Long-term Effects of Folic Acid Fortification and B-vitamin Supplementation on Total Folate, Homocysteine, Methylmalonic Acid and Cobalamin in Older Adults

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ABSTRACT

Objective: To investigate the long-term effects of the Canadian folic acid fortification program in older adults' whole blood cell folate (folate) and cobalamin (Cbl) status, including homocysteine (tHcy) and methylmalonic acid (MMA), with and without voluntary B-vitamin intake, from 1997 to 2004.

Methods: Cohort of community-dwelling volunteer older adults. Clinical and biochemical data, including intake of B-vitamin supplements, were obtained at 2- to 2.5-year intervals and divided in 4 periods. Random coefficients (mixed effects) models were used to estimate the linear trend in folate and to compare levels of biochemical parameters between periods. All models were estimated by restricted maximum likelihood as implemented in PROC MIXED of SAS V8.2.

Results: Folate levels increased continuously at a yearly rate of 234 ng/mL (95% CI 213-254; $p < 0.001$) and had not plateaued by the last period when 84% of subjects without B-vitamins had elevated folate. Homocysteine did not remain suppressed. Elevated tHcy was as prevalent in the last study period as in the first. No significant deficits of Cbl or increases of MMA were observed, but MMA levels tended to increase with time in subjects without B-vitamins. B-vitamin supplements significantly affected all results, reducing tHcy and MMA levels.

Conclusion: In this population, fortification with folic acid has resulted in cumulative increases of folate with no long-term reduction in tHcy or changes in Cbl or MMA. Possible deleterious effects of cumulative increases of folate, and beneficial effects of B-vitamin supplements in reducing tHcy and MMA, should be investigated.

Key words: Folate; cobalamin; homocysteine; methylmalonic acid; aged; fortification

La traduction du résumé se trouve à la fin de l'article.

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The folic acid fortification of grain products was mandated in Canada in 1998, although it began earlier and was nearly complete by mid-1998.^{1,2} The program, similar to the one implemented in the US, is aimed at preventing neural tube defects. Shortly after implementation, an increase of folate³⁻⁵ and a decrease of tHcy^{6,7} were observed. Homocysteine levels increase with age and renal dysfunction⁸ and are associated with vascular disease, Alzheimer's disease and osteoporosis;⁹⁻¹³ the expected lowering of tHcy in older adults with the fortification was, therefore, desirable.

Older adults are at high risk of Cbl deficiency, as suggested by the high prevalence of elevated MMA.¹⁴ Cbl deficits result in elevated MMA and tHcy. Since Cbl and folate are cofactors in the methylation cycle, changes in folate levels might affect Cbl function. We investigated folate and Cbl status through the fortification years from 1997 to 2004, and analyzed the changes in folate, Cbl, tHcy and MMA in a cohort of older adults with and without voluntary B-vitamin supplementation.

METHODS

Participants and data collection

The protocol was approved by the University Research Ethics Board. After providing informed consent, participants were included in the cohort as previously described.^{14,15} Briefly, systematic clinical history and treatments (including vitamin concentrations), physical exam and laboratory investigations were obtained in 281 older adults living in the community, with no neurological, psychiatric, renal or metabolic disease. At baseline, participants were 73 (± 4.9) years old, 75.4% female and had 13 (± 3.5) years of education. Data collection began in October 1997 and follow-up data were collected in two waves, the first between September 2000 and March 2002, and the second from October 2002 through November 2004. B-vitamin intake was recorded throughout.

We have considered August 1998 as the beginning of the full fortification period.^{2,16} Data collection time was categorized into four periods reflecting the fortification status during the baseline collection time and the two waves of follow-up: Period 1: partial fortification (October

TABLE I

Subject Characteristics by Period of Analysis

Characteristic	Period 1 Visit 1 <01AUG98 (n=131)	Period 2 Visit 1 01AUG98-31JUL00 (n=147)	Period 3 Visit 2 01AUG00-31JUL02 (n=148)	Period 4 Visit 3 ≥01AUG02 (n=134)
B-vitamins - %	27	32	44	49
Folic acid use - %	19	24	33	38
Dose (mg) - mean* (range)	0.635 (0.10-1.00)	0.543 (0.10-1.00)	0.531 (0.10-1.00)	0.638 (0.05-4.00)
Cbl Oral use - %	25	30	39	46
Dose (µg) - mean* (range)	21.6 (3.0-66.5)	22.1 (3.0-100.0)	37.1 (3.0-125.0)	73.2 (3.0-1300.0)
Cbl injection use - %	1	1	4	8
Age - mean (range)				
All	72.4 (65-89)	73.8 (65-83)	75.0 (65-91)	77.1 (69-94)
With B-vitamins	73.1 (66-89)	74.6 (65-82)	75.3 (65-91)	77.1 (69-93)
Without B-vitamins	72.1 (65-82)	73.4 (65-83)	74.7 (65-85)	77.1 (69-94)
Male - %				
All	25	24	30	30
With B-vitamins	20	13	28	32
Without B-vitamins	27	29	31	28
Creatinine clearance				
All	0.85 (0.36-1.51)	0.86 (0.24-1.76)	0.80 (0.33-1.36)	0.82 (0.21-7.35)
With B-vitamins	0.79 (0.36-1.09)	0.86 (0.24-1.25)	0.82 (0.33-1.28)	0.87 (0.29-7.35)
Without B-vitamins	0.87 (0.49-1.51)	0.86 (0.36-1.76)	0.79 (0.35-1.36)	0.78 (0.21-1.54)

B-vitamins - % = Percentage of subjects taking B-vitamins oral supplements, Cbl = cobalamin.

Mean daily dose and range was calculated from subjects taking B-vitamin supplements only. Information on B-vitamin supplementation was collected at each visit from all participants (concentration and daily intake of B-vitamins folic acid and Cbl during the 3 months preceding the visit).

Creatinine clearance was calculated by the formula: $[(140 - \text{age}) \times \text{weight} / 72 \times \text{creatinine}] \times 0.81$ for females). There were no significant differences in creatinine clearance over the 4 periods ($p=0.53$).

1997 to July 31, 1998 inclusive); Period 2: complete fortification (August 1, 1998 to July 31, 2000); Period 3: first follow-up (August 1, 2000 to July 31, 2002); and Period 4: second follow-up (August 1, 2002 to November 30, 2004).

Laboratory investigations

THcy and MMA were determined as described.^{14,17,18} Whole blood cell folate (folate) levels were determined by a chemiluminescent enzyme immunoassay (Immulate® folic acid) until April 2001, and by competitive binding assay using folate binding protein after whole blood hemolysis (Elecys® System 2010, Roche) from April 2001 to the present. The regression coefficient between the two assays was 0.915. Cbl and creatinine determinations were performed by standard methods. Folate and Cbl had a precision of <6% CV (coefficient variation) at 750 nmol/L for folate and 156 pmol/L for Cbl (between-day internal quality control). Creatinine clearance was calculated by the formula: $[(140 - \text{age}) \times \text{weight} / 72 \times \text{creatinine}] \times 0.81$ for females).

Statistical analysis

Scatter plots of folate, tHcy, Cbl, and MMA values over time were performed with plotting characters identifying the subjects' use of B-vitamins at the time of the determination. A non-parametric lowess smoother¹⁹ was overlaid to help

visualize the population-averaged trends by supplementation status. THcy, MMA and Cbl exhibited a strong positive skewness and were modelled on the natural log scale. After modelling, these variables were transformed back to their original scale, providing estimates of geometric means and 95% confidence intervals.

A random coefficients model with random subject intercepts and slopes was used to estimate the linear trend in folate over the study period. THcy, MMA and Cbl levels were compared between periods by a mixed effects model that accounted for within-subject correlation by allowing an unstructured within-subject covariance between assessments 1, 2 and 3. Age and sex were controlled for in all models. Some analyses have stratified by B-vitamins (yes/no) while others have controlled for it. This model accounts for the dependence induced by the repeated measures by modelling the covariance within a subject between visits. While the variance may be different at the three visits, and the covariance may differ between each pair of visits, it is assumed that this covariance structure is similar across all subjects.

Pearson's partial correlation coefficient was estimated between the logs of tHcy and folate for each period after controlling for age, sex and B-vitamins use.

All models were estimated by restricted maximum likelihood, as implemented in PROC MIXED of SAS V8.2.²⁰ Dunnett's

method²¹ for comparing multiple groups to a control was used when comparing period 1 to periods 2-4.

RESULTS

From October 1997 to November 2004, 605 determinations from 281 subjects were performed. This analysis uses 560 (93%) determinations from 275 (98%) subjects, with all data available. The number of subjects contributing one, two and three evaluable determinations was 96 (35%), 73 (27%) and 106 (39%), respectively. Subjects' characteristics are described in Table I.

Figure 1a shows a strong increasing trend in folate throughout the study regardless of B-vitamins intake, with folate levels not reaching a plateau 6 years after fortification started. Among participants not taking B-vitamins, the mixed model estimated a folate level of 624 nmol/L (95% CI 580-668) in 1997-98, with an average annual increase of 234 nmol/L (95% CI 213-254; $p<0.001$) per year. For subjects taking B-vitamins, the expected value for the same period was 283 nmol/L (95% CI 204-362; $p<0.001$) greater, and the average annual increase was 61 nmol/L (95% CI 30-93; $p=0.001$) greater than among subjects without B-vitamins. The sequential reduction in the remaining unexplained variance in folate was: $R^2=20\%$ due to B-vitamins intake,

Figure 1. Scatter plots of folate (a) tHcy (b) Cbl (c) and MMA (d) over time with overlaid non-parametric lowess smoother to visualize population's trends by B-vitamin intake

Folate in nmol/L (a), homocysteine in μmol/L (tHcy) (b), cobalamin in pmol/L (Cbl) (c), and methylmalonic acid in nmol/L (MMA) (d) from 1997 to 2004 in participants taking B-vitamin supplements (+) and those without supplements (o). Time periods are marked by vertical dotted lines.

Average (all participants) changes per year were only significant for folate concentrations. The estimated increase in folate concentration was 234 nmol/L (95% CI 213-254; $p < 0.001$) per year, throughout the study (mixed model analysis).

$R^2=61\%$ due to calendar year, $R^2=3\%$ due to the interaction between calendar year and B-vitamins intake, and $R^2=35\%$ due to subject, where random subject variance/covariance terms include intercept, calendar year and their covariance. Age and sex were not significantly associated with the expected value at the beginning of the fortification period or the annual rate of change thereafter (all $p > 0.2$). Enhancing the linear model by adding a second order quadratic time term did not significantly improve the fit ($\chi^2=3.5$, $df=2$, $p=0.17$) and residual diagnostics did not demonstrate a substantial lack of fit to the linear model. The between-subject rate of change over

time using subjects' initial data was similar to the within-subject rate of change over the study period, suggesting that the increase in folate was related to calendar time rather than within-person study time.

Table II shows the unadjusted biochemical parameters and percentage of subjects with abnormal levels by period and B-vitamins intake. Figure 1 shows the trends over the continuous time scale. Adjusted tHcy levels decreased from period 1 to period 2 by 11.7% (95% CI 6.6-16.6; $p < 0.001$). By period 3, the adjusted tHcy decrease was 5.6% (95% CI 0.9-10.1; $p=0.017$), and by period 4, the adjusted tHcy decrease was only 3.1% ($p=0.403$).

Participants taking B-vitamins had lower tHcy in each time period than those not ($p < 0.001$ for each period). Pooling data across all periods, the expected tHcy level was 13.2% (95% CI 9.8-16.4; $p < 0.001$) lower for subjects on B-vitamins compared to those not, 8.2% (95% CI 1.6-15.3; $p=0.015$) higher for men than for women, and increased by 1.7% (95% CI 1.1-2.2; $p < 0.001$) with each year of age. The effect of these covariates was not significantly different between periods (all $p > 0.2$ for covariate by period interactions). After controlling for gender, age and B-vitamin supplements, there was no significant change in the creatinine clearance over the four periods

TABLE II

Comparison of Unadjusted Geometric Means (and Range) of Laboratory Determinations between Period 1 and Periods 2-4

Lab Value	B-vitamins Supplements	Period 1 <01AUG98	Period 2 01AUG98-31JUL00	Period 3 01AUG00-31JUL02	Period 4 ≥01AUG02
N	Combined	131	147	148	134
	No	96	100	83	69
	Yes	35	47	65	65
Folate	Geometric mean (95% CI) [% >1300 nmol/L]				
	Combined	591 (547 - 638) [5%]	772 (722 - 825) [12%]*	1407 (1331 - 1487) [59%]*	1950 (1851 - 2053) [92%]*
	No	523 (482 - 567) [3%]	670 (624 - 721) [4%]	1206 (1130 - 1287) [40%]	1662 (1556 - 1776) [84%]
	Yes	815 (713 - 932) [11%]	1034 (935 - 1144) [28%]	1693 (1574 - 1822) [83%]	2319 (2189 - 2457) [100%]
tHcy	Geometric mean (95% CI) [% 14 µmol/L]				
	Combined	10.1 (9.6 - 10.7) [10%]	9.1 (8.7 - 9.5)* [5%]*	9.6 (9.3 - 10.0) [7%]	10.2 (9.7 - 10.6) [9%]
	No	10.6 (10.0 - 11.3) [13%]	9.6 (9.1 - 10.2) [7%]	10.4 (9.9 - 10.9) [8%]	11.2 (10.5 - 11.9) [16%]
	Yes	8.9 (8.2 - 9.7) [3%]	8.1 (7.6 - 8.6) [2%]	8.7 (8.2 - 9.2) [6%]	9.1 (8.7 - 9.5) [2%]
MMA	Geometric mean (95% CI) [% >271 nmol/L]				
	Combined	213 (197 - 231) [25%]	200 (186 - 215)* [26%]	199 (187 - 211) ¶ [16%]	236 (222 - 251) [32%]*
	No	223 (202 - 246) [29%]	212 (193 - 233) [33%]	208 (192 - 226) [19%]	259 (238 - 282) [43%]
	Yes	190 (169 - 214) [14%]	176 (160 - 193) [11%]	188 (171 - 205) [12%]	212 (195 - 230) [20%]
Cbl	Geometric mean (95% CI) [% <165 pmol/L]				
	Combined	274 (252 - 298) [16%]	265 (245 - 287) [17%]	380 (352 - 411)* [3%]	411 (378 - 447)* [2%]
	No	247 (223 - 272) [20%]	236 (216 - 258) [22%]	314 (287 - 345) [5%]	320 (291 - 353) [3%]
	Yes	360 (315 - 410) [3%]	338 (293 - 389) [6%]	475 (425 - 531) [2%]	539 (482 - 603) [0%]

N = number of subjects taking B-vitamins supplements. B-vitamins supplements = supplements containing folic acid and/or Cbl.

[%] = percentage of subjects with abnormal levels for each determination.

Folate = whole blood folate, tHcy = homocysteine, MMA = methylmalonic acid, Cbl = cobalamin.

p<0.001, ¶ p=0.01.

Across all periods, participants on B-vitamin supplements had significantly higher folate and Cbl, and lower tHcy and MMA levels than participants not taking B-vitamin supplements (see results).

TABLE III

Adjusted Geometric Means and Change from Period 1 of Laboratory Determinations for All Subjects Combined

Lab Value	Period 1 <01AUG98	Period 2 01AUG98-31JUL00	Period 3 01AUG00-31JUL02	Period 4 ≥01AUG02
tHcy				
Geometric Mean ¹ (95% CI)	10.8 (10.3 to 11.3)	9.5 (9.1 to 9.9)	10.2 (9.8 to 10.6)	10.4 (10 to 10.9)
% Δ from period 1 ² (95% CI) ³	Referent	-11.7 (-16.6 to -6.6)	-5.6 (-10.1 to -0.9)	-3.1 (-8.7 to 2.8)
Adjusted p-value ⁴	Referent	<0.001	0.017	0.403
MMA				
Geometric Mean ¹ (95% CI)	235.3 (218.8 to 253)	209.8 (196 to 224.7)	228 (213.9 to 243)	250.3 (233.7 to 268.1)
% Δ from period 1 ² (95% CI) ³	Referent	-10.8 (-17.3 to -3.8)	-3.1 (-9.5 to 3.7)	6.4 (-2.5 to 16)
Adjusted p-value ⁴	Referent	0.001	0.526	0.212
Cbl				
Geometric Mean ¹ (95% CI)	254.3 (233.5 to 277.1)	235.5 (217.3 to 255.2)	299.6 (276.8 to 324.3)	343.6 (314 to 375.9)
% Δ from period 1 ² (95% CI) ³	Referent	-7.4 (-16.9 to 3.1)	17.8 (6.7 to 30)	35.1 (20 to 52.1)
Adjusted p-value ⁴	Referent	0.211	<0.001	<0.001

Folate = whole blood folate, tHcy = homocysteine, MMA = methylmalonic acid, Cbl = cobalamin.

1. Expected geometric mean for a 75-year-old female without B-vitamins supplementation.

2. Controlling for age, gender and B-vitamins use (including folic acid and/or Cbl). Adjusting for creatinine clearance did not affect the results.

3. Simultaneous 95% confidence intervals by Dunnett's method.

4. Adjusted by Dunnett's method for comparing multiple groups to a control. Period 1 data have been assigned as control (referent).

(p=0.53, 85% data available). The trend in tHcy was unchanged after adjusting for creatinine clearance (data not shown).

Cbl levels did not change significantly during the first two periods, but increased by 17.8% (95% CI 6.7-30; p<0.001) in period 3 and 35.1% (95% CI 20-52.1; p<0.001) in period 4 (Figure 1c and Table III). Subjects found to have low Cbl levels were treated with Cbl.

MMA levels (Figure 1d and Table II) showed a similar pattern to tHcy, in spite of the increasing levels of Cbl. MMA decreased by 10.8% (95% CI 3.8-17.3; p=0.001) from periods 1 to 2, but increased to levels higher than in period 1

by periods 3 and 4 (non-significant) (Table III). Subjects on B-vitamins had lower MMA levels by 12.0% (95% CI 6.9-16.9; p<0.001) than those not, and MMA levels increased by 2.3% (95% CI 1.4-3.3; p<0.001) per year of age. Sex was not significantly associated with MMA. Mean MMA levels and percentage of subjects with high MMA were not significantly different between periods 1 and 4.

After controlling for age, sex and B-vitamins, Pearson's partial correlations suggested a negative association between tHcy and folate only in period 1 (r=-0.32, p<0.001). There were no significant correlations in periods 2-4.

DISCUSSION

These results show that in this cohort of community-living older adults, the folic acid fortification program has resulted in continuously increasing levels of folate that had not reached a plateau 6 years after fortification was started. Increases in folate levels post-fortification have been unanimously found in all countries where fortification is mandatory³⁻⁶ and it has exceeded predictions.^{22,23} Contrary to results in another elderly population,²⁴ this analysis shows that with the current fortification standards, folate levels in the elderly are still increasing significantly years after for-

tification, and that by period 4, 84% of subjects not taking B-vitamins supplements and 100% of those taking them have levels of folate above the pre-fortification normal range (>1300 nmol/L). This would not have been detected if only serum folate was measured. This increase in folate levels is worrisome in view of recent reports of possible associations with colorectal adenomas,^{25,26} its effects on some drug levels²⁷ and on cognitive function when combined with low Cbl levels.²⁸

High folate should have resulted in a sustained decline in tHcy, but this was not the case. Moreover, we only found correlation between folate and tHcy levels in the first period when folate levels were still relatively low, indicating that further fortification will not result in lowering of tHcy as previously suggested.²⁸ There might be a threshold of folate beyond which tHcy levels are not affected.

B-vitamins supplements had a significant effect on all parameters. Compared to subjects not taking B-vitamins, those taking them had higher folate and Cbl, and lower tHcy and MMA throughout all periods, and only 2% had elevated tHcy by period 4. For any given level of folate, subjects taking B-vitamins had lower tHcy than those not. These results support previous data^{29,30} suggesting that oral Cbl decreases tHcy levels and the prevalence of elevated tHcy in a population with high folate.

Cbl levels were significantly higher and low Cbl (<165 pmol/L) less frequent among subjects taking B-vitamins. In spite of fewer subjects with low Cbl levels, there was a trend increase in mean MMA levels and percentage of persons with high MMA (>271 nmol/L) among subjects not taking B-vitamins. The effects of B-vitamins on MMA levels were similar to the effects seen in tHcy. Subjects on B-vitamins had lower MMA levels and were less likely to have elevated MMA than subjects not taking B-vitamins. Although our results do not support previous suggestions that increases in folic acid result in Cbl deficiency in older adults,^{7,31,32} the observed trend on MMA is worrisome and indicates the need for longer follow-up. Moreover, it appears that the small amounts of Cbl in over-the-counter B-vitamins result in significant reductions of tHcy and MMA levels.

Limitations of this study include the variation in number of assessments and the timing of follow-up between subjects. Although the statistical approach efficiently handles highly unbalanced data sets, biases could occur if the missing data pattern is related to the outcomes. The cohort population includes only independently community-living older adults recruited in one geographical area of the country exclusively.

In conclusion, in this population, the long-term effects of the Canadian folic acid fortification program include continuously raising levels of folate, transitory decreases in tHcy with return to baseline levels three years after fortification, and a trend increase in MMA levels. B-vitamins supplementation results in significantly lower tHcy and MMA levels regardless of folic acid fortification.

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RÉSUMÉ

Objectif : Étudier les effets à long terme du programme canadien d'enrichissement en acide folique sur la concentration de folates et de cobalamine (Cbl) dans le sang total des personnes âgées, y compris l'homocystéine (tHcy) et l'acide méthylmalonique (MMA), avec et sans apport volontaire en vitamine B, entre 1997 et 2004.

Méthode : Cohorte bénévole de personnes âgées résidant dans la communauté. Les données cliniques et biochimiques, y compris les apports en vitamine B, ont été obtenues à 2 ans ou 2,5 ans d'intervalle et divisées en 4 périodes. Des modèles à coefficients aléatoires (effets mixtes) ont servi à estimer la tendance linéaire pour les folates et à comparer les niveaux des paramètres biochimiques d'une période à l'autre. Tous les modèles ont été estimés par le maximum de vraisemblance restreint tel que mis en œuvre avec PROC MIXED de SAS, version 8.2.

Résultats : Les niveaux de folates ont régulièrement augmenté de 234 ng/mL par année (IC de 95 % = 213-254; $p < 0,001$) et ne s'étaient pas encore stabilisés durant la dernière période, où 84 % des sujets sans apport supplémentaire en vitamine B affichaient des niveaux élevés. La suppression de l'homocystéine n'a pas persisté. Une tHcy élevée était aussi courante pendant la dernière période d'étude que pendant la première. On n'a observé aucun déficit significatif en Cbl ni augmentation du MMA, mais les niveaux de MMA ont eu tendance à s'accroître avec le temps chez les sujets sans apport supplémentaire en vitamine B. Les suppléments de vitamine B ont eu un effet significatif sur tous les résultats, en réduisant les niveaux de tHcy et de MMA.

Conclusion : Dans la population à l'étude, l'enrichissement en acide folique a entraîné des augmentations cumulatives de folates sans diminution à long terme de la tHcy ni changements dans les niveaux de Cbl ou de MMA. Les effets néfastes éventuels des hausses cumulatives de folates, et les effets bénéfiques des apports supplémentaires en vitamine B pour réduire la tHcy et le MMA, méritent d'être examinés.

Mots clés : folates; cobalamine; homocystéine; acide méthylmalonique; personnes âgées; enrichissement

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