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Received October 3, 2012
Revised November 20, 2012
Accepted December 31, 2012

Research Article

LC-MS/MS identification of the one-carbon cycle metabolites in human plasma

The one-carbon cycle is composed of four major biologically important molecules: methionine (L-Met), *S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAH), and homocysteine (Hcy). In addition to these key metabolites, there are multiple enzymes, vitamins, and cofactors that play essential roles in the cascade of the biochemical reactions that convert one metabolite into another in the cycle. Simultaneous quantitative measurement of four major metabolites can be used to detect possible aberrations in this vital cycle. Abnormalities in the one-carbon cycle might lead to hyper- or hypomethylation, homocystinemia, liver dysfunction, and accumulation of white-matter hyperintensities in the human brain. Previously published methods describe evaluation of several components of the one-carbon cycle, but none to our knowledge demonstrated simultaneous measurement of all four key molecules (L-Met, SAM, SAH, and Hcy). We describe a novel analytical method suitable for simultaneous identification and quantification of L-Met, SAM, SAH, and Hcy with LC-MS/MS. Moreover, we tested this method to identify these metabolites in human plasma collected from patients with multiple sclerosis and healthy individuals. In a pilot feasibility study, our results indicate that patients with multiple sclerosis showed abnormalities in the one-carbon cycle.

Keywords:
LC-MS/MS / Multiple sclerosis / One-carbon cycle DOI 10.1002/elps.201200536

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1 Introduction

One-carbon metabolism plays a pivotal role in methylation, amino acid metabolism, and nucleoside and polyamine synthesis. This fundamental cycle potentially contributes to the etiology of several diseases such as cancer, cardiovascular diseases, depression, schizophrenia, and disorders of the nervous system. L-Methionine (L-Met), *S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAH), and homocysteine (Hcy) are integral components of the one-carbon cycle that can be used as check points within the system. Synthesis of these metabolites is vitamin-, enzyme-, and cofactor-dependent. For instance, the enzyme methionine adenosyltransferase (MAT) converts L-methionine to SAM in the presence of ATP. SAM is further metabolized into SAH and in the presence of SAH hydrolase SAH is converted to Hcy. Hcy can be converted back to methionine in the presence of methionine synthase, vitamin B_{12}, and folate; formed into cystathionine in the presence of cystathionine beta synthase (CBS) and vitamin B_6; or transformed into Hcy thiolactone by methionyl-tRNA synthetase enzyme (Fig. 1).

SAM is an essential molecule in the metabolism of every living species. It is a principal methyl group donor, and a regulator of transcription and translation processes, protein function, and membrane integrity. SAH is a potent inhibitor of many methylation reactions, a precursor to Hcy, and an indicator of cardiovascular disease. Hcy is a sulfur-containing amino acid that is potentially toxic to neurons. It promotes excitotoxicity by stimulation of *N*-methyl-D-aspartate receptors, and damages neuronal DNA [1]. Elevated Hcy levels are associated with neurodegenerative conditions such as multiple sclerosis, Alzheimer's, Parkinson's disease, psychiatric disorders, and stroke.

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Abbreviations: CBS, cystathionine beta synthase; **CSF**, cerebrospinal fluid; **Hcy**, homocysteine; **KW**, Kruskal–Wallis; **L-Met**, L-methionine; **MAT**, methionine adenosyltransferase; **PPMS**, primary progressive multiple sclerosis; **RRMS**, relapsing remitting multiple sclerosis; **SAH**, *S*-adenosylhomocysteine; **SAM**, *S*-adenosylmethionine; **SPMS**, secondary progressive multiple sclerosis

Colour Online: See the article online to view Figs. 1 and 2 in colour.

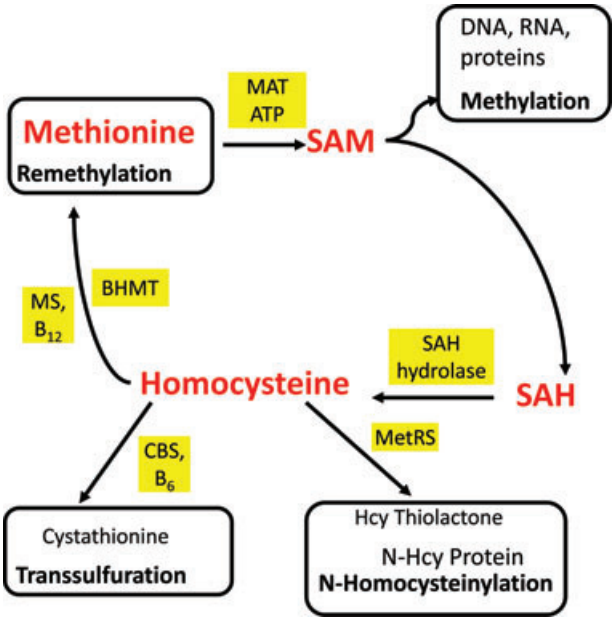


Figure 1. Simplified diagram of the one-carbon cycle. Enzymes and cofactors are highlighted in yellow. ATP, adenosine triphosphate; B_{12}, vitamin B_{12}; B_6, vitamin B_6; II; MetRS, methionyl-tRNA synthetase; MS, methionine synthase.

Several methods such as HPLC [2, 3], stable-isotope dilution LC-MS/MS [4–6], fluorescence polarization assay [7, 8], and colorimetric enzymatic methods [9] have been developed to quantify some of the one-carbon cycle metabolites in human samples. However, most of the studies concentrated on only one or two components of the cycle, and none to our knowledge simultaneously investigated all four metabolites with LC-MS/MS. We developed an extraction procedure for SAM and SAH separately from L-Met and Hcy that allowed simultaneous detection of four metabolites in one LC-MS/MS analysis. Two tubes were run per sample at the same time. One tube contained SAM and SAH extraction and second tube—L-Met and Hcy. This method was tested on human plasma samples collected from healthy individuals and patients with multiple sclerosis.

2 Materials and methods

2.1 Samples

All samples were collected according to the approved Institutional Review Board protocol with patient consent. Blood samples were drawn from the antecubital vein into heparin-coated tubes. All study participants fasted for 12 h before the blood was collected. Plasma was separated immediately, acidified, and stored at –80°C until analysis. Total plasma volume (1 mL) was equally separated into two tubes and used for SAM, SAH, Hcy, and L-Met measurements.

2.2 Materials

Acetic acid, acetonitrile, SAM, SAH, L-Met, and Hcy were obtained from Sigma-Aldrich, St. Louis, MO. The internal standards SAM-d3 (*S*-adenosyl-L-methionine-d3), Hcy-d8 (DL-homocystine-3,3,3′,3′,4,4,4′4′-d8), and L-Met-d3 (L-Methionine-D3) were obtained from CDN Isotopes, Quebec, Canada. SAH-d4 (*S*-adenosylhomocysteine-d4) was obtained from Cayman Chemical Company (Ann Arbor, MI). All internal standards were prepared as 100 μM stock solutions, and were stored at –80°C until use.

2.3 Plasma sample preparation for SAM and SAH measurements

SPE phenylboronic acid columns (Agilent technologies, Santa Clara, CA) and a vacuum manifold were used. The phenylboronic columns were activated with 2 mL of ACN: 1% acetic acid mixture (70:30 v/v). Prior to sample application, the phenylboronic matrix was conditioned with 2 mL of a 50 mM Na_2HPO_4, pH 7.4 buffer. Plasma (500 μL) was acidified with 50 μL of 1 M acetic acid. Base (40 μL of 1 M NaOH) was used to adjust the pH of the sample to 7.4 to selectively bind SAM and SAH *cis*-diol groups to the phenylboronic matrix. Plasma was centrifuged at 12 000 × *g* for 5 min at 4°C to pellet insoluble fragments. Supernatants were transferred into fresh tubes, and the total volume was adjusted to 1 mL with dH_2O. One microliter of each SAH-d4 and SAM-d3 stock internal standard was added to samples before applying the mixture onto the phenylboronic matrix. Water-soluble, nonbound impurities were eluted from the column with 2 mL of 50 mM Na_2HPO_4. SAM and SAH were eluted with 500 μL of an ACN: 1% acetic acid mixture (70:30 v/v). The sample volume was reduced to 100 μL in a speed-vacuum centrifuge. Extraction efficiency was calculated by comparing peak areas of spiked sample with that of standards. The mean recovery for SAM and SAH was 95.4 and 90.2%, respectively.

2.4 Plasma Sample preparation for L-Met and Hcy measurements

Plasma (500 μL) was mixed with 0.5 μL of Hcy-d8 stock solution and 1 μL of 200 mM DTT for 10 min at room temperature; L-Met-d3 stock solution (3 mL) was added. Samples were mixed with ACN in a 1:1 (v/v) ratio, and the mixture was vortexed and centrifuged at 12 000 *g* for 5 min. The supernatant was transferred into a new tube, and the pellet was mixed with 500 μL of methanol; that mixture was vortexed and centrifuged. The supernatants were combined and cleared with centrifugation. The volume of the supernatant was reduced to ca. 100 μL with a speed vacuum centrifuge. The mean recovery for L-Met and Hcy was 97 and 99.1%, respectively.

A

Analyte	MRM transition (m/z)	Declustering Potential DP (eV)	Collision Energy CE (eV)	Collision exit potential Cxp (eV)
L-Met	150/133	26	13	8
D_3 L-Met	153/136	26	13	8
D_4 Hcy	140/94.2	16	17	6
Hcy	136/90.2	16	17	6
SAM	399/250	31	23	18
D_3 SAM	402/250	31	23	18
SAH	358/136	40	25	10
D_4 SAH	389/138	40	25	10
The collision entrance potential was identical for all samples (10eV)				

B

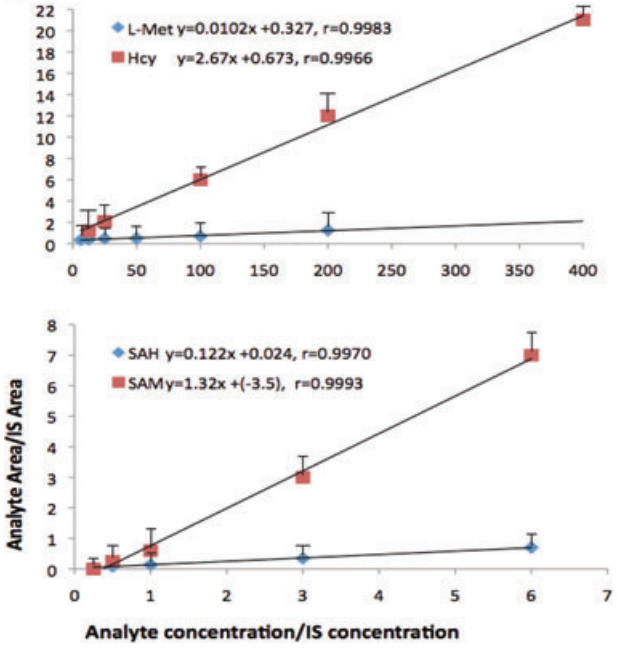


Figure 2. (A) Compound-dependent mass spectrometer instrumental parameters for one-carbon cycle metabolites. (B) Calibration curves for L-Met, Hcy, SAH, and SAM.

2.5 Methods

LC-MS/MS experiments used an LC-20AD system (Shimadzu, Kyoto, Japan) coupled to a Sciex API 4000 triple quadrupole MS/MS system (Applied Biosystems, Foster city, CA) that used the positive-ion mode and a turbo ion-spray with gas1, gas2, and curtain gas pressures set at 50, 30, and 10 psi, respectively. The source was heated to 600°C. Quantitation was performed with the multiple reaction-monitoring mode. Multiple reaction-monitoring conditions were optimized for eight channels. Specific compound-dependent and instrument-dependent MS/MS parameters for each analyte/internal standard are given in Fig. 2A. All mass spectrum parameters were optimized with direct infusion. Data were acquired and processed with the Analyst software (Applied Biosystems/MDS Sciex, Foster city, CA).

The samples (20 μL) were injected onto a preequilibrated Atlantis T3 3 μm column (3.0 × 15.0 mm) attached to a Sentry 2.1 × 10 mm guard column (Waters, Milford, MA), and analytes were eluted with a mobile phase composed of 0.1% acetic acid in water (A) and ACN with 0.1% acetic acid (B) at a flow rate 0.4 mL/min. The LC elution conditions were: 0 min 100% A, 5 min 5% B, 10 min 70% B, 12 min 90% B, 12.1 min 100% A. The retention times were 2.4, and 8.5 min for SAM and SAH (Supporting Information 1), and 2.6 and 4 min for Hcy and L-Met, respectively (Supporting Information 2).

Calibration curves were obtained by plotting ratios of the peak area (calibrator/internal standard) against the concentrations of the calibrator. Linear regression analysis was used to verify the linearity of the calibration curves (Fig. 2B). Analytes and internal standards were diluted in deproteinized human plasma diluted with 0.1% acetic acid to concentrations of 0.25, 0.5, 1, 3, and 6 for SAM and SAH, and 12.5, 25, 50, 100, 200, 400, and 600 ng/mL for L-Met and Hcy. For SAM and SAH, two calibration curves were created.

2.6 Enzyme-linked immunosorbent assay

Levels of vitamins B_6 and B_{12} were measured with ELISA (TSZ Scientific) per manufacturer's instructions.

2.7 Measurements of MAT activity

Extracts were obtained from peripheral blood mononuclear cells (PBMCs). For kinetic analyses, we used [^{14}C] L-Met (57.9 mCi/mmol) supplemented with cold L-Met at different concentrations (1.25–80 μM). Reaction velocity is expressed as units/mg protein, where 1 Unit = 1 nmol of adenosylmethionine/h [10]. K_m and V_{max} were calculated with GraphPad Prism software.

2.8 Statistical analysis

Statistical testing was performed with SAS statistical software (version 9.1.3 service pack 4). The primary statistical test performed consisted of the Kruskal–Wallis (KW) nonparametric analysis of variance, which provides an analysis of mean ranks rather than an analysis of means. When the results indicated a significant KW result, a post-hoc multiple comparison procedure was performed with a SAS macro implementation, which performs a Dunn's multiple comparison procedure specifically designed for the KW procedure [11].

3 Results

Plasma collected from a healthy individual was used for method optimization. The initial simultaneous analysis of four key metabolites of the one-carbon cycle resulted in a low recovery for Hcy and L-Met. Analysis of total Hcy via isotope

Table 1. The one-carbon cycle metabolites (mean ± SD) in plasma of multiple sclerosis patients and healthy controls

Multiple sclerosis type	Age range (y)	Gender M/F	Disease duration (y)	SAM (nMol/L)	SAH (nMol/L)	SAM/SAH ratio	Hcy (μMol/L)	L-Met (μMol/L)	B_6 (ng/mL)	B_{12} (pg/mL)
PPMS $n = 4$	40–62	4/0	3–12	191.9* (± 67.5)	7.7* (± 2.9)	24.8*	34.8* (± 5.0)	19.0* (± 2.9)	47.1* (± 7.4)	310.3* (± 71.8)
SPMS $n = 5$	49–53	4/1	6–20	115.3* (± 16.7)	6.5* (± 2.1)	17.2*	19.8* (± 7.0)	20.2* (± 7.4)	54.8* (± 4.9)	415.6* (± 64.1)
RRMS $n = 5$	42–58	3/2	2–20	54.5 (± 7.2)	8.6* (± 1.7)	6.4	10.2 (± 1.8)	26.1* (± 1.8)	60.1 (± 6.5)	458.4* (± 46.2)
Control $n = 10$	40–61	4/6	—	53.1 (± 21.6)	15.2 (± 0.66)	2.1	8.2 (± 1.0)	34.3 (± 2.1)	73.3 (± 5.2)	568.3 (± 17.2)

*Significantly different from controls, $p < 0.05$.

dilution LC-MS/MS requires a complete equilibration of the isotopically labeled internal standards and a reproducible extraction from the biological samples. Hcy disulfides must be released from covalent attachments to endogenous proteins, and reduced to the monomeric form for accurate quantitation. SAM and SAH are present in human plasma at nanomolar concentrations, and, therefore, additional steps are required for successful detection. In addition, SAM can be stabilized with acetic acid [5]. Based on this information, the sample preparation process was divided into two discrete steps.

In step one pH of the samples was adjusted to 7.4 with 1 N sodium hydroxide. SAM and SAH were extracted from acidified plasma with Bond Elute PBA SPE cartridges. Both compounds were retained on the column via reversible covalent bonds until the elution step.

In step two, the Hcy disulfides were reduced to Hcy monomers with DTT, internal standards for Hcy and L-Met metabolites were added, samples were precipitated with ACN and methanol, and supernatant was analyzed.

Compound-specific MS/MS parameters for each metabolite and its internal standard were optimized with a direct-infusion method (Fig. 2A).

LODs for SAM and SAH were estimated in samples from the analyte's peak height and the noise in the chromatogram. The minimal detectable concentration of the analytes in samples was 6 and 2.5 nmol/L for SAH and SAM, respectively. For Hcy and L-Met, the minimal detectable concentration was 3 and 5 μmol/L, respectively. Calibration curves were linear over a concentration range of 2.5–200 ng for SAM and SAH, and of 25–600 ng for Hcy and L-Met (Fig. 2B). The coefficient of linear correlation (r^2) was ≥0.98 for all cases.

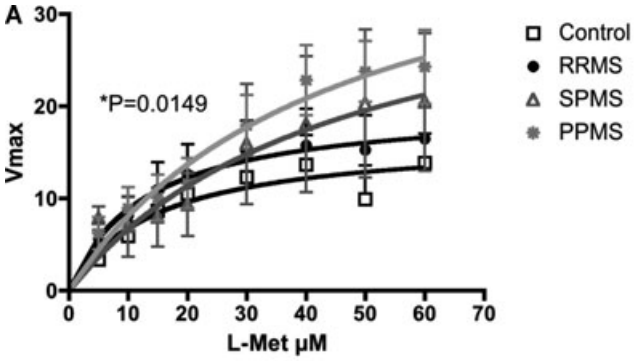
Following method optimization, we processed nine additional control samples and obtained similar results for L-Met, SAM, SAH, and Hcy values. Mean values ± SD for healthy individuals were: L-Met = 34.3 ±2.1 μM, SAM = 53.1 ± 21.6 nM, SAH = 15.2 ± 0.66 nM and Hcy = 8.2 ± 1.0 μM. These values fall within a normal range previously reported by others [4].

Next, we processed plasma collected from multiple sclerosis patients and compared the measurements of these four metabolites to those obtained from healthy gender-matched control subjects. Multiple sclerosis patients were divided into three groups: relapsing remitting (RRMS, $n = 5$), secondary progressive (SPMS, $n = 5$), and primary progressive (PPMS, $n = 4$) (Table 1). Thus, four metabolites were measured in four different groups.

Patients with progressive disease (SPMS and PPMS) had higher Hcy and SAM in their plasma and lower L-Met and SAH. No significant differences were detected in Hcy levels between patients with RRMS disease and healthy controls (Table 1). As stated earlier, production of these metabolites is enzyme- and cofactor-dependent. Therefore high levels of SAM and Hcy could be a direct result of vitamin deficiency or enzymatic malfunction. Thus, we measured levels of vitamins B_6 and B_{12} in plasma and the activity of MAT in peripheral blood mononuclear cells. We also measured the SAM/SAH ratio, which might be an indicator of cellular methylation. All groups of patients had higher SAM/SAH ratios compared to controls, with the highest ratio present in PPMS samples (Table 1). A normal SAM to SAH ratio of 2.1 was detected in the control group; the RRMS group ratio was 6.4, SPMS = 17.2 and PPMS = 24.8.

There are two MAT enzymes that are present in humans: MAT-I/III and MAT-II. The MAT-I/III isoform is liver-specific, whereas MAT-II is expressed in all tissues. MAT-II enzyme catalyzes SAM synthesis from L-Met and ATP (Fig. 1). Plasma was extracted from blood collected from fasting individuals. Peripheral blood mononuclear cell were stored and later used for MAT-II extraction. Kinetic assays were obtained for each sample, and data were averaged per group. Normal Michaelis–Menten kinetic curves were observed in control samples, with K_m and V_{max} average values of 14.51 and 16.62, respectively (Fig. 3A). Data from PPMS and SPMS patients had K_m and V_{max} values significantly higher in comparison to RRMS or controls. The Michaelis constant (K_m) for L-Met was 44.2 μM in PPMS and 46.19 μM in SPMS (Fig. 3, inset table). The RRMS group had a $K_m = 12.85$ and $V_{max} = 20.19$, which was not significantly different from controls ($K_m = 14.51$ and $V_{max} = 20.19$).

High Hcy and low L-Met values for patients with progressive multiple sclerosis pointed to a possible aberration either in remethylation process (vitamin B_{12}-dependent) or in transsulfuration process (vitamin B_6-dependent). Thus, we measured levels of both vitamins in these samples. Multiple sclerosis patients had lower levels of B_{12} in their plasma in comparison to controls (568.3 ± 17.2 pg/mL), Fig. 3B, Table 1. The lowest levels of B_{12} were detected in the PPMS group (310.1 ± 71.8 pg/mL). SPMS patients had a mean value of 415.6 ± 64.1 pg/mL, and RRMS patients had 458.4 ± 46.2 pg/mL of vitamin B_{12}. Those data indicated a decrease of remethylation of Hcy to L-Met. In addition, PPMS and SPMS patients had statistically lower levels of vitamin B_6



	Control	RRMS	SPMS	PPMS
Vmax	16.62	20.19	37.64	43.98
Km	14.51	12.85	46.19	44.20

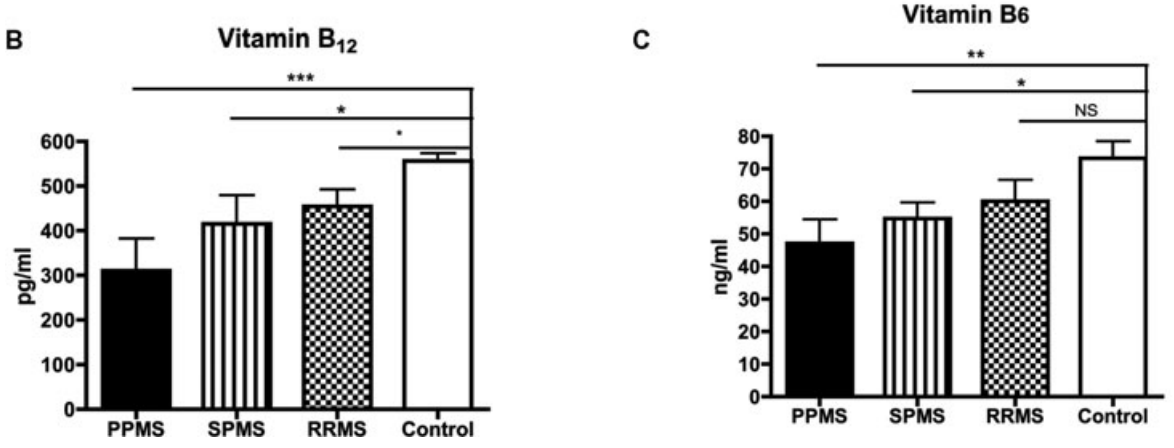


Figure 3. (A) MAT-II enzyme kinetics. (B) Vitamin B12 levels in plasma of patients and controls. (C) Vitamin B6 levels in plasma of patients and controls. NS = not significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

(47.1 ± 7.4 ng/mL and –54.8 ± 4.9 ng/mL, respectively) in comparison to controls (73.3 ± 5.2 ng/mL, Fig. 3C, Table 1). Patients with RRMS did not significantly differ from healthy controls in levels of vitamin B_6 (60.1 ± 6.5 ng/mL), Fig. 3C. Thus, as hypothesized earlier, high Hcy levels correlate with low levels of vitamins B_6 and B_{12} in multiple sclerosis patients, but not in controls. There were only three parameters in which RRMS were significantly different from controls. These patients had lower levels of L-Met, SAH, and B_{12} (Table 1). All other parameters were within the normal range.

4 Discussion

Each component of the one-carbon cycle plays a significant role in multiple biochemical reactions. The key checkpoints that we measured suggest an impaired pathway, which could potentially be remediated with increased vitamin or food intake. Therefore, the method that we describe could be useful as a starting point to create an initial metabolic analysis of human plasma. Simultaneous LC-MS/MS analysis of four key metabolites of the one-carbon cycle indicated an impaired remethylation and transsulfuation processes in progressive multiple sclerosis patients. With this method, we detected higher L-Met levels in controls compared to patients. The lowest L-Met levels were identified in PPMS. Under normal conditions, methionine synthetase ("MS" in Fig. 1) recycles Hcy to L-Met; these data might explain the correlation between higher L-Met and lower Hcy levels in controls. However, in patients with progressive multiple sclerosis the results were opposite: high Hcy and low L-Met levels. Increased Hcy levels are associated with cognitive impairment in multiple sclerosis [12]. Importantly, Hcy levels in our study correlate with previously reported Hcy values for patients with multiple sclerosis and controls [13–16]. Hcy levels did not differ significantly between RRMS patients and controls.

Alterations in SAM and SAH are associated with several neurological and cognitive diseases. In this study, we detected an inverse correlation between SAM and SAH concentrations. The SAM concentration trended higher in the plasma of PPMS and SPMS patients compared to controls (Table 1), whereas the concentration of SAH was almost twice as high in controls compared to disease samples (Table 1). In the one-carbon cycle, SAH is hydrolyzed to Hcy. One might expect that higher levels of SAH would result in higher Hcy. In this study, lower SAH levels were found in progressive multiple sclerosis patients. This result might be due to increased activity of SAH hydrolase, which would increase Hcy levels, while leaving SAH lower than expected. Interestingly, in our control population, higher SAH concentrations did not result in an elevation of Hcy levels, probably due to the rapid Hcy conversion back to L-Met.

In comparison to other studies, SAM concentrations in this study measured with LC-MS/MS in plasma samples from healthy controls is within the range previously reported by Struys et al. [4], and are slightly lower than that detected by Stabler and Allen [6]. Struys et al. also measured SAM and SAH concentrations in the cerebrospinal fluid (CSF) obtained from children with an unknown neurological disorder. The SAM concentrations in CSF were more than threefold higher, and the SAM/SAH ratio was tenfold higher compared to plasma from healthy individuals. Two other studies [5, 6] reported higher SAM values for healthy controls. Some variation is expected between gender and age of the study subjects. For example, SAM and SAH were reportedly higher in females [9] compared to males, and SAM/SAH ratios were lower in males [17]. Although multiple sclerosis is more prevalent in women, our cohort of patients was mostly male. In addition, the difference in concentrations might be explained by variations in the methods such as sample acidification before or after storage and the choice of HPLC buffer.

The SAH plasma concentration of healthy subjects in our study is comparable with that obtained by Gellekink et al. [5] and Stabler and Allen [6]. The SAM/SAH ratio in control human plasma is within previously reported values that range between 2.7 and 8.5 [2, 4–6]. The RRMS patients had a SAM/SAH ratio of 6.3, which is within normal range. However, in progressive forms of multiple sclerosis, the SAM/SAH ratio was higher (24.8 for PPMS and 17.1 for SPMS) (Table 1). Unfortunately, current data on the SAM/SAH ratio in plasma samples collected from patients with neurodegenerative diseases are limited. In the CSF of children with unknown neurological disorders [18], the ratio (about 22) correlates with SAM/SAH ratios for multiple sclerosis patients in this study.

High Hcy and SAM and low L-Met and SAH levels in disease samples pointed to aberrations in remethylation and transsulfuration. Indeed, multiple sclerosis patients had depressed levels of vitamins B_6 and B_{12} as well as increased MAT-II enzyme velocity (V_{max}) and K_m constant compared to controls. Taken together, these data create a metabolic picture that can potentially explain the elevated levels of Hcy in progressive multiple sclerosis patients. Specifically, if one considers that vitamin B_{12} is a critical cofactor for betaine-homocysteine methyltransferase, then an inefficiency of this enzymatic reaction would result in low levels of L-Met and high levels of Hcy (impaired remethylation process). Also, low levels of vitamin B_6 would reduce the metabolism of Hcy into cystathionine via CBS, to thereby hinder transsulfuration pathway.

Interestingly, the most significant differences in metabolites of the one-carbon cycle were found in patients with progressive disease. RRMS patients might have long remission periods before the disease strikes again. These patients had lower L-Met and B_{12} levels and normal Hcy and B_6 values. Therefore, it is possible that remethylation of Hcy to methionine was impaired, whereas conversion of Hcy to cystathionine was not affected. SPMS and PPMS patients experience continued worsening of the symptoms with no remissions in the disease course. Because these patients had significantly lower levels of B_{12} and B_6 vitamins, it is quite possible that remethylation and transsulfuration reactions were both impaired. These two groups of patients with progressive disease were statistically different from controls in all measured metabolites.

In conclusion, we developed a novel, reproducible, and sensitive analytical method for the simultaneous measurement of four one-carbon cycle metabolites in human plasma. When this method was applied to a metabolic analysis of multiple sclerosis patients, we uncovered aberrations in remethylation and transsulfuration pathways. Considering that levels of Hcy and L-Met can be manipulated with food intake and vitamin supplementation, metabolic analysis of the one-carbon cycle is very useful to provide the clues to cost-effective therapies for patients with progressive multiple sclerosis and possibly other diseases.

The authors thank Mr. Haibao Wan, M.S. for his excellent technical assistance. This research is funded by the Department of Veterans Affairs, VA Merit Review Award to Michael C. Levin, M.D.

The authors have declared no conflict of interest.

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