

Impairment of homocysteine metabolism in patients with retinal vascular occlusion and non-arteritic ischemic optic neuropathy

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Abstract

Mild hyperhomocysteinemia is established as an independent risk factor for atherothrombotic disease, including ocular pathologies such as retinal vascular occlusion and non-arteritic ischemic optic neuropathy (NAION). Low intake or low status of B-vitamins explains elevated total homocysteine (tHcy) concentrations only in part. The underlying cause for disturbed homocysteine metabolism requires further insight. We investigated whether the combined determinations of plasma tHcy, methylmalonic acid (MMA) and cystathionine provide more information on the causes of impaired homocysteine metabolism as compared with vitamin B₁₂, vitamin B₆ and folate in patients with ocular ischemic vascular disease. A total of 51 hyperhomocysteinemic (>12 μmol/L) patients with retinal vascular occlusion (n=42) and NAION (n=9) were included. Mild renal dysfunction was an important determinant of tHcy, indicated by the positive correlation between creatinine and tHcy (r=0.47, p=0.001). The assessment of MMA in addition to tHcy identified at least 12 out of 51 patients (23%) who were most likely to have a functional vitamin B₁₂ deficiency. An additional 14 patients (27%) with elevated MMA and cystathionine levels also had slightly elevated concentrations of creatinine, pointing to the need for discrimination between renal dysfunction and vitamin B₁₂ deficiency in this group. In contrast, measurement of cystathionine is very sensitive for renal dysfunction and this marker was strongly relat-

ed to serum creatinine (r=0.56, p<0.001) and to tHcy (r=0.50, p<0.001). Measurement of the vitamins folate, vitamin B₁₂ and vitamin B₆ in plasma did not provide sufficient information on intracellular disturbances in homocysteine metabolism. In conclusion, the metabolites homocysteine, cystathionine and MMA are sensitive indicators and valuable for discrimination of the underlying cause of mild to moderate hyperhomocysteinemia, with implications for therapeutic targeting.

Keywords: cystathionine; folic acid; homocysteine; methylmalonic acid; renal function; retinal vascular occlusion; vitamin B₁₂.

Introduction

Non-arteritic ischemic optic neuropathy (NAION) and retinal vascular occlusion are severe ocular pathologies and common causes of visual impairment. Patients older than 50 years of age are primarily affected and the visual deficits are frequently persistent. The underlying pathomechanisms may include endothelial damage and dysfunction, arteriosclerotic alterations of the vessel wall with turbulent and/or insufficient blood flow, intraluminal thrombus formation and embolism. Consequently, arterial hypertension, diabetes mellitus, smoking, increased plasma lipoprotein(a) levels and hyperviscosity have been identified as risk factors (1–3). Nevertheless, the overall risk is only partially explained by these risk factors and others remain to be identified.

Mildly elevated plasma levels of total homocysteine (tHcy) are associated with atherosclerosis, myocardial infarction, carotid artery stenosis, venous thrombosis and stroke, suggesting a central role of homocysteine in the pathogenesis of atherothrombotic disease (4–7). In fact, we have previously reported on the role of elevated plasma tHcy concentrations as a risk factor for vascular eye diseases, including NAION and retinal vascular occlusion (8–11). The prevalence of hyperhomocysteinemia in these patients, as defined by the 95% percentile, is approximately 20% (8–11). Furthermore, homocysteine appears to owe at least part of its risk to synergistic interactions with other risk factors, such as arterial hypertension, hyperlipidemia and smoking (6, 7).

The sulfur-containing amino acid homocysteine is an intermediate product derived from methionine metabolism. Intracellularly, homocysteine is reconverted to methionine by the enzyme methionine synthase (5-methyltetrahydrofolate homocysteine

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methyltransferase; EC 2.1.113) through the remethylation pathway. Alternatively, homocysteine can undergo irreversible degradation to cystathionine and on to cysteine and α -ketobutyrate through the transsulfuration pathway. Both sequential reactions of the transsulfuration pathway are catalyzed by the vitamin B₆-dependent enzymes cystathionine β -synthase (CBS; EC 4.2.1.22) and γ -cystathionase (EC 4.4.1.1), respectively. The balance between the two catabolic pathways is tightly regulated under normal conditions by S-adenosylmethionine through allosteric enzyme activation of CBS and methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.20), respectively (12).

Vitamin B₁₂ acts as a cofactor for methionine synthase in the remethylation pathway by using 5-methyltetrahydrofolate (5-MTHF) as a methyl donor. Deficiencies of either folate or vitamin B₁₂ result in increased tHcy concentrations. Vitamin B₁₂ is also a cofactor for methylmalonyl-CoA mutase (EC 5.4.99.2), which converts methylmalonyl-CoA to succinyl-CoA; hence, deficiency of vitamin B₁₂ results in elevated concentrations of methylmalonic acid (MMA). Consequently, elevated levels of MMA have been suggested to indicate functional vitamin B₁₂ deficiency, whereas measurement of vitamin B₁₂ in plasma is a poor indicator of functional vitamin B₁₂ status with low sensitivity and specificity (13). The plasma concentration of MMA is considered a relatively sensitive and specific marker for vitamin B₁₂ status, but elevated concentrations are also observed in renal insufficiency (14). Plasma concentration of homocysteine is a sensitive, but not a specific, marker for vitamin B₁₂ deficiency because homocysteine also increases in folate deficiency and in renal insufficiency. Individual diagnostic use of homocysteine and cystathionine measurements is limited because elevated levels do not distinguish between folate and vitamin B₁₂ deficiency, and both markers are influenced by functional renal status, vitamin B₆ and age (15–19). In the absence of vitamin deficiencies, however, cystathionine levels are closely associated with renal disease (19).

Thus, the combined use of tHcy, MMA and cystathionine may offer a higher specificity and sensitivity in diagnosing B-vitamin deficiencies. The present study aimed to investigate whether the determination of plasma MMA and/or cystathionine can provide more accurate and sensitive information on the causes of hyperhomocysteinemia, as compared to the assessment of plasma folate and B-vitamins, in patients with ocular ischemic vascular disease.

Subjects and methods

Subjects

A total of 51 patients with retinal vascular occlusion (n=42) or NAION (n=9) were included in the retrospective study. All study participants were seen at the Department of Ophthalmology and were eligible for enrolment if plasma tHcy concentrations were higher than 12 μ mol/L. The study was approved by the local Ethics Committee and all probands

gave written informed consent. Criteria for diagnosis of NAION included sudden visual loss, optic disc edema followed by optic atrophy, relative afferent pupillary defect, and visual field defects consistent with ischemic optic neuropathy. Retinal vascular occlusion comprised patients with either retinal vein or artery occlusion. Retinal vein occlusion was defined by intraretinal hemorrhages and venous dilation in the segment drained by the affected vein. Diagnosis of retinal artery occlusion was made by ophthalmoscopic fundus examination revealing superficial retinal whitening in the distribution of the retinal artery involved. Exclusion criteria for all patients included any history of retinal vasculitis and intake of vitamins and/or medications known to interfere with vitamin and homocysteine metabolisms.

Methods

Blood samples were drawn from an antecubital vein between 07:00 and 08:00 h after an overnight fast of 8 h. EDTA-blood for homocysteine determination was processed immediately, centrifuged at 4°C (3000 \times g for 10 min) and stored at -70°C until analysis. Measurements of plasma tHcy, cystathionine and MMA were performed using a modified capillary gas chromatography/mass spectrometry method according to Allan et al. (20), and Stabler et al. (16) (capillary gas chromatograph 6890 with a mass selective detector 5793, Hewlett Packard, Waldbronn, Germany). Plasma folate and vitamin B₁₂ concentrations were determined with an Abbott AxSym analyzer using a microparticle enzyme immunoassay (vitamin B₁₂) or "ion capture" technology (folate). Vitamin B₆ was analyzed by an online derivatization according to the procedure of Kurioka et al. (21) using a stable bond column instead of the graphitic carbon column and a slight modification of the mobile phase. Creatinine was measured using the Jaffe method without deproteinization (Roche Diagnostics, Mannheim, Germany) on a Hitachi analyzer.

Statistical analysis

Statistical analyses were conducted using the software package SPSS (version 11.0 for Windows, SPSS Inc., Chicago, IL, USA). All continuous variables were skewed and are therefore presented either as median (5–95th percentiles) or as geometric mean (SD). Differences between groups were assessed by ANOVA and this was followed by a Tamhane test if the ANOVA was significant. Correlations between different variables were tested using the Spearman ρ test and backward regression analysis was applied to find predictors of each of the metabolites. Logarithmic transformation was applied for statistical tests that required normal distribution of data. Values of $p < 0.05$ were considered to be statistically significant.

Results

A total of 51 subjects were investigated, 27 men and 24 females, with a mean age of 75.7 (53.6–86.5) years. The biochemical characteristics of the study population are shown in Table 1. As there were no statistically significant differences between the sexes, all results are presented in combined fashion.

Four groups were defined according to plasma concentrations of MMA and cystathionine using conventional cut-off values (Table 2). A total of 12 patients

Table 1 Characteristics of the study population.

Parameter	All	Males (n=27)	Females (n=24)	p
Age, years	75.7 (53.6–86.5)	75.5 (48.9–83.9)	77.7 (60.5–89.4)	0.050
tHcy, $\mu\text{mol/L}$	16.9 (13.8–34.8)	16.1 (13.1–35.1)	17.5 (14.0–35.4)	0.553
MMA, nmol/L	281 (132–908)	298 (116–1249)	245 (138–703)	0.422
Cystathionine, nmol/L	321 (174–1270)	293 (134–1235)	401 (179–1559)	0.314
Folate, nmol/L	7.9 (4.4–15.9)	7.7 (4.3–21.2)	8.6 (4.4–14.2)	0.985
Vitamin B ₁₂ , pmol/L	282 (117–609)	284 (107–1154)	277 (169–640)	0.463
Vitamin B ₆ , nmol/L	24.3 (8.0–122.4)	21.9 (7.4–149.3)	26.4 (13.2–111.9)	0.201
Creatinine, $\mu\text{mol/L}$	106.1 (70.7–196.2)	114.9 (83.1–206.9)	92.8 (64.1–205.5)	0.123

Data are median (5th–95th) percentiles. p-Values are according to ANOVA test. tHcy, total homocysteine; MMA, methylmalonic acid.

had normal concentrations of MMA (≤ 271 nmol/L) and normal cystathionine (≤ 301 nmol/L) and served as the control group (group 1). All patients in group 1 had normal creatinine, and mild hyperhomocysteinemia (mean tHcy 15.8 $\mu\text{mol/L}$) was associated with normal mean concentrations of B-vitamins, although four patients had low plasma folate (< 7 nmol/L) and three patients had low plasma vitamin B₁₂. Groups 2–4 consisted of patients with elevated levels of either MMA and/or cystathionine. Importantly, no significant differences were evident for all groups compared with the control group for age, folate, vitamin B₁₂ and vitamin B₆ plasma concentrations.

Elevated MMA levels (> 271 nmol/L) were found in 26 subjects (groups 2 and 3), but only five subjects had vitamin B₁₂ concentrations suggestive of vitamin B₁₂ deficiency (< 156 pmol/L) and only four subjects had serum creatinine higher than 168 $\mu\text{mol/L}$. Patients in group 3 (isolated elevation of MMA) had normal mean serum creatinine, which indicates that MMA elevation was most likely related to impaired vitamin B₁₂ status rather than renal insufficiency.

Patients in group 4 (elevated cystathionine) had higher serum concentrations of creatinine compared to group 1, indicating that renal function was a major determinant of the cystathionine elevation in group 4.

A total of 14 individuals (group 2) had combined elevated levels of cystathionine and MMA, and had also significant higher tHcy and creatinine compared to the control group (tHcy 20.7 vs. 15.8 $\mu\text{mol/L}$; creatinine 128.6 vs. 84.5 $\mu\text{mol/L}$). These data indicate

that, in addition to vitamin B₁₂ deficiency, renal function was a major confounding factor that determined concentrations of the metabolites in group 2.

In general, lower mean serum concentration of creatinine was present in patients with lower cystathionine levels (groups 1 and 3), whereas mean serum creatinine was markedly increased in patients with elevated cystathionine (groups 2 and 4). The influence of renal function on plasma concentrations of cystathionine was confirmed by a relatively strong correlation between concentrations of creatinine and cystathionine ($r=0.56$, $p<0.001$). Furthermore, concentrations of cystathionine also correlated to tHcy ($r=0.50$, $p<0.001$) and age ($r=0.40$, $p=0.005$).

Backward regression analysis confirmed that serum creatinine ($\beta=0.318$, $p=0.023$) was the main determinant of tHcy concentrations in this group of hyperhomocysteinemic patients (Table 3). Vitamin B₁₂ was a significant predictor of concentrations of MMA, and both serum creatinine and tHcy were predictors of cystathionine concentrations in this group of hyperhomocysteinemic patients (Table 4). Importantly, determination of folate, vitamin B₁₂ and vitamin B₆ were not predictive of elevated concentrations of tHcy (Tables 2 and 4).

To further analyze the effect of renal function on metabolites, the study population was divided according to median creatinine (median 106.1 $\mu\text{mol/L}$, Table 4). Mildly elevated creatinine was associated with significantly higher tHcy and cystathionine levels, confirming the diagnostic value of cystathionine and

Table 2 Groups according to metabolic changes.

Parameter	Group 1/control Normal MMA/ normal Cyst	Group 2 Elevated MMA/ elevated Cyst	Group 3 Elevated MMA/ normal Cyst	Group 4 Elevated Cyst/ normal MMA
Number	12	14	12	13
Age, years	69.4 (10.9)	73.3 (10.4)	72.2 (6.8)	79.5 (4.8)
tHcy, $\mu\text{mol/L}$	15.8 (1.9)	20.7 (6.0)*	16.9 (4.0)	18.3 (7.7)
MMA, nmol/L	174 (54)	435 (271)*	453 (272)*	293 (41)
Cystathionine, nmol/L	215 (29)	621 (422)*	230 (52)	353 (176)*
Folate, nmol/L	9.0 (5.8)	8.8 (2.4)	7.5 (3.8)	8.5 (3.1)
Vitamin B ₁₂ , pmol/L	275 (119)	310 (342)	203 (124)	274 (116)
Vitamin B ₆ , nmol/L	29.0 (31.2)	24.1 (10.0)	24.3 (42.9)	24.3 (30.9)
Creatinine, $\mu\text{mol/L}$	84.5 (13.8)	128.6 (50.0)*	97.4 (16.4)	108.0 (25.0)*

Data are geometric mean (SD). * $p<0.05$ compared to group 1 (normal MMA/normal Cyst). Cyst, cystathionine; tHcy, total homocysteine; MMA, methylmalonic acid.

Table 3 Results from multiple backward regression analyses.

Dependent variable	Constant	β coefficient [95% CI]*
tHcy	0.289	Creatinine 0.318 [0.045–0.591]
Cystathionine	0.074	Creatinine 0.832 [0.237–1.428] tHcy 0.626 [0.006–1.246]
MMA	2.404	Vitamin B ₁₂ -0.398 [-0.740 to 0.055]

*All p-values were <0.05. Other factors included in the regression analyses were age, sex, creatinine, vitamin B₁₂, vitamin B₆, folate, tHcy, MMA and cystathionine. tHcy, total homocysteine; MMA, methylmalonic acid.

tHcy measurement for diagnosis of renal dysfunction in the absence of folate and vitamin B₁₂ deficiency.

Importantly, determination of folate, vitamin B₁₂ and vitamin B₆ did not provide any information on the underlying cause of impaired metabolism (Tables 2 and 3).

Discussion

Mild hyperhomocysteinemia is an established risk factor for vascular disease (4–7), including retinal vascular occlusion and NAION (8–11, 22, 23). Patients with NAION and retinal vascular occlusion typically present with significantly higher plasma homocysteine levels than controls (8–11, 22, 23), but the underlying causes of impaired homocysteine metabolism can be manifold. In the present study we investigated the value of measuring cystathionine and MMA for identifying the cause of hyperhomocysteinemia compared to measuring concentrations of B-vitamins in patients with ocular vascular ischemic diseases. Our results showed that serum concentrations of the vitamins did not explain mild to moderate hyperhomocysteinemia in these patients. Moreover, elevated concentrations of homocysteine were most often explained through mild renal impairment and/or functional vitamin B₁₂ deficiency. In contrast, determination of folate, vitamin B₁₂ and vitamin B₆ in plasma did not provide sufficient information on intracellular disturbances in homocysteine metabolism. Importantly, the concentration of cystathionine was sensitive for renal function in the absence of folate and

vitamin B₆ deficiency. Furthermore, higher concentrations of cystathionine were related to higher homocysteine levels (Table 3), which may be explained by increased production of cystathionine as an intermediate product of homocysteine transsulfuration catabolism.

Homocysteine is a sensitive marker of mild renal impairment within normal reference limits for creatinine (24), and total plasma concentrations are partly explained by (physiological) deterioration of renal function with age in healthy subjects (25). Similar data are presented in the literature regarding concentrations of MMA and cystathionine (26). Hyperhomocysteinemia is frequent in patients with renal insufficiency and may indicate metabolic disturbances that contribute to excess cardiovascular complications in kidney disease. In turn, diet-induced chronic hyperhomocysteinemia was shown in an animal model to induce vascular remodeling and tubulointerstitial injury in the kidney, thus closing the cycle with further increase in tHcy (27). Mild renal abnormalities may exist in the early stages of atherogenesis, preceding the onset of overt clinical manifestation (28). Therefore, early detection of hyperhomocysteinemia and management of its causes may be reasonable approaches to reduce the risk of further renal and cardiovascular damage in patients at risk.

We used creatinine as a marker of renal function. Although more sensitive markers are available, such as cystatin C, the determination of creatinine sufficiently discriminates between functional states. This is clearly supported by our findings of early and sensitive changes in homocysteine and cystathionine due to incipient renal dysfunction, as indicated by significant differences in creatinine levels.

In our study, renal function was a stronger determinant of homocysteine than low vitamin B₁₂ status, confirming previous reports (19). Elderly individuals with increased creatinine may have increased homocysteine, even in the high-normal range of serum folate (29). These subjects require higher concentrations of B-vitamins to maintain normal cellular vitamin status, as indicated by MMA and homocysteine. The higher demand is supported by the observation of responsiveness of metabolites to vitamin treatment in renal dysfunction (30).

Our results have also shown that measuring MMA in conjunction with homocysteine may help in diag-

Table 4 Groups according to renal function.

Parameter	Creatinine < median	Creatinine \geq median
Number	25	26
Age, years	75 (49–88)	78 (54–87)
tHcy, μ mol/L	16.1 (13.7–24.0)	18.9 (13.3–36.8)*
MMA, nmol/L	246 (115–1027)	310 (134–1115)
Cystathionine, nmol/L	244 (174–1231)	413 (143–1505)*
Folate, nmol/L	7.7 (4.1–16.1)	8.0 (4.6–21.1)
Vitamin B ₁₂ , pmol/L	224 (132–553)	312 (107–1232)
Vitamin B ₆ , nmol/L	22.5 (8.2–151.2)	27.6 (7.9–105.5)
Creatinine, μ mol/L	88.4 (64.5–97.2)	132.6 (106.1–217.9)*

Median creatinine, 106.1 μ mol/L. Data are median (5th–95th) percentiles. *p < 0.01 (ANOVA test). tHcy, total homocysteine; MMA, methylmalonic acid.

nosing vitamin B₁₂ deficiency, especially in patients with normal renal function (i.e., group 3). In line with this, at least 12 out of 51 hyperhomocysteinemic patients (24%; group 3) had at least two metabolic markers (homocysteine, MMA) indicative of vitamin B₁₂ deficiency.

In patients with mild impairment of renal function, it is difficult to determine how much of the elevation of homocysteine, MMA and cystathionine is caused by renal insufficiency and how much is caused by vitamin deficiency (group 2) (31). As we suggested previously, vitamin deficiencies cannot be excluded in this case and a significant reduction in the metabolites after vitamin administration is the only reliable metabolic sign of pre-treatment deficiency (26). The causes of hyperhomocysteinemia in group 1 (mean tHcy 15.4 μmol/L) are not clear. We did not investigate *MTHFR* C677T polymorphism, but homozygosity for this mutation could be one probable explanation, especially when associated with marginal folate and vitamin B₁₂ status.

Elevated plasma cystathionine levels are useful in the diagnosis of folate, vitamin B₆ and vitamin B₁₂ deficiency (16), as they indicate a shift towards transsulfuration under conditions of impaired remethylation (such as in deficiencies of vitamin B₁₂, folic acid and in renal dysfunction) (16, 18). The limitation of isolated cystathionine measurement is that it cannot discriminate between folate and cobalamin deficiency (in the presence of normal vitamin B₆). It then follows that cystathionine is a useful marker of renal function only when vitamin deficiencies can be excluded. MMA is a sensitive indicator of intracellular vitamin B₁₂ deficiency. Because of limited specificity, however, MMA should be used as marker of vitamin B₁₂ status only with normal renal function and folate levels preferably above 5 mg/L (18).

In conclusion, this study demonstrates that cystathionine and MMA are sensitive indicators of metabolic perturbations and valuable for discrimination of the underlying cause of hyperhomocysteinemia in patients with ocular ischemic vascular disease. Measurement of MMA is a useful marker of vitamin B₁₂ status in subjects with normal renal function, and elevated MMA in patients with mild renal insufficiency does not exclude vitamin deficiency. Cystathionine is sensitive for mild renal impairment. Measurement of folate, vitamin B₁₂ and vitamin B₆ in plasma, however, does not provide sufficient information on disturbances in homocysteine metabolism.

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