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Metabolism Clinical and Experimental 54 (2005) 1–14

Metabolism
Clinical and Experimental

www.elsevier.com/locate/metabol

Homocysteine, glycine betaine, and *N*,*N*-dimethylglycine in patients attending a lipid clinic

Michael Lever[a], Peter M. George[a,*], Warwick J. Dellow[a], Russell S. Scott[b], Stephen T. Chambers[c]

[a]*Biochemistry Unit, Canterbury Health Laboratories, PO Box 151, Christchurch, New Zealand*
[b]*Department of Preventative Cardiology, Christchurch Hospital, Christchurch, New Zealand*
[c]*Department of Infectious Diseases, Christchurch Hospital, Christchurch, New Zealand*

Received 27 May 2003; accepted 3 July 2004

Abstract

We recruited nondiabetic subjects (n = 158) attending a lipid disorders clinic, a subset of whom (n = 46) had established cardiovascular disease. Glycine betaine, *N*,*N*-dimethylglycine, and carnitine were measured in fasting plasma and urine samples. The concentrations and excretions were related to known cardiovascular risk factors in multivariate regression models. The relationships between homocysteine and plasma and urinary glycine betaine were highly significant ($P < .002$), comparable with the known relationships with folate and plasma creatinine. The regression coefficient for plasma glycine betaine was consistently approximately −0.1 in 5 different regression models (3 best-subsets and forward and backward stepwise regression models) for predicting homocysteine using 23 variables. Plasma glycine betaine was higher in males than in females, and the difference was associated with a difference in percentage of body fat. Its concentration included a constant factor of approximately 20 μmol/L that was independent of any of the variables investigated here. In the total group, body fat, homocysteine, and carnitine were significant predictors of plasma glycine betaine. Carnitine, an important betaine that is involved in lipid metabolism positively correlated with both homocysteine and glycine betaine. In our sample, the urinary excretion of glycine betaine was outside the reference range in 14 of the 158 subjects and the betaine fractional clearances were above the reference range in 23 subjects. Fractional clearance correlated strongly with plasma homocysteine ($r = 0.50$), and this relationship may be stronger in patients with known vascular disease. Urinary loss of glycine betaine may contribute to hyperhomocysteinemia and the development of cardiovascular disease.

1. Introduction

Homocysteine is a nonprotein amino acid that is metabolized via 1 of 2 methionine-conserving methylation pathways, or by catabolism of its carbon skeleton, the first step being conversion to cystathionine (Fig. 1). In most tissues, the methylation of homocysteine is catalyzed by methionine synthase, with N^5-methyltetrahydrofolate as the methyl donor. This derives from N^5,N^{10}-methylenetetrahydrofolate, a reduction catalyzed by the enzyme N^5,N^{10}-methylenetetrahydrofolate reductase. The second methylation pathway is catalyzed by the zinc metalloenzyme betaine–homocysteine methyltransferase (BHMT). Glycine betaine is the methyl donor, and the products are methionine and *N*,*N*-dimethylglycine. BHMT, found mostly in the liver and kidney (Fig. 1) [1], is subject to feedback inhibition by *N*,*N*-dimethylglycine and (to a lesser extent) methionine [2].

Elevations in plasma total homocysteine concentrations have been identified as a risk factor for atherosclerotic disease in the coronary, cerebral, and peripheral vessels and for arterial and venous thrombosis [3-7]. Factors that cause elevated plasma total homocysteine concentrations include genetic defects [8-10], certain drugs, renal impairment, age, and nutritional deficiencies. A number of studies have shown an inverse relationship between plasma total homocysteine concentrations and circulating folate [11,12], vitamin B_6, and vitamin B_{12} [13-15].

The role glycine betaine has in the metabolism of homocysteine and its involvement in hyperhomocysteinemia is poorly understood. One reason is that the methods for measuring glycine betaine in human plasma and urine are not widely available. Glycine betaine metabolism is altered in diabetes mellitus [16-18] and in premature vascular disease [19]. Thus, it seems plausible that perturbations in

* Corresponding author. Tel.: +64 3 364 0336; fax: +64 3 364 1460.
E-mail address: peter.george@cdhb.govt.nz (P.M. George).

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doi:10.1016/j.metabol.2004.07.007

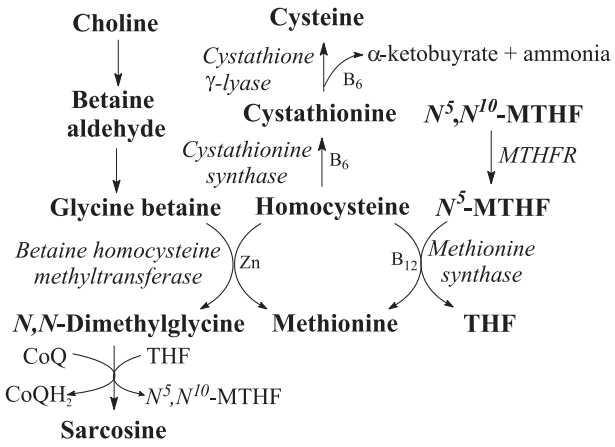

Fig. 1. Homocysteine metabolism. Homocysteine can be methylated to methionine via the enzymes BHMT or methionine synthase, the latter using N^5-methylenetetrahydrofolate as the methyl donor. MTHFR indicates methylenetetrahydrofolate reductase; MTHF, methylenetetrahydrofolate; THF, tetrahydrofolate; CoQ, coenzyme Q_{10}. The multiple pathways by which methionine is converted back to homocysteine during biologic methylations are not shown.

glycine betaine metabolism could contribute to hyperhomocysteinemia and be associated with the pathophysiology of cardiovascular disease (CVD). To test the plausibility of this hypothesis, we examined a population of patients attending a lipid disorders clinic. We expected that this population would have more patients with multiple cardiovascular risk factors, and hence, would have larger variances than a random population sample. We compared the plasma concentration and urinary excretions of glycine betaine and its metabolites with plasma homocysteine and other known risk factors. Carnitine is also a betaine (Fig. 2) that is transported by many of the same systems that transport glycine betaine [20-22]. It could affect the activity of BHMT or betaine transport or the expression of their genes. We have previously shown that its metabolism correlates with glycine betaine metabolism in diabetic patients [18]. Therefore, we included carnitine and its main metabolite (acetylcarnitine) in our study.

2. Materials and methods

2.1. Subjects

One hundred seventy consecutive subjects attending the adult lipid disorders clinic at Christchurch Hospital, New Zealand, were enrolled in the study. The hospital's Ethics Committee approved the study protocol, and all patients gave informed consent.

Data were obtained for each patient regarding date of birth, sex, diabetes, weight, body mass index (BMI), percentage of body fat, and current medication. The body fat ratio was measured by bioelectrical impedance analysis using a body fat analyzer (TBF-501, Tanita Corporation, Tokyo, Japan). Established CVD was defined as previously diagnosed angina, stroke, vascular lesions (identified by ultrasound or angiography), angioplasty, or coronary artery bypass graft.

All participating subjects attended in the morning after 12 to 14 hours of overnight fasting. Paired blood and urine samples were collected for laboratory assessment.

Because the existing reference ranges for betaine were based on nonfasting samples, we collected morning fasting specimens from a group of volunteers (n = 12, 8 men and 4 women) and then collected a nonfasting specimen from each later in the same day. In addition, fasting plasma betaine and dimethylglycine concentrations were measured in a group (n = 29, 18 men and 11 women) of healthy subjects.

2.2. Laboratory methods

Glycine betaine, *N*,*N*-dimethylglycine, and carnitine were measured in plasma and urine by high-performance liquid chromatography (HPLC) based on the method of Lever et al [23]. Sample (20 μL) was added to 1 mL of extraction solvent (methanol/acetonitrile 1:10 vol/vol) in a 1.5-mL microfuge tube and mixed thoroughly. Approximately 0.1 g of anhydrous sodium sulfate (AnalaR grade, BDH Laboratories, Poole, England) was added and vortex mixed for 1 hour, followed by centrifugation for 5 minutes at 11 000 rpm (Biofuge 15, Heraeus Corporation, Germany). A 250-μL aliquot of supernatant was transferred to a 1.5-mL microfuge tube for derivatization using 2-naphthacyltriflate [24]. First, 50 μL of 2-naphthacyltriflate solution (1 mmol/L in acetonitrile) was added. After 5 minutes, a 5-μL aliquot of $Mg(OH)_2$ suspension (0.1 g MgO/1 mL H_2O) was added and the sample was mixed for 5 minutes, followed by centrifugation for 5 minutes at 11 000 rpm. A 200-μL aliquot of supernatant was transferred into an HPLC vial. Derivatives were separated using an isocratic HPLC system consisting of a pump (Perkin-Elmer LC 410 BIO, Perkin-Elmer Instruments Division, Norwalk, Conn), autoinjector (Jasco AS-950, Jasco Corp, Japan), and UV detector set at 249 nm (Linear UVIS 200, Linear Instruments Corp, Reno, Nev). Separations were made on an Alusphere 250 × 4 mm alumina column (E. Merck, Darmstadt, Germany). The mobile phase consisted of 3.5% vol/vol water in acetonitrile

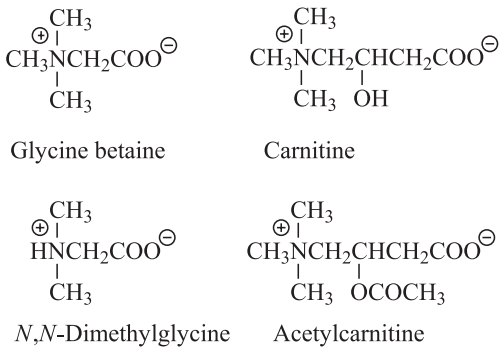

Fig. 2. Molecular structures of glycine betaine and carnitine (also a betaine) and their metabolites, *N*,*N*-dimethylglycine and *O*-acetylcarnitine, at physiological pH.

containing triethylammonium succinate buffer (10 mmol/L succinic acid and 3.8 mmol/L triethylamine), and the flow rate was 1 mL/min. Chromatograms were collected and integrated using Delta for Windows version 5 (DataworX Pty Ltd, Brisbane, Australia).

The respective intra- and interassay coefficients of variation for glycine betaine and *N*,*N*-dimethylglycine were calculated from pooled plasma (n = 10) and were glycine betaine, 3.3% and 4.8%; *N*,*N*-dimethylglycine, 5.0% and 11.8%.

The following components were measured in an International Accreditation New Zealand–registered medical laboratory. Creatinine was measured in plasma and urine using the classical Jaffé reaction on the fully automated Abbott Aeroset Analyzer (Abbott Laboratories, Abbott Park, Ill). Estimation of plasma homocysteine was carried out by a fluorescence polarization assay on the semiautomated Abbott IMx Analyzer (Abbott Diagnostic Division, Abbott Laboratories). The method measures the total concentration of thiol, disulfide, mixed disulfide, and protein-bound forms of homocysteine in the sample to give the total plasma homocysteine concentration. Hyperhomocysteinemia was defined as plasma total homocysteine > 15 μmol/L [25,26]. Serum vitamin B_{12} and red blood cell (RBC) folate concentrations were measured by separate competitive immunoassays on an automated Chemiluminescence ACS:180 Analyzer (Chiron Diagnostics Corporation, East Walpole, Mass). Plasma glucose was measured by a hexokinase-based method on the Abbott Aeroset Analyzer (Abbott Laboratories). Total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were measured in plasma on the Abbott Aeroset Analyzer (Abbott Laboratories). Low-density lipoprotein (LDL) cholesterol was calculated. Apolipoproteins A1 and B (ApoA1 and ApoB, respectively) were measured in plasma using the Beckman Array Protein System (Beckman Instruments Inc, Fullerton, Calif). Urine albumin was measured by a competitive immunoenzymometric assay [27].

2.3. Statistical analyses

Analysis was performed using SigmaStat for Windows 3 (SPSS Science, Chicago, Ill). To measure the strength of association between variables, raw data were tested for normality and log-transformed when necessary. Best-subsets, forward stepwise, and backward stepwise linear regression models were used to identify independent predictors of plasma total homocysteine, betaine, and betaine metabolite concentrations: urine data were log-transformed for these analyses. The best criterion for best-subsets regression was the adjusted r^2, which takes into account the number of independent variables: The 3 best models and the forward and backward stepwise regression models provided 5 models. Independent variables that were significant in all models and with consistent coefficients were accepted as showing a physiologically significant relationship with the dependent variable. The 24 variables used in these models were age, serum vitamin B_{12}, red cell folate; plasma creatinine, glucose, cholesterol, and triglycerides; HDL and LDL cholesterol; ApoA and ApoB; weight, BMI, and percentage body fat; plasma glycine betaine, dimethylglycine, carnitine, acetylcarnitine, and total homocysteine; and the excretions of glycine betaine, dimethylglycine, carnitine, acetylcarnitine, and albumin (expressed as the logs of their urinary concentrations divided by the urinary creatinine concentrations). Because the lipid measurements, weight, percentage of body fat, and BMI could be expected to be interrelated, variance inflation was tested. This only significantly affected the models in the cases of predicting plasma dimethylglycine in women (these results are not reported here) and of predicting plasma glycine betaine in the patients with known vascular disease. Associations were also tested using Pearson product moment correlation and confirmed by estimating Spearman rank order correlation coefficient. Independent Student *t* test and Mann-Whitney rank sum tests were used to determine the differences between groups.

3. Results

3.1. Study population

The 170 subjects originally enrolled into the study included 12 subjects who had been previously diagnosed with type 2 diabetes mellitus. Preliminary analysis demonstrated that these diabetic subjects had significantly higher urine glycine betaine concentrations (diabetic median, 33.7 mmol/mol creatinine; range, 4.7-82.1 mmol/mol creatinine; nondiabetic median, 7.4 mmol/mol creatinine; range, 1.3-346.4 mmol/mol creatinine; $P < .001$). Six of the diabetic subjects were above the reference range (> 32.5 mmol/mol creatinine [17]) and only one was below the median for the normal population. These results are consistent with previous reports [17,18], and all diabetic subjects were excluded from further analyses.

Of the remaining 158 subjects, there were 75 men and 83 women having a mean age 52.8 ± 12.0 SD years and ranging between 18.0 and 81.0 years.

Twenty-seven percent of the population were receiving one or more lipid-lowering agents as treatment of dyslipidemia (atorvastatin, n = 20; fluvastatin, n = 4; simvastatin, n = 1; bezafibrate, n = 17; gemfibrozil, n = 1). Other medications that may affect homocysteine metabolism included thiazide diuretics (n = 8) and carbamazepine (n = 1). Ten patients were on multivitamin supplements, which included vitamin B_{12}, vitamin B_6, folic acid, or vitamin C. No patients were on methotrexate or other folate antagonists.

Vascular disease had been established in 46 patients (24 men and 22 women; mean ± SD age, 60.7 ± 9.0 years; range 38-81). For comparison, 33 of these were matched for sex and age, with 33 patients without CVD (mean ± SD age, 57.3 ± 7.8 years; range, 37-75). It was not possible to

match all 46 patients because there was not a sufficient number of older patients in the group without vascular disease.

3.2. Biochemical characterization

3.2.1. General biochemical results

Results from laboratory tests were within the reported reference ranges (Table 1) with a few exceptions. As expected in a cohort attending a lipid disorders clinic, most had some form of dyslipidemia. Seventeen patients had mild homocysteinemia (fasting plasma total homocysteine > 15 μmol/L). Six patients had elevated plasma creatinine (> 0.11 mmol/L).

3.2.2. Glycine betaine, dimethylglycine, and carnitine

Plasma and urine concentrations of glycine betaine, *N*,*N*-dimethylglycine, and carnitine are shown in Table 2. Glycine betaine concentrations were lower than previously reported [16,17], where mean results were 34.0 μmol/L for normal women and 47.0 μmol/L for normal men. This is similar to the mean of 35.9 μmol/L (2 SD range, 17.6-73.3 μmol/L) reported for 60 blood donors who not analyzed by gender [28]. If the data we previously reported by gender [16,17] are used to calculate an overall reference range comparable with the report on the blood donor group [28], our mean is 35.3 μmol/L (2 SD range, 16.4-76.2 μmol/L, n = 88), which is an excellent agreement. The plasma *N*,*N*-dimethylglycine concentrations were within the reported reference range [19]. These previous reports [16,17] were based on nonfasting samples, whereas this study is based on fasting samples. To confirm an effect of fasting, specimens from volunteers (n = 12, average age 59.3 years) were compared with nonfasting specimens collected later in the same day. In every case, the nonfasting specimen had a higher plasma glycine betaine content than the fasting specimen (range of difference, 0.6-18.9 μmol/L), with mean ± SEM difference of 7.6 ± 1.5 μmol/L. For dimethylglycine, the difference (0.76 ± 0.42 μmol/L) was not significant. These differences are small compared with the interindividual variation.

Table 1
Fasting plasma concentrations in 158 nondiabetic subjects

	Median	Mean ± 2 SD	Observed range
Plasma creatinine (mmol/L)	0.07	0.05-0.12	0.05-0.16
Plasma total homocysteine (μmol/L)	9.7	6.9-15.2	5.3-27.6
Plasma glucose (mmol/L)	5.2	4.2-6.4	4.0-7.3
RBC folate (nmol/L)	662	424-893	248-1088
Serum vitamin B_{12} (pmol/L)	292	199-448	124-850
Serum triglycerides (mmol/L)	2.0	1.1-4.7	0.7-17.6
Total cholesterol (mmol/L)	6.8	5.6-8.2	4.4-9.7
HDL cholesterol (mmol/L)	1.21	0.5-1.9	0.5-2.4
LDL cholesterol (mmol/L)	4.6	3.4-6.9	0.1-7.4
Total cholesterol/HDL cholesterol	5.8	4.0-8.9	1.4-14.1
ApoA1 (g/L)	1.24	0.96-1.63	0.53-2.04
ApoB (g/L)	1.34	1.01-1.73	0.69-2.24

Mean and 2 SD ranges were calculated on log-transformed data, reported above after being back-transformed to original units.

Table 2
Fasting plasma and urine concentrations for glycine betaine, *N*,*N*-dimethylglycine, the dimethylglycine/glycine betaine ratio, and carnitine (n = 158)

	Median	Mean ± 2 SD	Observed range
Plasma betaines (μmol/L)			
Glycine betaine	22.2	13.3-34.2	6.3-51.3
N,*N*-Dimethylglycine	1.9	1.1-3.4	0.2-6.7
Carnitine	33.1	22.1-48.4	12.7-76.7
Urine betaine (mmol/mol creatinine)			
Glycine betaine	7.4	3.1-29.7	1.3-346.3
N,*N*-Dimethylglycine	3.4	0.7-10.6	0.02-61.4
Carnitine	8.7	2.0-24.4	0.2-95.1

Mean and 2 SD ranges were calculated on log-transformed data, reported after back-transformed to original units.

The fasting plasma betaine and dimethylglycine concentrations in the group (n = 29, average age 59.9) of healthy subjects ranged from 14.7 to 61.1 μmol/L for glycine betaine (mean 33.5 μmol/L) and from 1.2 to 10.7 μmol/L for dimethylglycine (mean 4.1 μmol/L). These results are consistent with previously published ranges. The plasma concentrations of glycine betaine and dimethylglycine in our study population tended to be lower ($P < .001$ by both *t* test and the Mann-Whitney rank sum test) than in the normal fasting subjects. The difference between the plasma betaine results on the study group and in published reference ranges [2,17,19,28] can be attributed to a lower plasma betaine concentrations trend in the study group, plus a difference between fasting samples collected in this study and nonfasting samples.

The sex difference in plasma glycine betaine concentrations that was previously found in nonfasting normal subjects was also found in these fasting patient samples. The mean ± SD concentration for men was 27.2 ± 9.2 μmol/L (n = 75), significantly greater ($P < .001$) than the mean for women, which was 19.9 ± 7.0 μmol/L (n = 83).

3.2.3. Excretion of glycine betaine and dimethylglycine

Fourteen patients (6 men and 8 women) had abnormally high glycine betaine excretion (>32.5 mmol/mol creatinine [17]). We excluded the possibility that these were undiagnosed diabetic patients, but 3 of the 14 also had elevated fasting plasma glucose concentrations (6-6.8 mmol/L). Fifteen of the remaining 144 patients had impaired fasting glucose, with plasma glucose between 6.1 and 6.8 mmol/L. One had a value of 7.3, who was nondiabetic (6.9) on repeat. Thus, none of the 158 patients were diabetic. None of the patients with high glycine betaine excretion had established renal disease, and only 2 were being treated with thiazides.

Fractional clearances of glycine betaine and dimethylglycine were also calculated (Fig. 3), showing that while both are largely resorbed, glycine betaine is more efficiently conserved. There were 23 subjects with glycine betaine clearances above the upper limit of the reported reference range, which is 7.1% [17]. Patients with established CVD

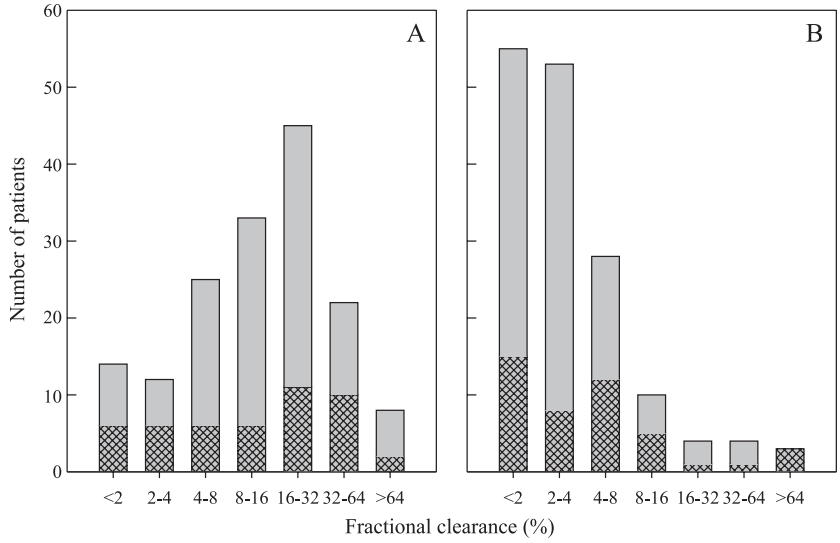

Fig. 3. Numbers of patients with fractional clearances (percentage of creatinine clearance) in different ranges. Fractional clearances of dimethylglycine (A) and glycine betaine (B) in patients attending a lipid clinic. Cross-hatched bars, Subjects with known vascular disease. For glycine betaine fractional clearance, the upper limit of the reference range for healthy subjects is 7.1% [17].

(marked by cross-hatching in the figure) were possibly overrepresented in this group. These subjects with high glycine betaine clearance were compared with the rest of the sample population. There were no significant differences in plasma dimethylglycine or creatinine between the 2 groups, but the high betaine clearance group had higher plasma total homocysteine ($P = .036$, Mann-Whitney rank sum test), the medians being 10.8 (high-excretion group) and 9.5 μmol/L. The plasma glycine betaine may have been different ($P = .060$), the medians being 19.5 (high-excretion group) and 23.3 μmol/L.

The group (n = 14) with abnormal betaine excretion was different in several respects (Table 3) from the remaining subjects (n = 144) with normal betaine excretion (<32.5 mmol/mol creatinine). Nine of these 14 had established vascular disease, a significantly ($P = .017$) larger proportion than the 37 of 144 with normal excretion. The high-excretion group had significantly elevated plasma homocysteine ($P < .001$), although folate levels were not significantly different in the 2 groups (Table 3). Thus, high excretion, rather than clearance, better defined a clinically interesting group of patients, none of whom had diabetes or renal impairment. The high excretion of glycine betaine is associated with an elevated excretion of dimethylglycine, which represents a further loss of glycine betaine.

3.2.4. Effect of vascular disease

When the 33 patients with established vascular disease, who could be matched to patients without known vascular disease, were compared, it was found that there were no statistically significant differences. This was despite the mean glycine betaine excretion in the vascular disease group (29.9 mmol/mol creatinine) being nearly 3 times that in the control group (10.8). However, this difference is attributable to a few extreme values in the vascular disease group. A much larger study would be needed to establish the significance of the observed difference. Dimethylglycine excretion has been reported [19] to be elevated in a selected group of patients with abnormal methionine loading tests and established CVD. Comparison of the paired groups were consistent, with this also being true of this patient group (mean

Table 3
Characteristics of patients with high betaine excretion

Characteristic (units)	Median value for patients by betaine excretion		Significance (P)
	High (n = 14)	Normal (n = 144)	
Age (years)	58.5	54	.015
Weight (kg)	75.9	78.6	NS
Body fat (%)	43	34	NS
Plasma creatinine (mmol/L)	0.075	0.070	NS
Plasma glucose mmol/L)	5.2	5.2	NS
Plasma total homocysteine (μmol/L)	14.4	9.4	<.001
Plasma glycine betaine (μmol/L)	20.8	22.4	NS
Plasma dimethylglycine (μmol/L)	2.4	1.9	NS
Plasma carnitine (μmol/L)	49.7	32.6	.001
Plasma acetylcarnitine (μmol/L)	8.8	7.9	NS
Serum vitamin B_{12} (pmol/L)	265	296	.021
RBC folate (nmol/L)	683	667	NS
Urine glycine betaine excretion (mmol/mol creatinine)	67.1	7.1	(selected)
Urine dimethylglycine excretion (mmol/mol creatinine)	8.7	3.2	<.001
Urine carnitine excretion (mmol/mol creatinine)	9.4	8.8	NS
Urine acetylcarnitine excretion (mmol/mol creatinine)	3.2	3.3	NS
Urine albumin (g/mol creatinine)	0.95	0.70	NS

Significance tested by Mann-Whitney rank sum test. Other biochemical characteristics and other variables (including lipids, weight, BMI, etc) were not significantly different between the 2 groups ($P > .1$ in all cases).

excretions 5.4 ± 0.8 with CVD, 3.7 ± 0.5 without CVD), although the data are not conclusive ($P = .085$).

3.3. Predictors of plasma homocysteine

3.3.1. Predictors of homocysteine in multiple regression models

Independent predictors of the plasma total homocysteine concentration were identified by best-subsets regression (using r^2 adjusted for the number of variables as the criterion) on 23 variables and by both forward and backward stepwise regression. The r^2 values were 0.560 (14 variables) in a best-subsets model, 0.520 (9 variables) in a backward stepwise regression model, and 0.481 (6 variables) in a forward stepwise regression model. Five independent models were considered, the first 3 best-subsets models and forward and backward regression models. A high proportion of the variance in the plasma homocysteine concentrations was explained by a variance in the predictor measures that were included in all the analyses. Four variables appeared as highly significant ($P \leq .002$) predictors in all models, with consistent coefficients (Table 4). Plasma glycine betaine concentration and urine excretion of glycine betaine were 2 of these predictors, showing a close association between plasma homocysteine and betaine metabolism. There was a negative association between plasma betaine and homocysteine and a positive relationship between betaine excretion and homocysteine. This is consistent with a relationship between plasma homocysteine and glycine betaine fractional clearance, and these are highly correlated ($r = 0.499$, $P < .0001$).

Thus, glycine betaine metabolism has a relationship with homocysteine that is comparable with the known relationships with folate and creatinine. This was supported by numerous models in which specific variables were omitted (glucose, albumin excretion, and blood lipids) and in which the same 4 variables were consistently strongly significant predictors; in these plasma, glycine betaine is often significant at $P < .001$. No other variable consistently appeared as a significant predictor (Table 4), although carnitine and acetylcarnitine appear as significant predictors in many models, with a positive relationship between plasma concentrations and homocysteine. Variables that did not appear as significant predictors of plasma total homocysteine in any of the models included dimethylglycine, blood lipids and lipoproteins, urine albumin, and body weight and fat. Models were also obtained with the plasma dimethylglycine–to–glycine betaine ratio tested as an independent variable, in place of the separate metabolites. The ratio did not appear as a stronger predictor of homocysteine than plasma glycine betaine alone (P = .004-.005). The B_{12} concentration and age appeared as significant predictors in some models, but not as consistently as carnitine.

Models were also generated for the population remaining (n = 112) after excluding all patents receiving drugs (including fibrates and therapeutically prescribed folate) known to affect plasma homocysteine. The results were the same as on the total population. Because the variance in folate decreased, its contribution in this population was less than urinary glycine betaine excretion (for glycine betaine excretion, $t = 4.9$, compared with $t = -3.7$ for folate, $t = 4.2$ for plasma creatinine, and $t = -3.1$ for plasma glycine betaine).

Models on the population with normal betaine excretion (Table 3, n = 144) did not show glycine betaine excretion as a significant predictor of homocysteine, although plasma glycine betaine was still significant ($P = .01$). For the high-

Table 4
Predictors of plasma total homocysteine

Variable (units)	Best-subsets regression		Forward stepwise regression		Backward stepwise regression	
	Coefficient	P	Coefficient	P	Coefficient	P
Consistently significant predictors						
Red cell folate (nmol/L)	−0.0069 ± 0.0016	<.001	−0.0082 ± 0.0016	<.001	−0.0072 ± 0.0016	<.001
Plasma creatinine (mmol/L)	80.4 ± 17.4	<.001	83.7 ± 14.5	<.001	76.1 ± 16.2	<.001
Plasma glycine betaine (μmol/L)	−0.109 ± 0.034	.002	−0.095 ± 0.029	.001	−0.106 ± 0.032	.001
Glycine betaine excretion [log (glycine betaine/creatinine)]	2.69 ± 0.65	<.001	2.88 ± 0.59	<.001	2.52 ± 0.70	<.001
Possibly significant predictors						
Serum vitamin B_{12} (pmol/L)	−0.0051 ± 0.0026	.056	NS	NS	−0.0050 ± 0.0026	.050
Plasma carnitine (μmol/L)	0.078 ± 0.029	.008	NS	NS	0.094 ± 0.028	.001
Plasma acetylcarnitine (μmol/L)	0.102 ± 0.070	.148	0.185 ± 0.068	.007	NS	NS
Plasma glucose (mmol/L)	0.67 ± 0.48	.167	1.33 ± 0.49	.007	NS	NS
Carnitine excretion [log(carnitine/creatinine)]	−1.53 ± 0.72	.034	NS	NS	−1.63 ± 0.68	.018
Acetylcarnitine excretion [log(acetylcarnitine/creatinine)]	1.64 ± 0.57	.005	NS	NS	1.72 ± 0.56	.003
Age (y)	0.045 ± 0.023	.058	NS	NS	0.058 ± 0.023	.013

Three multivariate models for predicting plasma total homocysteine concentrations. Excretions were entered as $\log_{10}$ of the metabolite-to-creatinine ratio (mmol/mol creatinine in each case). When the variable was eliminated from the analysis ($P > .05$), "NS" is recorded. Coefficients ± SE are given in the units of the variables.

betaine-excretion group (n = 14), glycine betaine excretion was the strongest predictor of plasma homocysteine, supporting the conclusion that these form a distinct group of patients with an elevated vascular risk.

3.3.2. Univariate correlation between homocysteine and betaine metabolites

With such complex interactions among variables, univariate correlations can mislead. For example, despite the results in Table 4, there was no apparent correlation between plasma glycine betaine and homocysteine ($r = -0.03$, $P = .70$). Plasma carnitine and homocysteine correlated ($r = 0.28$, $P < .001$), as did plasma dimethylglycine and homocysteine ($r = 0.18$, $P = .02$) and plasma creatinine and homocysteine ($r = 0.42$, $P < .0001$). This apparent contradiction is explained by the positive correlations between glycine betaine and carnitine ($r = 0.37$, $P < .001$), dimethylglycine ($r = 0.31$, $P < .001$), plasma creatinine ($r = 0.25$, $P = .0015$), and other variables. These positively correlate with homocysteine, and hence, mask the negative relationship between glycine betaine and homocysteine that emerges from the multivariate analysis. To illustrate the masking effect, a multiple linear regression model gives the equation (all units μmol/L; $r^2 = 0.24$):

$$\begin{aligned}(\text{plasma homocysteine}) = {} & 89(\text{plasma creatinine}) \\ & - 0.10(\text{plasma glycine betaine}) \\ & + 0.09(\text{plasma carnitine}) + 3.2\end{aligned}$$

with the glycine betaine coefficient significant at $P = .004$. Adding additional variables such as plasma dimethylglycine increases the significance, and removing either creatinine or carnitine decreases it.

Homocysteine and glycine betaine excretion were correlated ($r = 0.39$, $P < .001$).

3.3.3. Predicting homocysteine in patients with vascular disease

When multiple linear regression was used to predict plasma homocysteine from just the 4 most significant predictors (Table 4), $r^2 = 0.42$. Folate, creatinine, and betaine excretion were all significant variables at $P < .001$, with plasma glycine betaine significant at $P = .05$. When this regression was carried out on the 33 matched patients with and without known vascular disease, folate and creatinine were again significant ($.013 < P < .037$) in both groups. However, betaine excretion was only a significant term in the group with vascular disease ($P = .01$). When the whole group (n = 46) of patients with known vascular disease was considered, folate was not a significant predictor. The urinary excretion of glycine betaine remained significant ($P = .018$), and plasma cholesterol and ApoB were consistently significant predictors ($P < .005$). Age was not a significant predictor, despite the wide range of ages in this group (38-81 years).

3.3.4. Sex differences in homocysteine prediction

There were differences between the sexes in the association between the metabolism of homocysteine and betaines, and the most significant variables were different in the male and female groups. Best-subsets regression gave r^2 of 0.62 for women (n = 83) and 0.72 for men (n = 75). In women, all models (including stepwise regressions) showed folate and creatinine as the most significant predictor variables ($P < .001$) for plasma homocysteine, and plasma glycine betaine and glycine betaine excretion were not consistently significant. Of the other variables, acetylcarnitine excretion ($P = .009$-$.025$) and plasma glucose ($P = .002$-$.014$) were more significant in women than in the total group. However, in men, the most consistently and highly significant predictor was the urine excretion of glycine betaine ($P < .001$ in all models), and only plasma creatinine was comparably significant in some models. Folate ($P = .005$-$.011$) was also significant in all models. The fractional clearance of glycine betaine correlated with plasma homocysteine in both men ($r = 0.64$, $P < .0001$) and women ($r = 0.45$, $P < .0001$).

3.4. Predictors of glycine betaine excretion and concentration in plasma

3.4.1. Prediction of plasma glycine betaine by multiple regression

Independent predictors of the plasma glycine betaine concentration and urinary excretion of glycine betaine (expressed as the log of the ratio of urine glycine betaine to creatinine) were also identified by best-subsets and by both forward and backward stepwise regression. The r^2 values were 0.45 (11 variables) in a best-subsets model and 0.40 (6 variables) in an identical model obtained by both backward and forward stepwise regression. Four models were considered, the first 3 best-subsets models and the 1 obtained by forward or backward regression. The most consistently significant predictors of plasma glycine betaine concentration (Table 5) were percentage of body fat, plasma carnitine, carnitine excretion, and plasma homocysteine. The last was significant at $P < .001$ (with a coefficient of approximately -0.6) in all except the stepwise regression model. This further illustrates the close association between glycine betaine and homocysteine metabolism. Some lipid-related variables were significant in 2 or more models (Table 5); both percentage of body fat and BMI were significant in all models, and LDL cholesterol and ApoB were significant in the best-subsets models. Despite the expected relationships between some of these variables, the variance inflation factors showed that this did not compromise the models obtained. All models included a highly significant constant term of approximately 20 μmol/L glycine betaine; plasma glycine betaine was the only variable studied for which the constant was consistently significant. Variables that were not significant predictors in any model included urinary glycine betaine excretion, folate, and age.

Table 5
Predictors of plasma glycine betaine and urine betaine excretion

Variable (units)	Best-subsets regression		Forward stepwise regression		Backward stepwise regression	
	Coefficient	*P*	Coefficient	*P*	Coefficient	*P*
(A) Plasma glycine betaine						
Consistently significant predictors:						
Plasma carnitine (μmol/L)	0.248 ± 0.075	.001	0.224 ± 0.070	.002	0.224 ± 0.070	.002
Plasma total homocysteine (μmol/L)	−0.616 ± 0.178	<.001	−0.391 ± 0.163	.018	−0.391 ± 0.163	.018
Carnitine excretion [log(carnitine/creatinine)]	−5.97 ± 1.76	<.001	−4.26 ± 1.41	.003	−4.26 ± 1.41	.003
Body fat %	−0.344 ± 0.074	<.001	−0.350 ± 0.074	<.001	−0.350 ± 0.074	<.001
Constant (μmol/L)	20.9 ± 5.6	<.001	19.9 ± 4.6	<.001	19.9 ± 4.6	<.001
Possibly significant predictors						
Plasma acetylcarnitine (μmol/L)	0.355 ± 0.180	.050	0.527 ± 0.173	.003	0.527 ± 0.173	.003
Plasma LDL cholesterol (mmol/L)	1.47 ± 0.68	.034	NS	NS	NS	NS
Plasma ApoB (g/L)	−6.2 ± 2.6	.017	NS	NS	NS	NS
BMI	0.413 ± 0.190	.032	0.413 ± 0.192	.033	0.413 ± 0.192	.033
(B) Urine excretion of glycine betaine						
Consistently significant predictors						
Plasma carnitine (μmol/L)	0.0084 ± 0.0033	.013	0.0087 ± 0.0026	<.001	0.0080 ± 0.0026	.002
Plasma tot. homocysteine (μmol/L)	0.0250 ± 0.0083	.003	0.0176 ± 0.0073	.017	0.0235 ± 0.0076	.003
Dimethylglycine excretion	0.448 ± 0.061	<.001	0.433 ± 0.062	<.001	0.441 ± 0.061	<.001
Possibly significant predictors						
Red cell folate (nmol/L)	0.00038 ± 0.00017	.030	NS	NS	0.00037 ± 0.00017	.030
Carnitine excretion [log(carnitine/creatinine)]	0.188 ± 0.073	.012	0.131 ± 0.060	.032	0.135 ± 0.060	.026

Three multivariate models for predicting the following: A, Plasma glycine betaine concentrations. Excretions were entered as $\log_{10}$ of the metabolite-to-creatinine ratio (mmol/mol creatinine in each case). When the variable was eliminated from the analysis ($P > .05$), "NS" is recorded. Coefficients ± SE are given in the units of the variables. The 2 stepwise regression procedures gave the same model. B, Urine glycine betaine excretion.

3.4.2. Plasma creatinine and betaine

Because plasma creatinine has a significant univariate correlation with glycine betaine ($r^2 = 0.06$), but does not appear as a significant predictor in any of the models, the relationship between these variables was further investigated (Table 6). The major predictors from Table 5 were sequentially added in a series of multiple linear regression models, each addition decreasing the apparent significance of the creatinine contribution until it became insignificant. There was no consistency in the coefficients for creatinine in these equations. Thus, the relationship between creatinine and glycine betaine is probably the converse of the case considered previously: a univariate correlation that appears to be strong because both variables correlate in the same direction with other variables, although the connection in this case is probably very indirect. This illustrates the

Table 6
Effect of including variables in plasma glycine betaine predictions

	Plasma creatinine + total homocysteine	+ Plasma carnitine	+ Plasma dimethylglycine	+ Carnitine excretion	+ Body fat
Constant (μmol/L)	15.0	9.7	8.1	11.7	22.2
t(*P*)	4.8 (<.001)	3.1 (.002)	2.6 (.009)	3.4 (<.001)	4.7 (<.001)
Plasma creatinine (μmol/L)	161	120	102	84	26
t(*P*)	3.8 (<.001)	2.9 (.004)	2.6 (.011)	2.1 (.038)	0.6 (.55)
Plasma homocysteine (μmol/L)	−0.38	−0.53	−0.58	−0.53	−0.43
t(*P*)	−1.95 (.053)	−2.9 (.004)	−3.3 (.001)	−3.0 (.003)	−2.4 (.016)
Plasma carnitine (μmol/L)		0.29	0.27	0.30	0.30
t(*P*)		4.7 (<.001)	4.4 (<.001)	4.9 (<.001)	4.9 (<.001)
Plasma dimethylglycine (μmol/L)			2.1	1.7	1.6
t(*P*)			3.4 (<.001)	2.7 (.008)	2.5 (.013)
Carnitine excretion [log (mmol/mol)]				−3.3	−3.5
t(*P*)				−2.3 (.022)	−2.5 (.012)
Body fat (%)					−0.18
t(*P*)					−3.1 (.002)
r^2	0.09	0.20	0.26	0.28	0.33

First column: multiple linear regression parameters for plasma glycine betaine (μmol/L) as a function of plasma creatinine (μmol/L) and total homocysteine (μmol/L). Shown are the coefficients and the *t* values (with the corresponding probabilities in parentheses). Subsequent columns show the effects of adding further variables (Table 4) such as plasma carnitine (μmol/L), plasma dimethylglycine (μmol/L), log (urinary carnitine excretion) [log(mmol/mol)]. The last line shows the r^2 value for each regression model.

importance of including as many as possible relevant variables when using regression models to identify associations and justifies the conservative approach of requiring relationships to consistently appear in several different independent models.

3.4.3. Multiple regression prediction of plasma glycine betaine

Five models were obtained for urinary glycine betaine excretions, with r^2 = 0.55 (13 variables) by best-subsets regression, 0.50 (5 variables) by backward stepwise regression, and 0.48 (4 variables) by forward stepwise regression. The strongest predictor of urinary glycine betaine excretion was dimethylglycine excretion, possibly a result of common renal transport. Plasma homocysteine and carnitine concentrations also were significant in all models, whereas red cell folate and carnitine excretion appeared to be significant in most models. The close association between glycine betaine metabolism and homocysteine is further supported by these models, more so because dimethylglycine is a product of homocysteine metabolism (Fig. 1). Plasma glycine betaine concentration and age were not significant predictors in any model.

3.4.4. Sex differences in predictors of plasma and urine betaine

Regression models treating male and female subjects as separate groups had different patterns. In both sex groups, all models for predicting plasma glycine betaine had highly significant constant terms (23.7 ± 9.3 μmol/L in men, 24.3 ± 6.9 μmol/L in women) that were not significantly different between the sexes. Plasma total homocysteine was consistently a significant predictor variable in both sexes, with a negative coefficient. ApoB was only significant (P <.001) in men, but curiously, ApoA1 was a significant predictor in men (P = .017) with a positive coefficient (+23.6 ± 9.6) and in women (P = .008) with a negative coefficient (−8.0 ± 2.9). These values are from the best-subsets model, but other models gave similar estimates. LDL cholesterol was a significant predictor in men only. In neither sex was the percentage of body fat a significant predictor, in contrast with the result on the total population (Table 5). There is therefore an implication that the sex difference in plasma glycine betaine is associated with differences in lipid metabolism between genders. Sex differences were also apparent in models for predicting the urinary excretion of glycine betaine. The association with dimethylglycine excretion

Table 7
Dimethylglycine and carnitine: predictors of plasma concentrations and urine excretion

Variable (units)	Best-subsets regression		Forward stepwise regression		Backward stepwise regression	
	Coefficient	P	Coefficient	P	Coefficient	P
(A) Plasma dimethylglycine						
Plasma glycine betaine (μmol/L)	0.019	.083	0.034	<.001	0.031	.001
Plasma homocysteine (μmol/L)	0.039	.092	0.064	.003	0.057	.007
ApoB (g/L)	−1.19	<.001	NS	NS	−0.83	.006
Carnitine excretion [log(carnitine/creatinine)]	−0.41	.034	−0.42	.027	−0.43	.019
(B) Urine excretion of dimethylglycine						
Plasma carnitine (μmol/L)	−0.0083	.011	−0.0080	.013	−0.0080	.013
Acetylcarnitine excretion [log(acetylcarnitine/creatinine)]	0.144	.024	0.155	.017	0.155	.017
Glycine betaine excretion [log(glycine betaine/creatinine)]	0.72	<.001	0.67	<.001	0.67	<.001
(C) Plasma carnitine						
Plasma glycine betaine (μmol/L)	0.33	<.001	0.39	<.001	0.33	<.001
Plasma homocysteine (μmol/L)	0.67	<.001	0.68	<.001	0.60	.002
Plasma acetylcarnitine (μmol/L)	0.88	<.001	0.84	<.001	0.83	<.001
Plasma triglycerides (μmol/L)	1.93	.006	2.32	.001	1.91	.008
Glycine betaine excretion [log(glycine betaine/creatinine)]	6.2	.008	NS	NS	5.7	.013
Carnitine excretion [log(carnitine/creatinine)]	8.4	<.001	9.6	<.001	8.6	<.001
Acetylcarnitine excretion [log(acetylcarnitine/creatinine)]	−6.5	<.001	−7.5	<.001	−6.3	<.001
Dimethylglycine excretion [log(dimethylglycine/creatinine)]	−4.7	.012	NS	NS	−4.6	.015
(D) Urine excretion of carnitine						
Plasma glycine betaine (μmol/L)	−0.0143	<.001	−0.0156	<.001	−0.0156	<.001
Plasma homocysteine (μmol/L)	−0.023	.013	−0.025	.004	−0.025	.004
Plasma carnitine (μmol/L)	0.0154	<.001	0.0132	<.001	0.0132	<.001
LDL Cholesterol	0.103	.003	0.082	.004	0.082	.004
Acetylcarnitine excretion [log(acetylcarnitine/creatinine)]	0.425	<.001	0.397	<.001	0.397	<.001
Glycine betaine excretion [log(glycine betaine/creatinine)]	0.325	<.001	0.346	<.001	0.346	<.001

Three multivariate models for predicting the following: A, Plasma dimethylglycine concentrations. Excretions were entered as $\log_{10}$ of the metabolite to creatinine ratio (mmol/mol creatinine in each case). When the variable was eliminated from the analysis (P > .05), "NS" is recorded. B, Urine dimethylglycine excretion. The 2 stepwise regression procedures gave the same model. C, Plasma carnitine concentrations. D, Urine carnitine excretions. The 2 stepwise regression procedures gave the same model.

was highly significant in both genders, but the highly significant association with plasma total homocysteine was apparent in men only, where it appeared at $P < .001$ in all 5 models tested. In the male group, all models also showed percentage of body fat, folate, and urinary carnitine excretion as significant predictors ($.01 < P < .05$), whereas in the female group, plasma carnitine was a consistently significant predictor.

3.4.5. Betaines in vascular disease

In the subgroup (n = 46) with known vascular disease, blood lipids (cholesterol, triglycerides, and LDL cholesterol) consistently appear as a factor ($P < .001$, not independent of each other) in predicting plasma glycine betaine. There is a positive coefficient for triglycerides and LDL cholesterol and a negative coefficient for cholesterol. No relationship with age is seen. These relationships are consistent with the observed trend toward lower plasma glycine betaine concentrations in this lipid clinic cohort compared with normal subjects.

3.5. N,N-Dimethylglycine and carnitine

None of the models obtained for predicting plasma dimethylglycine on the total population were as strongly predictive as the models for predicting homocysteine and glycine betaine; r^2 were from 0.225 to 0.324. No variables were consistently significant ($P < .05$) in all models (Table 7), although plasma glycine betaine and plasma total homocysteine were consistently either significant or close to significant ($P < .1$) predictors in all models. Excretion of dimethylglycine was most strongly predicted by the excretion of glycine betaine, and to a lesser extent, by plasma carnitine and by the excretion of acetylcarnitine. Univariate correlations between homocysteine and both plasma dimethylglycine ($r = 0.18$, $P = .02$) and urinary dimethylglycine excretion ($r = 0.31$, $P < .001$) were significant.

More than 50% of plasma carnitine variance ($r^2 = 0.53$) was explained by a 10-variable best-subsets model. Similar results were obtained by backward stepwise ($r^2 = 0.52$, 9 variables) and forward stepwise ($r^2 = 0.47$, 6 variables) regression. Plasma acetylcarnitine, glycine betaine, and homocysteine were all consistently significant (Table 7), as were urinary carnitine excretion and urinary acetylcarnitine. In view of the metabolic function of carnitine, it is not surprising that plasma triglyceride concentration was a consistently significant predictor of plasma carnitine, although notwithstanding the consistent results from multivariate regression, plasma carnitine did not appear to directly correlate with plasma triglycerides ($r = 0.07$, $P = .42$). Plasma carnitine correlated with homocysteine ($r = 0.28$, $P < .001$), with plasma glycine betaine ($r = 0.37$, $P < .001$), with HDL cholesterol ($r = -0.21$, $P = .008$), and body weight ($r = 0.20$, $P = .012$).

Urinary carnitine excretion was predicted with $r^2 = 0.50$ by best-subsets and with $r^2 = 0.44$ by forward and backward stepwise regression, which gave the same model. All 4 models included 4 predictor variables with $P < .001$. These were plasma glycine betaine, plasma carnitine, and the urinary excretions of acetylcarnitine and glycine betaine. Other variables that were significant in all models were LDL cholesterol and plasma total homocysteine (Table 7).

4. Discussion

4.1. Glycine betaine is a predictor of plasma total homocysteine

In this cross-sectional study of patients attending a lipid clinic, we have shown that there is a close association between glycine betaine metabolism and fasting plasma total homocysteine. This shows up in both directions. Plasma glycine betaine concentrations and the urinary excretion of glycine betaine are both predictive factors for homocysteine, and homocysteine is a predictive factor for plasma glycine betaine concentrations and for the urinary excretion of glycine betaine. These associations are of comparable significance to the well-known relationship between homocysteine and folate. The strong relationships found here may underestimate those to be expected in a population not receiving drugs, where urinary excretion of glycine betaine may be the strongest determinant of plasma homocysteine. This relationship with excretion may depend on the inclusion of a subset of the population that has an abnormal excretion of glycine betaine and who appears to have an increased risk of vascular disease. The sex differences also show that the study (and also presumably the normal) population is not homogeneous, and much remains to be clarified in the control of betaine, homocysteine, and one-carbon metabolism in different population groups.

Plasma dimethylglycine and dimethylglycine excretion have weaker, although significant, associations with plasma total homocysteine. These results are consistent with the findings of a case-controlled study reported by Lundberg et al on the relationship between glycine betaine metabolism and vascular disease [19]. The study of Lundberg et al was limited by its small size (13 subjects and 15 controls), with the subjects selected on the basis of an abnormal methionine load test as well as vascular disease. The study of Lundberg et al showed (more conclusively than in the present study) that these patients had an increased urinary excretion of *N*,*N*-dimethylglycine, compared with a normal control group, and consistent with the present data, there was a trend toward increased urinary excretion of glycine betaine. It is likely that the increase in plasma and urine concentrations of *N*,*N*-dimethylglycine, a product of BHMT-catalyzed homocysteine methylation, is driven by high plasma total-homocysteine concentrations, causing accumulation of *N*,*N*-dimethylglycine in plasma and urine [19]. This is possibly exacerbated by the requirement for folate for maximal activity of dimethylglycine dehydrogenase, although dimethylglycine may be oxidized without

tetrahydrofolate [29]. The trend to lower plasma glycine betaine concentrations in the study population is expected, given that it was recruited from patients attending a lipid clinic, and elevated blood lipids are associated with lower circulating glycine betaine. We conclude that the association between betaine metabolism and homocysteine is comparable with, and independent of, the association between RBC folate (a marker of folate stores) and the fasting plasma total homocysteine, which has been shown in previous studies to be an important determinant of plasma total homocysteine concentrations [11,12]. It is noteworthy that urinary loss of glycine betaine is, at most, only weakly associated with folate, but has a similar power to predict plasma total homocysteine.

4.2. Dimethylglycine and folate

Folate is an essential cofactor in the metabolism of homocysteine to methionine by methionine synthase, but tetrahydrofolate also enhances the demethylation of *N*,*N*-dimethylglycine [29]. Most dimethylglycine is demethylated rather than excreted (Fig. 3), although in patients excreting abnormal amounts of glycine betaine, there is a further loss of betaine as dimethylglycine (Table 3). The observed relationships between RBC folate and *N*,*N*-dimethylglycine in plasma is consistent with the requirement for folate in dimethylglycine metabolism, and the fact that the high-betaine-excretion group are folate-replete shows that the cause of the abnormality needs to be sought elsewhere. In vitro studies indicate that *N*,*N*-dimethylglycine concentrations of >3 μmol/L would significantly inhibit BHMT activity [2], suggesting that the higher concentrations often seen in both patients and normal subjects would tend to limit flux through this pathway.

Impaired renal function is a cause of elevated fasting plasma total homocysteine concentrations although the mechanism by which this occurs has not been defined. Renal excretion is a trivial part of plasma total homocysteine clearance, and altered metabolism is likely to be a much more important mechanism. Depletion of BHMT, which is normally abundant in the kidney (as well as the liver), during renal failure may limit homocysteine metabolism through this pathway and accumulation of *N*,*N*-dimethylglycine may decrease enzyme activity further [30], but the relative importance of these, or other mechanisms, has not been determined. Undiagnosed renal disease is an unlikely reason for high betaine and dimethylglycine excretion because the subjects had both normal plasma creatinine and normal urine albumin excretion (Table 3).

4.3. Functions of glycine betaine

Circulating glycine betaine is conserved and remains constant for each individual subject, even under stress [31], both in the short and long term. Major factors controlling these set-point concentrations are independent of any of the variables considered here and are constant between groups in this study (Table 5), as suggested by the highly significant constant term in prediction models. In vivo, the feedback inhibition of BHMT may serve to protect systemic plasma glycine betaine concentrations. This conservation suggests an important physiological role for glycine betaine, which has known functions related to cell volume regulation [32,33]. Many cells (including some in the liver) accumulate betaine to intracellular concentrations manyfold greater than the circulating levels, the betaine acting as an osmolyte [32-36], thus, also providing a tissue reserve of glycine betaine. Circulating glycine betaine concentrations are maintained in patients fed enterally [17], and in normal subjects it rapidly return to normal after intake, showing that the control system involves an endogenous source and rapid metabolism or transport from circulation. Depletion of the total body reserves of glycine betaine, thus, could impair the ability of tissues to respond to osmotic stress. Metabolically, glycine betaine is a major source of methyl groups [37]. Methyl group deficiency has multiple metabolic consequences [38], for example, impairing DNA methylation [39] and lipoprotein biogenesis [40] (where there is a suggestive gender difference [41] in view of the gender differences observed here). It also exacerbates the toxic effects of uremia [42]. This provides another mechanism whereby depletion of the reserves of glycine betaine could have multiple pathological consequences. Cell volume regulation is a fundamental cellular requirement, and BHMT appears more likely to be involved in the regulation of the supply of methyl groups than methionine synthase (to judge from its greater subjection to metabolic control [43]).

4.4. Why carnitine?

Carnitine is a betaine that is essential for fatty acid catabolism and was included in this study because we have previously observed an interaction with glycine betaine in diabetic patients [18]. This may reflect chemical similarity (Fig. 2) with interaction through common transporter systems [20-22]. A direct effect on BHMT is unlikely because we have recently confirmed [44] previous reports [45] that carnitine is neither a substrate nor inhibitor in vitro, but we cannot preclude its being a modulator of *BHMT* gene expression. Another connection is that carnitine biosynthesis is dependent on the supply of methyl groups.

A connection between carnitine and homocysteine does not appear to have been reported previously, and the positive association is interesting because carnitine is widely sold as a nutritional supplement specifically as a “heart-protective nutrient.”

4.5. Control of betaine metabolism

This study was limited by its cross-sectional design, and we have not established that excessive loss of glycine betaine in the urine was causally related to elevated plasma total homocysteine concentrations. However, there are plausible metabolic explanations for linkage between glycine betaine loss and elevated plasma total homocysteine concentrations

(Fig. 1). Known controls include the feedback inhibition of BHMT by dimethylglycine and, to a lesser extent, methionine [2] and the regulation of the *BHMT* gene by the supply of these substrates [46,47]. Circulating betaine levels are maintained under stresses that affect its transport into and out of tissues [31], and because BHMT is regulated by the betaine supply, a lowered betaine supply will lead to down-regulation of this enzyme, sustaining circulating betaine by decreasing the methylation of homocysteine.

There are probably underlying hormonal control processes, which could also affect carnitine metabolism, that provide another possible indirect connection. The expression of *BHMT* appears to be regulated by several hormones [43], positively by corticosteroids and negatively by thyroid hormones [43,48] and possibly insulin [43]. Insulin also appears to bind to BHMT [49]. There are consensus binding sites for steroid hormone receptors associated with the *BHMT* gene [47], which may have a role in the observed gender differences and the effect of corticosteroids [43]. Carnitine also has associations with thyroid hormone action [50,51]. In view of the changed betaine metabolism in diabetic subjects [17,18], hormone control of betaines is an obvious subject for investigation. It appears that *BHMT* is more subject to control than folate-dependent homocysteine methylation [43], and thus may be important in the regulation of the supply of methyl groups from the pool of glycine betaine in tissues.

Since this paper was first submitted, Holm et al [52] have shown that plasma betaine is a determinant of post–methionine load homocysteine in patients with CVD in subjects not supplemented with B vitamins, providing further evidence of a connection between betaine homeostasis and circulating homocysteine.

4.6. Effect of dietary betaine

The urinary losses observed in the high-betaine-excretion patients (Table 3) correspond to approximately 0.5 to 1.5 times the estimated dietary glycine betaine intake (Slow and Cressey, unpublished data). This loss must impair the ability of these patients to remethylate homocysteine, and we consider the value of increasing their intake of betaine and choline. Glycine betaine is present in high concentrations in cereals, beets, and some seafood [53,54]. The inefficiency of betaine supplementation as a means of lowering homocysteine is not a contraindication, because (unlike supplementary folate) glycine betaine is consumed when homocysteine is methylated. We have shown in a rat model [55] that the rate of homocysteine methylation, through the BHMT-mediated pathway, can be increased by several orders of magnitude with only a modest (<30%) and short-lived fall in circulating homocysteine, the methionine presumably being rapidly converted back to homocysteine by glycine methylation [56]. Nevertheless, it has been shown that long-term supplementation with plausible dietary amounts of betaine has a detectable lasting effect on circulating homocysteine concentrations [57].

To sustain lowered homocysteine, a constant supply of additional betaine is needed, because it is consumed by irreversible conversion to dimethylglycine. Plasma levels are only modestly affected by betaine intake because betaine is used as an osmoregulator in many tissues. The betaine-excreting patients present the converse of patients receiving betaine therapy. The normal constant supply of betaine is being lost; hence, homocysteine methylation is likely to be compromised. The lowered availability of betaine itself is likely to have pathological consequences. Thus, increased urinary glycine betaine loss may identify a subset of patients who would benefit from betaine supplementation or from increasing the dietary intake of glycine betaine or its precursor choline.

4.7. Conclusions

In conclusion, within this study population, we have established a novel and independent association between renal loss of glycine betaine and elevated plasma homocysteine concentrations, which is as significant as reduced red cell folate. This direct loss of glycine betaine is associated with increased excretion of dimethylglycine, which is evidence that metabolism of glycine betaine is simultaneously increased, increasing the total loss of a major osmoregulator and reservoir of methyl groups. Because the plasma concentration of glycine betaine is also independently and inversely related to homocysteine, the urinary loss may be a significant stress on the patients' metabolism. Over several studies, we have found 55 of a total of 166 diabetic subjects with above the upper limit of normal glycine betaine excretion (32.5 mmol/mol creatinine), but the present group are nondiabetic patients without overt renal disease and the abnormal excretion may by a different mechanism. These should be followed prospectively in a separate study.

The cause of glycine betaine loss remains to be established. Patients who also excrete excessive glycine betaine may be from a distinct population, because when they are excluded, urine glycine betaine excretion is no longer a significant predictor of circulating homocysteine. However, this is circular reasoning, because there is no obvious independent criterion for their distinction from the total group (Table 3), pointing to the need for further work. When, for example, patients with abnormal concentrations of other metabolites such as homocysteine are removed, many well-established relationships disappear as a result of artificially decreasing the variance of the component (data not shown). Indeed, it is possible that one mechanism by which elevated homocysteine causes pathological change is by depleting glycine betaine, thus reducing the supply of methyl groups and compromising osmoregulation. There is clearly a need for further studies. In the meantime, dietary intervention or supplementation to adjust betaine and choline intake should be considered in the management of patients with mild homocysteinemia as well as those with homocystineuria.

Acknowledgments

This study was funded by the Health Research Council of New Zealand, with contributions from the Canterbury Medical Research Foundation and the Lotteries Grant Board. The authors thank Dr Chris Frampton for statistical advice and criticism.

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