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ORIGINAL CONTRIBUTION

Vitamin C-related nutrient–nutrient and nutrient–gene interactions that modify folate status

Mark Lucock · Zoë Yates · Lyndell Boyd · Charlotte Naylor · Jeong-Hwa Choi · Xiaowei Ng · Virginia Skinner · Ron Wai · Jeremy Kho · Sa Tang · Paul Roach · Martin Veysey

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Abstract
Purpose Folate-related nutrient–nutrient and nutrient–gene interactions modify disease risk; we therefore examined synergistic relationships between dietary folic acid, vitamin C and variant folate genes with respect to red cell folate status.
Methods Two hundred and twelve subjects were examined using chemiluminescent immunoassay, PCR and food frequency questionnaire to determine red cell and serum folate, 14 folate gene polymorphisms, dietary folate (natural and synthetic) and vitamin C.
Results When examined independently, synthetic PteGlu correlates best with red cell folate at higher levels of intake ($p = 0.0102$), while natural $5CH_3$-H_4-$PteGlu_n$ correlates best with red cell folate at lower levels of intake ($p = 0.0035$). However, dietary vitamin C and $5CH_3$-H_4-$PteGlu_n$ interact synergistically to correlate with red cell folate at higher levels of intake ($p = 0.0005$). No interaction between dietary vitamin C and PteGlu was observed. This 'natural' nutrient–nutrient interaction may provide an alternative to synthetic PteGlu supplementation that is now linked to adverse phenomena/health outcomes. On its own, vitamin C also correlates with red cell folate ($p = 0.0150$) and is strongly influenced by genetic variation in TS, MTHFR and MSR, genes critical for DNA and methionine biosynthesis that underpin erythropoiesis. Similarly, dietary vitamin C and $5CH_3$-H_4-$PteGlu_n$ act synergistically to modify red cell folate status according to variation in folate genes: of note, heterozygosity for 2R3R-TS ($p = 0.0181$), SHMT ($p = 0.0046$) and all three MTHFR SNPs ($p = 0.0023$, 0.0015 and 0.0239 for G1793A, C677T and A1298C variants, respectively) promote a significant association with red cell folate. Again, all these genes are critical for nucleic acid biosynthesis. Folate variants with the strongest independent effect on folate status were C677T-MTHFR ($p = 0.0004$) and G1793A-MTHFR ($p = 0.0173$).
Conclusions $5CH_3$-H_4-$PteGlu_n$ assimilation and variant folate gene expression products may be critically dependent on dietary vitamin C.

Keywords Antioxidants · Ascorbic acid · Folic acid · Polymorphism · Vitamin · Bioavailability

M. Lucock (✉) · Z. Yates · L. Boyd · C. Naylor · J.-H. Choi · X. Ng · J. Kho · S. Tang · P. Roach
School of Environmental and Life Sciences, University of Newcastle, Brush Rd, PO Box 127, Ourimbah, NSW 2258, Australia
e-mail: mark.lucock@newcastle.edu.au

V. Skinner · R. Wai · M. Veysey
Teaching and Research Unit, Central Coast Local Health District, PO Box 361, Gosford, NSW 2250, Australia

Introduction

Native dietary folate is in the form of reduced folylmono- or folylpolyglutamic acids. This contrasts with synthetic folic acid, which in the form of pteroylmonoglutamic acid (PteGlu) is fully oxidised and does not occur in nature.

Following increased discretionary use and/or Government-mandated fortification, PteGlu has become a major dietary component [1, 2]. Its bioavailability is up to 100 % [3] compared with an estimated 50 % for naturally occurring food folate [4]. There are many reasons for this: native food folates are sensitive to heat, and food preparation can result in destruction of 50–95 % of the folate content of food [5]. Indeed, lability is additionally influenced by pH, O_2, metal ions and antioxidant levels [6].

The natural reduced folylpolyglutamates, which include substituted or unsubstituted dihydro- and tetrahydro-forms that are found in foods, are extremely unstable [7]. Reduction at the 5, 6, 7 and 8 positions of the pteridine ring renders the molecule sensitive to oxidative cleavage at the C_9–N_{10} bond. Irreversible scission at this C_9–N_{10} locus yields a pteridine residue and *p*-aminobenzoylglutamate (P-ABG) [8, 9], which are nutritionally inert [10]. Although folates that carry one-carbon substituents at the N-5 or N-10 position have increased stability, they are still susceptible to oxidative loss [7]. Despite this, 5-methyltetrahydrofolate$_n$ ($5CH_3$-H_4-$PteGlu_n$) along with some formyl derivatives are the major natural forms of food folic acid. We therefore recently characterised the physicochemical properties that mono and triglutamate forms of $5CH_3$-H_4-$PteGlu_n$ are subjected to in human gastric juice [11, 12]. It was demonstrated that the antioxidant properties of vitamin C are likely to be critical in sparing highly labile trace levels of the natural $5CH_3$-H_4-$PteGlu_n$ vitamer. Vitamin C may be either a dietary co-nutrient, or may be present at high concentrations in gastric juice due to its transfer from blood plasma against a concentration gradient in the order of approximately 5:1 [12].

The molecular mechanism for stabilising and/or salvaging $5CH_3$-H_4-$PteGlu_n$ is based on the concomitant presence of vitamin C and $5CH_3$-H_4-$PteGlu_n$ in the stomach lumen. At typical postprandial pH, vitamin C prevents degradation of $5CH_3$-H_4-$PteGlu_n$ to 5-methyldihydrofolate$_n$ ($5CH_3$-5,6-$H_2PteGlu_n$) and onward reaction to the C_9–N_{10} scission product P-ABG, while additionally promoting the salvage of $5CH_3$-H_4-$PteGlu_n$ from $5CH_3$-5,6-$H_2PteGlu_n$. This is important because the oxidation product, $5CH_3$-5,6-$H_2PteGlu_n$, is considered to be a major dietary form of the vitamin following food preparation. Our findings [11] have indicated a clear differential between mono- and polyglutamate stability, since, irrespective of the modulatory effect of pH, or the presence of ascorbic acid, $5CH_3$-$H_4PteGlu_1$ is inherently more stable than $5CH_3$-H_4-$PteGlu_3$. However, these data also clearly showed that ascorbic acid stabilises both mono- and triglutamate forms of methylfolate in human gastric juice and hence plays a key role in the bioavailability of natural polyglutamate forms of the vitamin [11].

Folic acid is a water-soluble B-vitamin that performs one-carbon transfers through a series of interrelated biochemical reactions including critical ones involved in the elaboration and expression of DNA [13–15]. These metabolic reactions are modulated by many single nucleotide polymorphisms (SNPs) that modify phenotype, including several important clinical phenotypes [16]. The relevance of understanding factors governing folate bioavailability and performing research that makes a distinction between natural and synthetic forms of the vitamin is based on potentially negative findings related to the use of PteGlu. The consensus view has been that PteGlu supplementation offers numerous health benefits, ostensibly in relation to prevention of birth defects [17]. However, emerging evidence suggests that increased population exposure to PteGlu may also have a negative impact with respect to specific developmental and degenerative disorders, including colorectal, prostate and breast cancers, cognitive decline and a range of other conditions [16, 18]. In the context of cancer, its effect seems temporal: despite preventing tumour initiation, folate seems to facilitate progression of precancerous lesions [19]. While it is not possible to distinguish between the relative effects of native and synthetic folic acid in neoplastic disease, new evidence has recently emerged that raises concerns about widespread adoption of PteGlu as a fortificant: Synthetic PteGlu saturates human dihydrofolate reductase leading to unmetabolised PteGlu in the circulation with evidence that it may have a potential antimetabolite effect [20–22] including disruption of embryonic development at higher levels [23]. Additionally, the UV scission product of PteGlu is a potentially geno-toxic pterin moiety [24]. Therefore, with these and other concerns in mind, we have examined the relationship between dietary intake of vitamin C, natural $5CH_3$-H_4-$PteGlu_n$ and synthetic PteGlu, and explored how these nutrients on their own, or in concert, modify red cell folate as a surrogate for overall folate status. Moreover, we have examined how key folate genotypes modify the relationship between vitamin C intake, folate intake and red cell folate status and investigated a series of completely novel nutrient–nutrient and nutrient–gene interactions, which may be relevant to human health.

Materials and methods

Subjects

Participants ($n = 212$; 57 % women and 43 % men) were recruited from a gastroenterology clinic at Gosford Hospital (NSW) and were undergoing routine screening for colonic pathology. Subjects were between 18 and 89 years of age at the time of examination (overall mean age 61.8) and were mentally competent to complete a food frequency questionnaire (FFQ) interview. Patients under the age of 18, those with severe intellectual disabilities and any patients highly dependent on medical care were excluded. Furthermore, patients receiving high-dose vitamin B_{12} injections or on antifolate chemotherapy were, again, excluded. No other inclusion/exclusion criteria were applied. Local Human Research Ethics Committee approval was given and informed consent obtained prior to

volunteers participating in the study. It is important to note that examination of this population took place prior to the phasing in of mandatory fortification of the Australian diet with PteGlu in September 2009.

Food Frequency Questionnaire for intake of native methylfolic acid, synthetic folic acid and vitamin C

Estimated daily intake of nutrients was assessed by interviewer administered FFQ. The questionnaire was extensive, covering 225 food items and all food groups. Subjects were also asked to provide a list of all supplements they were taking and were asked about these during the FFQ interview.

The FFQs were analysed using Foodworks™ 2.10.146 (Xyris Software, Brisbane, QLD, Australia). This package uses a number of food databases to cover the majority of foods consumed by Australians. These include the AusFoods (brands), Aus Nut (base foods) and the New Zealand—Vitamin and Mineral Supplements 1999 databases.

The average daily intake of all vitamers of folic acid included the average daily intake from foods and supplements and is referred to as 'dietary folate'. Total dietary folate intake (μg per day) was calculated by adding the daily average value from Foodworks™ with any additional amounts from supplemental sources. Synthetic folic acid in the form of PteGlu was estimated by adding any PteGlu containing vitamin supplements with breakfast cereal sources of PteGlu and any other known sources, such as Sustagen® powder or fortified drinks/juices. Natural folate as $5CH_3$-H_4-$PteGlu_n$ was estimated by adding together only natural forms of folate from foods such as fruits, vegetables and grains. Although mandatory PteGlu fortification of bread flours took effect on 19 September 2009 in Australia, all samples in this study were collected prior to this date and should be considered as a prefortification data set despite ever increasing discretionary use of PteGlu in recent years.

The Foodworks™ database package was also employed for estimation of vitamin C intake. Three main sources of dietary vitamin C were taken into account in order to give a total amount per day: neutraceuticals, fruit juices and natural sources. Each participant's supplementary intake was reviewed for vitamin C content and included, for example, multivitamins and iron and vitamin C tablets. As Foodworks™ only has values for the natural form of vitamin C in foods, and since all commercial fruit juices contain added ascorbic acid, a separate vitamin C measurement for these fruit juices was calculated when participants noted these in their FFQ. As a consequence of this, for each participant, a total vitamin C intake value, a total neutraceutical vitamin C intake value and a total commercial fruit juice vitamin C intake value was calculated. The vitamin C intake from non-natural sources was subtracted from the total value to enable calculation of the vitamin C intake from natural sources, although for this study, only the total dietary vitamin C values were used.

Folate assay

Serum and red cell folate were measured using chemiluminescent immunoassay (Access Immunoassay System, Beckman Instruments, Inc). The analysis laboratory's normal reference range was 370–1,050 nmol/L for red blood cell (RBC) folate and 5–21 nmol/L for serum folate. With increased discretionary use of PteGlu in supplemental form over recent years, an increasing number of individuals present with blood folate values at or above the upper calibration range of 2,500 nmol/L for RBC folate and 45 nmol/L for serum folate. Therefore, data have been examined taking account of this since these extraordinarily high values would not be possible to achieve through consumption of natural sources of the vitamin alone. In the present study, 202 individuals of 212 fell within the RBC folate calibration range.

Folate genetics

Genomic DNA from all subjects was examined for the common C677T-MTHFR, A1298C-MTHFR, G1793A-MTHFR, G80A-RFC, A2756G-MS, A66G-MSR, 19 bpDHFR, C1561T-GCP II, C776-TCN2, 844ins68-CBS, TSER-2R3R, TSER-3RG>C, 1494del6-TS and C1420T-SHMT SNPs typically using PCR followed by restriction enzyme digestion and gel electrophoresis (excluding 19 bpDHFR that uses a nested primer allele-specific strategy for genotype scoring). A full description of all the primer sequences used for PCR detection of common folate metabolism associated genes is provided in Table 1.

C677T-MTHFR

Primers were used to generate a 198-bp MTHFR amplicon. The C → T nucleotide substitution at bp 677 creates a *Hinf*I recognition site. If the T allele is present, *Hinf*I digests the 198-bp amplicon into 175- and 23-bp fragments [25].

A2756G-MS

Primers were used to amplify a unique 265-bp sequence which, when digested with the restriction enzyme *Hae*III, yields a 265-bp fragment for the wild type: 265-, 180- and 85-bp fragments for the heterozygote; and 180- and 85-bp fragments for the homozygous recessive genotype [26].

Table 1 Description of forward and reverse primers used for the PCR based detection of 14 common folate metabolism related genes

Gene	Forward primer	Reverse primer
Description of primers used for PCR detection of common folate metabolism associated genes		
C677T-MTHFR	5′-TGAAGGAGAAGGTGTCTGCGGGA-3′	5′-AGGACGGTGCGGTGAGAGTG-3′
A1298C-MTHFR	5′CTTTGGGGAGCTGAAGGACTACTAC-3′	5′-CACTTTGTGACCATTCCGGTTTG-3′
G1793A-MTHFR	5′-CTCTGTGTGTGTGTGTGCATGTGTGCG-3′	5′-GGGACAGGAGTGGCTCCAACGCAGG-3′
A2756G-MS	5′-GGTGTGTTCCCAGCTGTTAGATG-3′	5′-GACACTGAAGACCTCTGATTTGAAC-3′
A66G-MSR	5′-GCAAAGGCCATCGCAGAAGACAT-3′	5′-GTGAAGATCTGCAGAAAATCCATGTA-3′
C1561T-GCP II	5′-CATTCTGGTAGGAATTTAGCA-3′	5′-AAACACCACCTATGTTTAACA-3′
G80A-RFC	5′-AGTGTCACCTTCGTCCC-3′	5′-TCCCGCGTGAAGTTCTTG-3′
C776G-TCN2	5′-GTCAGGTGCTGGAACACCTAG-3′	5′-GTGCCAGACAGTCTGGGAAG-3′
C1420T-SHMT	5′-AGAGTTCAAGGAGAGACTGGCAG-3′	5′-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′
TSER 2R3R	5′-GTGGCTCCTGCGTTTCCCCC-3′	5′-GGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′
TSER 3R G>C	The same primers for TSER 2R3R were used to determine this genotype—see full details under methods	
1494del6-TS	5′-CAAATCTGAGGGAGCTGAGT-3′	5′- CAGATAAGTGGCAGTACAGA-3′
844ins68-CBS	5′-CTGGCCTTGAGCCCTGAA-3′	5′-GGCCGGGCTCTGGACTC-3′
19 bp-DHFR	Primer A: 5′-CCACGGTCGGGGTACCTGGG-3′, primer B: 5′-ACGGTCGGGGTGGCCGACTC-3′ and primer C: 5′-AAAAGGGGAATCCAGTCGG-3′	

A1298C-MTHFR

Primers were used to generate a 163-bp MTHFR product. This amplicon was digested with the restriction enzyme *Mbo*II to yield 56-, 31-, 30-, 28- and 18-bp fragments for the wild type and 84-, 31-, 30- and 18-bp fragments for the homozygous recessive genotype [27].

19 bp-DHFR

Allele-specific primers were used for oligonucleotide amplification. Primers A, B and C were employed in a nested primer allele-specific protocol to generate either a 113-bp amplicon (primers A and C) or 92-bp amplicon (primers B and C) and thus permit detection of the 19-bp deletion [28].

G80A-RFC

Following oligonucleotide amplification, if the G allele was present in the amplicon, it was digested with the restriction enzyme *Cfo*I to yield three fragments: 125, 68 and 37 bp. The variant allele abolishes a restriction site leading to two fragments: 162 and 68 bp [29].

A66G-MSR

Primers were used to generate a DNA product containing an artificial *Nde*I restriction site. Subsequent to *Nde*I digestion, a DNA fragment of 66 bp remains uncut in the G allele, but is cleaved into 44- and 22-bp fragments in the presence of the A allele [30].

C1561T-GCP II

Primers produce a 244-bp amplicon that can be digested with the restriction enzyme *Acc*I. The 1561TT genotype renders two oligonucleotide fragments of 141- and 103-bp, respectively, while the 1561CC genotype yields only a single fragment of 244 bp [31].

G1793A-MTHFR

Primers were used to amplify a 310-bp amplicon. The 1793GG wild type can be digested by restriction enzyme *BsrB*I, yielding two fragments of 233 and 77 bp. The 1793AA homozygous recessive genotype remains uncut, leaving a single 310-bp band [32].

C776G-TCN2

Primers were used to generate a 364-bp oligonucleotide fragment [33, 34]. The restriction enzyme *ScrF*I recognises the cutting site in the 776GG genotype and cuts the amplicon into 238- and 126-bp fragments. For the 776CC genotype, one single 364-bp band can be visualised in the gel.

844INS68-CBS

Primers were employed to amplify a 184-bp amplicon. DNA from an individual without the insertion shows a 184-bp product, whereas that from an individual heterozygous for the insertion shows a 252-bp band in addition to the expected 184-bp product [35].

C1420T-SHMT

Primers were used to generate a 215-bp fragment. PCR products were cut by restriction enzyme *Ear*I into 126- and 89-bp fragments when the wild-type 1420CC was present. The 1420TT genotype remains as a single 215-bp oligonucleotide fragment [36].

TSER 2R3R

Primers were employed to amplify a 243-bp PCR product. The allele with only two repeats produces a 215-bp band, whereas the allele with three repeats generates a 243-bp fragment. Therefore, homozygous individuals show a 243-bp band, heterozygous individuals show 243- and 215-bp bands and homozygous recessive individuals exhibit a 215-bp fragment [37].

TSER 3R G>C

The same primers for TSER 2R3R were used to determine this genotype. Five different genotypes (2R/3Rc, 2R/3Rg, 3Rc/3Rc, 3Rc/3Rg and 3Rg/3Rg) were scored based on PCR fragment patterns obtained by using the restriction enzyme *Hae*III; the 2R allele produces 13-, 28-, 44-, 45-, 47- and 65-bp fragments: 3Rc generates 13-, 44-, 45-, 47- and 94-bp bands; 3Rg has 13-, 28-, 44-, 45-, 47- and 65-bp fragments [37, 38].

1494del6-TS

Primers targeted amplification of a fragment in the 3′-UTR of the TS gene containing the polymorphism within the 6-bp deletion allele. The presence of the 6-bp allele creates a *Dra*I restriction site. The deletion allele shows a 152-bp band, and the wild-type allele shows 70- and 88-bp fragments after digestion [39].

Statistics

All statistical analysis was performed using the JMP program for Windows (version 8.0; SAS Institute Inc., Cary, NC, USA). Relationships between key variables and related parameters were analysed using standard least squares analysis (including stepwise regression) taking account of any interactions as appropriate. Stepwise regression was performed in a mixed direction with significant probability [0.250] for a parameter to be considered as a forward step and entered into the model or considered as a backward step and removed from the model. Mallow's Cp criterion was used for selecting the model where Cp first approaches p variables. Descriptive statistics were calculated with data tabulated and presented as appropriate.

Results

Nutrient–nutrient interactions

This analysis is based on an examination of the relationship between three dietary components: vitamin C, $5CH_3$-H_4-$PteGlu_n$ and PteGlu (including interactions between dietary vitamin C and dietary folate vitamers) and red cell folate, the standard haematologic index of overall folate status.

Since discretionary use of PteGlu has increased dramatically over recent years, blood levels can be quite high. For this reason, nutrient–nutrient interactions have been examined in three ways: Taking account of all red cell folate values, taking account of red cell values below the median value and taking account of red cell values at or above the median value. In addition, data have been analysed taking account of the complete data set (i.e. inclusive of subjects with a RBC folate $\geq$ 2,500 nmol/L—that is beyond the calibration range) and excluding any subjects with a RBC folate $\geq$ 2,500 nmol/L. Taking account of all subjects ($n = 212$), the median RBC folate was 848 nmol/L. After excluding subjects with a RBC folate $\geq$ 2,500 nmol/L or serum folate $\geq$ 45 nmol/L (beyond calibration range), the median RBC folate value was 837 nmol/L.

Vitamin C is an antioxidant that protects natural folate coenzymes from oxidative degradation. With this physicochemical interaction in mind, Table 2 shows how vitamin C intake predicts red cell folate, most notably in subjects who are below the median red cell folate value ($p = 0.0150$). Furthermore, Table 3 demonstrates that there is a significant, positive association between intake of dietary vitamin C and natural folic acid as $5CH_3$-H_4-$PteGlu_n$, although there was no such relationship between intake of dietary vitamin C and synthetic (fully oxidised) PteGlu ($p \leq 0.0001$ and 0.3009, respectively).

In further support of a nutrient–nutrient interaction that has dietary vitamin C exerting an effect on red cell folate level either directly or indirectly via reductive protection of dietary $5CH_3$-H_4-$PteGlu_n$ (or both), Table 4 shows that there is a clear relationship between dietary $5CH_3$-H_4-$PteGlu_n$ intake and red cell folate status in individuals with below median red cell folate values, but not above the median value ($p = 0.0035$ and 0.7909, respectively). Of particular interest is the converse finding with intake of dietary PteGlu: A significant relationship exists between dietary PteGlu intake and red cell folate status in individuals with above median red cell folate values, but not below the median value ($p = 0.0102$ and 0.1045, respectively). To gain further clarity on these observations, Table 5 examines the interactive effect of crossing vitamin C intake with either dietary $5CH_3$-H_4-$PteGlu_n$ or PteGlu in respect of any association with red cell folate. The table shows that dietary vitamin C and $5CH_3$-H_4-$PteGlu_n$ intake

Table 2 Relationship between vitamin C intake and red cell folate status analysed using standard least squares regression analysis

Inclusive of subjects with a RBC folate ≥ 2,500 nmol/L						Excludes subjects with a RBC folate ≥ 2,500 nmol/L					
All subjects		Subjects ≥ median RBC folate		†Subjects < median RBC folate		All subjects		Subjects ≥ median RBC folate		Subjects < median RBC folate	
p (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2
0.018 (0.379, 0.159)	0.03	NS	–	0.015 (0.295, 0.119)	0.06	0.061 (0.258, 0.137)	0.02	NS	–	see†	see†

Data are examined inclusive and exclusive of individuals with a RBC folate ≥ 2,500 nmol/L. Input variables were limited to vitamin C intake and red cell folate status

Table 3 Analysis of the relationship between intake of dietary vitamin C and natural and synthetic forms of dietary folic acid using standard least squares regression

Inclusive of subjects with a RBC folate ≥ 2,500 nmol/L					
Dietary total folic acid		Dietary $5CH_3$-H_4-$PteGlu_n$		Dietary synthetic PteGlu	
p (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2
0.0016 (0.236, 0.074)	0.05	<0.0001 (0.168, 0.033)	0.11	NS	–

Input variables were limited to vitamin C intake and either natural or synthetic folate

interact to predict red cell folate in all subjects and subjects who have above the median red cell folate value ($p = 0.0011$ and 0.0005, respectively). This remains significant when subjects with a RBC folate ≥ 2,500 nmol/L are excluded. No interactive effect between intake of vitamin C and PteGlu with respect to red cell folate was detected.

These observations therefore lead to a consensus view that in the presence of a low red cell folate level, vitamin C and dietary $5CH_3$-H_4-$PteGlu_n$ (but not dietary PteGlu) are key independent determinants of systemic folate status. Furthermore, at high red cell folate status, vitamin C and dietary $5CH_3$-H_4-$PteGlu_n$ are not independent determinants of systemic folate status; rather, they act synergistically, with a particularly strong interactive effect evident between the two dietary nutrients in predicting red cell folate status ($p = 0.0005$). PteGlu is a strong independent predictor of red cell folate but does not interact with vitamin C at all to predict red cell folate (this is the case for all subjects and for those with a red cell folate above or below the median value).

The observation that natural dietary folate is important in predicting red cell folate at low concentrations on its own, and interacts with vitamin C at higher concentrations to predict red cell folate, raises an interesting question as to whether these effects reflect two phenomena: a generalised antioxidant effect of vitamin C that maintains active cellular folates in the reduced state and one that synergistically improves $5CH_3$-H_4-$PteGlu_n$ bioavailability by salvaging oxidised $5CH_3$-H_2-$PteGlu_n$ back to reduced $5CH_3$-H_4-$PteGlu_n$ in the gastric lumen. The bioavailability phenomenon in human stomach acid is well described[11,12]; however, if vitamin C is important in maintaining cellular folate in the reduced state for optimal metabolic flux in one-carbon transfer reactions, it is reasonable to assume that the relationship between dietary vitamin C and red cell folate might be modulated by key folate polymorphisms. This has not been explored before, and the following results are an examination designed to address whether such nutrient–gene interactions take place.

Nutrient–gene interactions

With dietary vitamin C clearly being important in determining red cell folate levels (see above), a key objective was to see how specific folate SNPs might alter the antioxidant requirement for maintaining red cell folate status. Therefore, 14 common polymorphisms of folate metabolism were examined to see how they influenced this relationship.

Table 3 shows that intake of dietary vitamin C and $5CH_3$-H_4-$PteGlu_n$ is closely associated ($p < 0.0001$; $r^2 = 0.11$). However, to ensure that vitamin C intake was not a simple surrogate measure of $5CH_3$-H_4-$PteGlu_n$ intake, both dietary vitamin C and dietary $5CH_3$-H_4-$PteGlu_n$ were examined independently to see how each polymorphism of folate metabolism influenced the relationship between dietary vitamin intake and red cell folate status. Furthermore, although PteGlu intake showed no relationship to vitamin C intake, for completion, the intake of this

Table 4 Examination of the relationship between the intake of folic acid (as either the synthetic or natural form) and red cell folate status using standard least squares regression analysis

Type of dietary folic acid	Inclusive of subjects with a RBC folate ≥ 2,500 nmol/L						Excludes subjects with a RBC folate ≥ 2,500 nmol/L					
	All subjects		Subjects ≥ median RBC folate		†Subjects < median RBC folate		All subjects		Subjects ≥ median RBC folate		Subjects < median RBC folate	
	p (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2	*p* (slope estimate, SE)	r^2
$5CH_3$-H_4-$PteGlu_n$	NS	–	NS	–	0.0035 (0.372, 0.125)	0.08	0.0033 (0.822, 0.276)	0.04	NS	–	see†	see†
PteGlu	<0.0001 (0.724, 0.164)	0.09	0.0102 (0.469, 0.179)	0.06	NS	–	0.0002 (0.546, 0.145)	0.07	0.0334 (0.319, 0.148)	0.05	see†	see†

Data are examined inclusive and exclusive of individuals with a RBC folate ≥ 2,500 nmol/L. Input variables were limited to these three parameters

Table 5 Examination of the interactive effect of crossing vitamin C intake with dietary folic acid (as either the synthetic or natural form) in respect of the association with red cell folate status; r^2 (*p*, slope estimate, SE). Analysis conducted using standard least squares regression

Type of dietary folic acid as an interactive factor with vitamin C intake	Inclusive of subjects with a RBC folate ≥ 2,500 nmol/L			Excludes subjects with a RBC folate ≥ 2,500 nmol/L		
	All subjects	Subjects ≥ median RBC folate	†Subjects < median RBC folate	All subjects	Subjects ≥ median RBC folate	Subjects < median RBC folate
Vitamin C × $5CH_3$-H_4-$PteGlu_n$	0.08 (0.0011, −0.006, 0.002)	0.12 (0.0005, −0.009, 0.002)	NS	0.06 (0.0504, −0.004, 0.002)	0.09 (0.0262, −0.005, 0.002)	see†
Vitamin C × PteGlu	0.12 (0.0652, 0.002, 0.001)	NS	NS	NS	NS	see†

Analysis has been conducted using standard least squares regression and takes account of individuals inclusive and exclusive of a RBC folate ≥ 2,500 nmol/L. Output is based on two input variables used to generate their interactive effect on red cell folate, that is, a separate analysis for natural dietary folate × vitamin C vs red cell folate and synthetic dietary folate × vitamin C vs red cell folate

Table 6 Folate related genotypes that exhibit a significant association between dietary vitamin intake (vitamin C, $5CH_3$-H_4-$PteGlu_n$ and PteGlu) and red cell folate status (all subjects). *p*, r^2, slope estimate and SE, respectively, have been determined using standard least squares regression analysis. Brackets show number of observations

Polymorphism	Dietary vitamin C			Dietary $5CH_3$-H_4-$PteGlu_n$			Dietary PteGlu		
	Wildtype	Heterozygote	Recessive	Wildtype	Heterozygote	Recessive	Wildtype	Heterozygote	Recessive
1494del6-TS	NS (95)	0.0005, 0.125, 1.242, 0.344 (93)	0.0045, 0.386, 2.358, 0.721 (19)	NS (95)	NS (95)	NS (19)	0.0024, 0.094, 0.594, 0.191 (95)	0.0053, 0.081, 0.939, 0.329 (95)	0.0402, 0.225, 1.980, 0.891 (19)
2R3R-TS	0.0027, 0.143, 1.496, 0.477 (61)	NS (101)	NS (43)	NS (61	NS (103)	NS (43)	0.0170, 0.093, 0.940, 0.383 (61)	0.0223, 0.051, 0.751, 0.323 (103)	0.0042, 0.183, 0.590, 0.195 (43)
3RGC-TS[a]	Only 1 of 6 genotypes significant: 3Rc/3Rg $p = 0.0027$, 0.318, 2.241, 0.671 (26)			0 of 6 genotypes significant			3 of 6 genotypes significant: 2R/2R $p = 0.0042$, 0.183, 0.590, 0.195 (43); 2R/3Rg $p = 0.0138$, 0.139, 2.052, 0.798 (43); 3Rc/3Rg $p = 0.0365$, 0.170, 1.224, 0.553 (26)		
C1420T-SHMT	0.0304, 0.044, 0.606, 0.276 (107)	NS (79)	NS (21)	NS (107)	NS (81)	0.0254, 0.236, 1.358, 0.560 (21)	0.0007, 0.105, 0.644, 0.184 (107)	0.0038, 0.101, 0.993, 0.333 (81)	NS (21)
19 bp-DHFR	NS (72)	0.0560, 0.038, 0.576, 0.298 (97)	0.0245, 0.133, 1.380, 0.588 (38)	NS (74)	NS (97)	0.0279, 0.127, 1.074, 0.469 (38)	0.0555, 0.050, 0.735, 0.378 (74)	0.0004, 0.123, 0.745, 0.204 (97)	NS (38)
C1561T-GCP II	0.0144, 0.032, 0.387, 0.157 (188)	NS (19)	N/A (0)	0.0550, 0.019, 0.661, 0.342 (190)	NS (19)	N/A (0)	0.0001, 0.090, 0.706, 0.164 (190)	NS (19)	N/A (0)
G80A-RFC	NS (73)	0.0372, 0.047, 0.599, 0.283 (93)	0.0026, 0.210, 2.178, 0.676 (41)	NS (73)	NS (93)	0.0034, 0.191, 1.572, 0.506 (43)	0.0003, 0.172, 0.763, 0.199 (73)	0.0342, 0.048, 0.798, 0.371 (93)	NS (43)
G1793A-MTHFR	NS (189)	NS (16)	N/A (2)	0.0277, 0.025, 0.736, 0.332 (191)	NS (16)	N/A (2)	0.0001, 0.079, 0.688, 0.171 (191)	NS (16)	N/A (2)
C677T-MTHFR	0.0081, 0.075, 0.622, 0.230 (92)	NS (94)	NS (21)	NS (93)	NS (94)	NS (22)	0.0187, 0.059, 0.583, 0.244 (93)	0.0005, 0.113, 1.220, 0.340 (94)	NS (22)
A1298C-MTHFR	0.0141, 0.058, 0.896, 0.359 (104)	NS (87)	NS (16)	0.0187, 0.053, 1.081, 0.452 (105)	NS (88)	NS (16)	0.0067, 0.069, 0.545, 0.197 (105)	0.0001, 0.170, 1.195, 0.285 (85)	NS (16)
A2756G-MS	0.0307, 0.034, 0.388, 0.178 (138)	NS (63)	NS (6)	0.0099, 0.048, 0.952, 0.364 (139)	NS (64)	NS (6)	0.0001, 0.104, 0.718, 0.180 (139)	0.0418, 0.065, 0.861, 0.414 (64)	NS (6)
A66G-MSR	NS (47)	0.0032, 0.090, 1.033, 0.341 (95)	NS (65)	NS (48)	NS (95)	NS (66)	NS (48)	0.0014, 0.104, 0.920, 0.280 (95)	0.0050, 0.117, 0.614, 0.211 (66)
844ins68-CBS	0.0498, 0.022, 0.306, 0.154 (175)	0.0462, 0.130, 1.538, 0.739 (31)	N/A (1)	NS (177)	0.0093, 0.211, 2.819, 1.012 (31)	N/A (1)	0.0001, 0.121, 0.762, 0.155 (177)	NS (31)	N/A (1)
C776G-TCN2	0.0001, 0.381, 2.391, 0.500 (39)	NS (111)	0.0131 (57)	NS (39)	NS (112)	0.0161, 0.099, 1.575, 0.634 (58)	0.0139, 0.153, 1.090, 0.422 (39)	0.0001, 0.129, 0.783, 0.194 (112)	NS (58)

p, r^2, slope estimate and SE, respectively, have been determined using standard least squares regression analysis. To help show where associations may be particularly meaningful, the number of observations is provided in brackets. Findings are based on a single input variable (either dietary vitamin C, natural folate or synthetic folate) versus red cell folate. These three regression analyses were performed by genotype (wildtype, heterozygote or homozygote recessive) for each of the individual genes

[a] Six possible genotypes exist: 2R/2R, 2R/3Rc, 2R/3Rg, 3Rc/3Rc, 3Rc/3Rg, 3Rg/3Rg

synthetic folate was also examined to see whether folate SNPs can alter its association with red cell folate levels. Table 6 lists the genotypes that exhibited a significant association between dietary vitamin intake (vitamin C, $5CH_3$-H_4-$PteGlu_n$ and PteGlu) and red cell folate status. It is quite clear from this table that dietary PteGlu had such a profound association with red cell folate status and that this overwhelmed/masked any influence that genotype might have had on the relationship; that is, all wildtypes and almost all heterozygotes within the 14 SNPs examined exhibited a significant association between PteGlu and red cell folate status. This is perhaps not surprising; however, of greater interest is the difference in points of significance between dietary vitamin C and red cell folate status, compared with dietary $5CH_3$-H_4-$PteGlu_n$ and red cell folate status. Data suggest that vitamin C may be particularly important in maintaining red cell folate status for individuals with any of the three thymidylate synthase polymorphisms examined. By contrast, none of the three thymidylate synthase polymorphisms influence the relationship between $5CH_3$-H_4-$PteGlu_n$ intake and red cell folate status. This differential effect seems likely to be indicative that vitamin C intake is not a simple surrogate measure of $5CH_3$-H_4-$PteGlu_n$ intake. A similar scenario seems to exist for C677T-MTHFR and A66G-MSR, with the opposite effect existing for G1793A-MTHFR. All these aforementioned polymorphic loci are notable in being associated with increased risk of several clinically important phenotypes [16]. Additionally, the expression products of these genome-/methylome-related genes utilise substrates that need to be maintained in a reduced state.

Despite this differential effect, there are also several points of agreement in the relationship between dietary vitamin intake (vitamin C and $5CH_3$-H_4-$PteGlu_n$) and red cell folate status. In particular, A2756G-MS, A1298C-MTHFR, 19 bp-DHFR and to a certain extent G80A-RFC and C1561T-GCPII exhibit similar genotype effects in modulating any relationship between vitamin intake and red cell folate level.

Table 7 describes how the 14 folate-related genotypes influence the interaction between vitamin C intake and $5CH_3$-H_4-$PteGlu_n$ intake with respect to any association with red cell folate status. The generalised linear model with interaction demonstrates that this important antioxidant vitamin and labile reduced folyl vitamer may act in synergy with respect to 2R3R-TS, C1420T-SHMT and all three MTHFR SNPs in particular. In all these cases, a significant association with red cell folate is, for the most part, manifested only in heterozygotes. All the aforementioned genes encode expression products essential for nucleic acid biosynthesis, and so their respective variant proteins may benefit from a dietary intake that combines these two vitamins—not just in terms of maintaining red cell folate status, but for the elaboration of DNA as well. This nutrient interaction is also highly significant in wild-type G80A-RFC and A66G-MSR individuals and recessive 19 bp-DHFR subjects. It should also be noted that in rarer polymorphisms where the majority of individuals belong to a single genotype, significance may occur, but is probably not related to the mutation. This may be the case for wild-type 844ins68-CBS where $n = 175$. Clearly this reflects the cohort as a whole more than it does any genotype effect.

The major observation from Tables 6 and 7 is that dietary vitamin C on its own or as a consequence of interaction with $5CH_3$-H_4-$PteGlu_n$ intake clearly modifies red cell folate status according to variation in specific folate genes, while on its own, $5CH_3$-H_4-$PteGlu_n$ intake appears to be less important. PteGlu, by comparison, seems to be profoundly predictive of red cell folate status to the extent that it masks the subtlety of any genetic effects.

Although the composite effect of genes, dietary vitamin intake and folate status are the major theme of this study, folate gene variants are recognised as influencing folate status on their own. Therefore, for completion, the direct effect of the various folate-related genotypes on red cell folate status has been determined using two approaches; a stepwise regression model that took account of all 14 genes, and the ANOVA which looked at each gene individually. The results are described in Table 8 and indicate profoundly significant effects for C677T-MTHFR and G1793A-MTHFR on red cell folate status when the entire population was examined (both using a stepwise regression model and ANOVA). These SNPs remain significant in individuals with a red cell folate above the median value, with 19 bp-DHFR and 844ins68-CBS also becoming significant predictors of red cell folate levels in this group. In the group with a red cell folate value below the median value, G1793A-MTHFR (but interestingly not C677T-MTHFR), 844ins68-CBS and C776G-TCN2 are significant predictors of red cell folate level. It is worth noting that the latter two genes encode proteins that relate to homocysteine and vitamin B_{12}, respectively, rather than to folate per se.

Discussion

The findings presented here support a synergistic relationship between dietary $5CH_3$-H_4-$PteGlu_n$ and dietary vitamin C. This is consistent with a paradigm in which the antioxidant vitamin C is likely to be critical in sparing highly labile trace levels of the natural $5CH_3$-H_4-$PteGlu_n$ vitamer. This arises not simply because vitamin C is a dietary co-nutrient, but also because it is required at high concentrations in gastric juice where it accumulates from blood plasma against a concentration gradient [12]. In the

Table 7 The effect of folate related genotype on the interaction between vitamin C intake and $5CH_3$-H_4-$PteGlu_n$ intake with respect to any association with red cell folate status (all subjects)

Polymorphism	Dietary vitamin C × Dietary $5CH_3$-H_4-$PteGlu_n$		
	Wildtype	Heterozygote	Recessive
1494del6-TS	NS (95)	NS (93)	NS (19)
2R3R-TS	NS (61)	0.0181, 0.065, −0.007, 0.003 (101)	0.0491, 0.182, −0.008, 0.004 (43)
3RGC-TS[a]	Only 1 of 6 genotypes significant: 2R/2R $p = 0.0491$ (43)		
C1420T-SHMT	0.0485, 0.082, −0.005, 0.003 (107)	0.0046, 0.114, −0.009, 0.003 (79)	NS (21)
19 bp−DHFR	NS (72)	0.0491, 0.078, −0.005, 0.003 (97)	0.0153, 0.291, −0.009, 0.004 (38)
C1561T-GCP II	0.0005, 0.101, −0.007, 0.002 (188)	NS (19)	N/A (0)
G80A-RFC	0.0063, 0.113, −0.008, 0.003 (73)	0.0104, 0.132, −0.008, 0.003 (93)	NS (41)
G1793A-MTHFR	0.0258, 0.055, −0.005, 0.002 (189)	0.0023, 0.659, −0.021, 0.005 (16)	N/A (2)
C677T-MTHFR	0.0457, 0.117, −0.005, 0.002 (92)	0.0015, 0.118, −0.010, 0.003 (94)	NS (21)
A1298C-MTHFR	NS (104)	0.0239, 0.077, −0.006, 0.002 (87)	0.0309, 0.367, −0.036, 0.015 (16)
A2756G-MS	0.0037, 0.119, −0.007, 0.002 (138)	NS (63)	NS (6)
A66G-MSR	0.0034, 0.192, −0.018, 0.006 (47)	NS (95)	NS (65)
844ins68-CBS	0.0001, 0.103, −0.007, 0.002 (175)	NS (31)	N/A (1)
C776G-TCN2	0.0499, 0.481, −0.014, 0.007 (39)	NS (111)	NS (57)

p, r^2, slope estimate and SE, respectively, have been determined using standard least squares regression analysis. To help show where associations may be particularly meaningful, the number of observations is provided in brackets. The table displays output from a model that used two input variables to generate their interactive effect on red cell folate, that is, natural dietary folate × vitamin C vs red cell folate. This regression analysis was performed by genotype (wildtype, heterozygote or homozygote recessive) for each of the individual genes

[a] Six possible genotypes exist: 2R/2R, 2R/3Rc, 2R/3Rg, 3Rc/3Rc, 3Rc/3Rg, 3Rg/3Rg

gastric lumen at typical postprandial pH, vitamin C prevents the degradation of $5CH_3$-H_4-$PteGlu_n$ to $5CH_3$-5,6-$H_2PteGlu_n$ and the onward reaction to the C_9–N_{10} scission product P-ABG, while additionally promoting the salvage of $5CH_3$-H_4-$PteGlu_n$ from $5CH_3$-5,6-$H_2PteGlu_n$ [11, 12].

However, this facile reaction, which improves folate bioavailability, may not be the only way by which vitamin C impacts on folate metabolism. The data presented here indicate that vitamin C on its own, or as a consequence of interaction with $5CH_3$-H_4-$PteGlu_n$ intake, clearly modifies red cell folate status according to variation in specific folate genes. In particular, vitamin C alone may be important in maintaining red cell folate status for individuals with any of the three thymidylate synthase polymorphisms examined (1494del6-TS, 2R3R-TS, 3RGC-TS) and for C677T-MTHFR and A66G-MSR. By contrast, none of these polymorphisms influence the relationship between $5CH_3$-H_4-$PteGlu_n$ intake and red cell folate status. This differential suggests that vitamin C intake is not a simple surrogate measure of $5CH_3$-H_4-$PteGlu_n$ intake, but that more significantly, it appears that the antioxidative environment promoted by vitamin C is critical to the body's redox status and helps maintain the appropriate level of reduction of folate gene expression products and of the labile substrates and products of these various enzymes, which, importantly, all contribute to nucleotide biosynthesis and/or the methylome. Hence, vitamin C may promote optimal reaction conditions for enzymes encoded by the variant genes 1494del6-TS, 2R3R-TS, 3RGC-TS, C677T-MTHFR and A66G-MSR. Certainly, erythropoiesis within the bone marrow, and the conversion of dietary $5CH_3$-H_4-$PteGlu_n$ into red cell folate, depends on these three enzymes.

Similarly, there seems to be a synergistic interaction between vitamin C and $5CH_3$-H_4-$PteGlu_n$ in which red cell folate status is modified according to variation in specific folate genes. Once again, these are genes critical for nucleic acid biosynthesis; antioxidant vitamin C and the labile reduced folyl vitamer seem to act in synergy with respect to 2R3R-TS, C1420T-SHMT, and all three MTHFR SNPs, and interestingly, this is most evident where heterozygosity exists (Fig. 1).

Broadly speaking, while a synergy does seem to exist between natural forms of folate and vitamin C, no such nutrient–nutrient interaction exists between the synthetic form of folic acid and vitamin C. On its own, PteGlu is a powerful predictor of red cell folate and therefore, in all probability, masks any likely genotypic effects on this blood vitamin index. However, one of the interesting outcomes of this research is the finding that dietary PteGlu is only predictive at higher levels of red cell folate, while at lower levels of red cell folate status, dietary $5CH_3$-H_4-$PteGlu_n$ is predictive. Furthermore, the interaction of vitamin C and $5CH_3$-H_4-$PteGlu_n$ mimics PteGlu in being

Table 8 The effect of folate-related genotype on red cell folate status

Polymorphism	Inclusive of subjects with a RBC folate ≥ 2500 nmol/L		
	All subjects	Subjects ≥ median RBC folate	Subjects < median RBC folate
r^2 for whole model based on stepwise regression	0.175	0.287	0.237
1494del6-TS			
2R3R-TS			
3RGC-TS			
C1420T-SHMT	0.0551†		
19 bp-DHFR		0.0343†	
C1561T-GCP II		0.0501*	
G80A-RFC			
G1793A-MTHFR	0.0173†; 0.0007* (−597.8, 173.1)	0.0352†; 0.0007* (−303.5, 87.1)	0.0087†
C677T-MTHFR	0.0004†; 0.0001* (−217.5, 54.4)	0.0111†; 0.0009* (−216.8, 63.2)	
A1298C-MTHFR			
A2756G-MS			
A66G-MSR			
844ins68-CBS		0.0204†; 0.0219* (269.6, 115.7)	0.0143* (52.2, 20.9)
C776G-TCN2			0.0323†

p has been determined using a stepwise regression model that takes account of all 14 genotypes used as input variables (*p* designated by * followed in brackets by slope estimate and SE, respectively). This same relationship has also been examined by ANOVA looking at each genotype individually (*p* designated by †). The r^2 for the whole model based on stepwise regression is also provided

predictive at higher levels of red cell folate and generally enhances the strength of the relationship between the natural vitamer and red cell folate levels.

While some of these findings are quite novel, it is well established that folate genes can modify folate status. One of the genes consistently linked to an altered folate status is C677T-MTHFR. It is therefore perhaps unsurprising that when the folate variants were examined independent of dietary intake, the C677T- MTHFR SNP stood out as an exceptionally significant predictor of overall red cell folate status. It is worth noting that G1793A-MTHFR also seems to be important in this respect.

On a point-by-point basis, this work suggests:

1. PteGlu best predicts red cell folate at higher levels of intake, making it an effective supplement for improving folate status such as in NTD prevention.
2. $5CH_3$-H_4-$PteGlu_n$ best predicts red cell folate at lower levels of intake, typical of what would be expected to occur on an a priori basis knowing the relatively low abundance of this native vitamer in foods.
3. Dietary vitamin C and $5CH_3$-H_4-$PteGlu_n$ interact synergistically to predict red cell folate at higher levels of intake in the same way that PteGlu does when examined on its own. This was unexpected and represents a new and potentially valuable nutrient–nutrient interaction with the potential to provide an alternative to PteGlu supplementation which has been linked to adverse phenomena and health outcomes in recent years [16, 18, 21–23, 25].
4. On its own, vitamin C predicts red cell folate.
5. On its own, vitamin C is important in predicting red cell folate where variation in three genes (TS, MTHFR and MSR) occurs. These are genes critical for maintaining DNA and methionine biosynthesis which underpin erythropoiesis within the bone marrow. Vitamin C is also likely to help keep folate intermediates and the expression products of these three variant genes in a reduced state.
6. Dietary vitamin C and $5CH_3$-H_4-$PteGlu_n$ act in synergy to modify red cell folate status according to variation in specific folate genes. In particular, heterozygosity for 2R3R-TS, SHMT, and all three MTHFR SNPs affords a significant association with red cell folate. All these genes are once again critical in nucleic acid biosynthesis.
7. PteGlu is profoundly predictive of red cell folate status to the extent that it likely masks the subtlety of any genetic effects.
8. The folate variants with the most profound direct effect on overall folate status (independent of dietary intake) were C677T-MTHFR and G1793A-MTHFR.

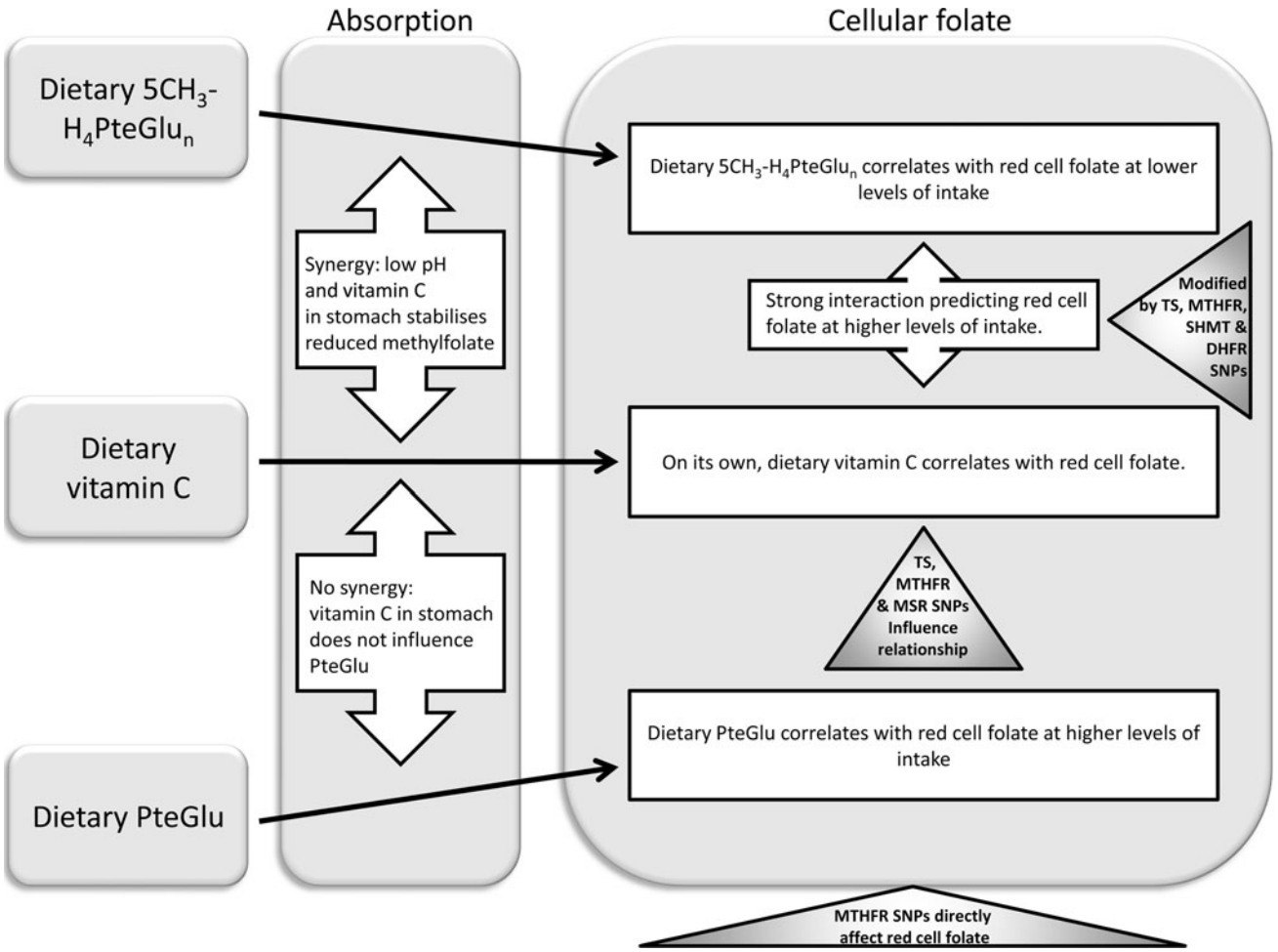

Fig. 1 Synergistic interactions (nutrient–nutrient and nutrient–gene) between vitamin C and folic acid

Ultimately, the significance of these nutrient–nutrient and nutrient–gene phenomena relates to the role of folate in cellular metabolism. Reduced folates are crucial for both deoxythymidine monophosphate (dTMP) and methionine biosynthesis. Intracellular folate in the form of the 5,10-methylenetetrahydrofolate$_n$ (5,10CH_2-H_4-PteGlu$_n$) coenzyme is critical for maintaining the structural integrity of DNA via dTMP formation. A shortage of 5,10CH_2-H_4-PteGlu$_n$ therefore leads to strand breaks because uracil (as dUMP) is misincorporated into DNA in place of dTMP. These strand breaks are an established antecedent of malignant transformation. Also, 5CH_3-H_4-PteGlu$_n$ dependent methionine biosynthesis contributes methyl groups to the methylome. This process governs gene expression, with some of these epigenetic DNA modifications (epimutations) being heritable. Changes to the methylome are also considered important in the pathoaetiology of cancer development [18]. Approximately half our methionine requirement is met by folate metabolism at the level of 5CH_3-H_4- PteGlu$_n$ [40], and so dietary folate is clearly important, with the implication that if extra vitamin C is present in the diet it might augment the methionine cycle. Increasingly, there is concern that synthetic PteGlu might act as an antimetabolite at critical folate enzymes where the avidity (*Km*) for the synthetic vitamer analogue leads to competition with the natural substrates for enzyme binding. This may occur for interactions involving dihydrofolate (H_2PteGlu) which has structural similarity to PteGlu. DHFR and MTHFR enzymes use H_2PteGlu as substrate and allosteric ligand, respectively, and so may be modulated in an unpredictable way by PteGlu [18, 20, 22, 23, 41, 42]. Recent work also suggests that synthetic PteGlu undergoes UV induced scission, yielding a potentially geno-toxic pterin that causes DNA damage in vitro. Natural 5CH_3-H_4-PteGlu does not have this property [24], implying an advantage to consumption of natural food sources of the vitamin.

Study limitations

This study draws attention to novel, putative nutrient–nutrient and nutrient–gene interactions. However, although in many cases we have evidence for a statistically

significant relationship between the response variable and predictor variable(s) ($p < 0.05$), r^2 values are low. Typically only around 10 % of the variation in the response variable can be explained by the regression model, depending on the nature of the relationship examined, although this does increase to around 50 % or more in some analyses.

For the association between vitamin C intake and red cell folate status, where any effect is likely to be small, due to vitamin C being but one minor component in a complex dietary interrelationship in which dietary folate, other B-vitamins and dietary antioxidants as well as gene variants are also major explanatory variables, 2–6 % of the variation is explained by regression analysis. Thus, a low r^2 is probably indicative of still uncharacterised independent variables that also have an effect on the dependent variable.

This is also true for the relationship between intake of dietary vitamin C and natural dietary folic acid, where only 11 % of the variation is explained by the model, even though significance is high ($p < 0.0001$). This variability is hardly surprising with the use of FFQs to characterise highly labile nutrients that will be unpredictable between similar food sources. The same is true for the relationship between dietary folate and red cell folate status where up to 9 % of the variation is explained by the model, but again with high significance ($p < 0.0001$). This level of data scatter remains typical, with up to 12 % variation explained by regression analysis when the interactive effect of crossing vitamin C intake with dietary folic acid is examined in respect of the association with red cell folate status ($p = 0.0005$).

When looking at how genotype influences the relationship between dietary vitamin intake and red cell folate status, data points move closer to values predicted by the regression equation and so the independent variables are a better predictor of the dependent variable. Here, typically 10–39 % of the variation is explained by the model with some highly significant p values. In the case of the genotype defined interaction between vitamin C intake and native methylfolate intake with respect to the association with red cell folate status, this increases to almost 66 % of the variation being explained by the regression model. Such findings are among the major outcomes of this study.

Therefore, while univariate impact can be low, multivariate impact is substantial in some instances. The data set is clearly complex, with many variables. But it does provide an interesting and novel perspective that fits with the biochemistry involved, although the authors recognise that the ideas put forward here would clearly benefit from a larger study to confirm and better define these relationships, particularly where r^2 values are low.

A further limitation of this study is potential heterogeneity within the subject group who were predominantly Caucasian with a mean age of 61.8 years (SD; 12.5 years). This could lead to erroneous interpretation of results. We therefore excluded patients receiving high dose vitamin B_{12} injections or antifolate chemotherapy. However, we recognise that a wide range of drugs might potentially interfere with the absorption, disposition and metabolism of vitamin C and folate. Despite possible variation arising from potential drug treatments, the data obtained fit what might be expected from knowledge of the vitamin biochemistry on an a priori basis. Nevertheless, this source of variability could be a contributory factor where low r^2 values were obtained.

Despite these study limitations, the outcome of further work in this area using larger, better defined populations may well offer an alternative to using PteGlu supplementation. This may be important given recent concerns relating to clinical conditions and aberrant biological phenomena associated with this synthetic vitamer.

Conflict of interest The authors declare that they have no conflict of interest.

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