Betaine for Nonalcoholic Fatty Liver Disease: Results of a Randomized Placebo-Controlled Trial

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Based on animal studies and pilot studies in humans, betaine, a methyl donor for the remethylation of homocysteine, may be a therapeutic agent for nonalcoholic steatohepatitis (NASH). We evaluated the safety and efficacy of betaine for patients with NASH and whether betaine positively modified factors postulated to be "second hits" and underlying mechanisms of NASH. We conducted a randomized placebo-control study of 55 patients with biopsy-proven NASH who received either oral betaine (20 g daily) or placebo for 12 months. Pre- and posttreatment variables were analyzed using the paired *t* test or Wilcoxon rank test. Treatment groups were comparable at baseline. Of the 35 patients (17 betaine, 18 placebo) who completed the study, 34 patients (16 betaine, 18 placebo) underwent posttreatment liver biopsy. Patients randomized to betaine had a decrease in steatosis grade. No intra- or intergroup differences or changes in nonalcoholic fatty liver disease activity score or fibrosis stage were noted. Elevations of insulin, glucose, and proinflammatory cytokines and the reduced antioxidant status noted in NASH patients did not improve with betaine therapy. The antiinflammatory agent adiponectin was significantly reduced in both groups and did not change with therapy. Lastly, S-adenosylhomocysteine was approximately twice normal and was not reduced by betaine therapy. *Conclusion:* Compared to placebo, betaine improved hepatic steatosis and may protect against worsening steatosis. High-dose betaine supplementation failed to reduce S-adenosylhomocysteine and did not positively affect any of the second hit mechanisms postulated to contribute to NASH that we studied. Although betaine has been proven effective in treating hepatic steatosis in several animal models, translating novel therapeutic options noted in animal studies to humans with NASH will prove challenging. (HEPATOLOGY 2009;50:1818-1826.)

Nonalcoholic fatty liver disease (NAFLD) is characterized by excessive accumulation of lipids in the livers of patients without clinical evidence of alcohol abuse.[1] Patients with nonalcoholic steatohepatitis (NASH), the intermediate stage of NAFLD, are at risk for fibrosis progression and poor clinical outcomes.[2,3] Proposed mechanisms for disease progression include impaired transmethylation (pathway depicted in Fig. 1), proinflammatory cytokines, oxidative stress, and insulin resistance. Despite a proposed role for oxidant stress in the pathogenesis of NASH, antioxidant approaches have had mixed results. Animal models of NAFLD have demonstrated reduced hepatic S-adenosylmethionine (SAM), the major methyl donor, with increased levels of both homocysteine and S-adenosylhomocysteine (SAH). Betaine (trimethylglycine), originally discovered in the juice of sugar beets, reduces circulating levels of homocysteine by facilitating its conversion to methionine, which also decreases SAH.

Betaine, derived from the oxidation of dietary sources of choline, is the only known alternative source of methyl groups for the conversion of homocysteine to methionine. Betaine can also substitute for SAM as a methyl donor for the direct methylation of phosphatidyletha-

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ER, endoplasmic reticulum; IL-1, interleukin-1; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, macrophage chemoattractant protein-1; PAI-1, plasminogen activator inhibitor-1, SAH, s-adenosylhomocysteine; SAM, s-adenosylmethionine; TNFα, tumor necrosis factor.

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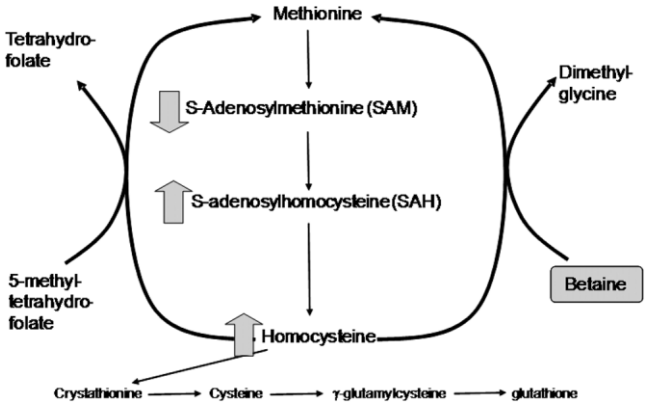

Fig. 1. Animal models of NASH demonstrate increased intrahepatic SAH with reduced intrahepatic SAM and betaine.

nolamine. This pathway serves as an alternative route of phosphatidylcholine formation. From these functions, betaine is thought to derive its beneficial effects on intermediary metabolism and alleviate defects in the methylation pathway caused by deficiencies or impaired function of the folate cycle and vitamin B_{12}.[4] Animal models have demonstrated that betaine could substitute for choline and function as an effective dietary methyl donor when dietary methionine is insufficient.[5] By demonstrating that betaine is a substance capable of reducing lipid accumulation in the liver, these experiments established betaine as a potentially effective lipotropic and attractive pharmacologic agent for the treatment of NAFLD.

In a pilot study of patients with NASH, betaine improved liver biochemistry and demonstrated an encouraging trend toward improvement in steatosis, necroinflammation, and fibrosis after 12 months of treatment.[6] On this basis, we hypothesized that betaine supplementation may attenuate hepatic necroinflammation and show improvement of biochemical parameters in patients with NASH.

We conducted a multicenter, randomized, double-blind, placebo-controlled trial safety and potential efficacy of 12 months of betaine anhydrous oral powder (Cystadane, Orphan Europe, Paris, France) 20 g daily versus placebo on liver biochemistries and the histologic features of NASH. The primary objective was to determine whether betaine therapy improved serum aminotransferases (percent change from baseline to month 12) and/or quality of life compared to placebo. Treatment success was defined as improvement by $\geq$50% or normalization of serum aminotransferases among individual patients and absence of treatment-related failure as defined by the occurrence of death, liver transplantation, the development of cirrhotic complications, and discontinuation of betaine for any reason. Secondary efficacy objectives included tolerance to study medication and a change in the composite and individual components of the histologic features of NASH as defined by Brunt et al.[7] The study was approved by the Institutional Review Board and Ethics Committee.

Patients and Methods

Study Design. This study was an investigator-initiated study sponsored by Orphan Medical (Minnetonka, MN) between two study sites (Mayo Clinic, Rochester, MN, and University of Florida, Gainesville, FL). Fifty-five consecutive patients with biopsy-proven NASH were enrolled after undergoing a thorough clinical evaluation. Fasting blood samples were drawn to measure liver biochemistries, plasma glucose levels, lipid profile, and complete blood counts. Liver biochemical parameters (i.e., serum alanine aminotransferase [ALT], aspartate aminotransferase [AST], alkaline phosphatase, total bilirubin, albumin), prothrombin time, hematologic parameters, fasting blood glucose, and lipids were measured at baseline and at the end of the study. ALT and AST were monitored every 3 months during the study. All women of child-bearing potential had a negative pregnancy test at enrollment and agreed to two methods of contraception while on study drug. Abdominal ultrasound was performed at entry to assess the liver parenchyma and exclude any complication(s) of chronic liver disease or portal hypertension. Screening for esophageal varices and/or hepatocellular carcinoma was performed if a patient had advanced fibrosis (i.e., fibrosis stage 3-4) on liver biopsy.

Initiation of any form of therapy that may confound study results (i.e., insulin-sensitizing agents, lipid lowering agents, S-adenosylmethionine, or vitamin E) from time of liver biopsy to randomization to study drug was not permitted. All participants were given appropriate nutritional and weight-loss advice but no additional weight loss strategies were offered. Subjects were randomly assigned in a 1:1 ratio to receive betaine anhydrous oral solution or a matching placebo.

The appropriate dose and interval of betaine administration have not been established.[8] For the treatment of homocystinuria, the usual adult dose is 6 g per day in two divided doses administered orally; however, doses up to 20 g per day have been utilized to decrease homocysteine levels in some patients.[9] We utilized a "top-down" dosing regimen so that a potential therapeutic benefit of betaine (if one existed) could be observed. All patients received anhydrous betaine (Cystadane, Orphan Europe) 20 g orally or a similar-appearing placebo in two divided doses per day for 12 months. Patients were contacted by phone after 2 weeks then monthly thereafter to ascertain adverse events and compliance. Patients were reevaluated at the

study site at 6 and 12 months following initiation of study drug with complete history, physical examination, laboratory studies, and completion of the Short Form 36 (SF-36) Health Survey. A liver histopathologist at each study site blinded determined the fibrosis stage for equal randomization of patients with advanced fibrosis between treatment groups. All pre- and posttreatment biopsies were reviewed by a single pathologist blinded to drug randomization, clinical response, and timing of the liver biopsy.

Inclusion Criteria. Men and women, 18-70 years old, who satisfied all of the following inclusion criteria were eligible to participate: 1) histologic features of NASH, defined as greater than 10% steatosis along with necroinflammatory changes (NAS score $\geq$4) regardless of fibrosis, within the previous 6 months; 2) elevation of ALT or AST at least 1.5 times normal on at least two different occasions within 6 months of enrollment; and 3) ethanol consumption of $<$20 g/day for women and $<$30 g/day for men. We defined aminotransferase elevation based on National Health and Nutrition Examination Survey (NHANES) III criteria; for men this corresponded to an AST $>$37 U/L or ALT $>$40 U/L, and for women an AST or ALT $>$31 U/L.[10]

Exclusion Criteria. Subjects were excluded from the study if any of the following criteria existed: 1) any form of chronic liver disease other than NAFLD; 2) women who were pregnant, lactating, or of childbearing potential and unwilling or unable to use adequate method(s) of contraception; 3) ALT or AST levels greater than 5 times the normal; 4) abnormal total bilirubin level or albumin, prolonged prothrombin time, or platelet count below the lower limit of normal; 5) serum creatinine level $\geq$1.5 mg/dL; 6) creatine kinase level $\geq$3 times the normal on baseline laboratory excluded from further study participation. Any patient anticipated to need liver transplantation within 1 year or with a comorbid medical condition(s) that might preclude successful completion of study was excluded. Treatment with any drugs known to cause hepatic steatosis (i.e., corticosteroids, high-dose estrogens, etc.) was not allowed. Therapy with approved drugs that may have potential benefit in the treatment of NAFLD (i.e., vitamin E, betaine, pioglitazone, rosiglitazone, pentoxifylline, gemfibrozil) was excluded. Use of metformin or other insulin-sensitizing agents for the treatment of diabetes mellitus were allowed only if patients were on stable doses at the time of inclusion liver biopsy and for the 6 months prior to study enrollment.

Study drug was discontinued if any of the following study-specific criteria emerged: 1) ALT or AST values $\geq$10 times normal; 2) symptomatic liver disease, as defined by the development of severe malaise, ascites, jaundice associated with direct hyperbilirubinemia, or a Child-Pugh score $\geq$5 in subjects with known cirrhosis; 3) abnormal serum albumin, total bilirubin, and/or prolongation of prothrombin time on two consecutive assessments; or 4) any reason based on the opinion of the investigator. After the withdrawal of the study drug, all patients continued study participation, including regularly scheduled visits and laboratory assessments.

Safety Evaluations. Patients were evaluated at the study center every 3 months and phone communication was made with each study participant on a monthly basis to assess compliance with study medication and development of any adverse events. The study medication was decreased by 3-g increments if symptoms of intolerance (i.e., nausea, vomiting, bloating, etc.) were reported. If a dose reduction occurred, the patient was maintained on their maximal tolerated dose of study medication for the remainder for the study. A statistician, not involved in the conduct of the study, provided an independent data safety monitoring committee which met on an interim basis and made recommendations about study continuation, with unblinded safety data including incidence of adverse events and laboratory results during the study.

Methods. Multiple serological tests were performed in NASH subjects before and after therapy as well as in an 11 member healthy control group at one timepoint. Members of the healthy control group had normal liver enzymes and no personal history of liver disease, obesity, or diabetes. Serum levels of SAH and homocysteine were assayed by the method of Capdevila et al.[11] using the Axis Homocysteine Enzyme Immunoassay Kit (Axis-Shield Diagnostics, Dundee, Scotland). The serum concentrations of SAM were assayed by reverse-phase HPLC by a modified method of Wang et al.[12] Serum methionine levels were measured by the modified method of De Antonis et al.[13]

Serum total antioxidant activity was measured with the Antioxidant Assay Kit (Cayman Chemical, Ann Arbor, MI) which evaluates the ability of antioxidants in the sample to inhibit the oxidation of 2,2′-azino-di-[3-ethylbenzthiazoline sulphonate] by metmyoglobin. Serum cytokines and adipokines were determined by multianalyte chemiluminescent detection using LINO*plex* Human Adipokine Panel A and B kits (Millipore, Billerica, MA) on the Luminex 100 IS system (Austin, TX). Specifically, tumor necrosis factor α (TNFα), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), macrophage chemoattractant protein-1 (MCP-1), adiponectin, leptin, insulin, resistin, and total plasminogen activator inhibitor-1 (PAI-1), were measured. Serumcytokeratin-18 whole (M65) and caspase-cleaved fragments (M30) were measured by enzyme-linked immunosorbent assay

(ELISA) (diaPharma, Columbus, OH) in healthy controls to further exclude active liver disease.

Statistical Methods. Data from both study sites were combined for the purpose of analysis. All analyses were conducted with SAS v. 8.2 or higher (Cary, NC). Statistical analysis was performed using analysis of variance (ANOVA) and paired *t* tests. A two-sided *P*-value of < 0.05 was considered statistically significant.

Sample Size and Power. NAFLD and/or significant liver disease due to NAFLD may exist with liver enzymes in the normal range[14] and that liver enzymes fluctuate in patients with NASH.[15] Given the insensitivity of serum aminotransferases as a measure of treatment response (as opposed to safety assessment), we elected to use histologic features of NAFLD as the "gold-standard" endpoint to define treatment response and to power the sample size for this study. Stratified randomization was executed in an attempt to balance each treatment group with respect to the presence or absence of bridging fibrosis or cirrhosis. Based on our pilot study,[6] we anticipated that at least 50% of patients randomized to betaine would have an improvement (at least 2 points in steatosis, inflammation grade and/or fibrosis stage) in liver histology compared to no more than 10% of those patients randomized to placebo. With 40 patients evaluable, the study would have at least an 80% power at an α-value of 0.05 (two-tailed) to detect a significant difference in histology response rate when betaine was compared to placebo. An enrollment of 46 patients (23 per group) was planned in anticipation of a 15% dropout rate.

All efficacy analyses were performed on the intent-to-treat population, which was defined as all randomized subjects who took at least 1 dose of double-blind medication and had at least 1 postbaseline measurement. The safety population was defined as all subjects randomized who took at least one dose of the study drug.

Endpoints. The primary efficacy endpoint (the percentage change from the baseline to month 12 ALT) was analyzed with a last observation carried forward algorithm for subjects who withdrew prematurely or missed an intermediate efficacy assessment. An analysis of covariance was used and the primary comparison of interest was the treatment group of betaine versus the placebo. In cases of severe nonnormality, nonparametric techniques were employed to analyze the data. The percentage changes from the baseline to month 12 in quality of life and histological endpoints were the secondary endpoints and they were analyzed with the same analysis of covariance model and summary statistics specified for the primary endpoint. The primary safety endpoint was the overall proportion of subjects with at least one ALT value at least 2 times normal for those subjects with normal ALT at the baseline or a doubling of the baseline ALT for those subjects with elevated ALT at the baseline (called an ALT event) within the 12 months of initiating the drug. The chosen biochemical safety endpoint for evaluation of potential drug-related hepatotoxicity was considered the simplest and most direct means of detecting a potentially meaningful difference between the treatment groups, with the clinical significance of such a difference being addressed with other parameters, including associated symptoms and concomitant bilirubin elevations. Descriptive statistics of ALT values at baseline, at each scheduled visit, and change from baseline to each scheduled visit were assessed.

Results

Enrollment. During the period between March 2003 and June 2005, 55 subjects were enrolled and randomly assigned to a treatment (26 betaine, 29 placebo). A dropout rate in excess of 15% was noted and enrollment was increased from 46 subjects (23 per group) to 55 patients in hopes of achieving the sample size necessary to maintain statistical power. Two patients who consented and were randomized to the placebo arm were subsequently noted not to meet all study exclusion criteria and were withdrawn from further study participation by the investigators. Overall, 17 versus 18 (65.4% versus 62.0%, $P =$ not significant [NS]) of subjects randomized to betaine and placebo, respectively, completed the study.

Demographics and Baseline Patient Characteristics. No differences between treatment groups were noted at baseline (Table 1). Twelve subjects (34%) with advanced fibrosis or cirrhosis enrolled in the study and were equally distributed between treatment groups. The majority of subjects in both groups were white, and the mean age was 47 years. The mean body mass index (BMI) was 33.6 kg/m^2.

Eighteen patients (33%) had evidence of type 2 diabetes (fasting glucose $>$126 mg/dL on at least two occasions) and were equally distributed between treatment groups. All patients with a known diagnosis of diabetes mellitus were on stable doses of insulin-sensitizing agent(s) at time of liver biopsy, study enrollment, and throughout the study period. Thirty-eight patients (69%) had hypertriglyceridemia (fasting triglyceride $\geq$200 mg/dL), 28 patients (51%) had hypercholesterolemia (fasting cholesterol $>$200 mg/dL), and 41 patients (75%) had low HDL-cholesterol levels (fasting HDL-cholesterol $<$50 mg/dL for women, $<$40 mg/dL for men). Twenty-two patients (40%) had hypertension (systolic blood pressure $\geq$140 and/or diastolic blood pressure $\geq$90 mmHg). All patients requiring oral insulin-sensitizing agents, insulin,

Table 1. Baseline Characteristics and Laboratory Values (All Randomized Subjects)

Parameter	Betaine (n = 27)	Placebo (n = 28)	*P*-value
Age (years)			
Mean (SD)	47.8 (11.4)	45.7 (13.4)	P = NS
Range			
Gender (n, [%])			
Male	12 (44%)	8 (29%)	P = NS
Female	15 (56%)	20 (71%)	
Race (n, [%])			
White	24 (89%)	25 (89%)	
Hispanic	2 (7%)	3 (11%)	
Other	1 (4)	0	P = NS
Body mass index (kg/m^2)			
Mean (SD)	32.4 (6.0)	34.7 (5.6)	P = NS
Median	31.4	35.0	
Range	26–48.5	19–45	
Diabetes mellitus	9 (33%)	9 (32%)	P = NS
Use of insulin sensitizing agents	9 (33%)	8 (28%)	P = NS
Hyperlipidemia	18 (67%)	20 (71%)	P = NS
Hypertension	11 (41%)	11 (39%)	P = NS
ALT (U/L)			
n	27	27	
Mean (SD)	106.3 (59.6)	86.0 (37.6)	
Range	36–292	35–156	P = NS
AST (U/L)			
n	27	27	
Mean (SD)	69.5 (34.8)	65.9 (34.3)	
Range	19–151	29–176	P = NS
Alkaline phosphatase (U/L)			
n	27	26	
Mean (SD)	121.5 (82.3)	117.6 (58.2)	
Range	25–311	48–208	P = NS
Total bilirubin (mg/dL)			
n	27	27	
Mean (SD)	0.78 (0.65)	0.64 (0.43)	
Range	0.2–3.0	0.1–2.4	P = NS
Albumin (g/dL)			
n	27	24	
Mean (SD)	4.4 (0.48)	4.4 (0.26)	
Range	3.6–5.2	3.9–5.0	P = NS

lipid lowering agents, and/or angiotensin II converting enzyme inhibitors for complications of the metabolic syndrome of insulin resistance, medications were on stable doses at the time of liver biopsy prior to randomization.

Eleven healthy controls were used as a comparison group for assays of methionine metabolism and cytokines. Healthy controls had a mean age of 44.3 ± 11.4 years, BMI of 24.9 ± 4.9, were on no medications, had no underlying health problems, and were employees of the University of Louisville. Although members of the control group did not have liver biopsies, these subjects were probably free from liver disease on the basis of normal mean liver enzymes, blood glucose, BMI, and cytokeratin-18 levels.

Clinical and Biochemical Results. Interval changes in serum aminotransferases during treatment at 3, 6, and 9 months and at the end of the study are summarized in Table 2. Of those who completed 12 months of therapy (betaine 17, placebo 18), no changes in weight, BMI, or serum aminotransferases or lipid or laboratory safety monitoring parameters (data not shown) were noted (Table 2). The proportion of patients who normalized AST and ALT was also not different between groups (Table 2).

Histology Results. Despite the lack of change in weight, BMI, or serum aminotransferases, patients randomized to betaine had a decrease in steatosis grade (−0.41 versus 0.67, $P < 0.005$) (Table 2). The proportion of patients in the betaine versus placebo group in whom steatosis improved by ≥1 grade was 29% versus 61%, $P < 0.01$; remained unchanged was 71% versus 22%, $P < 0.005$; and worsened by ≥1 grade was 0 versus 17%, P = NS (Fig. 2). Any change in steatosis could not be attributed to patient reports of drug-related nausea or weight loss during the study. Mean change in NAS score in betaine versus placebo group was −0.2 ± 0.9 versus −1.0 ± 2.6 (P = NS). In the betaine versus placebo groups, the NAS score improved by ≥1 point in 24% versus 61%, $P < 0.05$; remained unchanged in 42% versus 11%, P = NS; and worsened ≥1 point in 35% versus 28%, P = NS (Fig. 3). A change in fibrosis was not observed in either treatment group over the course of the study (data not shown).

Table 2. Mean Changes from Baseline in Patients Who Completed 12 Months of Therapy

Parameter	Betaine (n = 17)	Placebo (n = 18)	*P*-value
Δ Weight (kg)	−3.3 ± 3.1	0.62 ± 10.9	0.65
Δ ALT (U/L)	−32.1 (35.5)	−10.9 (44.5)	0.13
Mean % change (SD) in ALT			
3 months	n = 22	n = 20	0.97
	−9.7 (38.2)	−10.1 (31.4)	
6 months	n = 20	n = 21	0.46
	4.5 (64.8)	−7.1 (47.1)	
9 months	n = 18	n = 19	0.42
	5.5 (83.7)	−9.7 (45.6)	
12 months	n = 18	n = 19	0.13
	−20.0 (32.7)	−2.7 (47.4)	
Δ AST (U/L)	−19.8 (27.4)	−0.3 (44.7)	0.13
Mean % change (SD) in AST			
3 months	n = 22	n = 20	0.91
	10.1 (22.6)	−9.3 (25.5)	
6 months	n = 20	n = 21	0.52
	2.3 (38.2)	−4.8 (39.1)	
9 months	n = 18	n = 19	1.0
	−4.5 (29.9)	−4.4 (40.3)	
12 months	n = 18	n = 19	0.15
	−12.6 (24.4)	−1.7 (43.5)	
% Normalization of AST	8 (47%)	6 (33%)	0.50
% Normalization of ALT	5(29%)	5 (27%)	0.72
Δ in Steatosis grade (SD)	−0.4 (0.71)	0.7 (1.24)	0.46
Δ in NAS score (SD)	−0.2 (1.7)	−1.0 (2.6)	0.28
Δ in Lobular inflammation (SD)	0.12 (0.86)	0.28 (1.23)	0.28
Δ in Hepatocyte ballooning (SD)	0.35 (0.70)	0.0 (0.91)	0.21
Δ in Fibrosis stage (SD)	0.2 (0.9)	0.4 (1.6)	0.54

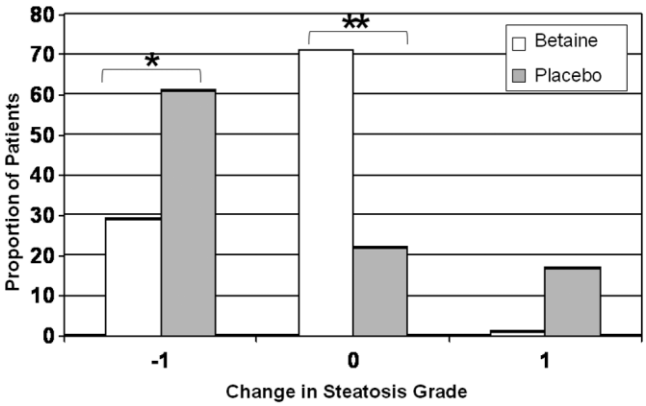

Fig. 2. In the betaine versus placebo group, a significant proportion of patients treated with betaine had no change in hepatic steatosis (71% versus 22%, $P < 0.01$) over the 1-year study period. However, more patients treated with betaine compared with placebo (71 versus 22%, $P < 0.005$) had no change in hepatic steatosis over the duration of the study. ($*P < 0.01$; $**P < 0.005$).

Short-form Health Survey Results. Twenty-four subjects (11 betaine, 13 placebo) completed and returned the SF-36 (Medical Outcomes Trust, Boston, MA) at baseline and after their participation in the study. In response to the specific question "Compared to *one year ago*, how would you rate your health in general *now*?," there was no difference between treatment groups in reporting an improvement, decrease, or no change in perceived physical, mental, and/or general health status.

Safety Results. No clinically apparent hepatotoxicity (ALT or AST $\geq 3\times$ baseline value) rise in total bilirubin value of $2\times$ normal, or severe adverse events was reported in either treatment group. The overall incidence of gastrointestinal adverse events (i.e., nausea, vomiting, abdominal bloating, and/or diarrhea) in those treated with betaine was 33% versus 9%, in those treated with placebo, $P < 0.05$. A comparable number of patients in each treatment arm were either noncompliant with study drug and/or voluntarily withdrew their participation. Eighteen subjects (nine in each group) voluntarily withdrew consent for adverse effects, noncompliance, and the "inconvenience" of taking study drug, or inability to continue study participation.

Transmethylation Pathway and Serum Antioxidant Status. Serum levels of methionine, SAM, SAH, and homocysteine were measured at baseline and after treatment in NASH subjects as well as in 11 healthy controls (Table 3). Because betaine could potentially affect serum antioxidant status, total serum antioxidant activity was also measured. At baseline, subjects with NASH had a significantly higher mean SAH level and a significantly lower mean serum antioxidant activity than healthy controls. Serum levels of methionine, SAM, and homocysteine were not different between the NASH subjects and the healthy controls at baseline. Betaine therapy significantly increased serum levels of methionine and SAM but did not affect SAH, homocysteine, or antioxidant activity. No changes were seen with placebo therapy.

Serum Cytokines and Adipokines. Serum levels of TNFα, IL-6, IL-8, MCP-1, insulin, adiponectin, and leptin were measured at baseline and after treatment in NASH subjects as well as in healthy controls (Table 4). At baseline, NASH subjects had increased mean serum insulin and leptin but decreased adiponectin compared to healthy controls. No differences were seen between NASH subjects at baseline and healthy controls in serum levels of TNFα, IL-6, IL-8, or MCP-1. However, there was a trend toward increased IL-8 in NASH subjects at baseline compared to healthy controls ($P = 0.071$). Neither betaine nor placebo treatment affected serum levels of any of these cytokines. Likewise, no differences were seen in mean serum IL-1, resistin, or total PAI-1 at baseline in the NASH subjects (versus healthy controls) or after either betaine or placebo treatment (data not shown).

Discussion

Several mechanisms have been proposed in the pathogenesis of NASH, including oxidative stress, endotoxins, cytokines, chemokines, and nutritional deficiencies. Increasing evidence suggests that altered methionine/folate metabolism contributes to the development of hepatic steatosis.[16] Betaine, an important human nutrient obtained from a variety of foods, is absorbed from the intestine and transported to the liver. In the liver, betaine serves as a methyl donor to homocysteine to form methionine, resulting in decreased concentrations of homocysteine and increased concentrations of methionine. The

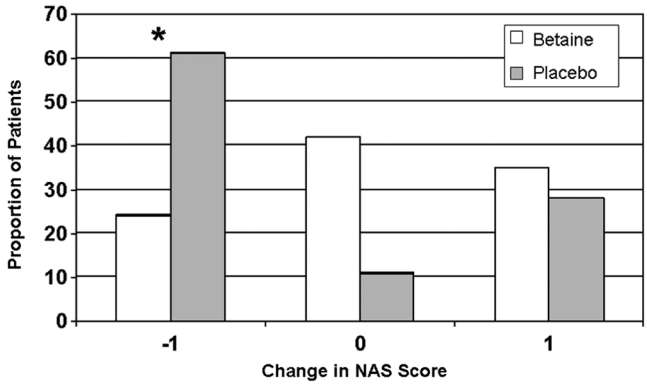

Fig. 3. Significantly fewer patients treated with betaine versus placebo (24% versus 61%, $P < 0.05$) improved the NAS score by ≥ 1 point; however, the proportion of patients with either no change or worsening NAS score was no different between groups (P = NS) ($*P < 0.05$).

Table 3. Transmethylation Pathway and Antioxidant Status

Analyte	Healthy Controls	NASH Baseline	Betaine Pretreatment	Betaine Posttreatment	Placebo Pretreatment	Placebo Posttreatment
Methionine (μmol)	18.8 (6.60)	22.1 (9.92)	19.2 (9.10)	29.0 (20.0)§	24.6 (10.2)	30.8 (15.1)
SAM (μmol)	0.0741 (0.0395)	0.129 (0.0905)	0.122 (0.0596)	0.211 (0.155)§	0.135 (0.116)	0.209 (0.227)
SAH (nmol)	33.8 (11.5)	60.4 (28.1)*	63.4 (28.4)	59.6 (16.2)	57.7 (28.4)	66.5 (35.5)
Homocysteine (μmol)	11.5 (3.58)	10.6 (3.12)	11.5 (3.05)	10.8 (2.02)	9.90 (3.08)	10.1 (3.73)
Antioxidants (mmol)	4.12 (0.267)	0.893 (0.409)*	0.735 (0.446)	0.703 (0.324)	1.02 (0.339)†	0.988 (0.295)‡

*$P<0.05$ for Healthy Controls vs. NASH Baseline.
†$P<0.05$ for Betaine Pretreatment vs. Placebo Pretreatment.
‡$P<0.05$ for Betaine Posttreatment vs. Placebo Posttreatment.
§$P<0.05$ for Betaine Pretreatment vs. Betaine Posttreatment.
$P<0.05$ for Placebo Pretreatment vs. Placebo Posttreatment.

consequent increase in SAM can trigger a cascade of events leading to activation of phosphatidylethanolamine methyltransferase pathway phosphatidylcholine synthesis, formation of very low density lipoprotein, export of triacylglycerol, and attenuation of fatty liver.[17-19] Decreased hepatic concentrations of homocysteine can attenuate endoplasmic reticulum (ER) stress, resulting in the down-regulation of proapoptotic genes and, thus, the attenuation of apoptosis, inflammation, and fibrosis.[20] Down-regulation of another ER stress gene, sterol response element binding protein-1, can reduce hepatic fatty acid synthesis, which may result in reduced fatty liver. Increased hepatic concentrations of SAM can activate cystathionine β-synthase and lead to the up-regulation of the transsulfuration pathway, increased synthesis of glutathione, and attenuation of oxidative stress.[21] Thus, betaine can potentially attenuate fatty liver disease by improving steatosis, inflammation, and fibrosis.

Although not extensively studied for NASH, betaine has been shown to attenuate alcoholic liver injury by increasing the concentrations of hepatic SAM and decreasing the concentrations of homocysteine and SAH in animal studies.[20,21] By increasing hepatic SAM levels, betaine may provide protection against liver injuries by: 1) increasing hepatic glutathione, a potent antioxidant; 2) down-regulating TNF-α and up-regulating IL-10 synthesis; and 3) by inhibiting the apoptosis of normal hepatocytes. An increase in hepatic SAM can restore the hepatic mitochondrial glutathione concentration,[22] which is critical for maintaining mitochondrial function and rescuing of mitochondria from free radical damage. In addition to protecting hepatocytes from oxidative stress, elevated glutathione concentrations have been shown to rescue hepatocytes from TNF-α toxicity, such as necrosis.[23]

Previous uncontrolled pilot studies could neither support nor refute the use of betaine for NAFLD. In a short-term prospective, randomized, double-blind therapeutic trial (n = 191 patients), oral administration of betaine glucuronate for 8 weeks reduced hepatic steatosis by 25%, reduced hepatomegaly by 8%, and significantly attenuated the hepatic concentrations of AST, ALT, and γ-glutamyltransferase in patients with NASH.[24] In a small pilot study, 1 year of betaine therapy improved AST ($P < 0.02$) and ALT ($P < 0.007$) as well as the degree of steatosis, necroinflammatory grade, and fibrosis stage.[6] In an ongoing study of NASH patients, betaine treatment attenuated ALT concentrations and improved the grades of steatosis, inflammation, and fibrosis.[25]

Data from this suggest that betaine at 20 g daily, although generally well tolerated, is not of value as a sole agent in the treatment of patients with NASH. Gastrointestinal side effects, which were unacceptable for patients with an otherwise asymptomatic condition, resulted in drug discontinuation for several subjects. These side ef-

Table 4. Serum Cytokines and Adipokines

Analyte	Healthy Controls	NASH Baseline	Betaine Pretreatment	Betaine Posttreatment	Placebo Pretreatment	Placebo Posttreatment
Insulin (pg/mL)	385.0 (201)	1801 (3429)*	1441 (1493)	1145 (755)	2085 (4450)	1101 (1829)
Adiponectin (μg/mL)	67.6 (62.2)	25.0 (25.2)*	18.8 (10.1)	16.5 (6.63)	31.1 (33.6)	32.2 (36.6)
Leptin (ng/mL)	11.9 (9.62)	31.3 (22.7)*	22.5 (19.8)	22.6 (18.9)	38.1 (23.2)	33.3 (21.2)
TNFα (pg/mL)	4.48 (1.78)	3.95 (2.02)	4.04 (2.51)	4.75 (3.35)	3.88 (1.63)	3.97 (2.66)
IL-6 (pg/mL)	1.44 (1.59)	6.27 (10.4)	3.46 (4.70)	1.81 (1.63)	8.48 (13.0)	7.06 (12.7)
IL-8 (pg/mL)	3.15 (2.34)	6.27 (5.05)	6.79 (6.01)	9.12 (8.55)	5.87 (4.34)	9.07 (11.2)
MCP-1 (pg/mL)	276 (121)	270 (100)	293 (125)	325 (138)	252 (75.2)	237 (80.5)

*$P<0.05$ for healthy controls vs. NASH baseline.
No significant differences were noted for betaine pre- and/or posttreatment vs. placebo pre- and/or posttreatment.

fects, as well as the inconvenience of taking a medication that necessitates suspension in solution, contributed to increased patient dropout and voluntary study withdrawal. However, of those patients who were able to tolerate the study medication and complete study participation, the use of betaine did not appear to impact quality of life measures.

Betaine did not improve liver enzymes or histology when compared with placebo. The lack of observed change in serum aminotransferases noted in our study could suggest that a change, if present, is transient and/or that serum aminotransferases fluctuate and are insensitive clinical endpoints of disease activity. Although betaine led to an improvement in serum aminotransferase levels as well as some histological features, which is in agreement with results of the uncontrolled pilot study,[6] a more significant improvement in hepatic steatosis as well as the NAS score was seen in the placebo group. Without a placebo (control) group, we would have wrongly concluded that betaine therapy can be associated with biochemical and histologic improvement in patients with NASH. The spontaneous biochemical and histologic improvements in the placebo group further emphasize the importance of including a control group in all future treatment studies of NASH. Given the dynamic nature of hepatic steatosis as well the potential influence of both dietary and physical activity on liver enzymes and histologic endpoints, future studies should rigorously evaluate such confounding variables which may contribute to improvement in disease endpoints in the placebo group.

Although serum SAH was elevated at baseline in subjects with NASH compared to healthy controls, no differences were seen in serum homocysteine, betaine, or SAM levels. Betaine treatment failed to significantly decrease serum homocysteine or SAH levels, but it did increase serum SAM levels. In animal models of NAFLD, betaine significantly attenuates hepatic steatosis and is associated with increases in SAM, methionine, SAH, homocysteine, and antioxidant activity. However, in humans, alternative metabolic and signaling pathways which influence SAH, homocysteine, and antioxidant activity may exist. For example, increased activation of hepatic AMP-activated protein kinase (AMPK) and attenuated lipogenic capability (enzyme activity and gene expression) in the liver may influence the downstream effect of betaine supplementation,[26] factors that may be influenced by different environmental and/or genetic factors among individuals.

Betaine treatment failed to improve the reduced serum antioxidant status and failed to attenuate the insulin resistance and adiponectin reduction in these subjects with NASH. Most proinflammatory cytokines typically elevated in NASH were normal in these subjects at baseline and were unchanged by betaine. The normal levels of proinflammatory cytokines may have been attributable to more quiescent degrees of necroinflammation and/or more advanced fibrosis in our study group. It is unknown if betaine is effective at other doses, or in subjects with elevated baseline homocysteine, more inflammation, or less liver fibrosis.

A few limitations in the study exist. First, detailed dietary and exercise diaries were not obtained over the course of the study. Second, it is unknown if the chosen dose of betaine was physiologically effective and/or if lower doses would have been better tolerated and thus more effective. Third, we did not specifically enroll patient with NASH who had elevated baseline homocysteine levels. Fourth, although the healthy control population used for measurement of methionine metabolism and cytokines lacked a known diagnosis of chronic liver disease, had normal liver enzymes, and were not obese, no diagnostic studies were performed to ensure the absence of hepatic steatosis. Fifth, the significant proportion of patients in this study with advanced fibrosis (i.e., stage 3-4 fibrosis) may have limited our ability to detect a treatment response if one was present. Lastly, due to increased dropout, the number of evaluable patients fell short of the calculated sample size necessary to detect a difference between groups. Therefore, the decreased number of evaluable patients at the end of the study raises a concern that our study may lack adequate power to detect a difference in response to treatment with betaine (or placebo). Any future clinical studies utilizing betaine must take into consideration several important factors such as number of patients, dose and duration of treatment, degree of disease severity, and bioavailability of compounds in their design.

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