Genetic modifiers of folate, vitamin B-12, and homocysteine status in a cross-sectional study of the Canadian population\textsuperscript{1–4}

John WR Zinck, Margaret de Groh, and Amanda J MacFarlane

\textbf{ABSTRACT}

\textbf{Background:} Genetic variation can cause variable responses to environmental stimuli. A number of single-nucleotide polymorphisms (SNPs) have been associated with B vitamin status or chronic diseases related to vitamin B-12 and folate metabolism.

\textbf{Objective:} Our objective was to identify associations between common SNPs in genes related to folate and vitamin B-12 metabolism or associated with B vitamin–related chronic diseases and biomarkers of nutrient status in a population exposed to folic acid fortification.

\textbf{Design:} A panel of 116 SNPs was sequenced by using the Sequenom iPLEX Gold platform in a sample of 3114 adults aged 20–79 y from the Canadian Health Measures Survey, cycle 1. Associations between these SNPs and red blood cell (RBC) folate, serum vitamin B-12, and plasma total homocysteine were determined.

\textbf{Results:} Twenty-one SNPs and 6 haplotype blocks were associated with RBC folate, serum vitamin B-12, and/or plasma homocysteine concentrations. Vitamin status was associated mainly with SNPs in genes directly involved in vitamin absorption/uptake (\textit{CUBN, CD320}, transport (\textit{TCN1, TCN2}), or metabolism (\textit{BHMT2, CBS, MTHFR, MUT, SHMT1}). Other SNPs included those in the \textit{DNMT2, DPEP1, FUT2, NOX4}, and \textit{PON1} genes.

\textbf{Conclusions:} We identified novel associations between SNPs in \textit{CD320} and \textit{DNMT2}, which had been previously associated with neural tube defects, and vitamin B-12 status, as well as between SNPs in \textit{SHMT1}, which had been previously associated with colorectal cancer and cardiovascular disease risk, and RBC folate status. These novel associations provide a plausible metabolic rationale for the association of these SNPs with B vitamin–related diseases. We also observed a novel association between an SNP in \textit{CUBN} with RBC folate and confirmed the association of a number of SNPs with B vitamin status in this large cross-sectional study.

\textbf{Keywords:} Canadian Health Measures Survey, folate, homocysteine, single-nucleotide polymorphisms, vitamin B-12

\textbf{INTRODUCTION}

B vitamins are a group of water-soluble vitamins that play important roles in cellular metabolism. Folate deficiency during pregnancy, due to diet or genetic predisposition, is associated with an increased risk of neural tube defects (NTDs); MIM 601634 (1, 2). Folate is involved in the transfer of one-carbon units in the de novo synthesis of thymidylate, purines, and methionine (3). Adequate folate consumption is therefore essential for the synthesis, stability, and repair of DNA and normal cell division, especially during times of rapid growth such as embryo development or cancer (3).

Vitamin B-12 deficiency results in megaloblastic anemia and neurodegeneration and, when left untreated, can lead to cognitive decline. It has also been associated with an increased risk of NTDs (4). Vitamin B-12 is required for the conversion of L-methylmalonyl-coenzyme A to succinyl-coenzyme A. Vitamin B-12 also acts as a coenzyme for methionine synthase (MTR) in the conversion of homocysteine to methionine, a folate-dependent reaction that creates the substrates for de novo nucleotide synthesis and S-adenosyl-methionine, a universal methyl donor. A methyl group from 5-methyltetrahydrofolate, produced by the enzyme methylenetetrahydrofolate reductase (MTHFR), can be transferred to homocysteine by MTR to form methionine and tetrahydrofolate. Conditions of folate and/or vitamin B-12 deficiency result in homocysteinemia (MIM 603174), a biomarker of vitamin B-deficiency that has been associated with endothelial injury, vascular inflammation, and increased risk of cardiovascular disease (CVD) (5).

Despite a number of informative genome-wide association studies and candidate gene analyses, the complex relationship between an individual’s genotype and his or her folate, vitamin B-12, or homocysteine status remains poorly understood. The most compelling evidence has been accumulated for the 677C>T single-nucleotide polymorphism (SNP) (rs1801133) in the \textit{MTHFR} gene, for which homozygosity of the minor \textit{T} allele has been associated with low folate status, increased homocysteine concentration, and elevated risk of NTDs (6–8). The \textit{TT} genotype results

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3\textsuperscript{ Supplemental Tables 1 and 2 are available from the “Supplemental data” link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

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5\textsuperscript{ Abbreviations used: CHMS, Canadian Health Measures Survey; CVD, cardiovascular disease; FDR, false-discovery rate; LD, linkage disequilibrium; MEC, mobile examination center; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; NTD, neural tube defect; RBC, red blood cell; SNP, single-nucleotide polymorphism.

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in a thermolabile MTHFR enzyme, reduced 5-methyltetrahydrofolate production, and hyperhomocysteinemia. Variants in other B vitamin metabolic genes, including methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), dihydrofolate reductase (DHFR), MTR reductase (MTRR), serine hydroxymethyltransferase 1 (SHMT1), transcobalamin II receptor (TCblR), and others, have also been associated with NTDs, colorectal cancer, and CVD, but their association with vitamin status is unknown (9–12).

Here we hypothesized that variants in genes related to folate and vitamin B-12 metabolism, or variants previously associated with folate and vitamin B-12-related diseases such as NTDs, are associated with vitamin status. We used a moderately high-throughput analysis to elucidate the association between 116 SNPs and red blood cell (RBC) folate, serum vitamin B-12, and plasma homocysteine status in a cross-sectional sample of 3114 adult Canadians from the Canadian Health Measures Survey (CHMS), cycle 1.

METHODS

Survey design and study population

The CHMS, cycle 1 is a comprehensive direct health measures survey that collects information on sociodemographic characteristics, risk factors, and health outcomes and includes blood, urine, and anthropometric measures (13). The CHMS, cycle 1 (n = 5604) is a representative sample of approximately 96.3% of the Canadian population aged 6–79 y living at home and residing in the 10 provinces and 3 territories. The CHMS, cycle 1 excluded persons living in remote areas or areas with a low population density, those on reserves or other aboriginal settlements, people in institutions, or full-time members of the Canadian Forces. The survey collected data from individuals over a 2-y period (2007–2009) at 15 sites with a minimum of 500 respondents for each sex from 5 age groups (6–11, 12–19, 20–39, 40–59, and 60–79 y). Collection sites were stratified into 5 regions to ensure national representation. In the CHMS, cycle 1, 88.3% of individuals who were initially contacted responded for the household questionnaire, 84.9% of those individuals reported to the mobile examination center (MEC), and 95.8% of the respondents at the MEC consented to DNA sampling. Our study population consisted of participants aged 20 y or older at the time of the MEC visit (n = 3114; women: n = 1632, men: n = 1482).

Ethics

All processes of CHMS, cycle 1 were reviewed and approved by the Health Canada Research Ethics Board. Participation in the survey was voluntary, and written informed consent was obtained from participants.

Demographic and metabolite data

Data collection for the CHMS, cycle 1 included a personal interview and a visit to the MEC. Individual demographic information was collected by Statistics Canada household interviewers during the CHMS personal interview. Blood was collected and hematocrit measured at the MEC. Plasma and serum were processed at the MEC, with whole blood maintained on ice and plasma processed within an hour of sampling. Samples were shipped frozen to the Health Canada Nutrition Laboratory for analysis. For RBC folate analysis, samples were thawed and diluted 1:26 with 0.5% ascorbic acid solution. After being mixed, hemolysates were allowed to stand for 180 min at room temperature. RBC folate and serum vitamin B-12 were analyzed by using the Immulite 2000 immunoassay (Siemens Canada), as described by the manufacturer. RBC folate was normalized to hematocrit. Plasma homocysteine was analyzed by using the Vitros 5,1 FS (OrthoClinical Diagnostics), as described by the manufacturer.

DNA was extracted from whole blood at the CHMS MEC by using ArchivePure DNA Blood Kits (5 Prime Inc.). DNA samples were shipped frozen and stored at −80°C at the Canadian National Microbiology Laboratory (Winnipeg, Manitoba, Canada).

SNP selection and genotyping

A panel of 116 SNPs was selected from genes involved in folate, vitamin B-12, and homocysteine metabolism, as well as genes associated with B vitamin–related chronic disease or NTD risk (12, 14–21). The panel of SNPs genotyped, the gene name/function, and sequencing call rate are given in Supplemental Table 1. SNPs were genotyped by using the iPLEX GOLD system (Sequenom) at the McGill University and Génome Québec Innovation Centre (Montreal, Quebec, Canada).

Marker quality was validated for standard quality controls by using PLINK (v1.07, http://pngu.mgh.harvard.edu/purcell/plink/; 22). All 116 SNPs had missing rates of <3%, minor allele frequencies of >1%, and no statistically significant deviation from Hardy–Weinberg expectations (Supplemental Table 1). All successfully genotyped SNPs were used in the analyses.

Because allele and phenotype frequencies are known to vary among populations of different genetic ancestry, population stratification can confound the association between a phenotypic trait and a genetic marker. This can result in false associations in cases where observed genetic associations are biased by and/or coincide with ancestral lineages. To identify any instances of population stratification, we performed a principal component analysis in GenAlex (v6.5, http://bioinformatics.oxfordjournals.org/content/28/19/2537; 23) to identify potential association bias due to ancestral population structure among samples. Separate principal component analyses were performed for each metabolite data set and for dichotomous (case-control) subsets, assuming the cases and controls represent separate populations.

Linkage disequilibrium (LD) among SNP markers was assessed by using Haploviev (v4.2, https://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploviev/; 24). Because of the presence of multiple, high-LD SNPs from single genes, we constructed haplotype blocks to perform multimarker associations (Figure 1 and Supplemental Table 2). SNP haplotype blocks were constructed and haplotype phases were identified by using Haplotype Block Estimation in PLINK (v1.07) (22).

A multistep analysis plan was developed a priori to examine the association between single markers and nutrient status by using a dichotomous (case-control) and a continuous method. This method was repeated for the haplotype markers to assess the associations between markers in high LD and nutrient status.
synthase; MTRR, methionine synthase reductase; SHMT1, serine hydroxymethyltransferase 1; SLC19A1, solute carrier family 19 (folate transporter), member protein 366.

(40) Type I errors. Only associations that passed the multiple-comparison tests, with the Bonferroni correction, were considered statistically significant. The direction of these associations. To correct for multiple comparisons, we subjected all associations to Benjamini and Hochberg (26) FDR multiple-testing corrections were calculated with PLINK (v1.07) (22).

FIGURE 1 Linkage disequilibrium relations (D') between SNPs tested for association with red blood cell folate, serum vitamin B-12, and homocysteine concentrations. In the panel of 116 SNPs, 60 SNPs in 18 genes had statistically significant levels of linkage disequilibrium with other markers. ADA, adenosine deaminase; BHMT2, betaine–homocysteine S-methyltransferase 2; CBS, cystathionine β-synthase; DHFR, dihydrofolate reductase; FOLR2, folate receptor 2; FPGS, polyglutamate synthase; FUT2, fucosyl-transferase 2; MFTC, mitochondrial folate transporter/carrier; MMAB, methylmalonic aciduria (cobalamin deficiency) cblB type; MTHFD, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylene tetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; SHMT1, serine hydroxymethyltransferase 1; SLC19A1, solute carrier family 19 (folate transporter), member protein 366.

Case-control association analysis
A case-control design was used to test associations between genotype and defined cutoffs for a given metabolite. The minor allele was considered the risk allele for all analyses, and the major allele was the reference group. Metabolite cutoffs were selected before genotyping. For RBC folate, we defined cutoffs as deficient (<320 nmol/L, n = 13) vs. all others (n = 3035), low (20th percentile; n = 608) vs. all others (n = 2440), and high (80th percentile; n = 623) vs. all others (n = 2425). Serum vitamin B-12 cutoffs were deficient (<148 pmol/L, n = 121) vs. all others (n = 2870) and less than adequate (<220 pmol/L, n = 720) vs. all others (n = 2271). The plasma homocysteine cutoff was high homocysteine (>13 μmol/L, n = 221) vs. all others (n = 2871).

Quantitative association analysis
Because of the continuous nature of the metabolite data, the user-defined case-control associations may not have adequately highlighted all associations between SNP markers and trends in the metabolite concentrations. Therefore, we also performed a second set of analyses to examine quantitative associations between SNPs and phenotypic distribution. Significance (Wald test asymptotic P value), r^2, and Wald test t distribution were used to estimate the quantitative associations between metabolite concentrations and genotypes. Quantitative associations were performed by using the linear quantitative trait association analysis with PLINK (v1.07) (22). Benjamini and Hochberg (26) FDR multiple-testing corrections were calculated with PLINK (v1.07) (22).

Multimarker analysis
In addition to single SNP analyses, we performed multimarker associations by using haplotype block analyses. Haplotype-based associations for both case-control and quantitative traits were performed with PLINK (v1.07) (22) by using the same parameters as the single-marker analyses. To obtain a measure of significance for multiple testing in haplotype analysis, we performed 10,000 permutations. Multiple-testing correction was performed by using FDR (26) corrections in PLINK (v1.07) (22).
No statistically significant patterns of population stratification were observed, likely because of the low number of non-Caucasian participants \((n = 186)\) and their varied ancestry. We did not observe any racial predisposition to cases or controls in any of the association tests. We identified 15 SNPs in 10 genes in the case-control analysis and 14 SNPs in 11 genes in the quantitative analysis that were statistically significantly associated with metabolite status or concentrations \((\text{Tables 2 and 3, respectively})\), the association between both SNPs and RBC folate suggest that the minor alleles of both markers (rs1979277, rs2273028) are in high LD of the folate in the quantitative analysis (Table 5). The high LD of the component markers in the \(\text{SHMT1}\) haplotype and the observed association between both SNPs and RBC folate suggest that the minor alleles of both markers \((\text{rs1979277, rs2273028})\) are inherited together. The \(\text{MTHFR H2 TG CCT}\) \((f: 22.7\%); \text{OR: 0.75, FDR P: 0.006}) haplotype was less likely to have high folate status \((\text{Table 4})\) and was negatively associated with RBC folate \((T: -3.10, \text{FDR P: 0.038})\).

### Genetic associations

#### RBC folate

\(\text{CUBN rs780635}\) was associated with higher risk of deficiency \([\text{allele frequency } (f): 34.6\%; \text{OR: 1.26, FDR P: 0.039}]\) or low \((\text{OR: 1.25, FDR P: 0.039})\) RBC folate status \((\text{Table 2})\). Individuals with \(\text{MTHFR rs1994798 } (f: 41.2\%); \text{OR: 1.30, FDR P: 0.005})\) were more likely to have high folate status \((\text{RBC folate } > 80\text{th percentile})\), whereas those with \(\text{MTHFR rs9651118 } (f: 22.4\%); \text{OR: 0.76, FDR P: 0.032})\) were less likely to have high folate status. No statistically significant quantitative associations between the tested SNPs and RBC folate were observed after FDR correction.

#### Serum vitamin B-12

In both the vitamin B-12 case-control and quantitative analyses \((\text{Tables 2 and 3, respectively})\), the \(\text{FUT2 rs492602 and rs602662}\) were statistically significantly associated with a lower risk of deficient \([f: 45.8\%, 48.0\%]; \text{OR: 0.60, 0.61}; \text{FDR P: 0.012, 0.013}, \text{respectively})\) or low \([\text{OR: 0.71, 0.74}; \text{FDR P: } 1.1 \times 10^{-5}\] or low (OR: 0.60, FDR P: 0.032) were negatively associated with RBC folate status.
4.0 × 10^{-4}, respectively) vitamin B-12 status and were associated with higher serum vitamin B-12 concentrations (T: 6.40, 5.93; FDR P: 2.1 × 10^{-8}, 2.1 × 10^{-8}, respectively). The FUT2 AG haplotype was associated with a higher risk of vitamin B-12 deficiency (f: 53.2%, OR: 1.59, 0.001) or less than adequate (OR: 1.33, FDR P: 3.8 × 10^{-5}) vitamin B-12 status (Table 3) and had a negative association with serum vitamin B-12 (Table 5; T: -6.02, FDR P: 3.6 × 10^{-8}). On the other hand, the FUT2 GA haplotype was positively associated with serum vitamin B-12 (f: 44.1%, T: 6.34, FDR P: 4.9 × 10^{-5}). This suggests that the 2 SNP components of the haplotype may be inherited together (AG: A is inherited with G and vice versa).

**TABLE 2**

<table>
<thead>
<tr>
<th>Phenotypic association/gene</th>
<th>SNP (rs)</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
<th>FDR P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate deficient (&lt;320 nmol/L)</td>
<td>rs780635</td>
<td>1.26</td>
<td>1.1, 1.43</td>
<td>6.8 × 10^{-4}</td>
<td>0.039</td>
</tr>
<tr>
<td>20th percentile folate</td>
<td>rs780635</td>
<td>1.25</td>
<td>1.1, 1.43</td>
<td>6.7 × 10^{-4}</td>
<td>0.039</td>
</tr>
<tr>
<td>80th percentile folate</td>
<td>rs1994798</td>
<td>1.30</td>
<td>1.14, 1.47</td>
<td>4.6 × 10^{-5}</td>
<td>0.005</td>
</tr>
<tr>
<td>Vitamin B-12 deficient (&lt;148 pmol/L)</td>
<td>rs1801222</td>
<td>1.61</td>
<td>1.24, 2.09</td>
<td>3.0 × 10^{-4}</td>
<td>0.013</td>
</tr>
<tr>
<td>Vitamin B-12 below adequate (&lt;220 pmol/L)</td>
<td>rs602662</td>
<td>0.61</td>
<td>0.47, 0.8</td>
<td>3.0 × 10^{-4}</td>
<td>0.013</td>
</tr>
<tr>
<td>High homocysteine (&gt;13 μmol/L)</td>
<td>rs757874</td>
<td>1.42</td>
<td>1.11, 1.72</td>
<td>3.3 × 10^{-4}</td>
<td>0.006</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>SNP (rs)</th>
<th>r^2</th>
<th>T</th>
<th>P</th>
<th>FDR P</th>
</tr>
</thead>
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<tr>
<td>Vitamin B-12</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>rs1801222</td>
<td>0.008</td>
<td>-5.12</td>
<td>3.5 × 10^{-7}</td>
<td>1.3 × 10^{-5}</td>
</tr>
<tr>
<td>CD320</td>
<td>0.004</td>
<td>3.42</td>
<td>6.4 × 10^{-4}</td>
<td>0.012</td>
</tr>
<tr>
<td>DNMT2</td>
<td>0.005</td>
<td>3.75</td>
<td>2.0 × 10^{-4}</td>
<td>0.004</td>
</tr>
<tr>
<td>FUT2</td>
<td>0.011</td>
<td>5.93</td>
<td>3.4 × 10^{-6}</td>
<td>2.1 × 10^{-7}</td>
</tr>
<tr>
<td>TCN1</td>
<td>0.007</td>
<td>6.52</td>
<td>2.3 × 10^{-5}</td>
<td>6.4 × 10^{-4}</td>
</tr>
<tr>
<td>NOX4</td>
<td>0.004</td>
<td>-3.35</td>
<td>8.1 × 10^{-4}</td>
<td>0.014</td>
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</table>

<table>
<thead>
<tr>
<th>SNP (rs)</th>
<th>r^2</th>
<th>T</th>
<th>P</th>
<th>FDR P</th>
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<tr>
<td>Vitamin B-12</td>
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<tr>
<td>rs1801222</td>
<td>0.003</td>
<td>3.18</td>
<td>0.002</td>
<td>0.004</td>
</tr>
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<td>CD320</td>
<td>0.003</td>
<td>3.13</td>
<td>0.002</td>
<td>0.034</td>
</tr>
<tr>
<td>DNMT2</td>
<td>0.008</td>
<td>-4.90</td>
<td>1.1 × 10^{-6}</td>
<td>1.2 × 10^{-4}</td>
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<tr>
<td>FUT2</td>
<td>0.003</td>
<td>3.09</td>
<td>0.002</td>
<td>0.034</td>
</tr>
<tr>
<td>NOX4</td>
<td>0.006</td>
<td>-4.31</td>
<td>1.7 × 10^{-5}</td>
<td>9.7 × 10^{-4}</td>
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<tr>
<td>MUT</td>
<td>0.004</td>
<td>3.75</td>
<td>2.0 × 10^{-4}</td>
<td>0.008</td>
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<tr>
<td>TNF</td>
<td>0.005</td>
<td>3.63</td>
<td>3.0 × 10^{-4}</td>
<td>0.035</td>
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</table>

1The major allele was the reference group for determining the OR. For definitions of genes, see Figure 2. FDR, false discovery rate; SNP, single-nucleotide polymorphism.
**Table 4**

Haplotypes in statistically significant case-control phenotypic association with red blood cell folate, serum vitamin B-12, or plasma homocysteine status

<table>
<thead>
<tr>
<th>Association/haplotype block</th>
<th>Haplotype</th>
<th>Frequency</th>
<th>OR 95% CI</th>
<th>P</th>
<th>FDR P</th>
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<tbody>
<tr>
<td>80th percentile folate</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MTHFR H2</td>
<td>TGCCT</td>
<td>0.227</td>
<td>0.75</td>
<td>0.63, 0.87</td>
<td>3.0 × 10⁻⁴</td>
</tr>
<tr>
<td></td>
<td>CGTCT</td>
<td>0.186</td>
<td>1.28</td>
<td>1.16, 1.39</td>
<td>0.002</td>
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<tr>
<td></td>
<td>TA</td>
<td>0.285</td>
<td>1.23</td>
<td>1.07, 1.42</td>
<td>0.002</td>
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<tr>
<td>Vitamin B-12 deficient (&lt;148 pmol/L)</td>
<td>FUT2</td>
<td>AG</td>
<td>0.532</td>
<td>1.59</td>
<td>0.49, 0.72</td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>MTHFR H1</td>
<td>GC</td>
<td>0.044</td>
<td>2.04</td>
<td>1.91, 2.21</td>
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<tr>
<td>Vitamin B-12 below adequate (&lt;220 pmol/L)</td>
<td>FUT2</td>
<td>GA</td>
<td>0.441</td>
<td>0.75</td>
<td>0.48, 0.91</td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG</td>
<td>0.532</td>
<td>1.33</td>
<td>1.21, 1.43</td>
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<tr>
<td>High homocysteine (&gt;13 μmol/L)</td>
<td>BHMT2 H2</td>
<td>ACT</td>
<td>0.045</td>
<td>1.82</td>
<td>1.68, 1.99</td>
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</table>

*The most common haplotypes are the reference markers for case-control tests (see Supplemental Table 2 for haplotype block information). For definitions of genes, see Figure 2. FDR, false discovery rate.*

**Table 5**

Haplotypes in statistically significant quantitative phenotypic linear regression association with red blood cell folate, serum vitamin B-12, or plasma homocysteine concentrations

<table>
<thead>
<tr>
<th>Association/gene haploblock</th>
<th>Haplotype</th>
<th>β</th>
<th>r²</th>
<th>T</th>
<th>P</th>
<th>FDR P</th>
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<tbody>
<tr>
<td>Folate</td>
<td>MTHFR H2</td>
<td>TGCCT</td>
<td>58.74</td>
<td>0.004</td>
<td>3.30</td>
<td>0.001</td>
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<td></td>
<td>SHMT1</td>
<td>CG</td>
<td>49.33</td>
<td>0.003</td>
<td>3.10</td>
<td>0.002</td>
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<tr>
<td>Vitamin B-12</td>
<td>FUT2</td>
<td>GA</td>
<td>23.85</td>
<td>0.012</td>
<td>6.34</td>
<td>4.9 × 10⁻⁹</td>
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<tr>
<td></td>
<td></td>
<td>AG</td>
<td>22.48</td>
<td>0.012</td>
<td>6.02</td>
<td>3.6 × 10⁻⁸</td>
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<tr>
<td>Homocysteine</td>
<td>BHMT2 H2</td>
<td>ACT</td>
<td>0.921</td>
<td>0.007</td>
<td>4.78</td>
<td>3.4 × 10⁻⁵</td>
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<tr>
<td></td>
<td>CBS</td>
<td>CAAA</td>
<td>0.317</td>
<td>0.003</td>
<td>3.18</td>
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<td>MTHFR H2</td>
<td>TATCT</td>
<td>0.265</td>
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*For definitions of genes, see Figure 2. See Supplemental Table 2 for haplotype block information. FDR, false discovery rate; T, Wald test.*

**Discussion**

In this cross section of the adult Canadian population, we found evidence that 21 SNPs in 12 genes (Tables 2 and 3 and Supplemental Table 1) and 6 haplotype blocks from 5 genes (Tables 4 and 5) were associated with RBC folate, serum vitamin B-12, and/or plasma homocysteine status (Figure 2). We observed statistically significant associations between nutrient status and SNPs in genes directly involved in the uptake or metabolism of these nutrients, as well as nutrient status and SNPs previously shown to be associated with NTDs or cancer and CVD risk.

MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate required for homocysteine remethylation to form methionine. MTHFR polymorphisms have been associated with cancer, NTD, and congenital heart defect risk (6–8, 27, 28). The MTHFR 677C>T SNP (rs1801133; MTHFR exon 5) has previously been associated with lower RBC folate status and higher total homocysteine (17, 20, 29–31) because of thermolability and decreased activity of the variant enzyme. MTHFR rs1801133 was common in our sample and was associated with higher homocysteine status.
However, rs1801133 was not associated with lower RBC folate status, likely reflecting exposure of the population to mandatory folic acid fortification.

Two common intron variants, MTHFR rs1994798 and rs9651180, which were previously associated with NTD risk (32), were associated with RBC folate status. MTHFR rs9651180 was associated with lower and MTHFR rs1994798 higher RBC folate. Two MTHFR H2 haplotypes, representing combinations of rs1801133, rs9651180, rs1994798, and 2 other SNPs, were associated with RBC folate; CGCTC was associated with higher and TCCCT was associated with lower RBC folate. These data indicate that other common MTHFR polymorphisms are associated with folate status even in a population exposed to folic acid fortification.

SHMT1 catalyzes the reversible conversion of serine and tetrahydrofolate to glycine and 5,10-methylenetetrahydrofolate (33). The SHMT1 1420C>T SNP (rs1979277; exon 12) has been associated with protection from rectal cancer and with CVD risk in a gene-gene interaction with MTHFR rs1801133 (9, 34). SHMT1 rs1979277 reduces the sumoylation of SHMT1, impairing its translocation to the nucleus, suggesting that it reduces nuclear folate metabolism and thymidylate synthesis (35). The SHMT1 TA haplotype, which includes rs1979277, was associated with higher
RBC folate and the CG haplotype with lower RBC folate. Higher tissue folate has been observed in heterozygous SHMT1 knockout mice (36), but to our knowledge, SHMT1 rs1979277 has not been previously associated with folate status in humans.

Common variants in exon 2 of FUT2 (rs602662, rs492602, and the FUT2 haplotype) were associated with higher vitamin B-12 status, confirming other findings (17, 20, 37). Two hypotheses have been postulated to explain the association between FUT2 and vitamin B-12 status. The first proposes that FUT2 variants reduce H-type antigen production and function, which reduces the risk of Helicobacter pylori infection and its related gastritis-induced vitamin B-12 malabsorption (38, 39). The second hypothesis proposes that FUT2 variants increase gastric intrinsic factor secretion, a fucosylated glycoprotein required for vitamin B-12 absorption (40).

Transcobalamin 1 and transcobalamin 2 are the circulating vitamin B-12 binding proteins. Vitamin B-12 bound to transcobalamin 2 (holoTC) represents approximately 10–20% of circulating vitamin B-12. HoloTC is bioavailable as it undergoes regulated cellular uptake by receptor-mediated endocytosis. Vitamin B-12 bound to transcobalamin 1 (holohaptocorrin) represents 80–90% of vitamin B-12 in circulation and is unavailable for cellular uptake. TCN1 rs526934 (intron 8) and TCN2 rs757874 (intron 4) were associated with lower vitamin B-12 status, supporting previous findings (17, 20). The CHMS assessed vitamin B-12 status by using total serum vitamin B-12; therefore, the data suggest that polymorphisms in either cobalamin binding protein may modify this biomarker. Whether these polymorphisms have a meaningful effect on tissue vitamin B-12 stores remains to be determined.

CD320 is the receptor that specifically binds and internalizes holoTC by endocytosis. We observed a novel association between the low-frequency CD320 rs2336573, a missense SNP in exon 4, and higher vitamin B-12 status. Perhaps counterintuitively, this SNP has also been associated with maternal risk of NTD development (12). It could be hypothesized that the variant receptor results in reduced cobalamin uptake into tissues, as has been shown in cell culture models (41), and leads to tissue deficiency in the absence of serum deficiency or even serum enrichment.

Cubilin/intrinsic factor-cobalamin receptor is the vitamin B-12–intrinsic factor complex receptor in the distal ileum (42). CUBN variants have been associated with vitamin B-12 status, maternal NTD risk, and megaloblastic anemia 1, a rare autosomal-recessive disorder characterized by juvenile pernicious anemia (12, 16, 17, 20). We found that CUBN rs1801222 was associated with lower vitamin B-12 status; rs1801222 is a missense mutation in exon 8 that is predicted to decrease CUBN functionality, thereby lowering vitamin B-12 absorption. CUBN rs4748353, an intron variant, was associated with lower vitamin B-12 status. The low-frequency CUBN rs11254363 (intron 52), which has been associated with lower coronary artery disease risk (43), was associated with higher vitamin B-12 status.

We observed a novel association between the common CUBN rs780635, which is located in the 3′ untranslated region of the gene, and lower RBC folate. This association highlights the interaction between vitamin B-12 and folate metabolism. The vitamin B-12–dependent enzyme MTR is required to convert 5-methyltetrahydrofolate to monoglutamate (44). We observed statistically significant associations between the common CBS rs2851391 and rs4920037 intron variants and the CBS haplotype (CAA, containing rs2851391 and rs4920037), and increased homocysteine status, suggesting that these variants reduce CBS activity. CBS rs2851391 reduces gene transcription (46) and was previously associated with high homocysteine and increased risk of spina bifida (19). The common CBS rs2124459 (intron), which has been associated with higher serum folate (20), was associated with higher vitamin B-12 status in our study. The potential mechanism underlying its association with vitamin B-12 or folate status is unclear.

DNMT2/TRDMT1 is an aspartic acid transfer RNA methyltransferase with residual DNA methyltransferase activity (47). DNMT2 variants, including the intronic rs2295809, have been associated with decreased NTD risk and increased RBC folate (16, 28). We found that rs2295809 is also associated with higher vitamin B-12 status. Although the mechanism is not understood, the data suggest that overall improved B vitamin status could underlie its association with reduced NTD risk.

Mitochondrial enzyme methylmalonyl-CoA mutase (MUT) catalyzes the isomerization of methylmalonyl-CoA to succinyl-CoA. We observed an association between 2 common intronic MUT polymorphisms, rs4527943 and rs9473555, with higher plasma homocysteine, replicating other findings (48). Like MTR, which remethylates homocysteine, MUT is a vitamin B-12–dependent enzyme. Under conditions of high folate status, as is observed in the Canadian population (49), it has been suggested that the activities of these enzymes are negatively affected, resulting in both high homocysteine and high methylmalonyl-CoA (50). Genetic variation in these enzymes may also influence this relationship.

PON1 is an esterase for which reduced activity or genetic variants have been associated with increased risk of vitamin B-12 deficiency anemia, atherosclerosis, and CVD (5, 51). We observed an association between the low-frequency PON1 rs3917577 (3′ untranslated region) and higher vitamin B-12 status. The data suggest that genetic variants that reduce PON1 activity or potentially alter its translation may modify vitamin B-12 status.

The NOX4 rs11018628 and DPEP1 rs1126464 intronic variants were both associated with lower plasma homocysteine, confirming previous observations (48). NOX4, an NADPH oxidase, and DPEP1, a dipeptidase, are both expressed in the kidney (52–54). Although their roles in homocysteine metabolism remain unclear, they may be involved in renal handling and metabolism of homocysteine and cysteine.

Finally, BHMT2 is one of 2 betaine homocysteine methyltransferases that transfers a methyl group from betaine to homocysteine to form methionine (55). The BHMT2 haplotype (ACT) was associated with higher homocysteine, suggesting that this haplotype may have a functional effect on BHMT2 activity,
resulting in lower homocysteine remethylation and higher circulating homocysteine.

Our findings need to be interpreted in light of a few limitations. First, the sample population was predominantly Caucasian, limiting the generalization of the findings to other ethnicities. Second, several tested SNPs were found in low frequencies, below the power of detection for associations after multiple testing corrections. Third, the nature of the status biomarkers used may pose limitations in older (>50 y) individuals. Older individuals have been shown to be at risk for lower serum vitamin B-12, although not in this population (49), and higher plasma homocysteine (56), which could confound their association with SNPs. Finally, we have not considered nutrient intake, which could modify the association between SNPs and nutrient status.

Strengths of this study include the comprehensive panel of SNPs and a sample size large enough to examine common phenotypic and genotypic traits involved in B vitamin metabolism. In addition, we were able to identify associations between nutrient status and SNPs that had been associated with B vitamin-related diseases, even when the mechanism underlying these associations was unclear. By combining a dichotomous and quantitative approach, we were able to identify associations that may have otherwise been missed with a single approach.

We have identified associations between 21 SNPs and 6 haplotypes and RBC folate, serum vitamin B-12, and plasma homocysteine (Figure 1). We have established novel associations between genes previously associated with NTDs (CD320, DNMT2), colorectal cancer and CVD (SHMT1), and vitamin B-12 and folate status, respectively. These findings provide a plausible explanation for the relationship between variants in these genes and B vitamin–related chronic diseases. In addition, we confirmed previously observed associations between SNPs in MTHFR, BHMT2, CBS, CUBN, FUT2, PON1, NOX4, MUT, TCN1, and TCN2 and B vitamin nutrient status.

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