Journal of Diabetes and Metabolism

Tiihonen et al., J Diabetes Metab 2016, 7:7
DOI: 10.4172/2155-6156.1000692

Research article Open Access

Effect of Dietary Betaine on Metabolic Syndrome Risk Factors in Asian Males with Mild Fatty Liver

Kirsti Tiihonen[1*], Markku T Saarinen[1], Esa Alhoniemi[2], Nobuaki Mitsuya[3] and Goro Yamaki[4]

[1]*DuPont Nutrition and Health, Kantvik, Finland*

[2]*Avoltus Oy, Turku, Finland*

[3]*Danisco Japan Ltd., Tokyo, Japan*

[4]*Medical Corporation Kokoro to Karada no Genki Plaza, Japan*

[*]**Corresponding author: Dr.** Kirsti Tiihonen, DuPont Nutrition and Health, Global Health and Nutrition Science, Sokeritehtaantie 20, 02460 Kantvik, Finland, Tel: +358405543273; E-mail: kirsti.tiihonen@dupont.com

Received date: June 22, 2016; **Accepted date:** July 28, 2016; **Published date:** July 30, 2016

Abstract

Objective: Betaine (trimethylglycine) is a naturally-occurring osmolyte and methyl donor with reported effects on liver metabolism. The aim of this study was to evaluate the safety and the effects of betaine administration to metabolic risk factors in Asian males.

Methodology: Twenty Japanese males (30-59 years) with mild fatty liver were enrolled in this blinded, placebo-controlled, randomized, parallel trial to assess the effects of 3 g of daily betaine for 12 weeks on safety and metabolic biomarkers. Selection criteria included fatty liver disease, as assessed by an abdominal ultrasonogram (US), the practice of moderate alcohol consumption, liver performance status and body mass index (≤ 27). Abdominal computed tomography (CT) as well as blood and urine sampling were performed before and after the intervention period.

Results: No differences in the degree of hepatic steatosis or in the occurrence of adverse events were observed between the groups. Compared to the placebo group, the betaine group showed a significant increase in plasma betaine ($P<0.0001$) and high-density lipoprotein (HDL)-cholesterol ($P<0.05$) concentrations.

Conclusion: Low-dose betaine supplementation to subjects with mild fatty liver disease is safe and well tolerated. Betaine increased plasma HDL which may reflect the favorable effects of betaine on liver function-related metabolic metabolism in Asian population.

Keywords: Betaine; Trimethylglycine; Metabolic syndrome; Fatty liver, Lipid metabolism; HDL cholesterol; Asian males

Introduction

Abdominal obesity is known to be a risk factor for metabolic syndrome (MetS) but also lean individuals can develop MetS [1]. An epidemiological study by Kojima et al. [1] showed that even non-obese Japanese individuals with a BMI of less than 25 kg/m^2 accounted for half of the patients with fatty liver. They concluded that not only absolute BMI but also relative increase in the BMI may be involved in the increased prevalence of fatty liver seen in Asian population during the last decades.

Non-alcoholic fatty liver disease (NAFLD) occurs when fat is accumulated into the hepatocytes without excessive alcohol use. The risk of type 2 diabetes [2], cardiovascular disease [3], and advanced liver disease [4] is increased in subjects with NAFLD. Liver fat content is linearly correlated with all the metabolic syndrome risk factors independently of obesity [5]. Fatty liver overproduces glucose and VLDL resulting hyperglycemia, hypertriglyceridemia and a lowering of HDL cholesterol [6].

Weight reduction is the optimal treatment for NAFLD. In overweight, obese or type 2 diabetic subjects, a moderate weight loss of 8-14% of body weight results in a 40-80% decrease in liver fat content, when determined using proton magnetic resonance spectroscopy [7,8]. Anti-diabetic peroxisome proliferator-activated receptor-gamma (PPARγ) agonist therapy is also used in patients with impaired glucose tolerance or type 2 diabetes. This treatment has shown to reduce liver fat content by 40-50% within 4-6 months [9,10]. Recently, the roles of hepatoprotective nutrients such as antioxidants, n-3 polyunsaturated fatty acids, and betaine in attenuating fatty liver disease have also been discussed [11].

Betaine (glycine betaine, N,N,N-trimethyl glycine) is a naturally-occurring osmolyte and methyl donor [12] that is rapidly absorbed into the blood circulation [13]. The high concentration of betaine in whole grain flour is widely assumed to explain part of the beneficial effects of cereals. Konstantinova et al. [14] showed inverse association of serum betaine concentration with waist circumference, non-HDL cholesterol, triglycerides and blood pressure but positive association with HDL cholesterol in an epidemiological study.

In liver, betaine transfers its methyl group to homocysteine in a reaction catalysed by betaine-homocysteine methyltransferase (BHMT) and induces the formation of S-adenosyl methionine (SAM)

[15,16]. Betaine intake has been shown to inhibit the accumulation of triglycerides in hepatic tissue by recovering decreased intracellular SAM and glutathione (GSH) levels in rats with alcoholic fatty liver [17-19]. Moreover, betaine has been shown to alleviate high-sucrose [20] and high-fat [21,22] diet-induced pathological changes in mice liver. However, the association in humans having fatty liver disease remains as yet unclarified. Recently, it has been shown that high-dose betaine supplementation (20 g/d) results in a significant reduction in histologically determined liver fat content in patients with non-alcoholic steatohepatitis (NASH) [23].

In the present study, we examined how a low-dose betaine supplementation affects metabolic syndrome risk factors in Asian subjects, as well as the safety of this dose in a long-term use. For this purpose, we conducted intervention study on subjects with mild fatty liver disease as diagnosed by abdominal ultrasonogram (US). The effect of betaine on the liver fat and function were studied using computed tomography (CT) and biochemical markers.

Methods

Participants

The study protocol was examined and approved by the Medical Ethical Review Board of Kimura Hospital, Ota-ku, Tokyo. The intervention took place between July 2008 and November 2008 in the Medical Corporation Kokoro to Karada no Geneki Plaza, Japan following guidelines laid down by the Declaration of Helsinki. The purpose of the study was explained to all participants who gave their written informed consent to be included in the study.

A total of 20 Japanese males aged between 30 and 59 years were selected for the study. The selection criteria for fatty liver was based on abdominal ultrasonographic (US) findings, alcohol consumption habits, liver enzymes (aspartate transaminase (AST) alanine transaminase (ALT) gamma-glutamyl transferase (GGT)), grades 0-2 in national cancer institute institute common toxicity criteria (NCI-CTC, ver.2) and body mass index (BMI) ≤ 27 kg/m^2.

Exclusion criteria included liver damage which had advanced to cirrhosis and/or hepatic failure, fatty liver disease requiring medication, heavy alcohol consumption (of more than 100 g/day), and previous treatment for alcoholism or alcoholic organ damage. Other exclusion criteria included hepatitis B surface (HBs) antigen-positivity or hepatitis C virus (HCV) antibody-positivity, AST>200 U/l, ALT>225 U/l, GGT>400 U/l. Autoimmune diseases, serious renal disease, unstable eating habits and the customary lack of a breakfast, medical complications and/or a history of other serious diseases, and the simultaneous participation in other clinical studies were other grounds for exclusion. In addition, the use of any drugs, such as antihyperlipidemia drugs, which may affect liver function and liver enzyme levels, as well as the consumption of vitamin E, folic acid and/or vitamins B_6 and B_{12} preparations, led to exclusion from the study.

Study design

A randomized, double-blind, placebo-controlled, parallel study was conducted on 20 volunteers who were randomly allocated into either betaine or placebo groups. A study flow diagram is shown in Figure 1. The aim the study was to determine the long term effects of dietary betaine on safety and metabolic syndrome risk factors in Asian males.

At the screening visit, the subjects' background and health information was collated on a structured form. At the same visit the semi-quantified fatty liver diagnosis was based on the abdominal US findings. Their vital signs were measured and blood and urine samples were collected four weeks before the start of the trial, again at the actual start of the trial, and at 2, 4, 8 and 12 weeks after the test food intake. Liver fat content (liver/spleen CT ratio) as well as visceral and subcutaneous fat area were assessed by abdominal CT at the 0- and 12-week stages. Any adverse events were noted through observations and checks after the completion of each 2-, 4-, 8- and 12-week test food intake. An adverse event meant any of the undesirable or unintended signs (including abnormal laboratory values in a clinical test), symptoms, or diseases that have temporal relevance to the test food intake.

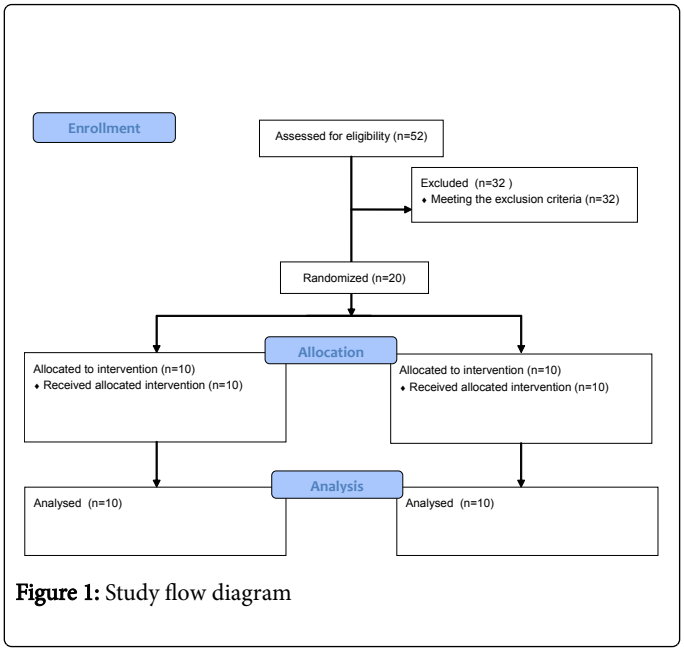

Figure 1: Study flow diagram

The subjects were instructed not to change any of their daily habits regarding alcohol consumption, smoking, physical activity or food intake and to keep a log once a week of all such events. The subjects were, however, not allowed to consume alcohol the day before, nor the test food on the day of, sample collection. In order to measure the fasting blood sugar level and the fasting insulin level, the subjects were instructed to fast (water drinking allowed) for 6 h before sample collection. The collected blood samples were centrifuged and the plasma stored at -20°C until analyzed.

Study products

The subjects received a 3 g dose of either betaine (Betafin BF®; Finnfeeds Finland Oy, Finland) or a placebo powder (maltodextrin; Syral AS, France). The dose was dissolved in 200 ml of water or a soft drink and orally consumed at once each day after breakfast for 12 weeks. Maltodextrin was used as a placebo as it is not presumed to cause significant metabolic effects at the level used and is indistinguishable from betaine powder.

Liver, visceral and subcutaneous fat measurements

Selection criteria for the study included the semi-quantified diagnosis of fatty liver based on the abdominal US findings, such as a

bright liver pattern with its resultant depth and attenuation, and a liver/kidney ratio of the echogenicity as established previously [24,25]. The measurement was performed with a Toshiba SSA-680A ultrasound system (Toshiba Medical Systems Corporation, Otawara, Japan).

Liver fat content (liver/spleen CT ratio) as well as visceral and subcutaneous fat area were assessed by abdominal CT at the 0- and 12-week stages. Special care was taken to perform the scanning in the same position before and after the intervention. Liver and spleen fat was measured using CT images with 5-6 mm thickness at T12–L1 as previously described [26,27]. CT images were obtained using a SOMATOM Emotion 6/16 slice configuration CT scanner (Siemens, Tokyo, Japan). To calculate the CT value, the radiologic density of the liver was first divided into four segments and the value of each segment was the average of the four separate regions. Simultaneously, CT values for the spleen were the average of the four regions. Major vessels such as the portal and the splenic vein were not included in the regions analysed. The liver and spleen CT values were used to calculate the liver fat index i.e. the liver-to-spleen (L/S-) ratio.

The visceral and subcutaneous fat area was scanned above and below the umbilical region using the same slice thickness as used in liver and spleen. To calculate the visceral and the subcutaneous fat area the bone image was used as a reference.

Blood analysis

Markers of liver, renal function and blood pressure: The following parameters in plasma were determined using specific enzymatic assay kits: AST (Iatro-LQ AST-JII), ALT (Iatro-LQ ALT-JII), lactate dehydrogenase (LDH)(Iatro-LQ LDH-II), GGT (Iatro-LQ gamma-GT-IIJ), alkaline phosphatase (ALP) (Iatro-LQ ALP-II), total bilirubin (Iatro-LQ T-bil Q), blood urea nitrogen (BUN)(Iatro-LQ UN-AII), creatinine (Iatro-LQ Cre-AII), uric acid (Iatro-LQ UA-II), total protein (Iatro TP-II), albumin (Iatro-fine ALB-II), CRP (Iatro CRP-EX). The Iatro assay kits were obtained from Mitsubishi Chemical Medience (Tokyo, Japan). Cholinesterase (ChE) was measured with a Serotec Che-CL assay kit (Serotec, Sapporo, Japan). Urinary protein, sugar, bilirubin, ketone bodies, pH and occult blood were determined by semiquantitative Uropaper III dipstick methods (Eiken Chemical, Tokyo, Japan).

Markers of lipid and sugar metabolism: The following parameters in blood plasma were determined with specific enzymatic assay kits: total cholesterol (Iatro-LQ T-Cho-AII), triglycerides (Iatro-LQ TG-II) and fasting blood sugar (Iatro LQ GLU). The Iatro assay kits were obtained from Mitsubishi Chemical Medience (Tokyo, Japan). HDL cholesterol and LDL cholesterol were measured with Cholestest N HDL and Cholestest LDL assay kits respectively (Sekisui Medical, Tokyo, Japan). Fasting immunoreactive insulin (F-IRI) was measured with an Architecht Insulin assay kit and glycated hemoglobin (HbA1c) was measured with a JCA-BM9030 analyzer (JEOL, Tokyo, Japan). Adiponectin concentration was measured with an enzyme immunoassay kit from Otsuka Pharmaceutical (Tokyo, Japan).

Markers of inflammatory, oxidative stress, hepatic fibrosis and fibrinolytic activities: Plasma high-sensitivity C-reactive protein (hs-CRP) was determined with an enzymatic assay kit (Iatro CRP-EX; Mitsubishi Chemical Medience, Tokyo, Japan). Plasminogen activator inhibitor type-1 (PAI-1, SERPIN E1) was measured with a latex photometric immunoassay, using an LPIA-t -PAI test kit (Mitsubishi Chemical Medience, Tokyo, Japan). Fibrinogen was determined with an ACL-TOP coagulation analyzer (Instrumentation Laboratory, Tokyo, Japan). Collagen type-IV was measured with an enzyme immunoassay kit from Daiichi fine chemical (Toyama, Japan). 8-hydroxydeoxyguanosine (8-OHdG) levels were determined using a specific enzyme immunoassay kit (Nikken SEIL, Japan Institute of the Control of Aging, Shizuoka, Japan).

Betaine and amino acid concentrations: Plasma betaine levels were determined as bromophenacyl ester derivatives with UV detection (at 254 nm) as previously described [28]. Amino acids were measured by high performance liquid chromatography using a JLC-300V amino acid analyzer (JEOL, Tokyo, Japan).

Markers of hematological function: Red blood cell, white blood cell, hemoglobin, hematocrit, and platelet counts and the differential count of leukocytes (i.e. neutrophils, eosinophils, basophils, lymphocytes, and monocytes) were measured with an XE-2100 hematology analyzer (Sysmex, Kobe, Japan).

Statistical Analysis

First, an exploratory data analysis method was used to get an overview of the differences between the placebo and the betaine groups over all parameters of the study. For each parameter, the difference between the placebo and the betaine groups was computed using raw score, which is defined as difference between the placebo and the treatment group means divided by the pooled standard deviation. The raw score was calculated separately for each time point and finally all the scores in different time points were averaged. The absolute value of the obtained statistic is an indicator of the difference between the treatments for a parameter. It was used to order the parameters so that the ones with the largest differences between the treatment groups over all time points can be easily detected. This method was applied to both original data (on absolute scale) and relative data. In the relative data, every value was calculated as the percentage of the baseline value of the corresponding subject.

The actual statistical analyses were performed using statistical software R version 3.2.2 [29] and NLME library [30]. Data obtained at day 0, just before the first intake of either betaine or the placebo, was designated as the baseline. The baseline comparisons were performed using t test, Mann-Whitney U test, or Chi-Squared test. The parameters were analysed using mixed models with fixed effect terms for treatment, time point (week), and their interaction. Baseline value and BMI were used as covariates of the models, and the random effect of the models was a subject-wise intercept term. For some parameters, there were only two time points, and fixed effects models with treatment as fixed effect and baseline and BMI as covariates were used instead. If necessary, parameter values were log-transformed prior to modelling in order to obtain good model fit to the data. The treatments were compared using model contrasts in all time points. All the p-values of all statistical analyses were adjusted using false discovery rate correction [31]. An adjusted p-value below 0.05 was considered as statistically significant.

Results and Discussion

Subject characteristics

The baseline characteristics of the study subjects are shown in Table 1. The betaine and control groups were comparable with respect to age, BMI, waist circumference, blood pressure, plasma lipid, glucose and insulin concentrations, liver enzyme levels, smoking habits and alcohol

consumption. Moreover there was no bias between the groups in the liver fat content, medical history or exercise levels (data not shown).

	Betaine group		Placebo group		p-value
	Mean	SD	Mean	SD	
Age (y)	42.8	3.3	46	6	0.75
BMI (kg/m^2)	24.4	1.3	25.4	1.7	0.75
Waist (cm)	86.7	3.3	88.2	4.5	0.87
fP-total cholesterol (mmol/l)	5.76	0.97	5.79	0.81	0.97
fP-HDL cholesterol (mmol/l)	1.34	0.28	1.22	0.2	0.85
fP-LDL cholesterol (mmol/l)	3.57	0.94	3.86	0.73	0.87
Systolic BP (mm Hg)	134.3	11.4	137.4	13.1	0.9
Diastolic BP (mm Hg)	85.2	11.4	89.6	12.3	0.87
fP-glucose (mmol/l)	5.13	0.53	4.88	0.5	0.85
fP-insulin (mU/l)	8.09	8.38	4.8	1.48	0.97
P-ALT (U/l)	48.3	16.4	60.1	35.4	0.87
P-AST (U/l)	28.1	5.8	33.3	13.4	0.85
AST/ALT-ratio	0.6	0.1	0.6	0.2	0.97
P-GGT (U/l)	104.2	58.9	90	72.9	0.87
P-ALP (U/l)	221.5	74.5	181.6	65.2	0.79
Alcohol consumption (g/day)	18.1	15	21	25.8	0.97
Smoking (n)	3		5		0.9

BMI-Body Mass Index; Fp-Fasting Plasma; HDL-High Density Lipoprotein; LDL-Low Density Lipoprotein; BP-Blood Pressure; ALT-Alanine Transaminase; AST-Aspartate Transaminase; GGT-Gamma-Glutamyl Transferase; ALP-Alkaline Phosphatase. Data is expressed as means and standard deviations, except smoking. No statistically significant difference was observed between the groups.

Table 1: Baseline characteristics of the study subjects.

The mean alcohol consumption in betaine group was 18.1 g/day (5-45.2) and 21.0 g/day (3.5-84) in placebo group. Majority of the study subjects (16/20) were light-moderate drinkers (mean 11.0 g alcohol/day) whereas minority was moderate-heavy drinkers (4/20, mean 53.6 g alcohol/day). The wide alcohol use range may have increased the variation in the responses. Based on the liver enzyme activities the study subjects were only at the upper end of normal range [32].

Exploratory data analysis: The exploratory data analysis method described in Statistical analysis was used to get an overview of the differences between the placebo and the betaine groups for all parameters of the study over all time points. The largest differences between the treatments in the parameters analysed using original data were in descending order: plasma betaine and HDL concentrations, white blood cell counts, creatinine concentration, visceral fat area, plasma adiponectin concentration and glycated hemoglobin (HbA1c) (Figure 2A)." However, the largest differences in parameters using relative data in descending order were plasma betaine and isoleucine concentrations, HbA1c, choline esterase activity, blood urea nitrogen (BUN), threonine and HDL concentrations (Figure 2B). Taking account of the absolute and relative approach the most constant differences between the treatments were increased plasma betaine and HDL concentrations in betaine treatment.

The statistically significant findings: Unlike the exploratory analysis approach, the statistical analysis compared the treatments separately in each time point. The analysis was performed using absolute values but by taking the baseline value into account in the modeling as a covariate. The statistically significant findings in descending order were betaine, HDL cholesterol, HbA1c, threonine, choline esterase, BUN and fibrinogen (Figure 3A, absolute scale and Figure 3B relative scale). The two topmost parameters contain the findings with adjusted p-value below 0.05: betaine at week 12 and HDL Cholesterol at week 12. For the other five parameters the non-adjusted p-value was below 0.05, but the adjusted p-value became non-significant for HbA1c (week 12), threonine (week 12), choline esterase (weeks 2 and 8), BUN (week 12), and fibrinogen (week 8). Although there were some non-significant changes in betaine group compared to placebo group the changes were within the range of reference values (HbA1c 4-5.6%; threonine 75-210 µmol/l; choline esterase 234-493 U/l; BUN 7-20 mg/dl; fibrinogen 150-400 mg/dl). The decreased trend of plasma choline esterase (butyrylcholinesterase, BChE) activity by betaine

supplementation in this study may reflect improved lipid metabolism in the liver [33,34].

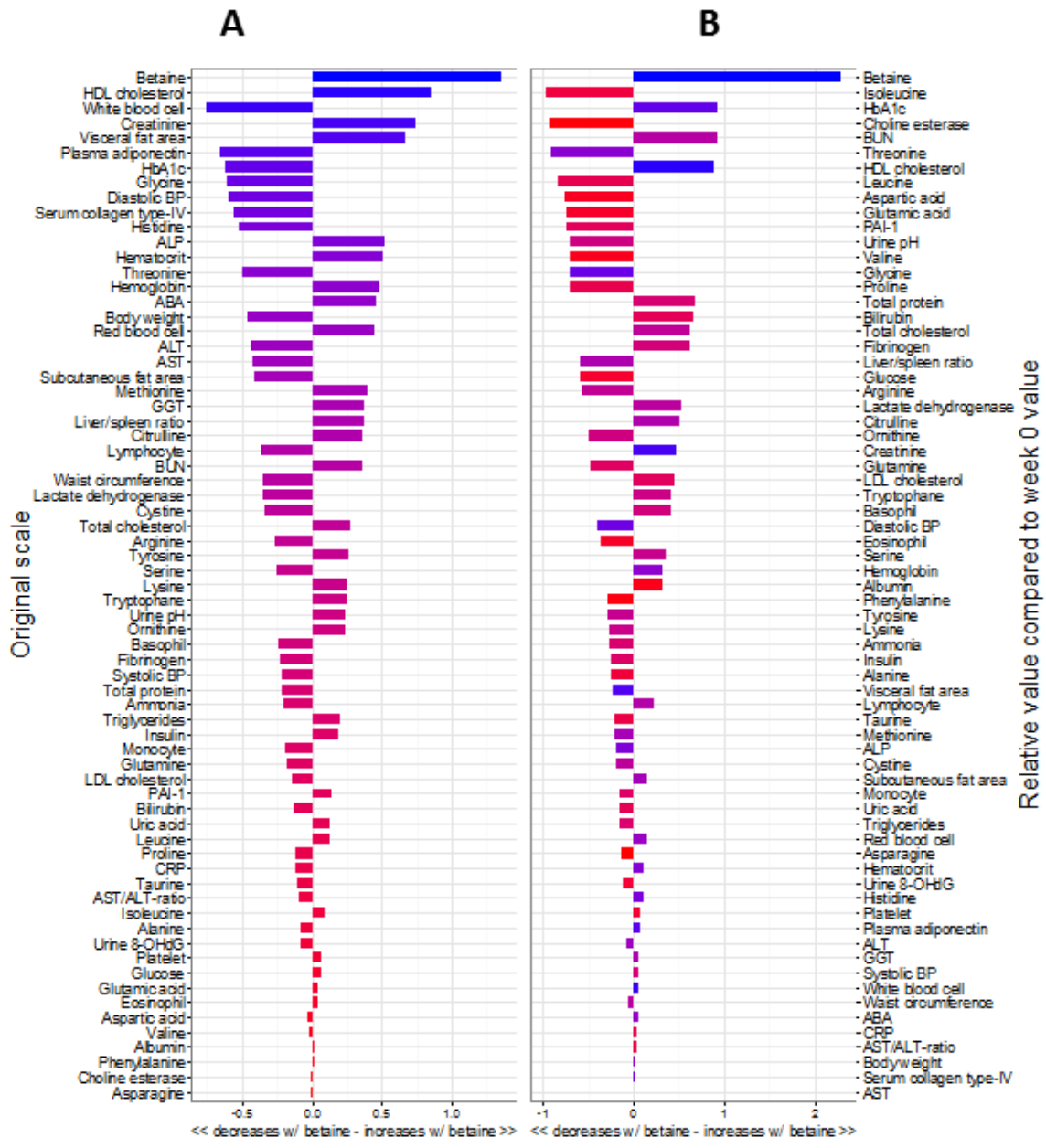

Figure 2: An illustration of the differences between the treatments for each parameter calculated using averaged raw score over all time points A) original data (on absolute scale) and B) relative data.

The width of the bar indicates the magnitude of the difference, and the direction shows the sign of the difference: positive value means that the value in the betaine group was larger than in the placebo group and vice versa. The parameters are shown in descending order so that the parameters with the largest differences over all time points are shown on top. The figures A) and B) are also linked so that each parameter has its unique color which is the same in both figures. This color coding reveals the fact that the order of the variables is somewhat dependent on the scale (absolute vs. relative) used.

Effect of betaine on body composition: The liver fat content (liver/spleen CT ratio) as well as visceral and subcutaneous fat area were assessed before and during the intervention by abdominal computed tomography. The liver-to-spleen (L/S) ratio as well as the visceral and subcutaneous fat area remained unchanged (Table 2) over the 12

weeks. However, the individuals selected for this study had only mild hepatic steatosis (L/S min-max) as in Japanese population the cut-off value of L/S ratio should be less than 1.1 to be diagnosed even mild steatosis [35].

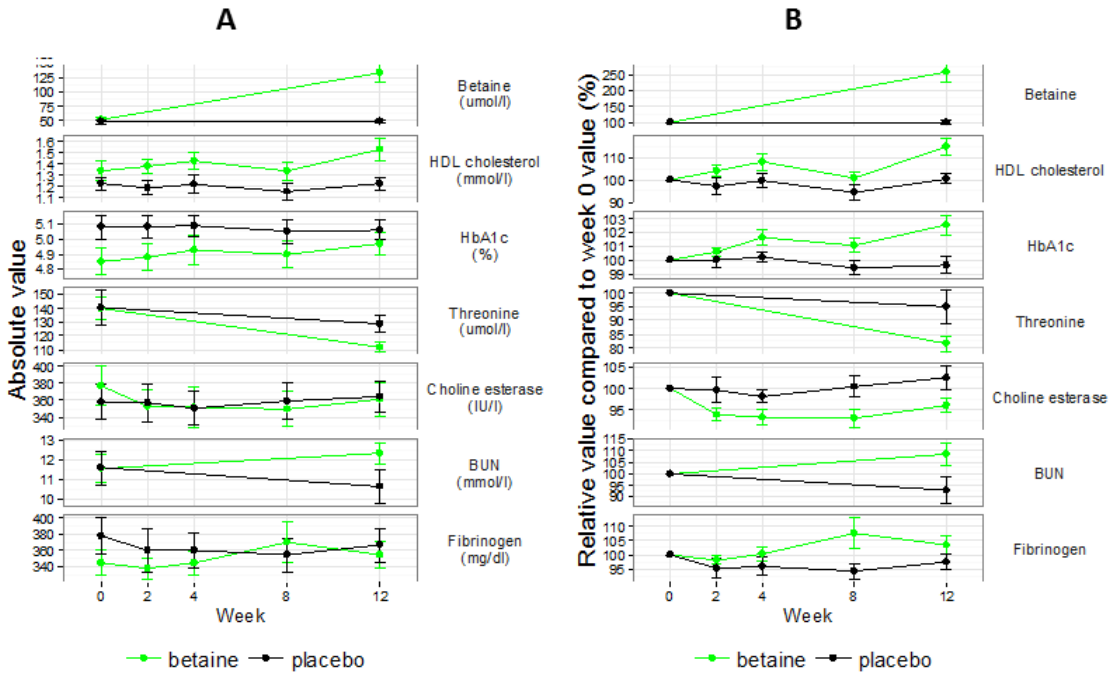

Figure 3: The illustration of the parameters with statistically significant findings. A) is based on the original data (on absolute scale) with respective units indicated in brackets and B) relative data where the values are subject-wise relative values compared to week 0 value. The treatments are shown in different colours, and the mean of each treatment at each week is shown with +/-SEM. The two topmost parameters contain the findings with adjusted p-value below 0.05: betaine at week 12 and HDL cholesterol at week 12. For the other five parameters the the adjusted p-value became non-significant for HbA1c (week 12), threonine (week 12), choline esterase (weeks 2 and 8), BUN (week 12), and fibrinogen (week 8).

The attenuation of liver fat by betaine supplementation was not seen using CT-imaging based diagnosis. Although the CT is widely used procedure for measuring lipid content in the liver its ability to accurately detect the degree of hepatic steatosis may be limited [36] especially in this study population with the wide alcohol use range and possibly varying degrees of inflammation [37]. In a recent study with biopsy-proven NASH patients, who received either oral betaine (20 g/day) or a placebo for 12 months, the degree of hepatic steatosis was significantly reduced in the betaine - as opposed to the placebo–group [23]. However, in that study there were no differences in terms of liver enzyme activity between the betaine and placebo groups.

It has recently been reported that the degree of hepatic steatosis can be most reliably and non-invasively quantified using proton magnetic resonance spectroscopy (^{1}H-MRI) [36]. In a large population-based study by Victor et al. [38], a diagnostic cut-off for increased liver fat (fatty liver disease) of more than 5.6% has been proposed [39]. It was strongly suggested that this MRI method be utilized in order to elucidate more detailed metabolic effects in this kind of human study. However, even more detailed information of the fatty liver could get using liver biopsies but this method has ethical limitations in routine clinical use.

Effect of betaine on markers of liver and renal function: DBP and SBP remained unchanged in both study groups over the 12 weeks (Table 2). Liver enzyme activities and other markers of liver and kidney function remained unchanged in both study groups over the 12 weeks intervention (Supplementary Tables 1 and 2). The same held true in the qualitative urinary analysis (protein, sugar, bilirubin, occult blood, ketone bodies) (data not shown).

Effect of betaine on lipid and glucose concentrations: Over the 12-week investigation period, no significant differences were observed between the betaine and placebo groups in terms of the plasma total cholesterol, triglyceride and LDL-cholesterol (Table 3). However, HDL cholesterol increased significantly in the betaine group alone ($p<0.05$), with no such significant change noted in the control group (Table 3). The HDL cholesterol concentrations were comparable between the two groups at baseline. Fasting blood glucose, HbA1c and insulin, concentrations remained unchanged in both study groups over the 12 weeks (Table 4).

The fatty liver overproduces VLDL particles [6,40], which leads to elevated triglyceride and low HDL cholesterol concentrations in humans. The present study shows that a low-dose betaine supplementation can increase in HDL in subjects with mild fatty liver, as compared to the placebo group, even after adjustment for age and BMI. However the betaine supplementation did not affect significantly total cholesterol, triglyceride, LDL-cholesterol, glucose, insulin, or liver enzyme concentrations over the 12-week intervention.

The previous epidemiological studies have indicated that plasma betaine concentrations are positively associated with plasma HDL concentrations [14].

	Betaine group		Placebo group		p-value
	Mean	SD	Mean	SD	
Body weight (kg)					
Baseline	69.1	5.2	72.21	8.2	*
2 weeks	69.2	5.6	72.38	8.2	*
4 weeks	69.5	5.1	72.09	8	*
8 weeks	69.5	5.3	72.72	8.1	*
12 weeks	69.5	5	72.81	8.1	*
BMI (kg/m^2)					
Baseline	24.4	1.3	25.35	1.7	*
2 weeks	24.3	1.3	25.38	1.7	*
4 weeks	24.5	1.2	25.29	1.7	*
8 weeks	24.5	1.3	25.48	1.7	*
12 weeks	24.5	1.4	25.53	1.6	*
Waist circumference (cm)					
Baseline	86.7	3.3	88.15	4.5	*
12 weeks	86.3	4.8	87.75	3.9	0.97
Subcutaneous fat area (cm^2)					
Baseline	131.8	29.1	149.29	39.5	*
12 weeks	132.6	35.9	145.06	41.7	0.9
Visceral fat area (cm^2)					
Baseline	93.9	16.9	80.26	22.7	*
12 weeks	92.6	16.4	80.62	20.7	0.85
Liver/spleen ratio					
Baseline	1.01	0.3	0.89	0.3	*
12 weeks	1.03	0.2	0.96	0.2	0.9
Systolic BP (mm Hg)					
Baseline	134.3	11.4	137.4	13.1	*
2 weeks	125.9	8.9	132.2	11.3	0.9
4 weeks	133.8	10.4	135.2	17	0.9
8 weeks	135.4	17.6	135.4	11.2	0.87
12 weeks	133.6	11.1	135.2	10.6	0.9
Diastolic BP (mm Hg)					
Baseline	85.2	9.2	89.6	12.3	*
2 weeks	78.8	10.6	87.3	10.4	0.63
4 weeks	82	7.7	86.1	12.5	0.97
8 weeks	81.7	10.7	88.3	11.7	0.79
12 weeks	81.2	7	87.5	8.3	0.85
BMI-Body Mass Index. No statistically significant difference was observed between the groups *Baseline and BMI were used as covariates of the linear models.					

Table 2: Effects of betaine vs. placebo on body weight, BMI, subcutaneous and visceral fat area and liver/spleen ratio during the intervention.

	Betaine group		Placebo group		p-value
	Mean	SD	Mean	SD	
fP-Total cholesterol (mmol/l)					
Baseline	5.76	0.97	5.79	0.81	*
2 weeks	5.95	1	5.75	0.68	0.85
4 weeks	6.08	0.95	5.77	0.63	0.75
8 weeks	5.99	0.97	5.73	0.74	0.78
12 weeks	6.32	0.8	5.93	0.69	0.63
fP-HDL cholesterol (mmol/l)					
Baseline	1.34	0.28	1.22	0.2	*
2 weeks	1.38	0.2	1.18	0.2	0.75
4 weeks	1.43	0.25	1.22	0.25	0.72
8 weeks	1.34	0.25	1.15	0.22	0.75
12 weeks¤	1.53	0.31	1.22	0.18	0.04
fP-LDL cholesterol (mmol/l)					
Baseline	3.57	0.94	3.86	0.73	*
2 weeks	3.73	0.93	3.86	0.78	0.9
4 weeks	3.76	0.94	3.83	0.71	0.87
8 weeks	3.65	1.06	3.86	0.83	0.97
12 weeks	3.98	0.79	3.93	0.71	0.78
fP-Triglycerides (mmol/l)					
Baseline	2.14	0.8	1.87	0.62	*
2 weeks	2.07	0.77	2.12	0.95	0.97
4 weeks	2.27	0.84	1.78	0.76	0.75
8 weeks	2.74	1.67	2.15	0.93	0.85
12 weeks	1.84	0.54	2.09	0.84	0.87
fp-Fasting Plasma. Data is expressed as means and standard deviations. * Baseline was used as a covariate of the linear models.					

Table 3: Effects of betaine vs. placebo on plasma lipids during the intervention

Moreover, high dietary betaine intake is inversely associated with the concentrations of inflammatory markers such as C-reactive protein

and tumor necrosis factor-α [41], indicating decreased risk markers for metabolic disorders. In addition, in a hyperhomocysteinemic mice model, betaine has been shown to reverse the atherogenic lipid profile characterized by decreased plasma triglyceride and increased HDL cholesterol concentrations [42]. However, the high betaine dose has been suggested to have an adverse effect on blood lipids [43-45]. Such effects have not been related to low-dose betaine supplementation (<6g/d) [45]. Although high HDL cholesterol levels are inversely associated to coronary heart disease [46] recent clinical trials on cholesteryl ester transfer protein (CETP) inhibitor has indicated that HDL function, rather than its concentration, determines its protection from atherosclerosis [47]. Thus, the analysis of HDL subpopulations displaying specific biological functions would be needed to show the atheroprotective potential of HDL [48].

	Betaine group		Placebo group		p-value
	Mean	SD	Mean	SD	
fP-Glucose (mmol/l)					
Baseline	5.12	0.54	4.87	0.51	*
2 weeks	4.84	0.35	5.05	0.47	0.72
4 weeks	4.82	0.35	4.81	0.19	0.9
8 weeks	5.1	0.79	4.96	0.25	0.97
12 weeks	5.01	0.51	5	0.47	0.9
HbA1c (%)					
Baseline	4.85	0.29	5.08	0.26	*
2 weeks	4.88	0.29	5.08	0.24	0.9
4 weeks	4.93	0.32	5.09	0.23	0.75
8 weeks	4.9	0.27	5.05	0.25	0.72
12 weeks	4.97	0.25	5.06	0.21	0.054
fP-Insulin (mU/l)					*
Baseline	8.09	8.38	4.8	1.48	0.97
8 weeks	11.27	11.89	7.14	3.82	0.9
12 weeks	6.39	6.55	11.61	15.87	0.75

fP-Fasting Plasma. Data is expressed as means and standard deviations. No statistically significant difference was observed between the group. *Baseline was used as a covariate of the linear models.

Table 4: Effects of betaine vs. placebo on glucose metabolism during the intervention

Effect of betaine on markers of inflammatory, oxidative stress, fibrosis and fibrinolytic activities: The concentrations of plasma hs-CRP, PAI-1, fibrinogen collagen type-IV and urinary 8-OHdG (Supplementary Table 3) remained unchanged in both study groups over the 12-week investigation period.

Effect of betaine on plasma amino acid and betaine concentrations: After 12 weeks, plasma betaine concentrations were significantly elevated from 52 ± 8 to 133 ± 53 μmol/l (mean ± SD, P=0.0001) in the betaine group, though no such increase was observed in the control group (from 48 ± 11 to 48 ± 10 μmol/l). Thus, betaine has good bioavailabilty as shown by the significantly elevated plasma betaine concentrations compared to the control group. There were no other significant differences in plasma amino acid concentrations during the intervention (Supplementary Table 4).

Effect of betaine on hematological function: There were no significant differences in hematological counts at baseline or any significant changes in the counts during the intervention (Supplementary Table 5).

Side effects: All the subjects in the present study completed the intervention. There were no differences between the study groups in the complaints of adverse events. Five non-serious adverse events occurred in three cases in the betaine group and 12 non-serious adverse events occurred in five cases in the placebo group. There was only one adverse effect (loose stool) in betaine group that could have causal relationship with the product. All the events were mild, and the symptoms resolved without interruption of the intervention.

There were no differences in blood or urine safety parameters between the betaine and placebo groups. Moreover, in contrast to the betaine intervention study with the healthy subjects by Schwab et al [49] in the present study there was no increase in PAI-1 concentration. This implies that low-dose betaine (3 g/day) supplementation is both safe and well-tolerated in mild fatty liver subjects. At moderate doses (6 g/d) betaine is generally well-tolerated. The side effects of high-dose betaine supplementation in humans may cause gastrointestinal discomfort, which occur in approximately one-third of subjects receiving high-dose daily betaine supplementation (20 g/d) [23].

Conclusion

As a conclusion betaine is safe and well tolerated, it has also good bioavailabilty as shown by the significantly elevated plasma betaine concentrations compared to the control group. Although there were no changes in liver fat content in the present study, an increase in HDL cholesterol in the betaine group may reflect favorable effects of betaine on liver function-related metabolism in Asian males who has increased risk for developing fatty liver. However, further studies are required to clarify the long term effect of betaine in liver function in more defined and larger Asian populations.

Acknowledgements

The authors are indebted to the participants of the study for their cooperation and assistance. The authors express the special gratitude to Professor Emeritus Matsuzaki (Tokai University) for his many valuable advices on the study protocol and interpretation of results. The authors thank also Anna Kotronen (University of Helsinki) for the fatty liver expertise, professor emeritus Mikko Salaspuro (University of Helsinki) for the comments, Stuart Craig and Elaine Krul (DuPont) for the comments and the language consultancy, Katriina Talvitie (Danisco) for the betaine analysis. Kazuomi Hamatsu (EPS Co., Ltd.), Maki Sanami (EPMint Co., Ltd.), and Mika Paulamäki (Danisco) are thanked for the practical arrangements. KT was involved in planning and designing the study, interpretation of data, and writing the manuscript, MS was involved in analysis of the data and writing the manuscript, EA contributed in the statistical analysis and data management, GY wrote the protocol, organized the study and acquisition of the data, MN was involved in planning the study and writing the manuscript. All authors read and approved the final manuscript.

Conflicts of Interests

KT, MS and NM are or have been employees of DuPont. DuPont manufactures and markets betaine. The authors declare no other conflict of interest regarding this study.

The study was funded by Danisco A/S, a company now owned by E. I. du Pont de Nemours and Company (commonly referred to as DuPont).

References

1. Kojima S, Watanabe N, Numata M, Ogawa T, Matsuzaki S (2003) Increase In the Prevalence of Fatty Liver In Japan Over the Past 12 Years: Analysis of Clinical Background. J Gastroenterol 38: 954-961.
2. Hanley AJ, Williams K, Festa A, Wagenknecht LE, D'Agostino RB, et al. (2004) Elevations in markers of liver injury and risk of type 2 diabetes: the insulin resistance atherosclerosis study. Diabetes 53: 2623-2632.
3. Targher G, Marra F, Marchesini G (2008) Increased risk of cardiovascular disease in non-alcoholic fatty liver disease: causal effect or epiphenomenon? Diabetologia 51: 1947-1953.
4. Ekstedt M, Franzen LE, Mathiesen UL, Thorelius L, Holmqvist M, et al. (2006) Long-term follow-up of patients with NAFLD and elevated liver enzymes. Hepatology 44: 865-873.
5. Kotronen A, Yki-Jarvinen H (2008) Fatty liver: a novel component of the metabolic syndrome. Arterioscler Thromb Vasc Biol 28: 27-38.
6. Adiels M, Taskinen MR, Packard C, Caslake MJ, Soro-Paavonen A, et al. (2006) Overproduction of large VLDL particles is driven by increased liver fat content in man. Diabetologia 49: 755-765.
7. Larson-Meyer DE, Heilbronn LK, Redman LM, Newcomer BR, Frisard MI, et al. (2006) Effect of calorie restriction with or without exercise on insulin sensitivity, beta-cell function, fat cell size, and ectopic lipid in overweight subjects. Diabetes Care 29: 1337-1344.
8. Petersen KF, Dufour S, Befroy D, Lehrke M, Hendler RE, et al. (2005) Reversal of nonalcoholic hepatic steatosis, hepatic insulin resistance, and hyperglycemia by moderate weight reduction in patients with type 2 diabetes. Diabetes 54: 603-608.
9. Belfort R, Harrison SA, Brown K, Darland C, Finch J, et al. (2006) A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. N Engl J Med 355: 2297-2307.
10. Tiikkainen M, Hakkinen AM, Korsheninnikova E, Nyman T, Mäkimattila S, et al. (2004) Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, insulin clearance, and gene expression in adipose tissue in patients with type 2 diabetes. Diabetes 53: 2169-2176.
11. Ratziu V, Zelber-Sagi S (2009) Pharmacologic therapy of non-alcoholic steatohepatitis. Clin Liver Dis 13: 667-88.
12. Craig SA (2004) Betaine in human nutrition. Am J Clin Nutr 80: 539-549.
13. Schwab U, Torronen A, Meririnne E, Saarinen M, Alfthan G, et al. (2006) Orally administered betaine has an acute and dose-dependent effect on serum betaine and plasma homocysteine concentrations in healthy humans. J Nutr 136: 34-38.
14. Konstantinova SV, Tell GS, Vollset SE, Nygard O, Bleie O, et al. (2008) Divergent associations of plasma choline and betaine with components of metabolic syndrome in middle age and elderly men and women. J Nutr 138: 914-920.
15. Williams KT, Schalinske KL (2007) New insights into the regulation of methyl group and homocysteine metabolism. J Nutr 137: 311-314.
16. Finkelstein JD, Martin JJ (1984) Methionine metabolism in mammals. Distribution of homocysteine between competing pathways. J Biol Chem 259: 9508-9513.
17. Varatharajalu R, Garige M, Leckey LC, Gong M, Lakshman MR (2009) Betaine protects chronic alcohol and omega-3 PUFA-mediated down-regulations of PON1 gene, serum PON1 and homocysteine thiolactonase activities with restoration of liver GSH. Alcohol Clin Exp Res 34: 424-431.
18. Kharbanda KK, Mailliard ME, Baldwin CR, Beckenhauer HC, Sorrell MF, et al. (2007) Betaine attenuates alcoholic steatosis by restoring phosphatidylcholine generation via the phosphatidylethanolamine methyltransferase pathway. J Hepatol 46: 314-321.
19. Barak AJ, Beckenhauer HC, Junnila M, Tuma DJ (1993) Dietary betaine promotes generation of hepatic S-adenosylmethionine and protects the liver from ethanol-induced fatty infiltration. Clin Exp Res 17: 552-555.
20. Song Z, Deaciuc I, Zhou Z, Song M, Chen T, et al. (2007) Involvement of AMP-activated protein kinase in beneficial effects of betaine on high-sucrose diet-induced hepatic steatosis. Am J Physiol Gastrointest Liver Physiol 293: G894-902.
21. Wang Z, Yao T, Pini M, Zhou Z, Fantuzzi G, et al. (2010) Betaine improved adipose tissue function in mice fed a high-fat diet: a mechanism for hepatoprotective effect of betaine in nonalcoholic fatty liver disease. Am J Physiol Gastrointest Liver Physiol 298: G634-642.
22. Kathirvel E, Morgan K, Nandgiri G, Sandoval BC, Caudill MA, et al. (2010) Betaine improves nonalcoholic fatty liver and associated hepatic insulin resistance: a potential mechanism for hepatoprotection by betaine. Am J Physiol Gastrointest Liver Physiol 299: G1068-7G1070.
23. Abdelmalek M, Sanderson SO, Angulo P, Soldevila PC, Liu C, et al. (2009) Betaine for nonalcoholic fatty liver disease: results of a randomized placebo-controlled trial. Hepatology 50: 1818-1826.
24. Joseph AE, Saverymuttu SH, Al-Sam S, Cook MG, Maxwell JD (1991) Comparison of liver histology with ultrasonography in assessing diffuse parenchymal liver disease. Clin Radiol 43: 26-31.
25. Celle G, Savarino V, Picciotto A, Magnolia MR, Scalabrini P, et al. (1988) Is hepatic ultrasonography a valid alternative tool to liver biopsy? Report on 507 cases studied with both techniques. Dig Dis Sci 33: 467-71.
26. Akabame S, Hamaguchi M, Tomiyasu KI, Tanaka M, Kobayashi-Takenaka Y, et al. (2008) Evaluation of vulnerable coronary plaques and non-alcoholic fatty liver disease (NAFLD) by 64-detector multislice computed tomography (MSCT). Circ J 72: 618-25.
27. Davidson LE, Kuk JL, Church TS, Ross R (2006) Protocol for measurement of liver fat by computed tomography. J Appl Physiol 100: 864-866.
28. Laryea Md, Steinhagen F, Pawliczek S, Wendel U (1998) Simple method for the routine determination of betaine and N,N-dimethylglycine in blood and urine. Clin Chem 44: 1937-1941.
29. R Development Core T (2015) A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria
30. Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RC (2015) Linear and Nonlinear Mixed Effects Models.
31. Benjamini Y, Hochberg Y (1995) Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) 57: 289-300.
32. Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC (2008) Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatology 47: 1363-1370.
33. Sridhar GR, Rao AA, Srinivas K, Nirmala G, Lakshmi G, et al. (2010) Butyrylcholinesterase in metabolic syndrome. Med Hypotheses 75: 648-51.
34. Iwamura K, Matsuzaki S, Masaru I (1980) An elevation of serum cholinesterase activity in fatty liver. Acta Hepatology Japan 21: 823-829.
35. Kan H, Kimura Y, Hyogo H, Fukuhara T, Fujino H, et al. (2014) Non-invasive assessment of liver steatosis in non-alcoholic fatty liver disease. Hepatol Res 44: E420-E427.
36. Celle G, Savarino V, Picciotto A, Magnolia MR, Scalabrini P et al. (1988) Is hepatic ultrasonography a valid alternative tool to liver biopsy? Report on 507 cases studied with both techniques. Dig Dis Sci 33:467-471.
37. Hamer OW, Aguirre DA, Casola G, Lavine JE, Woenckhaus M, et al. (2006) Fatty liver: imaging patterns and pitfalls. Radiographics 26: 1637-53.
38. Victor RG, Haley RW, Willett DL, Peshock RM, Vaeth PC, et al. (2004) The Dallas Heart Study: a population-based probability sample for the

multidisciplinary study of ethnic differences in cardiovascular health. Am J Cardiol 93: 1473-80.

39. Szczepaniak LS, Nurenberg P, Leonard D, Browning JD, Reingold JS, et al. (2005) Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. Am J Physiol Endocrinol Metab 288: E462-E468.
40. Adiels M, Westerbacka J, Soro-Paavonen A, Häkkinen AM, Vehkavaara S, et al. (2007) Acute suppression of VLDL1 secretion rate by insulin is associated with hepatic fat content and insulin resistance. Diabetologia 50: 2356-2365.
41. Detopoulou P, Panagiotakos DB, Antonopoulou S, Pitsavos C, Stefanadis C (2008) Dietary choline and betaine intakes in relation to concentrations of inflammatory markers in healthy adults: the ATTICA study. Am J Clin Nutr 87: 424-430.
42. Schwahn BC, Wang XL, Mikael LG, Wu Q, Cohn J, et al. (2007) Betaine supplementation improves the atherogenic risk factor profile in a transgenic mouse model of hyperhomocysteinemia. Atherosclerosis 195: e100-107.
43. Dalmeijer GW, Olthof MR, Verhoef P, Bots ML, Van der Schouw YT (2008) Prospective study on dietary intakes of folate, betaine, and choline and cardiovascular disease risk in women. Eur J Clin Nutr 62: 386-394.
44. Olthof MR, Van Vliet T, Verhoef P, Zock PL, Katan MB (2005) Effect of homocysteine-lowering nutrients on blood lipids: results from four randomised, placebo-controlled studies in healthy humans. PLoS Med 2: e135.
45. Schwab U, Torronen A, Toppinen L, Alfthan G, Saarinen M, et al. (2002) Betaine supplementation decreases plasma homocysteine concentrations but does not affect body weight, body composition, or resting energy expenditure in human subjects. Am J Clin Nutr 76: 961-967.
46. Di Angelantonio E, Sarwar N, Perry P, Kaptoge S, Ray KK, et al. (2009) Major lipids, apolipoproteins, and risk of vascular disease. JAMA 302: 1993-2000.
47. Barter PJ, Puranik R, Rye KA (2007) New insights into the role of HDL as an anti-inflammatory agent in the prevention of cardiovascular disease. Curr Cardiol Rep 9: 493-498.
48. Camont L, Chapman MJ, Kontush A (2011) Biological activities of HDL subpopulations and their relevance to cardiovascular disease. Trends Mol Med 17: 594-603.
49. Schwab U, Alfthan G, Aro A, Uusitupa M (2011) Long-term effect of betaine on risk factors associated with the metabolic syndrome in healthy subjects. Eur J Clin Nutr 65: 70-76.