Methionine and protein metabolism in non-alcoholic steatohepatitis: evidence for lower rate of transmethylation of methionine

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ABSTRACT

Hepatic metabolism of methionine is the source of cysteine, the precursor of glutathione, the major intracellular antioxidant in the body. Methionine also is the immediate precursor of SAM (S-adenosylmethionine) the key methyl donor for phosphatidylcholine synthesis required for the export of VLDL (very-low-density lipoprotein) triacylglycerols (triglycerides) from the liver. We have examined the kinetics of methionine, its transmethylation and trans-sulfuration with estimates of whole body rate of protein turnover and urea synthesis in clinically stable biopsy-confirmed subjects with NASH (non-alcoholic steatohepatitis). Subjects with NASH were more insulin-resistant and had significantly higher plasma concentrations of usCRP (ultrasensitive C-reactive protein), TNFα (tumour necrosis factor α) and other inflammatory cytokines. There was no significant effect of insulin resistance and NASH on whole body rate of protein turnover [phenylalanine Ra (rate of appearance)] and on the rate of urea synthesis. The rates of methylation of homocysteine and transmethylation of methionine were significantly lower in NASH compared with controls. There was no difference in the rate of trans-sulfuration of methionine between the two groups. Enteric mixed nutrient load resulted in a significant increase in all the measured parameters of methionine kinetics. Heterozygosity for MTHFR (5,10-methylenetetrahydrofolate reductase) (677C→T) did not have an impact on methionine metabolism. We speculate that, as a result of oxidant stress possibly due to high fatty acid oxidation, the activity of methionine adenosyltransferase is attenuated resulting in a lower rate of transmethylation of methionine and of SAM synthesis. These results are the first evidence for perturbed metabolism of methionine in NASH in humans and provide a rationale for the development of targeted intervention strategies.

INTRODUCTION

NAFLD (non-alcoholic fatty liver disease) constitutes a spectrum ranging from simple triacylglycerol (triglyceride) accumulation in the hepatocytes (hepatic steatosis) to steatosis with inflammation (steatohepatitis), fibrosis and cirrhosis [1–3]. On the basis of studies in humans and animal models, a pathophysiological
metabolic paradigm for the progression of NAFLD from steatosis to steatohepatitis has been proposed [3–6]. These findings suggest that hepatic steatosis is related to excessive delivery of fatty acids to the liver caused by an increased whole body rate of lipolysis due to systemic insulin resistance, coupled with increased hepatic de novo lipogenesis and an attenuated export of hepatic triacylglycerols [7,8]. In addition, due to high rate of fatty acid oxidation in the liver, there is increased oxidative stress leading to changes in mitochondrial function, depletion of ATP, DNA damage, lipid peroxidation, release of cytokines and consequently hepatic inflammation and fibrosis [9,10]. The increase in oxidative stress results in augmented consumption of the major intracellular antioxidant, glutathione and in lower hepatic glutathione levels. In addition, as a result of high fatty acid uptake by the liver coupled with higher de novo lipogenesis, there is a need for higher rate of phosphatidylcholine synthesis for VLDL (very-low-density lipoprotein) triacylglycerol export. The precursors of both glutathione and phosphatidylcholine, i.e. cysteine for glutathione and methyl groups from SAM (S-adenosylmethionine) for phosphatidylcholine, are produced during the metabolism of methionine in the liver.

Methionine, an essential amino acid required for protein synthesis, is the source of methyl groups for a number of methylation reactions such as methylation of nucleic acids, proteins, biogenic amines, phospholipids and for creatine synthesis etc. [11]. During its metabolism, methionine is converted into its active form SAM (Figure 1). Following SAM-dependent transmethylation, the product SAH (S-adenosylhomocysteine) is metabolized to adenosine and homocysteine. Homocysteine is either via the trans-sulfuration pathway converted into cystathionine or is re-methylated back into methionine. The methyl group required for the methylation of homocysteine is obtained from either the folate-dependent one-carbon pool (5-methyltetrahydrofolate) or from (non-vitamin-dependent) betaine in certain tissues. SAM is also converted into SAH by GNMT (glycine N-methyltransferase), an enzyme abundant in the liver [12,13]. The synthesis of SAM by SAM synthase is regulated by hypoxia [14], glutathione [15] and availability of methionine [16] and modified by oxidant injury [17] and redox state [18] of the cell. Although the effect of ATP availability on SAM synthesis is not known, an increase in SAM synthase activity in transfected Chinese-hamster ovary cells caused a depletion of ATP [17]. Whether a limited availability of ATP as a result of mitochondrial dysfunction in vivo can cause decreased synthesis of SAM is not known. A decrease in synthesis of SAM in MAT1A (methionine adenosyltransferase 1A)-knockout mice predisposes the animal to hepatic injury and more susceptible to choline-deficient diet induced fatty liver [19]. Decreased SAM biosynthesis is also a consequence of all forms of chronic liver injury [20].

We hypothesize that, as a result of oxidant insult and decreased availability of glutathione, there is attenuated activity of methionine adenosyltransferase which, in combination with lower ATP availability due to mitochondrial dysfunction, results in lower rate of synthesis of SAM. Consequently, the synthesis of phosphatidylethanolamine and VLDL export are reduced and this leads to hepatic steatosis. The low thiol redox state results in unbalanced ROS (reactive oxygen species) production and propagates the hepatic injury. Although such a metabolic paradigm can be inferred from studies...
in animals, or from indirect evidence in humans, direct evaluation of methionine metabolism has not been done in patients with non-alcoholic steatohepatitis. In the present study, we have quantified the whole body flux of methionine, its rate of transmethylation and trans-sulfuration, in biopsy-confirmed subjects with NASH (non-alcoholic steatohepatitis). In addition, the effect of insulin resistance with NASH on whole body rate of protein turnover was examined using a phenylalanine tracer. Since hepatic urea synthesis requires energy, the impact of mitochondrial dysfunction, if any, in NASH on the rate of urea synthesis was also quantified.

**MATERIALS AND METHODS**

**Subjects, study design and isotope administration**

Methionine, phenylalanine and urea kinetics were quantified in 15 subjects with NASH and compared with 19 healthy age–matched controls (Table 1). Subjects with NASH were recruited from the metabolic clinics at MetroHealth Medical Center and at the Cleveland Clinic. NASH was confirmed by liver biopsy according to the criteria of Kleiner et al. [21]. All subjects in the present study were evaluated by the investigators and were abstinent from alcohol for at least 6 months. Their possible remote consumption of alcohol was less than that suggested to cause liver injury.

Control subjects were recruited by advertisement. All healthy controls underwent a detailed history and clinical examination. A hepatic ultrasound study was performed in order to exclude hepatic steatosis. MTHFR (5,10-methyleneetetrahydrofolate reductase) polymorphism 677C→T was determined in all subjects. The study protocol was approved by the Institutional Review Boards at Cleveland Clinic and MetroHealth Medical Center. Written informed consent was obtained from all subjects after fully explaining the procedure.

Study subjects were kept on their usual diet except they were advised to take a minimum of 70 g of protein/day for 7 days prior to the tracer isotope study. Dietary compliance was monitored by the clinical nutritionist. Tracer kinetic studies were performed in the CRU (Clinical Research Unit) of the Cleveland Clinic (National Institutes of Health Clinical & Translational Science Award to Case Western Reserve University). Subjects reported to the CRU on the morning of the tracer study after an overnight fast of ~10 h. Following a physical examination including height and weight, the subject rested in a bed for the duration of the study. Two indwelling intravenous cannulae were placed, one in each superficial vein on the dorsum of the hand, one for the infusion of the isotopic tracer and the other for obtaining blood samples. The sample site was kept warm (by a thermostatically controlled heating pad) to obtain arterialized blood samples and kept patent by infusing isotonic saline solution. The study design is shown in Figure 2.

![Figure 2](image_url)

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**Table 1  Characteristics of the study population**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NASH (n = 15)</th>
<th>Controls (n = 19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (male/female) (n)</td>
<td>8/7</td>
<td>8/11</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.4 (11.7)</td>
<td>42.2 (12.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>104.3 (19.6)</td>
<td>76.6 (18.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>34.0 (6.2)</td>
<td>26.8 (5.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>2.2 (0.2)</td>
<td>1.9 (0.2)</td>
<td>0.001</td>
</tr>
<tr>
<td>HOMA</td>
<td>2.8 (2.3, 3.9)</td>
<td>0.7 (0.4, 1.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ALT (units/l)</td>
<td>54.0 (39.0, 91.0)</td>
<td>16.0 (14.0, 22.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triacylglycerols (mg/dl)</td>
<td>116.0 (91.0, 207.0)</td>
<td>68.0 (55.0, 104.0)</td>
<td>0.015</td>
</tr>
<tr>
<td>8-OHdG (mg/ml)</td>
<td>42.8 (19.5)</td>
<td>51.4 (20.5)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means (S.D.) (range), or medians (quartiles). BSA, body surface area; ALT, plasma alanine aminotransferase; 8-OHdG, 8-hydroxy-2-deoxyguanosine. NS, not significant.
A priming dose of $^{13}$C-labelled sodium bicarbonate (5 mg) was given to achieve an early steady-state enrichment in the bicarbonate/carbon dioxide pool. The actual rate of infusion was confirmed by gravimetrically measuring the rate of flow using the same tubing and equipment at the end of each study.

After a basal period (180 min), the response to a mixed-nutrient load was examined by giving 45 ml of oral Boost® High Protein drink (Nestlé) every 30 min for the next 180 min. Each 45 ml of Boost® contains $\sim 45$ kcal ($1$ kcal $\approx 4.184$ kJ) and $2.8$ g of protein.

Arterialized blood samples (6 ml) were obtained at 30-min intervals throughout the study. Blood samples were centrifuged immediately at $4^\circ$C, and the separated plasma was frozen at $-70^\circ$C until analysis. Breath samples for the measurement of $^{13}$C enrichment in the expired carbon dioxide were obtained at 30-min intervals, as described previously [22]. $\dot{V}CO_2$ (production of carbon dioxide) and $\dot{V}O_2$ (oxygen consumption) were measured using an open hood system (Viasys Encore, Cardinal Health).

Analysis of the enrichment data (see the Results section) showed an increase in $^{13}$C enrichment of plasma homocysteine following an enteral mixed-nutrient load, in the presence of decreasing $^{13}$C enrichment of plasma methionine. In order to confirm that the increase in $^{13}$C enrichment of homocysteine during feeding (180–360 min) was a true effect of feeding and not related to a lack of tracer equilibrium, we studied four subjects with NASH and eight healthy controls, only in the fasting state for 6 h. The tracer isotopic infusions were the same as above.

**Analytical procedures**

The clinical biochemical analyses were performed in the Cleveland Clinic Reference Laboratory. The concentration of tHcy (total homocysteine), glutathione, total cysteine and amino acids in the plasma and in the infusates were measured by HPLC [23,24].

All of the GC–MS analytical methods used have been reported from our laboratory previously [25,26]. Urea and amino acids in the plasma were separated using mixed-bed ion-exchange chromatography. $^{15}$N$_2$ enrichment of urea was measured as reported previously [27]. A heptafluorobutryl $n$-propyl ester derivative of methionine was prepared as described by Davis et al. [28]. Negative chemical ionization was used to monitor $m/z$ ratio of 367 ($m_0$), 368 ($m_1$), 369 ($m_2$), 370 ($m_3$) and 371 ($m_4$) in order to quantify unlabelled and labelled methionine. The $m/z$ 367 ($m_1$) represented the enrichment of L-[1-$^{13}$C]methionine, and the mass 370 ($m_3$) represented the enrichment of L-[C$_2$H$_3$]methionine. Multiple linear regression analyses were performed to calculate the relative enrichments and correction for natural abundance of $m_1$ ([1-$^{13}$C]tracer) and $m_3$ ([C$_2$H$_3$]methyl) methionine by using an in-house-developed software (Isomet, developed by J. Kim). The $^{13}$C enrichment of homocysteine in the plasma was measured as described by Davis et al. [28]. The enrichment of plasma phenylalanine was measured as described [26]. Enrichment of $^{13}$C in the carbon dioxide was quantified by isotope-ratio MS (Metabolic Solutions).

**Calculations**

The whole body $R_a$ (rate of appearance) of various amino acids was calculated by tracer dilution during isotopic steady state as described [29]. The rates of transmethylation and trans-sulfuration were calculated as described by Storch et al. [30] and MacCoss et al. [31], and detailed previously [25]. The methionine $R_a$ ($Q_a$), measured by [1-$^{13}$C]methionine tracer, represents methionine entering the circulation from proteolysis and that entering from exogenous sources (food). During the conversion of methionine into homocysteine, and back into methionine, the $^{13}$C on carboxyl carbon is retained (Figure 1). In contrast, the $^2$H-labelled methyl group is lost during the conversion of methionine into homocysteine and replaced by an unlabelled methyl group during the formation of methionine from homocysteine (re-methylation). Therefore the $R_a$ ($Q_a$), measured by the dilution of the methyl tracer, is the sum of methionine released from protein breakdown and the methionine that is produced by methylation of homocysteine. Therefore the difference between $Q_a$ and $Q_a$ is a measure...
of the rate of remethylation: $R_M = Q_M - Q_C$. The rate of trans-sulfuration was assumed to be equal to the rate of oxidation of methionine and was estimated from the $R_s$ of $^{13}$C in the expired carbon dioxide [32]. It is assumed that during the formation of cysteine, an equimolar quantity of $\alpha$-ketobutyrate is formed from cystathionine and is oxidized in the tricarboxylic acid cycle.

We did not correct the kinetic data for the intracellular enrichment of methionine, as measured by the enrichment of homocysteine in the plasma because of uncertainty regarding which intracellular pool such data represent. Therefore the reported estimates of methionine kinetics ($R_a$, transmethylation and trans-sulfuration) are lower than actual. As shown in the results and reported previously [25], feeding resulted in an increase in $^{13}$C enrichment of homocysteine, probably a consequence of suppression of intracellular protein breakdown. We did not adjust for possible retention of carbon dioxide. The use of a 20% tracer retention in the bicarbonate pool would only increase our estimates of trans-sulfuration by such magnitude.

### Statistical analysis

All values are reported as means ± S.D. or medians (25–75th percentile). Descriptive statistics were computed for all variables. These included means, S.D. and percentiles for continuous variables and frequencies for categorical factors. A univariable analysis was performed to assess differences between subjects with NASH and controls; Student’s $t$ tests and Wilcoxon rank sum tests were used for continuous variables and $\chi^2$ or Fisher’s exact tests for categorical factors. In addition, in order to assess association of subject group with phenylalanine and methionine kinetics while adjusting for BMI (body mass index), ANCOVA (analysis of covariance) was performed. Kinetic data during the fasting state were compared with those after feeding using paired Student’s $t$ tests. Correlations between various parameters were evaluated using Spearman’s correlation coefficients. For BMI, HOMA (homoeostatic model of insulin resistance) and CRP (C-reactive protein), linear regression analysis was done to assess interactions between these clinical characteristics and subject group. A $P < 0.05$ was considered statistically significant. All analyses were performed using SAS version 9.2 software.

### RESULTS

We studied clinically and biochemically characterized and biopsy-proven subjects with NASH (Table 1). The liver biopsy of all subjects with NASH showed evidence of cellular ballooning. Hepatic fibrosis was seen in five subjects. The NASH activity score was 5.6 ± 1.4. The subjects with NASH were obese, had higher BMI, higher body surface area, elevated plasma ALT (plasma alanine aminotransferase), triacylglycerols, had higher fasting insulin levels and higher measure of insulin resistance (HOMA), when compared with controls. The plasma concentration of 8-hydroxy-2-deoxyguanosine, a measure of oxidative damage, was not different between the groups. The plasma concentrations of usCRP (ultrasensitive CRP) and TNF-$\alpha$ were significantly higher in NASH (Table 2). In addition, several other inflammatory cytokines were significantly higher in subjects with NASH compared with controls. The plasma concentrations of folate, vitamin B12 and TSH (thyroid stimulating hormone) in both healthy controls and NASH were within normal range (results not shown).

There were ten heterozygotes and no homozygote for MTHFR (677C $\rightarrow$ T) polymorphism among subjects with NASH, and there were eight heterozygotes and one homozygote among the controls. The plasma concentrations of total cysteine, homocysteine and glutathione during fasting were not different between the controls and NASH subjects (Table 3). In response to enteral protein load, there was a significant decrease in total cysteine and increase in tHcy in the plasma in control subjects. In subjects with NASH,
Table 3  Plasma concentration of total glutathione, cysteine and homocysteine
Values are means (S.D.). *P = 0.014, **P = 0.003 and ***P = 0.001 compared with the fasted state, as determined using a paired Student’s t test.

<table>
<thead>
<tr>
<th>Group</th>
<th>Cysteine (μmol/l)</th>
<th>Homocysteine (μmol/l)</th>
<th>Glutathione (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NASH (n = 11)</td>
<td>404.1 (60.8)</td>
<td>403.5 (56.2)</td>
<td>8.8 (3.2)</td>
</tr>
<tr>
<td>Controls (n = 11)</td>
<td>381.8 (47.0)</td>
<td>366.7 (43.2)*</td>
<td>7.9 (0.9)</td>
</tr>
</tbody>
</table>

Table 4  Respiratory calorimetry
Values are means (S.D.).

<table>
<thead>
<tr>
<th>Group</th>
<th>$V_{O_2}$ (mmol · kg$^{-1}$ of body weight · h$^{-1}$)</th>
<th>$V_{CO_2}$ (mmol · kg$^{-1}$ of body weight · h$^{-1}$)</th>
<th>RER</th>
</tr>
</thead>
<tbody>
<tr>
<td>NASH (n = 15)</td>
<td>7.04 (0.74)</td>
<td>7.54 (0.95)</td>
<td>5.59 (0.66)</td>
</tr>
<tr>
<td>Controls (n = 19)</td>
<td>7.31 (1.97)</td>
<td>8.14 (0.90)</td>
<td>6.14 (0.63)</td>
</tr>
</tbody>
</table>

Table 5  Phenylalanine Ra (a) and urea Ra (b)
Values are means (S.D.). *P = 0.002, **P = 0.016, ***P = 0.01 and ****P = 0.004 compared with controls; †P = 0.001 compared with fasting.

(a) Phenylalanine Ra

<table>
<thead>
<tr>
<th>Group</th>
<th>Phenylalanine Ra (μmol · kg$^{-1}$ of body weight · h$^{-1}$)</th>
<th>Phenylalanine Ra (mmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NASH (n = 11)</td>
<td>30.0 (4.5)</td>
<td>35.9 (6.8)†</td>
</tr>
<tr>
<td>Controls (n = 10)</td>
<td>32.4 (5.6)</td>
<td>38.7 (6.6)†</td>
</tr>
</tbody>
</table>

(b) Urea Ra

<table>
<thead>
<tr>
<th>Group</th>
<th>Urea Ra (μmol · kg$^{-1}$ of body weight · h$^{-1}$)</th>
<th>Urea Ra (mmol/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NASH (n = 11)</td>
<td>208.6 (63.8)**</td>
<td>201.0 (54.6)****</td>
</tr>
<tr>
<td>Controls (n = 10)</td>
<td>333.2 (92.6)</td>
<td>297.9 (76.8)†</td>
</tr>
</tbody>
</table>

there was a significant increase in plasma homocysteine, and no change in plasma total cysteine concentration in response to protein feeding. There was no change in plasma glutathione in either group. The respiratory calorimetry findings are displayed in Table 4. There was no significant difference between the groups for $V_{O_2}$, $V_{CO_2}$ or the RER (respiratory exchange ratio) during fasting or during the fed state.

Phenylalanine kinetics

The phenylalanine $R_d$ was calculated by tracer dilution during isotopic steady state. The results on subjects studied both during fasting and in response to feeding are displayed (Table 5). There was no significant difference in weight-specific phenylalanine $R_d$ between the controls and NASH during fasting [NASH (n = 15), 30.9 ± 5.1 μmol · kg$^{-1}$ of body weight · h$^{-1}$; Controls (n = 18), 32.9 ± 5.7] or during feeding (Table 5), suggesting an unchanged rate of whole body protein breakdown. Total phenylalanine $R_d$ was significantly higher in NASH subjects, reflecting their higher body weight. However, phenylalanine $R_d$, adjusted for BMI, was not different between controls and NASH (ANCOVA). There was a significant negative correlation between plasma insulin (rho = 0.47, P = 0.042), HOMA (rho = 0.48, P = 0.038) and phenylalanine $R_d$, during fasting, in the control subjects only. A positive correlation between plasma bilirubin levels and phenylalanine $R_d$ during fasting was seen in both controls (rho = 0.59, P = 0.008) and in subjects with NASH (rho = 0.62, P = 0.044). In response to the mixed nutrient load, there was a significant increase in phenylalanine $R_d$ in both NASH and healthy controls (Table 5).

Urea synthesis

The rates of urea synthesis in subjects studied both during fasting and during the fed state are displayed (Table 5). The weight-specific rate of urea synthesis was significantly lower in subjects with NASH, both during fasting and in response to feeding. In response
to the feed, the rate of urea synthesis decreased ($\Delta 3.53 \pm 23.6\, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$) in all control subjects ($P < 0.001$). The response of NASH subjects was variable, namely a decrease in seven subjects and unchanged or an increase in four subjects. The rate of urea synthesis, adjusted for BMI, was not different in controls and subjects with NASH. There was a significant negative correlation between urea $R_a$ and usCRP both during fasting and following feed in the NASH group (fast: $\rho = -0.69$, $P = 0.004$; fed: $\rho = -0.78$, $P = 0.005$). Additionally, urea $R_a$ was also negatively correlated with the HOMA score during fasting in the NASH group ($\rho = -0.5$, $P = 0.05$).

**Methionine kinetics**

As with phenylalanine $R_a$, the methionine $R_a$ measured using the [1-$^{13}$C]methionine tracer was not different between NASH and controls both during fasting and in the fed state (Table 6). In contrast, the weight-specific methionine $R_a$, measured using [C$^{3}$H$_{3}$]methionine tracer was significantly lower during fasting in subjects with NASH. Although the fractional rate of trans-sulfuration of methionine measured by the appearance of $^{13}$C in expired CO$_2$ was higher during fasting in NASH (NASH, 14.2 $\pm 3.9\%$; controls, 10.5 $\pm 4.1\%$; $P = 0.047$), the actual rate of trans-sulfuration was not different between the two groups (Table 6). The rate of methylation of homocysteine ($R_m$, the difference between methionine $R_a$ quantified by the two tracers) and the rate of transmethylation of methionine ($T_m$) was significantly lower ($P < 0.03$) during fasting in subjects with NASH compared with controls. As anticipated, in response to feeding, there was a significant increase in all the measured parameters of methionine kinetics: $R_a$, trans-sulfuration, remethylation and transmethylation in both controls and in subjects with NASH.

**$^{13}$C enrichment of homocysteine**

We measured the $^{13}$C enrichment of homocysteine in order to estimate the enrichment of intracellular pool of methionine. During fasting, the ratio of $^{13}$C enrichment of plasma homocysteine and methionine was not different in the two groups (NASH, 0.36 $\pm 0.06$; controls, 0.32 $\pm 0.07$; $P = 0.16$). In response to mixed nutrient feeding, there was an increase in $^{13}$C enrichment of homocysteine in all subjects, suggesting a suppression of intracellular protein breakdown. In contrast, due to the entry of unlabelled methionine into the circulation, there was a significant decrease in $^{13}$C and C$^{3}$H$_{3}$ enrichment of plasma methionine. The net effect was a significant increase in the ratio of $^{13}$C enrichment of plasma homocysteine and methionine (NASH, 0.59 $\pm 0.07$; controls, 0.53 $\pm 0.07$; $P = 0.045$).

MTHFR (677 C$\rightarrow$T) heterozygosity had no impact on methionine metabolism, neither in controls nor in subjects with NASH (results not shown).

**DISCUSSION**

The results of the present study show that, in subjects with NASH, in association with insulin resistance, the whole body rate of protein turnover as well as the rate of urea synthesis, during fasting, were unchanged when compared with healthy controls. The rate of transmethylation of methionine was lower in NASH when compared with controls. There was no significant change in the absolute rate of trans-sulfuration of homocysteine, although the fractional rate of trans-sulfuration was higher in NASH. These findings should be examined in the context of insulin resistance and of insulin resistance plus steatohepatitis.

**Phenylalanine kinetics**

The weight-specific phenylalanine $R_a$ was not significantly different between the controls and subjects with NASH, both in the basal state and in response to enteral nutrient (protein) administration. The total phenylalanine $R_a$ was significantly higher in NASH. However, when adjusted for BMI, all differences became statistically not significant. In addition, a subgroup analysis of subjects matched for body weight did not show any significant difference in phenylalanine $R_a$ during fasting between controls and NASH (controls ($n = 11$), 2.6 $\pm 0.6$ mmol/h;
NASH \( (n = 10) \), 2.9 \( \pm \) 0.6; \( P = 0.23 \). These results suggest that insulin resistance in NASH, as measured by HOMA, does not appear to have any significant impact on the whole body rate of protein breakdown during fasting. These findings are similar to other studies where no difference in whole body rate of protein turnover was observed between lean and obese insulin-resistant subjects with and without glucose intolerance [33–36]. This is in contrast with the acutely induced insulin resistance by intravenous fatty acid infusion, where a decrease in the phenylalanine \( R_a \) across the leg was observed [37]. The fatty-acid-induced decrease in proteolysis may be a direct inhibitory effect of fatty acids on protein breakdown [38,39]. The negative correlation between plasma insulin levels and phenylalanine \( R_a \) in both controls and NASH. Although the exact reason for this observation cannot be discerned from the present results, the correlation may reflect the contribution of red blood cell (haemoglobin) turnover to phenylalanine \( R_a \). Only studies in subjects with high red blood cell turnover will confirm this hypothesis.

### Urea synthesis

The weight-specific urea \( R_a \) was significantly less in the NASH both during fasting and during the fed state. However, total rate of urea synthesis and the urea \( R_a \) adjusted for BMI were not significantly different among the two groups, suggesting no impact of steatohepatitis on urea synthesis. These findings are similar to those reported by us previously [22]. In response to feeding, as anticipated, there was a decrease in urea \( R_a \) in healthy subjects, probably mediated by an increase in insulin and decrease in glucagon levels [44–46]. In contrast, we did not observe a consistent suppression of urea synthesis in NASH, suggesting an impaired hepatic insulin action in NASH. This is in agreement with the significant negative correlation between urea \( R_a \) and HOMA in this group. The resistance to insulin action on urea synthesis is in contrast with our previous results where urea \( R_a \) decreased in response to change in hepatic redox induced by increase in hepatic fatty acid oxidation [22], suggesting a discordance between hormonal and redox regulation of urea synthesis in NASH. The negative correlation between usCRP and urea \( R_a \) in NASH may be related to the inflammation-induced insulin resistance and consequent impaired action of insulin on urea synthesis.

### Methionine metabolism in NASH

As reported by us previously [22], there was no significant difference in the plasma concentrations of cysteine, homocysteine and glutathione, during fasting among healthy controls and subjects with NASH (Table 3). The findings on the association between insulin resistance and plasma homocysteine levels in humans are conflicting. Acute hyperinsulinaemia in healthy humans caused a decrease in plasma homocysteine levels possibly due to acute suppression of proteolysis by insulin [47]. In contrast, streptozotocin-induced lack of insulin in the rat, by increasing the trans-sulfuration flux, resulted in a significant (30%) decrease in plasma homocysteine levels [48]. Treatment with insulin resulted in an increase in tHcy levels. A significant negative correlation was seen between plasma insulin levels [49], measures of insulin resistance [50,51] and plasma homocysteine concentration in healthy human subjects. In contrast, other studies in human have shown a positive [52,53] or no [54] significant correlation between tHcy levels and measures of insulin resistance. The positive correlation has been attributed to the confounding effect of associated low-grade inflammation [high CRP and IL (interleukin)-6] in these subjects [53]. Thus the observed lack of significant difference in plasma homocysteine levels in our subjects may also be related to the associated inflammation in subjects with NASH. In response to a mixed nutrient load, there was a significant increase in plasma tHcy levels in both groups. The increase in plasma homocysteine is consistent with the increase in transmethylation flux following a protein load (Table 6).

The mechanism for the decrease in plasma cysteine concentration following a protein load in the healthy controls is unclear. The lack of any change in the plasma glutathione levels following a nutrient load (Table 3) is in contrast with the results showing an increase in plasma glutathione in NASH in response to an intravenous intralipid infusion [22]. These differences, i.e. no response to enteral mixed nutrient and parenteral fatty acid load, reflect the difference in the magnitude of oxidative stress induced by the two interventions.

During fasting, there was no significant effect of insulin resistance and NASH on the whole body methionine \( R_a \) (Qc). These findings are similar to those for phenylalanine (Table 5) and add credence to these analyses. The whole body (primarily the liver) rate of remethylation of homocysteine and of transmethylation of methionine was significantly lower in subjects with NASH. The possible mechanisms of this decrease include: (i) a lower availability of methyl groups, not likely since the NASH subjects were all folate sufficient and had plasma folate levels in the normal range; (ii) a lower methylation demand, also not likely since the major reactions that require methyl groups, i.e. creatine synthesis and VLDL export, are not known to be attenuated in NASH; creatine synthesis is unlikely to
be changed in the presence of unchanged skeletal muscle mass, and VLDL triacylglycerol export is expected to be higher in subjects with obesity and insulin resistance; (iii) the lower rate of transmethylation is not likely to be related to impaired insulin action (insulin resistance), since both lack of insulin and insulin resistance in animal models cause an increase in the expression and activity of BHMT (betaine homocysteine methyltransferase) and therefore increase the remethylation of homocysteine [55,56]; in contrast, in human subjects with Type 1 diabetes, withdrawal of insulin was associated with lower rates of methylation of homocysteine, and insulin treatment normalized the transmethylation rate [57]; the reason for this discrepancy between animal and human findings is not clear, but may be related to the effect of counter-regulatory hormones in Type 1 diabetes, and to acute (12 h) withdrawal of insulin in humans [57] and to chronic lack of insulin in the animal model [55,56]; (iv) we propose that the lower rate of transmethylation in subjects with NASH is related to oxidant injury [4–7] and change in the redox state of the hepatocytes. The synthesis of SAM by SAM synthase is regulated by glutathione [58] and hypoxia [59], and modified by oxidant injury and redox state of the cell [17,18]. A decrease in the synthesis of SAM, as a result of MAT1A deletion in mice, predisposes the animal to hepatic injury and makes them more susceptible to choline-deficient diet-induced fatty liver [19]. We speculate that, as a consequence of high rate of hepatic fatty acid oxidation [22], there is a high rate of production of ROS and decreased availability of glutathione, resulting in lower activity of methionine adenosyl transferase and a lower rate of transmethylation.

Although the fractional rate of trans-sulfuration was higher in NASH, the total rate was not different from that in the control. The enzymes of the trans-sulfuration pathway, cystathionine β-synthase and cystathionine γ-lyase, are down-regulated by insulin and up-regulated by glucagon and glucocorticoids and by lack of insulin [45,52]. We had hypothesized that, in the presence of insulin resistance, and in the presence of higher demands for glutathione in NASH, the flux through this pathway would be increased. The unchanged rate of trans-sulfuration may simply reflect a compensated state, in order to meet the heightened demands for glutathione, in the presence of a lower rate of flux through the methionine cycle.

In summary, results from our present study show for the first time a lower rate of transmethylation of methionine in insulin-resistant subjects with NASH. These results point to the potential mechanism for hepatocellular injury in NASH and suggest a rationale for the use of SAM [60] and antioxidant therapy [61] for therapeutic interventions. It will be interesting to evaluate whether antioxidant therapy in NASH patients would increase the transmethylation of methionine and increase the rate of VLDL expression, or whether SAM supplementation would increase VLDL secretion.

**AUTHOR CONTRIBUTION**

Satish Kalhan was responsible for all aspects of the study, conceived the protocol, executed the clinical studies, supervised laboratory and data analysis, and wrote the manuscript. John Edmison helped in development and execution of the study. Susan Marczewski was responsible for all clinical aspects and data management. Srinivasan Dasarathy helped with subject recruitment and the clinical studies. Lourdes Gruca, Carole Bennett and Clarita Duenas did the laboratory analysis, and Rocío Lopez did the statistical analysis. All authors read the manuscript and approved its contents.

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**REFERENCES**


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