were highly collinear with 205 out of 284 women reporting the consumption of all three. Similarly, there was a relationship between ethnicity and fish consumption with Europeans (106/140 = 76%), "Other" (54/66 = 82%), and Chinese Asian (43/56 = 77%) reporting more fish consumption than South Asians (9/22 = 41%). No similar relationship was observed between ethnicity and meat or egg consumption; however, very few women report no egg consumption (5%) or meat consumption (6%). We thus included ethnicity but excluded fish intake from the initial full model during model selection.

For plasma total B12, backwards model selection resulted in five variables remaining in the best-fit linear regression model: age, gestational age, pre-pregnancy BMI, education, and use of B12 supplements (Table 2); and two variables remaining in the best-fit logistic regression model, ethnicity and use of B12 supplements (Table 3). For plasma MMA, backwards model selection resulted in four variables remaining in the best-fit linear regression model: gestational age, ethnicity, smoking during pregnancy, and egg consumption (Table 4); and three variables remaining in the best-fit logistic regression model, gestational age, ethnicity, and egg intake (Table 5).

Plasma total B12 concentration was positively associated with age, education, and B12 supplement use (Table 2). Conversely, there was a negative relationship between gestational age and pre-pregnancy BMI with plasma total B12 concentration. Multivariate logistic regression revealed that the odds of being B12 deficient were about 10-times higher for those who self-identified as South Asians relative to those who identified as European (with "Other" and Chinese Asian ethnicity not statistically different from European ethnicity) (Table 3). The odds of being B12 deficient were 69% lower for those who were taking B12 supplements.

Table 2. Predictors of plasma total B12 concentration in linear regression model.

	Univariate		Multivariate	
	β Coefficient (95% Confidence Interval)	p Value	Adjusted β Coefficient (95% Confidence Interval)	Adjusted p Value
Age (years)	3.05 (0.49, 5.6)	0.02	2.07 (−0.54, 4.67)	0.12
Gestational age (weeks)	−3.12 (−5.63, −0.61)	0.02	−2.96 (−5.41, −0.51)	0.02
Ethnicity				
European	reference	0.10		
Other	−5.74 (−38.8, 27.3)			
Chinese Asian	33.8 (−1.20, 68.8)			
South Asian	−26.7 (−77.4, 24.1)			
Pre-pregnancy body mass index (kg/m^2)	−3.75 (−6.74, −0.76)	0.01	−3.37 (−6.31, −0.42)	0.03
Smoking	−31.7 (−90.8, 27.3)	0.29		
Education				
Less than high school	reference	0.02	reference	0.13
High school degree	46.4 (−11.3, 104)		32.3 (−24.6, 89.2)	
University or trade school	69.2 (16.3, 122)		50.8 (−2.61, 104)	
B12 supplement use	43.9 (−6.4, 94.2)	0.09	42.1 (−6.92, 91.1)	0.09
Fish intake	13.2 (−17.2, 43.5)	0.39		
Meat intake	13.1 (−41.2, 67.4)	0.63		
Egg intake	41.4 (−19.5, 102.3)	0.18		

p values are from likelihood ratio tests.

Table 3. Odds Ratios (OR) for having B12 deficiency, defined as plasma total B12 concentration <148 pmol/L, in logistic regression model.

	Univariate		Multivariate	
	OR (95% Confidence Interval)	p Value	Adjusted OR (95% Confidence Interval)	Adjusted p Value
Age (years)	0.95 (0.89, 1.00)	0.07		
Gestational age (weeks)	1.03 (0.97, 1.09)	0.28		
Ethnicity				
European	reference	<0.0001	reference	<0.0001
Other	1.25 (0.57, 2.62)		1.41 (0.64, 3.04)	
Chinese Asian	0.39 (0.11, 1.08)		0.48 (0.13, 1.35)	
South Asian	8.90 (3.43, 24.7)		10.4 (3.90, 29.4)	
Pre-pregnancy body mass index (kg/m^2)	1.05 (0.98, 1.11)	0.17		
Smoking	1.07 (0.24, 3.51)	0.92		
Education				
Less than high school	reference	0.18		
High school degree	2.01 (0.58, 9.44)			
University or trade school	1.07 (0.33, 4.79)			
B12 supplement use	0.34 (0.14, 0.91)	0.03	0.31 (0.11, 0.86)	0.03
Fish intake	0.96 (0.50, 1.95)	0.91		
Meat intake	0.59 (0.21, 1.90)	0.35		
Egg intake	0.29 (0.10, 0.91)	0.03		

p values are from likelihood ratio tests.

Plasma MMA concentration was positively associated with gestational age, ethnicity, and smoking. South Asian women had higher MMA concentration on average than European, with no discernible difference between the other ethnicities. Conversely, there was a negative relationship between plasma MMA and egg intake; however, because egg intake was nearly universal, this should be interpreted with caution. The odds of having plasma MMA concentration >210 nmol/L were about 5-times higher for those who self-identified as South Asian compared to those who self-identified as European (with Chinese Asian and "Other" ethnicities not statistically different from European). For every increase in one week of gestational age, the odds of having MMA >210 nmol/L were increased by about 11%.

Table 4. Predictors of plasma methylmalonic acid (MMA) concentration in linear regression model.

	Univariable		Multivariable	
	β Coefficient (95% Confidence Interval)	p Value	Adjusted β Coefficient (95% Confidence Interval)	Adjusted p Value
Age (years)	−1.16 (−3.05 to 0.74)	0.23		
Gestational age (weeks)	2.61 (0.78 to 4.45)	0.01	2.88 (1.11 to 4.65)	0.002
Ethnicity				
European	reference	0.0007	reference	0.008
Other	−8.69 (−32.4 to 15.1)		−13.7 (−36.5 to 9.14)	
Chinese Asian	−13.8 (−39.0 to 11.3)		−11.4 (−35.7 to 13.0)	
South Asian	67.4 (30.9 to 104)		52.6 (15.8 to 89.4)	
Pre-pregnancy body mass index (kg/m^2)	0.57 (−1.64 to 2.79)	0.61		
Smoking	32.8 (−10.4 to 76.0)	0.14	40.7 (−1.05 to 82.4)	0.06
Education				
Less than high school	reference	0.12		
High school degree	−32.1 (−74.7 to 10.5)			
University or trade school	−40.4 (−79.4 to −1.42)			
B12 supplement	−8.74 (−45.8 to 28.3)	0.64		
Fish intake	−17.8 (−40.0 to 4.39)	0.12		
Meat intake	−24.3 (−63.9 to 15.5)	0.23		
Egg intake	−101 (−145 to −58.2)	<0.0001	−72.6 (−117 to −28.2)	0.002

p values are from likelihood ratio tests.

Table 5. Odds Ratios (OR) for having plasma methylmalonic acid (MMA) concentration >210 nmol/L in logistic regression model.

	Univariable		Multivariable	
	OR (95% Confidence Interval)	p Value	Adjusted OR (95% Confidence Interval)	Adjusted p Value
Age (years)	0.97 (0.91 to 1.02)	0.25		
Gestational age (weeks)	1.10 (1.03 to 1.17)	0.002	1.11 (1.04 to 1.19)	0.001
Ethnicity				
European	reference	0.001	reference	0.003
Other	0.70 (0.28 to 1.61)		0.59 (0.23 to 1.38)	
Chinese Asian	1.11 (0.47 to 2.45)		1.07 (0.45 to 2.41)	
South Asian	6.10 (2.37 to 16.2)		5.48 (1.95 to 15.8)	
Pre-pregnancy body mass index (kg/m^2)	1.01 (0.94 to 1.07)	0.84		
Smoking	0.66 (0.10 to 2.48)	0.57		
Education				
Less than high school	reference	0.42		
High school degree	1.20 (0.37 to 4.69)			
University or trade school	0.75 (0.26 to 2.77)			
B12 supplement	0.54 (0.21 to 1.59)	0.25		
Fish intake	0.74 (0.39 to 1.46)	0.38		
Meat intake	0.43 (0.16 to 1.29)	0.12		
Egg intake	0.21 (0.07 to 0.63)	0.006	0.36 (0.10 to 1.28)	0.11

p values are from likelihood ratio tests.

4. Discussion

Vitamin B12 sufficiency in pregnancy is essential for maternal and fetal wellbeing. Assessment of B12 status of pregnant women living on the west coast of Canada using both plasma total B12 and MMA has not previously been conducted. In light of this, we measured plasma total B12 and MMA in 320 pregnant women living in Vancouver, British Columbia. We found that 33% of the women had suboptimal B12 status and 18% were B12 deficient using total B12 as sole indicator. Overt B12 deficiency, defined as plasma total B12 <148 pmol/L and concurrent plasma MMA >370 nmol/L, was uncommon and found in only 1.6% of the study population. We observed lower total B12 and higher plasma MMA concentrations in South Asian women compared to other ethnicities, and determined that the odds of being B12 deficient and having elevated MMA concentration were about 10-times and 5-times higher,

respectively, for those who self-identified as South Asians relative to those who identified as European. The odds of being B12 deficient were 69% lower for those who were taking B12 supplements.

Comparing the results of studies investigating B12 status among different populations presents a number of challenges. Firstly, a wide variety of markers are used to measure B12 status, including serum/plasma total B12, MMA, total homocysteine, holotranscobalamin and total transcobalamin, and haptocorrin [24,25]. Secondly, different laboratory methods are used even for the same biomarker; for example, total B12 can be measured using microbiological [26] or immunoassays [10]. Thirdly, different cutoffs have been applied to define deficiency (e.g., for serum total B12 <125 pmol/L [27], <148 pmol/L [10,28], or even <180 pmol/L [29]). The cutoff for MMA, 210 nmol/L, was the reference value (95th percentile) of B12-replete adults with normal renal function (determined by serum creatinine) in the NHANES [22]. MMA concentrations >370 nmol/L is the "generally agreed on cutoff for elevated plasma MMA" [22] and was derived from the reference value (97.5th percentile) of healthy adults aged 40–68 years [30]. To date, there are no established cutoffs for B12 status during pregnancy for any of the B12 biomarkers. Accordingly, study findings should be compared with caution.

Two recent Canadian studies have reported the prevalence of B12 deficiency in pregnancy, with varying results. In the PREFORM study in Toronto (n = 368), the prevalence of suboptimal B12 status (serum total B12 <210 pmol/L) was 35% at 12–16 gestational weeks and 43% at delivery, and the prevalence of B12 deficiency (serum total B12 <148 pmol/L) was 17% and 38%, respectively [12]. In Vancouver, the prevalence of suboptimal B12 status and B12 deficiency was 21% and 10% in 264 women at 16 gestational weeks, and 35% and 23% in 220 women at delivery, respectively [13]; plasma MMA concentration was not measured in the study by Wu et al. [13]. In the PREFORM study, plasma MMA concentration was measured and the prevalence of functional B12 deficiency (defined as MMA >271 nmol/L) was two percent in early pregnancy and five percent at delivery [12]. In the present study, 26 (eight percent) of second- and third-trimester pregnant women had plasma MMA concentration >270 nmol/L. Notwithstanding the variable findings and the need for performance testing of B12 biomarkers during pregnancy, the prevalences of low total B12 concentration in pregnant Canadian women are high and highlight the importance of measuring B12 status early in pregnancy in order to develop interventions and prevent adverse maternal and fetal health outcomes.

In the present study, plasma MMA concentrations were positively associated with gestational age in these women. Differences in plasma total B12 concentrations were less pronounced and negatively correlated to gestational age. Because median MMA concentrations of women in their second (128 nmol/L) and third trimester (146 nmol/L) were well within the normal range (i.e., <210 nmol/L), the relationship we found between gestational age and MMA concentration does not appear to be clinically significant. Our findings are consistent with those of Murphy et al. [31], who reported increases in plasma MMA between the first and third trimester. In a longitudinal study in 406 Danish pregnant women, the prevalence of plasma total B12 concentrations <150 pmol/L (i.e., B12 deficiency) also increased over pregnancy, from 15% at 18 weeks of gestation, to almost 43% at 39 weeks of gestation [32]. Another prospective longitudinal study of pregnancy indicated that the prevalence of B12 deficiency increased between the second and third trimester from 8% (n = 3) to 35% (n = 12) in healthy pregnant women with B12 intake >RDA (2.6 μg/day) [28] supporting the need for pregnancy-specific cutoffs. The decrease in plasma total B12 during pregnancy could result from increased metabolic rate, active B12 transport across the placenta, and hemodilution. This suggestion is supported by the finding that plasma total B12 concentrations significantly increase postpartum, without supplementation [32,33]. Altering cutoffs for B12 biomarker concentrations in pregnant women will require extensive research to ensure the prevention of adverse health outcomes.

In our study, women who did not take supplements had double the risk of B12 deficiency compared to those who took B12-containing supplements (Table 3). Plasma total B12 concentration substantially differed between supplement users and non-users (Table 1); however, supplement use was not a significant predictor of plasma total B12 concentration (Table 2), which might be explained by

the low number (n = 23/320; 7%) of women not using supplements. The prevalence of supplement use in pregnant Canadian women was reported to be very high with 89% of women using supplements in late pregnancy [34]. The B12 content in prenatal supplements in Canada greatly varies from 2 µg/day to 12 µg/day; the majority (62%) of supplement users in this study reported to use prenatal supplements with a dosage of 2.6 µg/day. Supplement use however was neither a predictor of plasma MMA concentrations nor associated with a lower risk for having elevated MMA. This finding might indicate that the majority of the women in this study were B12 replete, as reflected by their MMA concentration, and supplemental B12 only affected circulating total B12. This would support the hypothesis and B12 absorption findings by Greibe et al. [33] that the decrease in circulating total B12 concentration throughout gestation does not reflect B12 deficiency but 'normal' pregnancy-related changes in total B12 that do not impair intracellular B12 status.

An important finding of our study is that the majority of South Asian pregnant women in our cohort had suboptimal B12 status (11.5%) or were B12 deficient (61.5%). Moreover, three out of five women with overt B12 deficiency were of South Asian ethnicity. Our findings are comparable to those of a recent study on pregnant women from urban South India reporting that 51% had plasma total B12 <150 pmol/L and 76% had elevated MMA concentrations (defined as plasma MMA >260 nmol/L; compared to 46% in the current study) [15]. Of those women, 42% had impaired B12 status (i.e., total B12 <150 pmol and MMA >260 nmol/L). The study found that not consuming yogurt or fish and being primiparous were predictors of poor B12 status [15]. Cultural and religious practices that promote vegetarianism inherent to certain Indian communities around the world place these groups at greater risk of B12 deficiency during pregnancy. In a study conducted in the US, Indian women had significantly lower median serum total B12 concentrations than did "other ethnicity" (combining whites, blacks, non-Indian Asians and Latin Americans) and 24% were B12 deficient (<180 pmol/L) [29]. A randomized controlled trial of B12 supplementation in South Asian immigrant women living in New Zealand reported that 48% of the cohort (n = 62) had serum total B12 <222 pmol/L [16]. Supplementation with 6 µg of cyanocobalamin per day for six months increased serum total B12 by 30% [16].

In contrast to South Asian immigrant women, our study showed that Asian pregnant women (mostly Chinese, Southeast Asian, Japanese, Korean, and Filipino) had the highest total B12 concentrations, and the lowest prevalence of B12 inadequacy. The risk of being B12 deficient was 50% lower in Asians than in women of European descent. Similar results have been observed in an elderly population, where Asian-Americans (Chinese, Korean, Vietnamese, Japanese, or Filipino origin) had significantly higher plasma total B12 concentrations compared to European whites [35].

Without collecting quantitative data on food product consumption, we do not know what the overall B12 intake was. In our study, meat or fish consumption did not have a significant effect on plasma total B12 concentrations. Egg and dairy intake was reported by 95% and 99%, respectively, of all women. Vogiatzoglou et al. [26] found that higher consumption of dairy products (especially milk) and fish increased plasma total B12 concentrations, but meat and eggs did not. Intake of fish, but not other animal source foods, decreased the risk of having inadequate B12 status in South Indian pregnant women [15]. Of the South Asians in the current study 59% reported eating no fish, 27% no meat, and 27% no eggs. Ninety-five percent of South Asian women used B12-containing prenatal vitamin supplements compared to 89% and 100% of European and Chinese Asian women, respectively. We hypothesize that low dietary B12 intake likely contributed to the poor B12 status observed in the South Asian women.

In our study, pre-pregnancy BMI was negatively correlated with plasma total B12 ($\rho = -0.184$; $p = 0.001$) but did not significantly influence the median values of either marker or the prevalence of B12 deficiency. We observed that B12-replete pregnant women had a tendency to have a lower median BMI value. Pre-pregnancy BMI was the strongest independent predictor of plasma total B12 concentration, as was gestational age. First-trimester BMI was inversely associated with third-trimester serum total B12 concentration in a retrospective case-control study of 344 UK pregnant

women who were not taking B12-containing supplements [36]. The BMI at 28 weeks of gestation was an independent predictor of circulating total B12 concentrations in UK pregnant women [37]. The Canadian Health Measures Survey 2007–2009 showed that the prevalence of B12 adequacy was lower in Canadian adults with obesity compared to normal weight and overweight adults of all age groups [10]. Being obese may alter B12 absorption, excretion or metabolism, but whether the relationship is casual [38] requires further investigation.

The strength of our research is the measurement of two biomarkers for B12 status assessment as per recommendation by an expert panel [17]. We however acknowledge the limitations of our study that include the convenience sampling and the lack of a sensitive dietary assessment tool to quantify dietary B12 intake. We also acknowledge the lack of hematological indicators, such as hemoglobin, mean corpuscular volume, or blood cell count, to investigate whether overt B12 deficiency, as observed in five women in this study, was associated with clinical outcomes. The original study did not include the measurement of hematological indicators and the retrospective analysis was not feasible for reasons of biospecimen requirements. We thus cannot conclude whether the overt B12 deficiency was of clinical concern. Women with overt B12 deficiency did not differ from the other 315 women in age (30.6 years versus 31.4 years, $p = 0.74$) or BMI (24.5 kg/m^2 versus 22.5 kg/m^2, $p = 0.42$); but reported lower intake of fish (40% versus 75%), meat (67% versus 94%), and egg (80% versus 95%). Nutritional status can be influenced by genetic modifiers of molecules involved in the digestion, absorption or metabolism of nutrients. Genetic variants related to B12 metabolism might explain some of the variation in our findings with respect to elevated MMA [39] and low total B12 concentration [40,41]; however, because we lack the women's consent for genotyping in this study, we were unable to investigate this.

The assessment of B12 status is challenged by the sensitivity and specificity limitations of individual biomarkers; we therefore followed the recommendations to using at least one direct and one functional indicator of B12 status [17] while respecting available sample volume and budget. We measured total B12, the most commonly used biomarker and direct indicator, and MMA, the more specific functional indicator of B12 deficiency compared to total homocysteine. The additional measurement of total homocysteine might not have allowed further insight to the B12 status of these women; in Canada, a folate-replete nation, elevated total homocysteine concentrations (defined as >13 μmol/L [22,42]) was observed in five percent of the general population [10] but not in pregnant Canadian women [12]. While MMA is considered the more specific indicator of B12 status, we acknowledge that MMA is influenced by renal function [43] and we lack the measurement of creatinine to control for renal function in the interpretation of elevated MMA concentration. Due to budget limits, we also did not include the measurement of folate that has an interdependent metabolic role with B12. The access to both total B12 and folate data would have allowed us to investigate the interaction of folate and B12 on biochemical outcomes. High folate status with low plasma total B12 concentration has been associated with higher MMA concentration in older adults [44–46], however not in younger adults [46]. The interaction of high folate and low B12 status on biochemical outcomes in pregnant women warrants further investigation.

5. Conclusions

Protecting women of reproductive age from B12 deficiency is important to the health of mothers and their offspring. Our study showed that a sample of pregnant women in Vancouver, Canada, had a high prevalence of low circulating total B12 concentrations, with 18% being classified as B12 deficient. However, overt B12 deficiency was present in only 1.6% of the sample. The observed discrepancy in the prevalence of B12 adequacy depending on the biomarker and cutoff used emphasizes the need for pregnancy-specific cutoffs and performance testing of B12 biomarkers during pregnancy. Independent of the biomarker used, individually or combined, South Asian pregnant women were at particular risk for B12 deficiency compared to other ethnic groups, and further study of this vulnerable group is required.

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