Homocysteine and methionine metabolism in ESRD: A stable isotope study

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Background. Hyperhomocysteinemia has a high prevalence in the end-stage renal disease (ESRD) population, which may contribute to the high cardiovascular risk in these patients. The cause of hyperhomocysteinemia in renal failure is unknown, and therapies have not been able to normalize plasma homocysteine levels. Insight into methionine-homocysteine metabolism in ESRD is therefore necessary.

Methods. Using a primed, continuous infusion of $^{[2H3]}$-Homocysteine, we measured whole body rates of methionine and homocysteine metabolism in the fasting state in four hyperhomocysteinemic hemodialysis patients and six healthy control subjects.

Results. Remethylation of homocysteine was significantly decreased in the hemodialysis patients: $2.6 \pm 0.2$ (SEM) vs. $3.8 \pm 0.3 \mu$mol · kg$^{-1}$ · hr$^{-1}$ in the control subjects ($P = 0.03$), whereas transsulfuration was not $2.5 \pm 0.3$ vs. $3.0 \pm 0.1 \mu$mol · kg$^{-1}$ · hr$^{-1}$ ($P = 0.11$). The transmethylation rate was proportionally and significantly lower in the ESRD patients as compared with controls: $5.2 \pm 0.4$ vs. $6.8 \pm 0.3 \mu$mol · kg$^{-1}$ · hr$^{-1}$ ($P = 0.02$). Methionine fluxes to and from body protein were similar.

Conclusions. The conversion of homocysteine to methionine is substantially (approximately 30%) decreased in hemodialysis patients, whereas transsulfuration is not. Decreased remethylation may explain hyperhomocysteinemia in ESRD. This stable isotope technique is applicable for developing new and effective homocysteine-lowering treatment regimens in ESRD based on pathophysiological mechanisms.

Hyperhomocysteinemia is an independent cardiovascular risk factor in end-stage renal disease (ESRD) [1] with a prevalence as high as 85 to 100% [2, 3]. Many dialysis patients are therefore at risk, necessitating the development and testing of adequate treatment regimens. Folic acid-containing regimens have been shown to be able to lower plasma homocysteine concentration in ESRD patients [3–9]. These studies have also demonstrated that normalization of plasma homocysteine occurs in only a small proportion of patients, an observation that is currently unexplained. To develop more effective therapies, further insight into the pathogenic mechanism of hyperhomocysteinemia in renal failure is essential.

Homocysteine is the transmethylation product of the essential sulfur-containing amino acid methionine [10]. S-adenosylmethionine (AdoMet) and S-adenosylhomocysteine (AdoHcy) are intermediates in this pathway. Homocysteine can be either remethylated to methionine or degraded through the transsulfuration pathway. There are two different remethylation pathways. The first requires 5-methyltetrahydrofolate as methyl donor and reduced folate as a cofactor. 5-Methyltetrahydrofolate is generated by a reaction catalyzed by 5,10-methylene-tetrahydrofolate reductase (MTHFR), of which a common, thermolabile, and less active variant resulting from a cytosine to thymidine point mutation at position 677 has been described [11]. The second remethylation reaction uses betaine as methyl donor. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine, which is subsequently cleaved into cysteine and α-ketobutyrate. Both reactions are irreversible and require the active form of vitamin B$_6$, pyridoxal 5'-phosphate, as a cofactor.

Possible pathophysiological mechanisms of hyperhomocysteinemia in renal failure focus on a decreased homocysteine metabolism in or outside of the kidney. Patients with chronic renal failure exhibit a substantial decrease in plasma homocysteine clearance after oral homocysteine loading [12]. The loss of urinary homocysteine excretion is an unlikely mechanism, as this is normally negligible [13, 14]. An impaired homocysteine transsulfuration in the kidney has been postulated by in vitro observations that homocysteine is transsulfurated in rat kidney tissue [15] and by the in vivo finding that

Key words: hemodialysis, breath, cardiovascular risk, hyperhomocysteinemia, renal failure.

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there is a net uptake of homocysteine in the normal rat kidney [16]. However, we have recently shown that the kidney does not extract homocysteine from the circulation in fasting humans with normal renal function [17].

To understand the pathophysiology of hyperhomocysteinemia in ESRD, it is necessary to quantitatively assess methionine transmethylation and homocysteine remethylation and transsulfuration in ESRD patients. In this study, we used a stable isotope tracer technique to determine whole body rates of use of methionine and homocysteine in ESRD patients and in healthy control subjects.

METHODS

Subjects

Six healthy control subjects (mean age 35 years, four males) and four ESRD patients (mean age 44 years, one male) on maintenance hemodialysis treatment participated in the study. Patients were on chronic standard bicarbonate hemodialysis three times per week. The mean time on dialysis was 36 months (range 4 to 82). All patients received one multivitamin tablet per day containing 2 mg pyridoxine, but no folic acid or vitamin B12. The control subjects did not use medication or vitamin supplements. Subjects’ characteristics are shown in Table 1.

The study protocol was approved by the ethics committee of the University Hospital Vrije Universiteit, and all participants gave their written informed consent.

Experimental design

We used doubly labeled methionine (L-[2H3-methyl-1-13C]methionine) as a tracer, according to the method described by Storch et al [18]. Because of the presence of four additional neutrons, this stable isotope has a molecular weight of m + 4 relative to natural methionine (m). The metabolism of methionine and homocysteine occurs in the intracellular compartment. We assumed that the intracellular and intravascular compartments are in rapid and complete isotopic equilibrium. In steady-state conditions, the appearance and disappearance of whole body methionine can be determined [18, 19].

Methionine is the only known precursor of homocysteine in humans, and when no dietary intake of methionine takes place, remethylation of homocysteine and endogenous protein breakdown are the only sources of methionine. The 2H3-methyl label is removed from methionine during transmethylation and thus [2H3-methyl-1-13C]methionine is converted to [1-13C]homocysteine. Homocysteine can be recycled to methionine by accepting a methyl group from either betaine or 5-methyltetrahydrofolate. Enrichment by 2H3 of the methyl groups of these two donors is negligible in tracer studies of short duration [18].

Remethylation results in the generation of m + 1 methionine, because the 13C atom of the carboxyl moiety of homocysteine remains intact. In contrast, during transsulfuration, the carboxyl moiety of [1-13C]homocysteine loses its 13C atom. When α-ketobutyrate is oxidized in the Krebs cycle, the label appears as 13CO2 in breath air after passage through the body bicarbonate pool. The m + 4 methionine tracer is diluted by methionine entering the pool via the diet, from homocysteine remethylation, and by free methionine entering from protein breakdown in the tissues. In steady state, the rate of appearance of methionine from these sources equals the rate of disappearance (that is, protein synthesis and transmethylation).
**Infusion protocol**

All subjects were kept on a fixed diet containing 1.0 g protein/kg body weight/day for three days prior to the study. The experiments were conducted after an overnight fast. Fasting was continued throughout the infusion period. Only small amounts of tap water given orally were allowed. The hemodialysis patients were studied one day prior to a regular midweek dialysis session. All subjects were kept in bed during the study period. At 8:00 a.m., two intravenous catheters were placed in a dorsal hand vein, one for infusion of substances and one in the contralateral hand for sampling. Arterialized blood samples were drawn from the dorsal hand vein after the hand was inserted in a heated hand box [20]. In the hemodialysis patients, blood samples were drawn from the arterial end of the arteriovenous fistula. Blood was collected in heparinized glass tubes, immediately placed on ice, and centrifuged for 10 minutes at 3000 r.p.m. at −4°C within 15 minutes. Plasma was stored at −30°C until analyzed. Samples of end-tidal–expired breath air were collected in a 15 ml Venoject® tube by instructing the subjects to exhale through a straw. During the last three seconds of expiration, the straw was withdrawn from the tube, which was immediately closed by the investigator.

Before each infusion, three baseline blood and breath samples were collected for the determination of natural abundances of methionine isotopes and 13CO2, respectively. During the infusion, blood and breath samples were taken at regular intervals (Fig. 1). After completion of the protocol, the intravascular catheters were withdrawn, and the subjects were allowed to eat.

Carbon dioxide production was measured during 30 minutes with a ventilated hood using an indirect calorimeter (2900 Metabolic Measurement Cart; SensorMedics, Yorba Linda, CA, USA). Internal calibration for carbon dioxide and volume were conducted before each infusion. Gas volumes were automatically corrected for temperature and air pressure.

**Tracer administration**

L-[3-2H3-methyl-1-13C]methionine (isotopic purity-dilabeled methionine 95 mole percentage excess, (MPE); 1,13C, 99 atom percentage (AP); 2H3-methyl, 99 AP 2H1; Mass Trace, Woburn, MA, USA) was dissolved in sterile saline solution, filtered twice (through 1.2 and 0.2 μm membrane filters), and used within 24 hours. NaH13CO3, (13C, 99 AP) was dissolved in sterile saline solution in 5 ml vials, which were steam sterilized at 121°C for 15 minutes. Both solutions were tested for pyrogens. At t = 0, priming doses were given of 13C-bicarbonate (5.9 μmol) and L-[3-2H3-methyl-1-13C]methionine (2.9 μmol ± kg⁻¹ in the control subjects, and 3.5 μmol ± kg⁻¹ in the dialysis patients, in order to adjust for their larger homocysteine pool size). Thereafter, a six-hour continuous infusion of L-[3-2H3-methyl-1-13C]methionine (1.8 and 2.2 μmol ± kg⁻¹ ± hr⁻¹ in controls and ESRD patients, respectively) was conducted with a calibrated precision infusion pump (Teruma, Tokyo, Japan). After having assessed in the first control subject that plateau enrichments were already obtained after 220 minutes, the infusion period was shortened to five hours in the remaining five healthy subjects. Plateau enrichments levels were calculated as the mean of the final five 20-minute interval samples of the infusion period.

**Laboratory analyses**

For isotopic analysis of methionine, the acetyl-3,5 bis-trifluoromethylbenzyl derivative was prepared from 500 μl plasma samples. Samples were purified with anion-exchange chromatography according to Stabler et al [21]. The dried elutes were derivatized with acetic anhydride (50 μl in 1 ml 1 m phosphate buffer, 40°C) and 3,5 bis-trifluoromethylbenzylbromide (10% in acetonitrile). The enrichments of [1-13C]methionine and [2H3-methyl-1-13C]methionine were measured by gas chromatography mass spectrometry (GCMS) with negative ion chemical ionization (with NH3) on a Hewlett-Packard (Palo Alto, CA, USA) 5989B quadruple GCMS machine. Selected monitoring was set to ~m/z = 190, 191, and 194 for the acetyl-bis.trifluoromethylbenzylbromide derivative of tracee (m) and tracer isotopomers ([1-13C]methionine, m + 1, and [2H3-methyl-1-13C]methionine, m + 4), respectively. Enrichments (in MPE) were calculated on the basis of the abundance relative to all measured methionine species: m + 0, m + 1, and m + 4 [18, 22]. Calibration curves obtained by measurement of standard mixtures containing weighed amounts of tracer and tracee were used to correct for minor instrument variation.

The 13C-enrichment of carbon dioxide in breath sam-
amples was measured on a dual-inlet isotope-ratio mass spectrometer (VG OPTIMA; Fisons Instruments, Middlewich, Cheshire, UK), against a reference gas of carbon dioxide, calibrated relative to PeeDee Bemelite. Isotope abundance for $^{13}$CO$_2$/12CO$_2$ was measured ± 0.0001 AP (N = 3). The geometric mean of the three baseline breath air $^{13}$CO$_2$ values was subtracted from each sample to establish a $^{13}$CO$_2$ enrichment as AP excess (APE).

At baseline, plasma total (that is, free and protein bound) homocysteine was measured by high-performance liquid chromatography (HPLC) with fluorescence detection according to the method of Ubink, Vermaak and Bissbort [23]. Baseline serum folate and vitamin B$_12$ levels were determined by radioassay (ICN Pharmaceuticals, Costa Mesa, CA, USA) and serum pyridoxal phosphate by fluorescence detection. It thus follows that:

$$Q_m = I_d + B + RM = S + TM \quad \text{(Eq. 3)}$$

and

$$Q_c = I_d + B = S + TS \quad \text{(Eq. 4)}$$

where $I_d$ is the tracee methionine intake via the diet (in this study, $I_d$ equals zero as subjects were fasting). B is methionine release from protein breakdown. RM is homocysteine remethylation. S is methionine incorporation in protein synthesis. TM is methionine transmethylating, and TS is the transsulfuration rate. Transsulfuration is calculated from the $^{13}$CO$_2$ excretion in breath air as follows:

$$TS = V^{13}$CO$_2 \times (1/[^{13}$C] methionine enrichment in plasma - 1/[^{13}$C] methionine enrichment in tracer infusion) \quad \text{(Eq. 5)}$$

in which $V^{13}$CO$_2$ equals carbon dioxide production (in $\mu$mol $\cdot$ hr$^{-1}$) * breath air $^{13}$CO$_2$ enrichment (in APE/100) * bicarbonate retention factor (assumed to be 0.72) [24].

As the $^{13}$C in the carboxyl moiety is not removed during transmethylation and remethylation, equations 3 and 4 can be rearranged into:

$$RM = Q_m - Q_c \quad \text{(Eq. 6)}$$

As methionine is the only precursor of homocysteine, the homocysteine disappearance (RM + TS) equals homocysteine appearance (TM); thus:

$$TM = RM + TS \quad \text{(Eq. 7)}$$

Methionine and homocysteine rates (in $\mu$mol $\cdot$ hr$^{-1}$) were expressed per kg body weight.

### Statistical analysis

The plateau isotopic enrichment levels were analyzed by visual inspection and analysis of variance. Values are expressed as mean ± SEM unless otherwise stated. Differences between patients and controls were compared with unpaired Student $t$-tests and Mann-Whitney tests as appropriate. Correlation coefficients were calculated with Pearson’s and Spearman’s tests as appropriate. To analyze sex, age, vitamin status, and uremia as possible determinants of the various flux rates, multivariate regression was performed in the total group after adding a factor “ESRD present (0) or absent (1)” to the model. A $P$ value < 0.05 was accepted as the level of significance.

### RESULTS

Plateau plasma methionine and breath air $^{13}$CO$_2$ enrichments were obtained in all individuals (analysis of variance, $P < 0.05$) with a mean of coefficient of variation of 7% ($N = 5$ samples). In the ESRD patients, the plateau was reached after 260 minutes and, in the control
Table 2. Rates of methionine and homocysteine metabolism in ESRD patients and control subjects in the postabsorptive state

<table>
<thead>
<tr>
<th>Subject</th>
<th>( Q_m )</th>
<th>( Q_c )</th>
<th>( RM )</th>
<th>( TS )</th>
<th>( TM )</th>
<th>( B )</th>
<th>( S )</th>
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<td>16.8 ± 1.2</td>
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<td>2.5 ± 0.3</td>
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<td>16.8 ± 1.2</td>
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</tr>
<tr>
<td>Mean ± SEM</td>
<td>21.7 ± 0.6</td>
<td>18.0 ± 0.5</td>
<td>3.8 ± 0.3</td>
<td>3.0 ± 0.1</td>
<td>6.8 ± 0.3</td>
<td>18.0 ± 0.5</td>
<td>15.0 ± 0.5</td>
</tr>
</tbody>
</table>

\( P \)

Abbreviations are: \( Q_m \), methionine methylflux; \( Q_c \), methionine carboxylflux; \( RM \), remethylation; \( TS \), transsulfuration; \( TM \), transmethylation; \( B \), methionine from protein breakdown; \( S \), methionine to protein synthesis.

Subjects, after 220 minutes. Conditions for mass spectrometry measurements were similar in both groups (isotopic enrichment ranged between 1 to 2 MPE for \([1-^{13}C]\)methionine, between 5 to 10 MPE for \([^{3}H_{2}-methyl-1-^{13}C]\)methionine, and breath air\(^{13}CO_2\) was >0.0020 APE in each subject). The carbon dioxide production was similar in patients and controls (ESRD, 2.60 ± 0.33; controls, 2.61 ± 0.09 ml · kg\(^{-1}\) · min\(^{-1}\), \( P = 0.97 \)).

Results for conversion rates in the methionine-homocysteine cycle calculated from the tracer study are presented in Table 2. Remethylation and transmethylation were both significantly decreased in the ESRD group, whereas transsulfuration, protein synthesis, and breakdown were not significantly different. Figure 2 depicts the individual results for remethylation and transmethylation in both groups. Remethylation and transmethylation rates were highly correlated when data from both groups were pooled (\( r = 0.95, P < 0.001 \)). The transsulfuration rate also showed a significant correlation with transmethylation (\( r = 0.74, P = 0.01 \)), but no significant correlation with remethylation.

Hyperhomocysteinemia was present in all ESRD patients, whereas the control subjects had plasma total homocysteine levels within the reference range (that is, \( \leq 15 \) \( \mu \)mol/liter). Serum folate, vitamin \( B_12 \) and vitamin \( B_6 \) levels were similar in both groups. None of the dialysis patients were homozygous for the MTHFR \( C677 \rightarrow T \) mutation. Multivariate analysis showed that lower remethylation and transmethylation were significantly related to being an ESRD patient (standardized \( r = 0.68, P = 0.03 \), and standardized \( r = 0.72, P = 0.02 \), respectively), but not to any of the \( B \) vitamins, sex, or age. The only significant determinant of transsulfuration was serum vitamin \( B_6 \) (standardized \( r = 0.64, P = 0.045 \)).

**Fig. 2.** Individual values of remethylation rates plotted against transmethylation rates. The regression line with its 95% confidence interval is given for the total group. Symbols are: (□) control subjects; (●) hemodialysis patients.

**DISCUSSION**

The results of this investigation are the first data on in vivo rates of methionine and homocysteine metabolism in patients with renal disease. At increased concentrations of total homocysteine in plasma of these patients, the homocysteine transsulfuration appeared not to be affected when compared with apparently healthy control subjects. In contrast, the methionine transmeth-


ylation and the homocysteine remethylation were proportionally decreased. In the healthy control subjects, the calculated postabsorptive rates of homocysteine remethylation, homocysteine transsulfuration, and methionine transmethylation were virtually identical to those reported by the group of Young et al applying the same isotope model and at similar dietary nitrogen intake of 1.0 g · kg⁻¹ · day⁻¹ [25].

In patients with ESRD, only a nonsignificant trend was observed toward lower rates of homocysteine transsulfuration compared with healthy control subjects. A closer analysis of the data in Table 2 shows that this trend is mainly due to the very low rate of homocysteine transsulfuration measured in patient 1. This patient also had the lowest vitamin B₆ concentration (11 nmol · l⁻¹). Because in the whole group of subjects serum vitamin B₆ was found to be a determinant of homocysteine transmethylation in the multivariate analysis, the tendency toward a lower rate of homocysteine transsulfuration in the ESRD patients may be related to their tendency of having a lower serum vitamin B₆ concentration. In that case, it can be concluded that ESRD per se does not affect the homocysteine transsulfuration. Irrespective of the level of total homocysteine in plasma in patients with ESRD, a significant and proportional decrease in the methionine transmethylation and homocysteine remethylation was observed. A remarkable finding was that the patient with polycystic kidney disease had the lowest remethylation and also the lowest plasma homocysteine, which could indicate that patients with polycystic kidney disease constitute a different patient population. This issue requires further investigation. Nevertheless, the results of this study indicate that an impaired whole body remethylation of homocysteine, and not its transsulfuration, is a plausible cause of hyperhomocysteinemia in hemodialysis patients. This may seem a somewhat unexpected finding, particularly in view of the data of Guttormsen et al [12]. They observed a significantly decreased clearance of total homocysteine from plasma in patients with chronic renal failure. The decreased clearance could not be influenced by the administration of folic acid. They concluded that the decreased clearance in patients with renal failure could not be due a defect in homocysteine remethylation and speculated that the decreased clearance of total homocysteine was brought about by decreased renal extraction of homocysteine. This speculation was based on findings in postabsorptive rats, showing net renal extraction an subsequent renal transsulfuration of homocysteine [15, 16]. We have, however, recently shown that such a mechanism is not of importance in humans, because no net renal extraction of homocysteine was observed in fasting humans [17]. It should be stressed that the findings in this study are true under the assumption that body compartments are in complete equilibrium and that they pertain to fasting steady-state conditions. Guttormsen et al studied bioavailability and distribution volume by oral and intravenous homocysteine (and not methionine) loading [12]. They found that folic acid therapy did not normalize total body homocysteine clearance after homocysteine loading. These findings do not necessarily contradict our data. In addition to an impaired homocysteine remethylation, which is best detected in the fasting steady state, renal failure patients may have a transsulfuration defect on homocysteine or methionine loading as well. Such a defect is not likely to be responsive to folate therapy.

On theoretical grounds, elevated fasting transmethylation could be postulated as a cause of hyperhomocysteinemia. When fasting transsulfuration is unchanged, remethylation would be elevated as well as a consequence. Our observations point to the opposite. Transmethylation is decreased, which is a counter-intuitive finding as less supply is an unlikely explanation for elevated plasma homocysteine levels. However, remethylation is also decreased and evidently more impaired than the consequential decrease in transmethylation.

Several factors may be implicated in the decreased homocysteine remethylation in ESRD. The most obvious cause is a low folate and/or vitamin B₁₂ status, but this was not the case in our patients. Alternative disturbances in folate metabolism that have been described in ESRD patients include an impaired transmembrane transport of folic acid [26] and a decreased activity of plasma folate conjugase, an enzyme that splits polyglutamate forms of folate into biologically more active oligoglutamates and monoglutamates [27]. Further evidence for a disturbed folate metabolism is provided by intervention studies that show that only folic acid-containing regimens are able to lower, but not normalize, plasma homocysteine levels in renal failure patients [3–9]. It remains to be elucidated whether the homocysteine remethylation rate in ESRD patients remains decreased during folic acid treatment. In addition, it can not be excluded that the concentration or the activity of the enzymes involved in homocysteine remethylation are altered in ESRD. Homozygosity for the thermolabile MTHFR variant has a similar frequency of approximately 10 to 15% in ESRD patients and in control subjects [28, 29], which makes this enzyme a priori an unlikely candidate to explain the 85 to 100% prevalence of hyperhomocysteinemia in ESRD. As none of the dialysis patients in our study were homozygous for the C677→T mutation, the decreased homocysteine remethylation could evidently not be explained by the thermolabile MTHFR enzyme variant. Also, abnormalities in the betaine-dependent remethylation pathway do not offer a plausible explanation, as betaine, when added to folic acid, does not further lower plasma homocysteine in hemodialysis patients [3]. Finally, regulatory factors in methionine-homocysteine metabolism may be altered in
ESRD. It has been proposed that a high AdoMet level decreases remethylation by inhibiting betaine homocysteine transferase and MTHFR [10]. High AdoHcy levels relative to AdoMet are thought to inhibit transmethylation reactions [30]. Recent research indicates that the AdoMet/AdoHcy ratio is decreased in erythrocytes [31] and plasma [32] of dialysis patients, especially as the result of elevated AdoHcy levels. A low AdoMet/AdoHcy ratio could be responsible for our observation of a decreased transmethylation rate, but is unlikely that the latter would cause hyperhomocysteinemia because it has been made plausible that the high erythrocyte AdoHcy levels are the result and not the cause of high plasma homocysteine levels [33]. Decreased transmethylation resulting from elevated AdoHcy levels has been associated with impaired erythrocyte membrane protein repair [31] and has also been proposed as a mechanism for other cellular abnormalities in chronic renal failure [34]. In this study, we indeed observed a significant decrease in transmethylation of methionine, but AdoMet and AdoHcy concentrations were not determined. This topic remains a subject for future research.

In conclusion, this study with [3H-methyl-1,13C]methionine shows that in hemodialysis patients with hyperhomocysteinemia, whole body homocysteine remethylation and methionine transmethylation are proportionally decreased as compared with healthy control subjects. In contrast, whole body homocysteine transsulfuration appeared to be unaffected when corrected for variation in the vitamin B12 status. Decreased homocysteine remethylation is a good candidate to explain hyperhomocysteinemia in hemodialysis patients.

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APPENDIX

Abbreviations used in this article are: AdoHcy, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; AP, atom percent; APE, atom percent excess; B, methionine from protein breakdown; CGN, chronic glomerulonephritis; ESRD, end-stage renal disease; GCMS, gas chromatography mass spectrometry; HUS, hemolytic uremic syndrome; MPE, mole percent excess; MTHFR, 5,10-methylene tetrahydrofolate reductase; PCR, polymerase chain reaction; Qm, methionine carboxyl-flux; Qn, methionine methyl-flux; R, remethylation; S, methionine to protein synthesis; tHcy, plasma total homocysteine; TM, transmethylation; TS, transsulfuration.

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