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Ratios of One-Carbon Metabolites Are Functional Markers of B-Vitamin Status in a Norwegian Coronary Angiography Screening Cohort[1–3]

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Abstract

Background: Functional (metabolic) markers of B-vitamin status, including plasma total homocysteine (tHcy) for folate and plasma methylmalonic acid (MMA) for vitamin B-12, suffer from moderate sensitivity and poor specificity. Ratios of metabolites belonging to the same pathway may have better performance characteristics.

Objective: We evaluated the ratios of tHcy to total cysteine (tCys; Hcy:Cys), tHcy to creatinine (Hcy:Cre), and tHcy to tCys to creatinine (Hcy:Cys:Cre) as functional markers of B-vitamin status represented by a summary score composed of folate, cobalamin, betaine, pyridoxal 5′-phosphate (PLP), and riboflavin concentrations measured in plasma.

Methods: Cross-sectional data were obtained from a cohort of patients with stable angina pectoris (2994 men and 1167 women) aged 21–88 y. The relative contribution of the B-vitamin score, age, sex, smoking, body mass index, and markers of renal function and inflammation to the variance of the functional B-vitamin markers was calculated by using multiple linear regression.

Results: Compared with tHcy alone, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre all showed improved sensitivity and specificity for detecting plasma B-vitamin status. Improvements in overall performance ranged from 4-fold for Hcy:Cys to ~8-fold for Hcy:Cys:Cre and were particularly strong in subjects with the common 5,10-methylenetetrahydrofolate reductase (MTHFR) 677CC genotype.

Conclusions: Ratios of tHcy to tCys and/or creatinine showed a severalfold improvement over tHcy alone as functional markers of B-vitamin status in Norwegian coronary angiography screenees. The biological rationale for these ratios is discussed in terms of known properties of enzymes involved in the catabolism of homocysteine and synthesis of creatine and creatinine. *J Nutr* 2017;147:1167–73.

Keywords: B-vitamins, one-carbon metabolites, functional markers, inflammation, renal function

Introduction

Functional biomarkers of vitamin status have been an important topic in nutritional science since the discovery of vitamins during the first half of the 20th century. Early examples involved measuring the activity of vitamin cofactor–dependent enzymes in tissue extracts either natively or as an "activation coefficient" after the addition of surplus vitamin (1, 2). Measuring metabolite excretions in urine after the ingestion of vitamins or other micro- or macronutrients was another strategy (3, 4). Such methods are cumbersome, however, and with the advent of highly sensitive quantitative assays, interest turned to direct measurements of vitamins in the circulation or to metabolites sensitive to vitamin status. A well-known example of the latter is the increase in plasma methylmalonic acid (MMA)[8] as a

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[3] Supplemental Figure 1 is available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
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[8] Abbreviations used: CBS, cystathionine β-synthase; CRP, C-reactive protein; GAMT, guanidinoacetate methyltransferase; Hcy:Cre, ratio of total homocysteine to creatinine; Hcy:Cys, ratio of total homocysteine to total cysteine; Hcy:Cys:Cre, ratio of total homocysteine to total cysteine to creatinine; HK, 3-hydroxykynurenine; KTR, kynurenine-to-tryptophan ratio; LC-MS/MS, LC–tandem MS; MTHFR, 5,10-methylenetetrahydrofolate reductase; PLP, pyridoxal 5′-phosphate; ROC, receiver operating characteristic; SAH, *S*-adenosyl homocysteine; SAM, *S*-adenosyl methionine; SDMA, symmetric dimethylarginine; tCys, total cysteine; tHcy, total homocysteine; WENBIT, Western Norway B-Vitamin Intervention Trial.

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consequence of low vitamin B-12 status affecting the adenosylcobalamin-dependent methylmalonyl-CoA mutase (5).

In the late 1980s it was shown that plasma total homocysteine (tHcy) concentrations increase at low folate status and decrease upon folate supplementation. Depending on the clinical setting and metabolic status of the individual, tHcy also responds to treatment with betaine, vitamin B-6, cobalamin, and riboflavin (6, 7). Studies show that tHcy in the general population is related to dietary intake (8) and plasma concentrations of all of the above vitamins (9, 10).

Although measurements of single metabolites in (stored) blood samples are convenient, the markers often suffer from inadequate sensitivity and lack of specificity. A number of factors not related to micronutrient status are known to affect both tHcy and MMA. The most important is probably renal function, which is often reduced in patient groups or cohorts in whom vitamin status may be clinically relevant (11, 12).

Recently, we proposed the tryptophan metabolite 3-hydroxykynurenine (HK) as a new marker for vitamin B-6 status (13). Subsequently, we showed that the sensitivity and specificity of this marker could be improved by taking the ratio of HK to its product xanthurenic acid, catalyzed by the pyridoxal 5′-phosphate (PLP)–dependent kynurenine aminotransferase (14). Such ratios are attractive because both substrate and product depend on cofactor status, improving sensitivity. Moreover, 2 metabolites along a pathway often share a number of determinants; hence, taking their ratio may attenuate those influences, resulting in enhanced specificity.

In the present article, we describe the ratios of tHcy to total cysteine (tCys; Hcy:Cys), tHcy to creatinine (Hcy:Cre), and tHcy to tCys to creatinine (Hcy:Cys:Cre). We compare these ratio-based indexes and tHcy as markers of B-vitamin status represented by a B-vitamin score composed of folate, cobalamin, betaine, PLP, and riboflavin. Finally, we discuss the biological and methodologic rationale for using the ratio-based markers as functional indicators of B-vitamin status.

Methods

Participants. The Western Norway Coronary Angiography Cohort consists of 4164 patients who underwent elective coronary angiography due to suspected stable angina pectoris between 2000 and 2004 (15). Approximately two-thirds of the cohort participated in the Western Norway B-Vitamin Intervention Trial (WENBIT), which evaluated the efficacy of lowering plasma tHcy by oral B-vitamin treatment to prevent future clinical events (16). The patients who did not participate in WENBIT are referred to as the Angio cohort in this report. In the present study, baseline data were used for the cross-sectional evaluation of the association of B-vitamin markers with circulating B-vitamins.

Laboratory analyses. Plasma concentrations of folate and cobalamin were measured by microbiological assays (17, 18), and tHcy and tCys were determined by GC–tandem MS (19). Betaine, choline, PLP, riboflavin, creatinine, neopterin, kynurenine, tryptophan, and cotinine were quantified by LC-tandem MS (LC-MS/MS) (20, 21). Creatinine was also measured in serum by the traditional Jaffe method. C-reactive protein (CRP) was measured in serum by using an ultrasensitive immunoassay (Behring Nephelometer II System N Latex CRP mono; Behring Diagnostics). All of the analyses except for CRP and creatinine (Jaffe) were performed at the laboratory of Bevital AS (Bergen, Norway; www.bevital.no). Details concerning handling and storage of blood samples before analysis have been described previously (15, 22). Briefly, baseline venous blood samples were drawn either 1–3 d before or immediately after coronary angiography and stored at −80 C until laboratory analyses.

Clinical data. Smoking status was based on self-reported smoking habits corrected by plasma cotinine (i.e., patients initially classified as former smokers, but with plasma cotinine ≥85 nmol/L, were reclassified as smokers) (23). We further classified smokers as "light" and "heavy" on the basis of a cotinine cutoff of 1200 nmol/L. BMI was obtained by dividing weight by squared height (kg/m^2). Estimated glomerular filtration rate/1.73 m^2 was calculated on the basis of the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula (24).

Statistical methods. All continuous variables were right-skewed, and therefore log-transformed before inclusion in parametric regression models. Differences in clinical and blood measurements between subcohorts of the Western Norway Coronary Angiography Cohort were evaluated by Fisher's exact test or partial Spearman's correlation adjusted for sex as appropriate. A B-vitamin score was calculated as the sum of the log-transformed and standardized [i.e., subtracting the mean and dividing by the SD (i.e., z score)] concentrations of folate, cobalamin, betaine, PLP, and riboflavin. Betaine was included in the score because of its relevance to homocysteine metabolism (25). For receiver operating characteristic (ROC) analysis we similarly calculated a renal function/inflammation score as the sum of log-transformed and standardized symmetric dimethylarginine [SDMA; a marker of kidney function (26, 27)], the kynurenine-to-tryptophan ratio (KTR), and neopterin. The association of each B-vitamin marker with a common set of predictors (regressors) was evaluated by using multiple linear regression. The relative importance of each regressor was assessed by using the algorithm "lmg," as described (28). In short, this algorithm evaluates all possible models and all sequences for addition to a regression model that can be applied to a given number of regressors. The impact of each regressor is then averaged over these models by using the percentage explained variance as a metric. The model used for evaluation of the functional B-vitamin markers consisted of SDMA, neopterin, KTR, CRP, smoking, BMI, age, and sex. In addition, indicator variables for subcohorts were included as applicable. With the use of the results of these calculations, we calculated performance indexes of the markers as follows: The amount of variation in the outcome explained by the B-vitamin score was taken as an indicator of sensitivity to the B-vitamin score; the ratio of this number to the total explained variation was taken as an indicator of specificity for the B-vitamin score; and the overall performance was defined as the product sensitivity × specificity. Notably, the terms "sensitivity" and "specificity" used in the context of the regression-based analyses are different from similar terms used in ROC analysis. We used R for Macintosh version 3.1.3 (29) for all statistical calculations, with the R packages "relaimpo" for relative importance of predictors and "pROC" for ROC analyses.

Results

Characteristics of the study cohort. The main characteristics of the study cohort are summarized in **Table 1**. The median age (62 y) and the proportion of current smokers (32.0% and 31.3%, respectively) were similar in the WENBIT and Angio subcohorts (Table 1). For other characteristics, there were notable differences: for example, the percentage of men was 79.7% among WENBIT participants compared with 59.4% in the Angio subcohort. A number of measures related to kidney function, including SDMA, creatinine, estimated glomerular filtration rate, and inflammatory markers CRP, KTR, and neopterin, indicated a generally less-healthy profile of the Angio subcohort. The Angio subcohort had higher concentrations of all B-vitamins except for riboflavin, which was largely explained by a higher proportion of vitamin supplement use. Finally, the variations in most blood variables were greater in the Angio subcohort (Table 1). Patients who reported taking vitamin supplements had a higher B-vitamin score but also a wider distribution of the score (**Supplemental Figure 1**).

TABLE 1 Baseline characteristics of participants in the WECAC[1]

	WECAC (n = 4161)		
	WENBIT (n = 2571)	Angio (n = 1590)	P^2
Age, y	62 (45, 77)	62 (43, 79)	0.56
Male sex, n (%)	2049 (79.7)	945 (59.4)	<0.001
Current smoking, n (%)	823 (32.0)	497 (31.3)	0.63
Vitamin supplement use, n (%)	565 (22.1)	527 (39.3)	<0.001
Fasting,[3] n (%)	902 (35.1)	205 (12.9)	<0.001
BMI, kg/m^2	26.5 (21.6, 33.5)	26.0 (20.5, 34.1)	<0.001
eGFR, mL · min^{-1} · 1.73 m^{-2}	80.7 (53.1, 102)	79.2 (45.1, 106)	<0.001
SDMA, μmol/L	0.580 (3.8, 9.2)	0.602 (3.8, 10.0)	<0.001
Creatinine, μmol/L	73.3 (53, 102)	75.5 (54.1, 115)	<0.001
Creatinine (Jaffe), μmol/L	90 (70, 117)	87 (69–123)	0.45
tCys, μmol/L	286 (234, 348)	298 (238, 374)	<0.001
B-vitamins			
Folate, nmol/L	9.9 (5.0, 28.1)	10.5 (4.7, 55.6)	0.01
Cobalamin, pmol/L	371 (170, 627)	415 (221, 827)	<0.001
Betaine, μmol/L	38.9 (23.6, 63.1)	39.4 (22.4, 64.7)	<0.001
Riboflavin, nmol/L	11.0 (4.5, 44.3)	11.7 (4.2, 56.2)	0.36
PLP, nmol/L	40.0 (18.7, 101)	43.8 (18.8, 177)	<0.001
B-vitamin score	−0.13 (−1.52,1.50)	0.03 (−1.44, 2.26)	<0.001
B-vitamin markers			
tHcy, μmol/L	10.3 (6.7, 17.6)	10.8 (6.9, 20.0)	<0.001
Hcy:Cys (×100)	3.55 (2.5, 5.7)	3.62 (2.5, 6.1)	<0.001
Hcy:Cre (×100)	13.8 (9.6, 22.3)	14.1 (9.2, 23.4)	0.98
Hcy:Cys:Cre (×10,000)	4.85 (3.4, 7.8)	4.76 (3.0, 8.0)	<0.001
Inflammatory markers			
CRP, mg/L	1.74 (0.3, 12)	1.82 (0.4, 13.3)	0.006
KTR, nmol/μmol	23.7 (15.8, 39.3)	24.1 (14.2, 44.0)	0.74
Neopterin, nmol/L	7.82 (5.2, 14.4)	8.83 (5.1, 19.4)	<0.001
MTHFR 677 genotypes, n (%)			
CC	1204 (49.6)	NA	
CT	1019 (42.0)	NA	
TT	203 (8.4)	NA	

[1] Values are medians (5th, 95th percentiles) unless otherwise indicated. All vitamins and metabolites were measured in plasma except for creatinine (Jaffe) and CRP, which were measured in serum. Angio, WECAC participants who did not participate in the WENBIT; Cre, creatinine; CRP, C-reactive protein; Cys, cysteine; eGFR, estimated glomerular filtration rate; Hcy, homocysteine; KTR, kynurenine-to-tryptophan ratio; MTHFR, 5,10-methylenetetrahydrofolate reductase; NA, not available; PLP, pyridoxal 5′-phosphate; SDMA, symmetric dimethylarginine; tCys, total cysteine; tHcy, total homocysteine; WECAC, Western Norway Coronary Angiography Cohort; WENBIT, Western Norway B-Vitamin Intervention Trial.
[2] P for difference between subcohorts by Fisher's exact test or partial Spearman's correlation adjusted for sex.
[3] Fasting at the time of blood draw.

Association profiles for tHcy, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre. Association profiles for the B-vitamin markers were obtained by using multiple linear regression and the algorithm "lmg" for relative importance of regressors as implemented in the R package "relaimpo." The B-vitamin score, composed of folate, cobalamin, betaine, PLP, and riboflavin, explained 9.0% of the variation in tHcy (**Figure 1**). As shown, the renal function marker SDMA, neopterin, and KTR also contributed a considerable percentage (18.5%) of the total explained variance of tHcy. By comparison, the ratio-based markers, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre, showed stronger associations with the B-vitamin score and weaker associations with most other regressors. In a parallel set of analyses, the B-vitamins were included independently in the regression model. As shown in Figure 1, folate was the dominant component of the B-vitamin score in terms of explaining the variance in tHcy and Hcy:Cys, whereas for Hcy:Cre and Hcy:Cys:Cre, larger relative contributions from the other vitamins and nutrients were observed. The inclusion of fasting status did not change the results appreciably and was therefore not included in the analyses.

Performance indexes overall and in subcohorts. **Table 2** shows a summary of performance indexes for the 4 B-vitamin markers. For tHcy, the sensitivity and specificity for the B-vitamin score was 9.0 and 24.5, respectively, resulting in an overall performance of 9.0 × 0.245 = 2.2. For the ratio-based markers, both the sensitivity and specificity parameters were greater, resulting in overall performances of 8.6, 13.4, and 18.0 for Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre, respectively (Table 2). Hcy:Cre and Hcy:Cys:Cre were also calculated by using creatinine measured by the Jaffe method. The performances of these ratios were 9.3 and 17.4, respectively. Finally, performance indexes were similar in the WENBIT and Angio subcohorts (Table 2).

ROC analysis. The analyses based on multiple linear regression assume a linear relation between predictors and outcome. Traditional ROC analysis is, by contrast, assumption free. We evaluated the markers according to how well they identified individuals with a B-vitamin score ≤5th percentile. The results are shown in **Figure 2**. The AUCs for tHcy, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre were 0.71, 0.79, 0.79, and 0.84, respectively. Similarly, we evaluated how well the markers identified individuals with a renal function or inflammation score ≥95th percentile. The results of this analysis were 0.85, 0.79, 0.49, and 0.63, respectively (Figure 2).

Performance according to MTHFR 677 genotype. We evaluated regression-based performance indexes within strata of 5,10-methylenetetrahydrofolate reductase (MTHFR) 677C→T genotypes for all markers. Performance indexes were strongly dependent on MTHFR 677 genotype (**Table 3**). Among the 4 markers, tHcy showed the strongest dependence, with performances of 1.0, 2.4, and 11.4 in the MTHFR 677 CC, CT, and TT subgroups, respectively. For the tHcy ratios, improvements in performance were largest in the CC followed by CT genotype group, resulting in more even performance across genotypes (Table 3). Notably, the performance of Hcy:Cys:Cre in the MTHFR 677 CC group was comparable to that of tHcy in the MTHFR 677 TT group (10.8 compared with 11.4, respectively).

Discussion

Principal findings. We evaluated the characteristics of tHcy and Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre as markers of B-vitamin status in 4614 individuals with stable angina pectoris. By using multiple linear regression and a B-vitamin score composed of folate, cobalamin, betaine, PLP, and riboflavin as the main predictor, we showed that the 3 ratio-based markers were more sensitive and specific as markers of circulating B-vitamin status. Depending on the cohort or stratum investigated, the tHcy ratios showed ≤11-fold improvement in performance compared with tHcy alone.

Contribution of the separate vitamins to the explanation of the markers. Among B-vitamins, folate has generally been found to be the strongest determinant of tHcy (30, 31). Accordingly, we found that folate was the dominant component of the B-vitamin score in terms of its association with tHcy, and this pattern was largely preserved for the tHcy-to-tCys ratio.

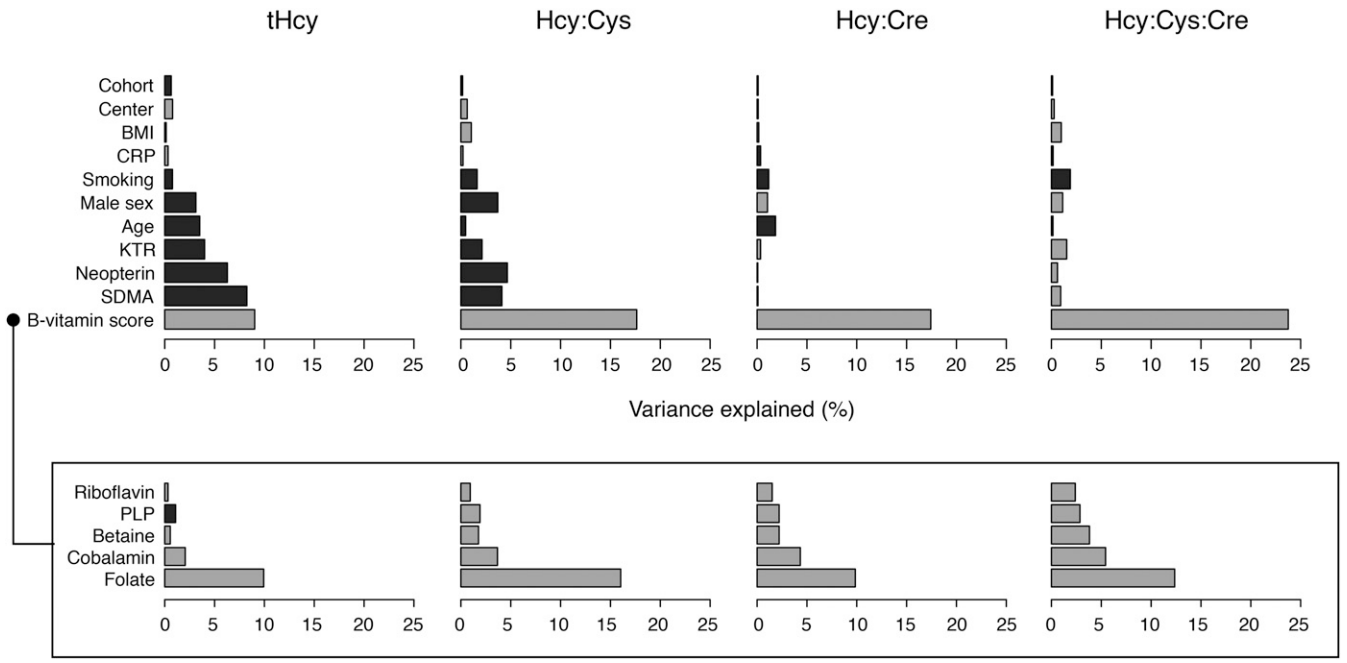

FIGURE 1 Association profiles for tHcy, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre evaluated in the Western Norway Coronary Angiography Cohort. The figure shows the relative contribution of a panel of predictors to the explanation of the variance of the functional B-vitamin markers. Explained variance for each predictor was estimated by multiple linear regression and the algorithm ''lmg'' according to the ''relaimpo'' package in R. Negative associations are shown in gray, and positive associations are shown in black. The figure also shows how each individual vitamin component of the B-vitamin score contributes to the explained variance of the functional markers. All vitamins and metabolites shown were measured in plasma except for CRP, which was measured in serum. Cre, creatinine; CRP, C-reactive protein; Cys, cysteine; Hcy, homocysteine; KTR, kynurenine-to-tryptophan ratio; PLP, pyridoxal 5′-phosphate; SDMA, symmetric dimethylarginine; tHcy, total homocysteine.

Notably, however, Hcy:Cre and Hcy:Cys:Cre also showed considerable association with the other vitamin and nutrient components of the B-vitamin score. Thus, rather than narrowly indicating folate (and vitamin B-12) status, Hcy:Cre and Hcy:Cys:Cre could be described as markers of general B-vitamin status, at least as far as the B-vitamins are involved in homocysteine metabolism.

Variation in performance across subcohorts. The study cohort was divided, by self-selection, into 2 subcohorts on the basis of participation in the WENBIT intervention study. Nonparticipants of the WENBIT study (Angio subcohort) had poorer kidney function and higher concentrations of inflammatory markers, but also higher concentrations of most B-vitamins; furthermore, the variability of these variables was also greater. Larger variation in B-vitamins may explain the higher sensitivity scores observed for all markers in the Angio cohort compared with the WENBIT cohort. At the same time, larger variations in renal function and inflammation-related variables are likely to have lowered the specificity score for the tHcy marker. In contrast, the specificity of the markers Hcy:Cre and Hcy:Cys:Cre did not change appreciably, resulting in no loss in overall performance for those markers in the Angio cohort compared with the WENBIT subcohort. These results serve as cross-validation for the markers, but also show the robustness of the tHcy ratios in detecting variation in B-vitamin concentrations against a background of considerable variation in renal function and inflammation variables.

Variation in performance across MTHFR 677C→T genotypes. We found that the performance, and thereby utility, of the markers was dependent on MTHFR 677 genotype. For tHcy,

TABLE 2 Performance indexes for tHcy, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre overall and by subcohort in the WECAC[1]

	Plasma tHcy			Plasma Hcy:Cys			Plasma Hcy:Cre			Plasma Hcy:Cys:Cre		
	All	WENBIT	Angio	All	WENBIT	Angio	All	WENBIT	Angio	All	WENBIT	Angio
Sensitivity,[2] %	9.0	8.9	10.1	17.6	17.0	19.3	17.4	16.1	19.6	23.8	22.4	25.6
Specificity,[3] %	24.5	28.5	22.9	48.5	52.6	45.2	76.6	74.5	78.0	75.1	76.6	72.6
Performance[4]	2.2	2.5	2.3	8.6	8.9	8.7	13.4	12.0	15.3	18.0	17.1	18.6
Performance ratio	1 (ref)	1 (ref)	1 (ref)	3.9	3.5	3.8	6.0	4.7	6.6	8.3	6.7	8.1

[1] Angio, WECAC participants who did not participate in the WENBIT; Cre, creatinine; Cys, cysteine; Hcy, homocysteine, ref, reference; tHcy, total homocysteine; WECAC, Western Norway Coronary Angiography Cohort; WENBIT, Western Norway B-Vitamin Intervention Trial.
[2] Defined as the percentage variation in the marker explained by the B-vitamin score.
[3] Defined as the variation in the marker explained by the B-vitamin score divided by the total explained variation (expressed as a percentage).
[4] Defined as sensitivity multiplied by specificity.

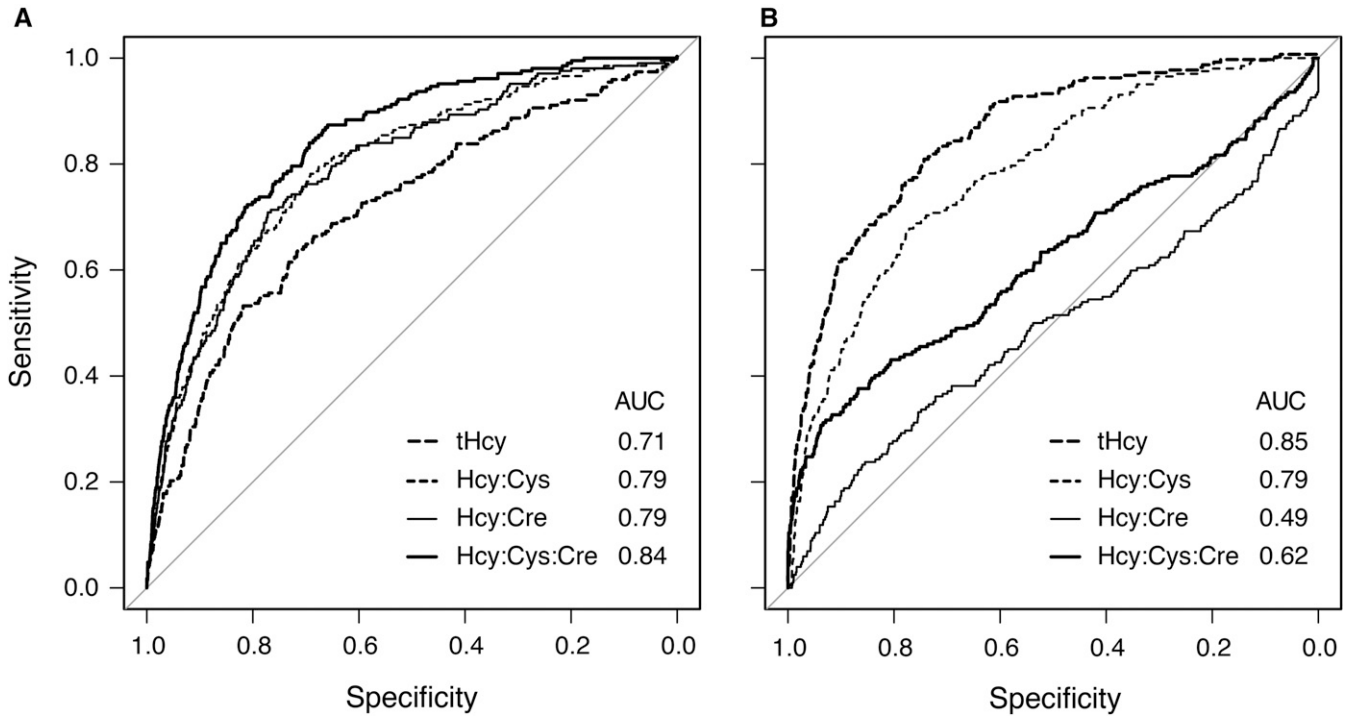

FIGURE 2 ROC analysis in patients in the Western Norway Coronary Angiography Cohort. The diagnostic accuracy of the markers tHcy, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre to detect a low (≤5th percentile) B-vitamin score (A) and poor kidney function or high inflammation (≥95th percentile) score (B) is shown. All vitamins and metabolites used in this analysis were measured in plasma except for C-reactive protein, which was measured in serum. Cre, creatinine; Cys, cysteine; Hcy, homocysteine; ROC, receiver operating characteristics; tHcy, total homocysteine.

good (>10) performance was found in the MTHFR 677 TT genotype group only. This finding is consistent with earlier reports that the relation of folate and cobalamin with homocysteine is considerably stronger in the MTHFR 677 TT group (32). A strong characteristic of the tHcy ratios was their decreased dependency on MTHFR 677 genotype. In particular, the Hcy:Cys:Cre marker showed good performance across all MTHFR 677 genotypes.

Proposed mechanisms for the reciprocal regulation of tHcy compared with tCys and creatinine by B-vitamin concentrations. **Figure 3** shows an outline of reactions that may be key in regulating the relative concentrations of tHcy, tCys, and creatinine. The metabolites homocysteine, Met, *S*-adenosyl methionine (SAM), and *S*-adenosyl homocysteine (SAH) make up the methionine cycle, which has 2 major net outlets: the conversion of homocysteine through cystathionine to Cys and the transfer of a methyl-group from SAM to guanidinoacetate to make creatine (which make up ~50% of all methylated products). The key regulator of these reactions is SAM through the positive allosteric regulation of cystathionine β-synthase (CBS) and as a substrate-limiting reactant in the production of creatine (33). In the fed state, SAM is generated from Met contained in the diet. During fasting, SAM requirements must be met through the remethylation of tHcy. Remethylation is, in turn, dependent on adequate folate, cobalamin, riboflavin, betaine, and PLP status (33, 34). If 1 or several of the B-vitamins are limiting, SAM production is impaired, resulting in reduced stimulation of CBS, and thereby a buildup of tHcy and a reduction in cysteine. In parallel, a direct substrate effect [i.e., the high SAM Michaelis constant of guanidinoacetate methyltransferase (GAMT) (34)] would explain reduced creatine and creatinine synthesis. According to this scheme, homocysteine and cysteine and creatinine are inversely related to B-vitamin status through the regulatory properties of SAM, whereas confounding influences such as renal function are likely to affect the 3 metabolites similarly. In

TABLE 3 Performance indexes for tHcy, Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre across MTHFR 677 genotypes in the WECAC[1]

	Plasma tHcy			Plasma Hcy:Cys			Plasma Hcy:Cre			Plasma Hcy:Cys:Cre		
	CC	CT	TT	CC	CT	TT	CC	CT	TT	CC	CT	TT
Sensitivity,[2] %	5.3	9.7	22.7	11.5	19.6	31.3	11.0	18.8	28.7	16.4	26.0	34.8
Specificity,[3] %	17.9	25.1	50.3	38.0	51.0	62.0	57.9	73.4	68.9	66.0	75.9	69.6
Performance[4]	1.0	2.4	11.4	4.4	10.0	19.4	6.4	13.8	19.8	10.8	19.7	24.2
Performance ratio	1 (ref)	1 (ref)	1 (ref)	4.6	4.1	1.7	6.7	5.7	1.7	11.3	8.1	2.1

[1] Cre, creatinine; Cys, cysteine; Hcy, homocysteine, MTHFR, 5,10-methylenetetrahydrofolate reductase; ref, reference; tHcy, total homocysteine; WECAC, Western Norway Coronary Angiography Cohort.
[2] Defined as the percentage variation in the marker explained by the B-vitamin score.
[3] Defined as the variation in the marker explained by the B-vitamin score divided by the total explained variation (expressed as a percentage).
[4] Defined as sensitivity multiplied by specificity.

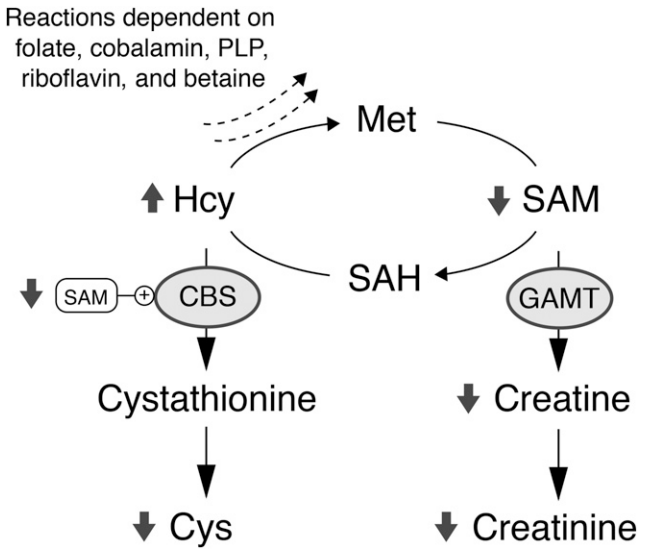

FIGURE 3 Proposed mechanisms for the reciprocal regulation of Hcy and Cys/creatinine by B-vitamins. The remethylation cycle is shown, consisting of the metabolites Hcy, Met, SAM, and SAH. The main net outputs of the cycle are from Hcy via cystathionine to Cys and the utilization of SAM in creatine synthesis (>50% of SAM utilization). Both of these reactions are controlled by SAM through allosteric positive regulation of CBS and by the high Km_{SAM} of GAMT. During the fed state, the main input to the cycle is Met. During fasting, Met, and therefore SAM, derives mainly from the remethylation of Hcy. Remethylation depends on several reactions each using ≥1 of folate, cobalamin, betaine, riboflavin, or PLP as a substrate or cofactor (33, 34). Up or down arrows symbolize proposed changes in metabolite concentrations when B-vitamins are limiting. CBS, cystathionine β-synthase; Cys, cysteine; GAMT, guanidinoacetate methyltransferase; Hcy, homocysteine; Km_{SAM}, Michaelis constant for SAM; PLP, pyridoxal 5′-phosphate; SAH, *S*-adenosyl homocysteine; SAM, *S*-adenosyl methionine.

aggregate, this could explain why Hcy:Cys, Hcy:Cre, and Hcy:Cys:Cre are more sensitive to B-vitamin status and less sensitive to other influences compared with any of its component metabolites.

Strengths and limitations. Strengths of the study include the well-characterized, large, and relatively homogenous study cohort in terms of ethnicity, social indicators, and age. The cohort included a large panel of variables recognized as relevant determinants or covariates of tHcy. The results were similar in 2 subcohorts, and power was adequate to assess performance indexes across MTHFR 677 genotypes. We obtained essentially the same results by using 2 alternative statistical methods, linear regression, and ROC analysis. A possible limitation was the regression model and ROC criteria used for comparing the markers. Additional predictors and criteria that were unknown, or unavailable, to us could be important for marker evaluation.

Our main test variable for evaluating the B-vitamin markers were the B-vitamin score made up of standardized folate, cobalamin, betaine, PLP, and riboflavin concentrations. Although this score may be conceived of as a robust measure of nutritional B-vitamin status, it is also dependent on the variability in the present study cohort, limiting the generalizability to other cohorts and populations. Many of the components of the B-vitamin score (e.g., folate, PLP, and riboflavin) are short-term indicators of B-vitamin status dependent on, for example, prandial status. This limits our ability to assess how well the markers reflect long-term B-vitamin status.

tHcy and tCys were analyzed in multiplex on the same analytical platform (GC–tandem MS); thus, an expected effect of taking their ratio is to reduce or eliminate variation from sample workup and system drift. Creatinine was measured on the same day by LC-MS/MS and on a different day by the traditional (Jaffe) method. As could be expected, Hcy:Cre and Hcy:Cys:Cre based on creatinine measured by LC-MS/MS performed somewhat better than ratios based on creatinine measured by the traditional method. Such limitations should be recognized if ratios are composed of analytes measured on different days, from different laboratories, by using a different methodology or sample matrix.

Our present understanding of the mechanisms relating B-vitamin status to ratios of homocysteine, cysteine, and creatine relies on a simple model that ignores the contribution of numerous additional influences and key enzymes such as arginine:glycine amidinotransferase, a recognized rate-limiting enzyme for creatine synthesis (35). Additional data and further studies are required, however, to determine if and how the current model should be revised or extended.

Implications. A marginal status of several B-vitamins often coexists (36), which underscores the potential utility of general B-vitamin markers. The tHcy ratios may be used as a first analysis and then complemented with markers specific to single vitamins. In this way, a broader nutritional context may be characterized, which could aid in the detection of dietary inadequacies or improve clinical assessment.

The determination of vitamin status by measurement of vitamin species in stored biospecimens poses a number of challenges related to low concentrations and stability, depending on sample matrix and storage temperature (37). tHcy, tCys, and creatinine are, in contrast, moderate- to high-abundance compounds with high stability during storage (37). The indexes described in this report should therefore be attractive in the context of epidemiologic research based on stored plasma or serum.

Summary. We have shown and quantified important limitations of tHcy as a functional marker of B-vitamin status. At the same time, we have shown that the performance of tHcy can be improved by severalfold by correcting for the closely related metabolites tCys, creatinine, or both, in simple ratios. We argue that these ratios are genuine functional markers of B-vitamin status, specifically folate, cobalamin, betaine, PLP, and riboflavin, on 2 grounds: *1*) the ratios envelop the 2 major routes of homocysteine metabolism and *2*) the regulatory properties of SAM explain the link between low B-vitamin status and a simultaneous increase in tHcy with decreases in tCys and creatinine. Further studies are required to evaluate these markers in the contexts of human nutrition and health.

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