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METABOLIC LIVER DISEASE

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Nonalcoholic steatohepatitis is associated with a state of betaine-insufficiency

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Abstract

Background and Aims: Nonalcoholic fatty liver disease (NAFLD) develops from a complex process, which includes changes in the liver methylome. Betaine plays a pivotal role in the regulation of methylogenesis. We performed a two-stage case–control study, which included patients with biopsy-proven NAFLD to explore circulating levels of betaine and its association with the histological spectrum. We also explored the association between a missense rs1805074, p.Ser646Pro variant in *DMGDH* (dimethylglycine dehydrogenase mitochondrial) and NAFLD severity (n=390).

Results: In the discovery phase (n=48), betaine levels were associated with the disease severity (*P*=.0030), including liver inflammation (Spearman R:−0.51, *P*=.001), ballooning degeneration (R: −0.50, *P*=.01) and fibrosis (R: −0.54, *P*=.0008). Betaine levels were significantly decreased in nonalcoholic steatohepatitis (NASH) in comparison with nonalcoholic fatty liver (NAFL). Further replication (n=51) showed that betaine levels were associated with advanced NAFLD (*P*=.0085), and patients with NASH had a 1.26-fold decrease in betaine levels compared with those with NAFL. The rs1805074 was significantly associated with the disease severity (*P*=.011).

Conclusion: NAFLD severity is associated with a state of betaine-insufficiency.

KEYWORDS

betaine, biomarkers, dimethylglycine dehydrogenase mitochondrial, DNA methylation, epigenetics, fibrosis, genetics, inflammation, metabolomics, nonalcoholic fatty liver disease, nonalcoholic steatohepatitis

Abbreviations: ALT, alanine-aminotransferase; AST, aspartate-aminotransferase; BMI, body mass index; CVD, cardiovascular disease; DMGDH, dimethylglycine dehydrogenase mitochondrial; HOMA, homeostasis model assessment; MAF, minor allele frequency; NAFLD, nonalcoholic fatty liver disease; NAFL, nonalcoholic fatty liver; NASH, nonalcoholic steatohepatitis; SAM, S-adenosylmethionine; SNPsingle nucleotide polymorphism.

1 | INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is characterized by abnormal lipid accumulation, predominantly triglycerides in the liver. The histological spectrum of NAFLD extends from a relatively benign nonalcoholic fatty liver (NAFL) characterized by steatosis to a more severe form represented by liver cell injury, a mixed inflammatory lobular infiltrate, hepatocellular ballooning and variable fibrosis named nonalcoholic steatohepatitis (NASH).[1] NAFLD is the leading cause of chronic liver disease worldwide;[2] in fact, its prevalence has reached global epidemic proportions, both in adults and children.[3] The development of NAFLD is a complex interplay of several processes including genetic susceptibility and environmental insults.[4]

Furthermore, emerging human data suggest that in addition to the metabolic syndrome (MetS)-related phenotypes, including insulin resistance (IR), epigenetic modifications in both genomic[5–8] and mitochondrial DNA[9] modulate NAFLD pathobiology. [7,10,11] In fact, these clinical studies provide robust evidence that NAFLD is associated with tissue-specific epigenetic modifications, which directly impact the regulation of the transcriptome.[5–7,9]

Recent experimental evidence also showed that NAFLD is associated with hepatic methionine deficiency and homocysteine elevation as a result of impaired homocysteine remethylation and an aberrancy in methyltransferases-mediated reactions.[12] Taken together, these evidences consistently demonstrate an aberrant pattern of DNA methylation in the liver of patients with NAFLD.

S-adenosylmethionine (SAM) is the major methyl donor in the cell. The liver is the major site for SAM- synthesis and degradation and SAM is involved in the regulation of the "methylome" dynamics during DNA methylation.[13] Methyl groups are usually delivered by dietary methyl donors, which mostly include methionine, choline, and betaine.[14] Betaine, (N,N,N-trimethylglicine, which refers to the amino acid glycine with three N-methyl groups) is a critical player in the pathway of methylogenesis. For instance, betaine controls the serum methionine levels by methylation of homocysteine, and also by producing dimethylglycine, which in turn controls the methy-group transference.[14] Moreover, betaine modulates the rate of methyl donor in the synthesis of many other metabolic pathways, including proteins and phospholipids.[14] Betaine is also an important regulator of SAM liver concentration; SAM availability is thought to regulate phosphatidylcholine (PC) synthesis by phosphatidylethanolamine N-methyltransferase (PEMT), the later normalizes VLDL production rates being determinant to exporting lipids from the liver.[14–17] Betaine/choline deficiency may decrease SAM availability and PC synthesis, promoting liver fat accumulation[14–17]; for instance, *PEMT* knockout mice have fatty liver and abnormal hepatic choline metabolite concentrations despite ingesting a recommended dietary intake of choline.[18] Experimental results demonstrated a protective role of betaine-homocysteine methyltransferase (BHMT) in homocysteine-induced injury in both cultured hepatocytes[16] and transgenic mouse models.[17]

Key points

- NAFLD is associated with tissue-specific epigenetic modifications, including an aberrant pattern of DNA methylation.
- Betaine, (N,N,N-trimethylglicine) is a critical player in the pathway of methylogenesis by controlling the serum methionine levels and by producing dimethylglycine, which in turn controls the methy-group transference.
- NASH is associated with decreased levels of betaine in circulation.
- The missense variant p.Ser646Pro (rs1805074) in *DMGDH* gene, which encodes for the mitochondrial dimethylglycine dehydrogenase, was significantly associated with the disease severity and circulating levels of dimethylglycine.

Of note, betaine is inversely associated with plasma triglycerides, LDL-cholesterol and apolipoprotein levels.[19] Betaine is also associated with biomarkers of systemic inflammation,[20] vascular function, and an overall risk of cardiovascular disease.[21]

Results from a recent genome-wide association study coupled with high-throughput metabolic profiling showed variants in the gene *DMGDH*, which encodes for the mitochondrial dimethylglycine dehydrogenase, are significantly associated with circulating levels of betaine and betaine-related metabolites, as well as metabolites of other metabolic pathways, including amino acids.[22]

We therefore hypothesized that NAFLD severity is associated with a state of "betaine-insufficiency". To test this, we performed a two-stage case–control study in patients with biopsy-proven NAFLD to explore the association between circulating levels of betaine and the spectrum of liver histology in NAFLD. In addition, we performed a candidate-gene association study on the role of a *DMGDH*-missense variant (rs1805074, p.Ser646Pro) in NAFLD severity.

2 | PATIENTS AND METHODS

2.1 | Patients and control subjects: selection criteria

This study was conducted in accordance with the guidelines of the 1975 Declaration of Helsinki. Written consent from individuals was obtained in accordance with the procedures approved by the Ethical Committee of each institution. The protocol was approved by the Comite de Etica Hospital Zubizarreta (protocol number: 104/HGAZ/09 and 89/100) and VCU-IRB (protocol numbers HM14081 and HM14427).

The exploration of circulating levels of betaine and its relationship with NAFLD severity was performed in two phases:(i) a discovery phase in subjects recruited in Buenos Aires, Argentina (n=48), and (ii) a replication phase in a cohort of subjects recruited in Richmond, VA,

USA (n=51). This study design allowed us to confirm the initial findings in an independent sample of patients with different environmental influences.

The candidate-gene association study was performed in a sample of 390 individuals under the same inclusion and exclusion criteria that of the case–control study on betaine levels. Participants included 138 controls and 252 patients with NAFLD proven by liver biopsy and recruited in Buenos Aires, Argentina. Complete details on selection criteria, anthropometric and biochemical evaluation, and liver biopsy are given in the Supporting Information.

2.2 | Histopathological evaluation

The degree of steatosis was assessed according to the system developed by Kleiner et al., based on the percentage of hepatocytes containing macrovesicular fat droplets.[23] NASH[24] and NAFLD Activity Score (NAS)[23] were defined as reported previously; a NAS threshold of 5 was used for further comparisons with variables of interest, NASH was defined as steatosis plus mixed inflammatory-cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory's hyaline, and any stage of fibrosis, including absent fibrosis.[23]

2.3 | Measurement of circulating levels of betaine and related metabolites

Serum metabolite measurements, including betaine and dimethylglycine, from patients and controls were obtained using high performance liquid chromatography-mass spectrometry (HPLC-MS/MS) method. Determinations in samples of patients from Argentina were performed in the Northwest Metabolomics Research Center Core facility (University of Washington, Seattle, WA, USA), while determinations in samples of patients from the VCU, Richmond, were done in Metabolon Inc, Durham, NC, United States. Details are given in the Supporting Information.

2.4 | Genotype and association analysis; power and sample size calculation

The selection of *DMGDH* single nucleotide polymorphism (SNP) for the association analysis was based on the results from the comprehensive exploration of genetic loci influencing human metabolism[22] and The Metabolomics GWAS Server available at http://metabolomics.helmholtz-muenchen.de/gwas/. Prioritization was given to variants with a minor allele frequency higher than 10%; hence, we focused our analysis on A>G rs1805074, a missense variant at position 646 (p.Ser646Pro) located in the forward strain, which was significantly associated with betaine levels (P=7.97e^{-18}), betaine/glutamine ratio (P=5.583e^{-19}), carnitine/betaine ratio (P=1.434e^{-18}) and tyrosine/betaine ratio (P=6.557e^{-18}) (http://metabolomics.helmholtz-muenchen.de).[22]

Using the CaTS power calculator for genetic association studies[25] and assuming a prevalence of NAFLD of 0.30 and a MAF of 0.30, our sample had 95% power for the additive genetic model. Further details are given in the Supporting Information.

2.5 | Statistical analysis

Complete details are given in the Supporting Information.

3 | RESULTS

The discovery study included 48 individuals, of whom 32 were patients with NAFLD and 16 were healthy controls. The participant characteristics are shown in Table 1. As expected, compared with the control group, patients with NAFLD showed most of the risk factors of the MetS, namely higher BMI, fasting glucose, insulin and HOMA-index, and CVD risk factors.

3.1 | NAFLD severity is associated with a state of "betaine-insufficiency"

In the discovery phase, we observed that the serum levels of betaine were significantly associated with the disease severity (regression analysis for an ordinal multinomial distribution P=.0030); also, the betaine levels in circulation were significantly reduced in patients with NASH in comparison with NAFL (Table 1). Moreover, betaine levels were inversely correlated with the degree of histological steatosis (Spearman R: −0.49, P=.004), inflammation (Spearman R: −0.51, P=.001), ballooning degeneration (Spearman R: −0.50, P=.01), and fibrosis stage (Spearman R: −0.54, P=.0008); accordingly, the serum levels of betaine were significantly and negatively correlated with the NAS score (Spearman R: −0.55, P=.005). Figure 1 shows betaine levels according to the scores of NAFLD histological lesions; statistical differences remain significant even after adjusting by BMI and HOMA-IR. Furthermore, we observed that circulating levels of betaine were significantly lower in NASH as compared with controls (P=.0037); however, there were no differences in the comparison between NAFL and the control group (Table 1).

We also observed that circulating betaine levels were significantly and inversely correlated with systolic blood pressure (Spearman R: −0.34, P=.04), and other parameters associated with the MetS, including body adiposity index (BAI) [26] (Spearman R: −0.35, P=.02) or leucocyte count as surrogate of systemic inflammation (Spearman R: −0.49, P=.01) but not with glucose metabolism.

The replication study included 51 individuals, of whom 44 were patients with NAFLD, and seven were healthy controls; the participants' characteristics are shown in Table 2. Also worthy of note are the serum levels of betaine, which were consistently associated with the histological severity of NAFLD (P=.0085); furthermore, patients with NASH compared with those with NAFL had a 1.26-fold reduction in serum betaine levels. The comparison between circulating levels of betaine between patients with NASH and healthy controls showed significant differences (P=.0026); however, there were no

TABLE 1 Clinical and biochemical evaluation of patients with NAFLD and healthy controls enrolled in the discovery study on circulating levels of betaine

Variable (mean ± SD)	Control	NAFL	NASH	*P* value* *P*<	*P* value** *P*<	*P* value# *P*<
Number of subjects	16	16	16	–	–	–
Female/male, %	50/50	50/50	59/41	NS	NS	NS
Age, years	47.5 ± 13	52 ± 8	51.7 ± 9	NS	NS	NS
BMI, kg/m^2	25 ± 2.5	30 ± 4.3	32 ± 7	.0008	NS	.001
Waist circumference, cm	85 ± 13	102 ± 9	107 ± 14	.0005	NS	.0002
Fasting plasma glucose, mg/dL	74 ± 7	97 ± 15	98 ± 22	.00004	NS	.0004
Fasting plasma insulin, mg/dL	6 ± 2	10 ± 6	14 ± 7	.05	NS	.001
HOMA-IR index	1.1 ± 0.4	2.4 ± 1.4	3.5 ± 2	.001	NS	.0003
SABP, mm Hg	113 ± 8.5	126 ± 17	127 ± 14	.03	NS	.01
DABP, mm Hg	69 ± 7.6	79 ± 9	81.5 ± 10	.01	NS	.008
Total cholesterol, mg/dL	221 ± 45	212 ± 48	228 ± 45	NS	NS	NS
HDL-cholesterol, mg/dL	55 ± 12	57 ± 29	48 ± 10	NS	NS	NS
LDL-cholesterol, mg/dL	143 ± 34	120 ± 40	133 ± 52	NS	NS	NS
Triglycerides, mg/dL	117 ± 68	161 ± 76	198 ± 117	.07	NS	.010
ALT, U/L	22 ± 8.5	53 ± 37	92 ± 84	.0004	.04	.000006
AST, U/L	19.5 ± 4.6	34 ± 14	58 ± 42	.0006	.03	.000002
GGT, U/L	39 ± 42	74 ± 48	71 ± 72	.02	NS	.02
AP, U/L	139.5 ± 57	237 ± 117	222 ± 127	.001	NS	.007
Histological features						
Degree of steatosis, %	–	38 ± 14	61 ± 18	–	.001	–
Lobular inflammation (0-3)	–	0.64 ± 0.67	1.09 ± 0.7	–	NS	–
Hepatocellular ballooning (0-2)	–	0.0 ± 0.0	0.82 ± 0.6	–	.01	
Fibrosis Stage	–	0.0 ± 0.0	1.06 ± 1.34	–	.02	
NAS	–	2.3 ± 1.1	5.75 ± 1.7	–	.0001	
Circulating betaine levels (normalized data from the peak areas detected by HPLC-MS/MS)	0.25 ± 0.90	0.44 ± 0.71	−0.61 ± 0.98	NS	.0016	.01
Circulating dimethylglycine levels (normalized data from the peak areas detected by HPLC-MS/MS)	0.17 ± 0.61	0.47 ± 1.02	−0.30 ± 1.03	NS	.04	NS

ALT and AST, serum alanine and aspartate aminotransferase, respectively; AP, alkaline phosphatase; BMI, body mass index; GGT, gamma-glutamyl-transferase; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment-insulin resistance; LDL-C, low-density lipoprotein cholesterol; NAFL, nonalcoholic fatty liver or simple steatosis; NAFLD, nonalcoholic fatty liver disease; NAS, NASH activity score; NASH, nonalcoholic steatohepatitis.

Results are expressed as mean ± SD. *Indicates comparisons between NAFL and controls, **denotes NAFL vs. NASH comparisons, and # pertains to the comparisons between NASH and controls. The *P* value reflects the statistical significance calculated using the Mann–Whitney *U*-test, with the exception of the female/male ratio, where the *P* value reflects the statistical significance calculated via a Chi-squared test. NS: nonsignificant.

differences in the comparison between NAFL and the control group (Table 2).

Figure 2 depicts the results of the ROC analysis on the performance of betaine in predicting the disease severity (NAFL vs NASH) in the discovery (panel A) and the replication (panel B) study, as well as in the joined dataset (panel C).

Of note, the decrease in circulating levels of betaine in NASH patients compared either with controls or NAFL patients remains significant after adjustment for main confounding factors, such as HOMA and BMI (*P*=.034).

3.2 | The missense variant p.Ser646Pro (rs1805074) was significantly associated with the disease severity

We further performed a candidate-gene association study in a larger sample to explore the role of the missense *p.Ser646Pro* (rs1805074) variant in the disease severity. The characteristics of patients and controls are shown in Table 3. On the basis of the previous knowledge of the role of this variant in the modulation of betaine-intermediate metabolites,[22] we reasoned that rs1805074 may be involved in NAFLD biology.

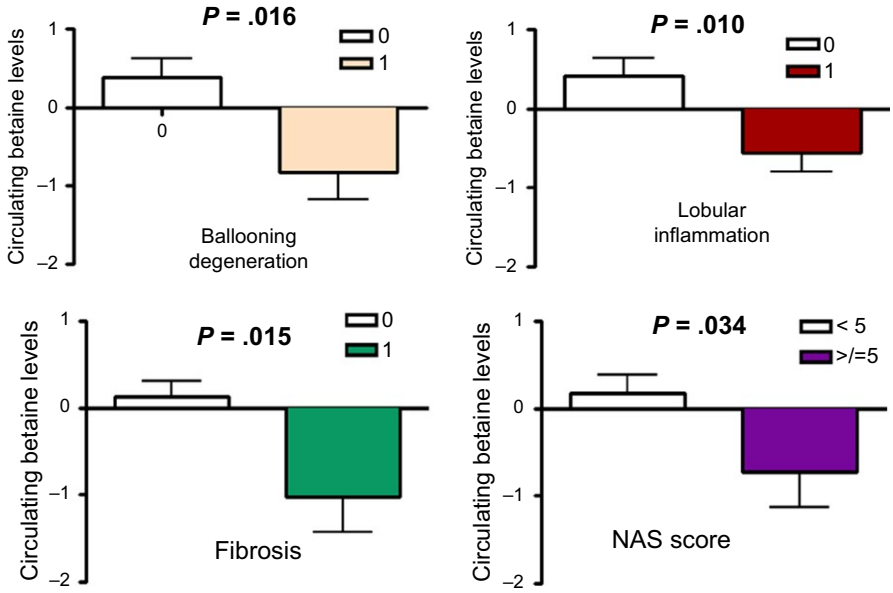

FIGURE 1 The association between circulating betaine levels and the histological severity of Nonalcoholic Fatty Liver Disease. Circulating betaine levels stand for normalized data (Z scores) from the peak areas detected by HPLC-MS/MS; each bar represents mean ± SE values. NAFLD histological lesions were scored as described in methods section[23,24]; for assessing the levels of betaine according to liver histology, were grouped the histological variables as follows: NAFLD activity score (NAS) threshold of 5: <5 vs ≥5; liver fibrosis: absent or mild (F0-F1) vs. moderate or severe (F2-F3); intra-acinar (lobular) inflammation and ballooning degeneration: absent (0) vs. present (1). *P* stands for values adjusted for log-transformed HOMA and BMI

For this purpose, we performed an ordinal multinomial distribution with Probit function by coding the histological grade as control subjects (NAFL and NASH), and observed that rs1805074 was significantly associated with the disease severity (*P*=.011), independent of sex and HOMA-IR. The allelic cumulative OR for the allele G: 0.693 95% CI: 0.509-0.944 (Cochran–Armitage test for trend *P*=.020, chi-sq.=5.391). This effect is mostly explained by the protection of the G allele on NASH vs. control risk (OR per allele: 0.88, 95% CI: 0.66-0.98, *P*=.0275).

Furthermore, the rs1805074 was significantly associated with circulating levels of dimethylglycine in both the additive (Spearman R: −0.353, *P*=.0148) and dominant (Spearman R: -.034, *P*=.0189) models of inheritance; in addition, an exploration of serum levels of dimethylglycine was performed in the subjects recruited in the discovery study. The distribution of genotypes according to the disease status in the candidate-gene association study is shown in Table 3.

4 | DISCUSSION

In this study, we demonstrated that NASH is associated with decreased levels of betaine in circulation, and this observation was validated in an independent study that included subjects who did not share environmental, ethnic and dietary habits (Figure 2D).

While the aim of this study was not focused on the role of betaine as potential biomarker, the area under the ROC to predict the presence of NASH was 0.755 in the joined analysis, which is comparable to other biomarkers used in the clinical setting to predict the presence of advanced liver disease, including the plasma levels of CK18 (caspase-cleaved cytokeratin 18 fragments: pooled sensitivity of 66% and a specificity of 82% in diagnosing NASH) [27] or circulating miR-122 (area under the ROC curve 0.714).[28]

Our findings are consistent with previous studies on animal models of diet-induced NAFLD. For instance, accumulated evidence from experimental studies in rodents consistently showed that betaine supplementation is able to ameliorate or even reverse fatty liver disease.[15,29–33] Furthermore, betaine supplementation was associated with increased SAM levels,[15] the improvement of inflammation and liver injury,[33] and the reversal of insulin resistance.[34]

The results from a randomized placebo-control study of 55 patients with a biopsy-proven NASH who had received either oral betaine (20 g daily) or a placebo for 12 months indicated that although betaine administration was not associated with changes in the non-alcoholic fatty liver disease activity score (NAS) or fibrosis stage, patients randomized to betaine had a decrease in steatosis grade.[35] From these results, it is then reasonable to suggest that betaine participates in the pathogenesis of NAFLD by modulating the transmethylation cycle and maintaining SAM levels, which probably explains the differences in the methylation state of genes involved either in fibrogenesis [6,8] or the metabolic pathways associated with the disease severity.[7,9]

Unfortunately, we do not have data on dietary habits or estimates of betaine dietary intake, which would have been of major interest to our study. Nevertheless, it was shown that betaine content in foods is not only variable but is dependable of different cooking methods, intestinal absorption and kidney function.[21] Results from a large cross-sectional study that involved 1628 participants from China, whose dietary pattern differ from those in the western populations and so their effect on the risk of the MetS,[36] showed that the severity of liver fat accumulation – as detected by liver ultrasound – was negatively correlated with serum betaine levels.[37] Together, these data suggest that – tough important – diet or dietary habits seem not to be involved in the association between betaine levels and the risk of NAFLD.

On the other hand, we observed that the missense *p.Ser646Pro* (T/C, rs1805074) variant located in the *DMGDH* locus was significantly associated with the disease severity and circulating levels of dimethylglycine. While the association between rs1805074 and NAFLD severity

TABLE 2 Clinical and biochemical evaluation of patients with NAFLD and healthy controls enrolled in the replication study on circulating levels of betaine

Variable (mean ± SD)	Control	NAFL	NASH	*P* value
Number of subjects	7	13	31	
Male/female, %	57/43	54/46	77/23	NS
Age, years	47.6 ± 9.8	54.7 ± 10	58.1 ± 9.2	.03
BMI, kg/m^2	29.0 ± 7.3	31.7 ± 6.3	34.2 ± 4.1	.05
Waist circumference, cm	92.3 ± 18.8	99.7 ± 19.3	105.5 ± 10.6	NS
Fasting plasma glucose, mg/dL	92.6 ± 8.5	95.2 ± 3.7	145.8 ± 62.6	.02
Fasting plasma insulin, Units	9.0 ± 4.6	15.6 ± 8.8	30.4 ± 14.9*,**	.001
HOMA-IR Index	1.2 ± 0.6	2.0 ± 1.1	4.2 ± 1.9*,**	.0004
SABP, mm Hg	126 ± 6.4	132 ± 13	138 ± 13.6	NS
DABP, mm Hg	77 ± 7.9	77 ± 10.3	73 ± 9.1	NS
Total cholesterol, mg/dL	187 ± 47	171 ± 34	189 ± 39	NS
HDL-C, mg/dL	55.7 ± 16.3	52.6 ± 15.1	45.1 ± 11.1	.09
LDL-C, mg/dL	117 ± 45	100.9 ± 27.6	116 ± 38.6	.48
Triglycerides, mg/dL	140.4 ± 53	106 ± 48.9	199 ± 131.5	.06
ALT, U/L	31.3 ± 17.6	42.1 ± 13	54.8 ± 27.5	.05
AST, U/L	25.4 ± 9.7	35.5 ± 27.4	40.3 ± 12	NS
AP, U/L	89 ± 16.1	74 ± 22.6	86 ± 30.9	NS
Histological features				
Degree of steatosis, grade		1.5 ± 1.0	1.9 ± 0.8	NS
Lobular inflammation (0-3)		1 ± 0.0	1.21 ± 0.6	NS
Hepatocellular ballooning (0-2)		0.2 ± 0.4	1.2 ± 0.5	<.0001
Fibrosis Stage		0.6 ± 0.96	2 ± 1.14	.0005
NAS		2.7 ± 0.85	4.3 ± 1.1	<.0001
Circulating betaine levels (normalized data from the peak areas detected by HPLC-MS/MS)	0.85 ± 0.55	0.46 ± 0.78	−0.35 ± 0.96*,**	.02
Circulating dimethylglycine levels (normalized data from the peak areas detected by HPLC-MS/MS)	0.04 ± 1.09	0.25 ± 0.87	−0.11 ± 1.00	NS

ALT and AST, serum alanine and aspartate aminotransferase, respectively; AP, alkaline phosphatase; BMI, body mass index; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment-insulin resistance; LDL-C, low-density lipoprotein cholesterol; NAFL, nonalcoholic fatty liver or simple steatosis; NAFLD, nonalcoholic fatty liver disease; NAS, NASH activity score; NASH, nonalcoholic steatohepatitis.

Continuous variables are reported as mean ±SD. Categorical variables are reported as counts (%). *Significant difference when compared with controls (Tukey's pairwise comparison $P<.05$). ** Significant difference when compared with NAFL (Tukey's pairwise comparison $P<.05$).

is a novel finding of our study, the association with dimethylglycine levels was previously reported in a large combined GWAS and exome analysis (P value=1.65×10^{-19}).[38] *DMGDH* encodes an enzyme involved in the catabolism of choline, which catalyzes the oxidative demethylation of dimethylglycine to form sarcosine. DMGDH is found as a monomer in the mitochondrial matrix, and uses flavin adenine dinucleotide and

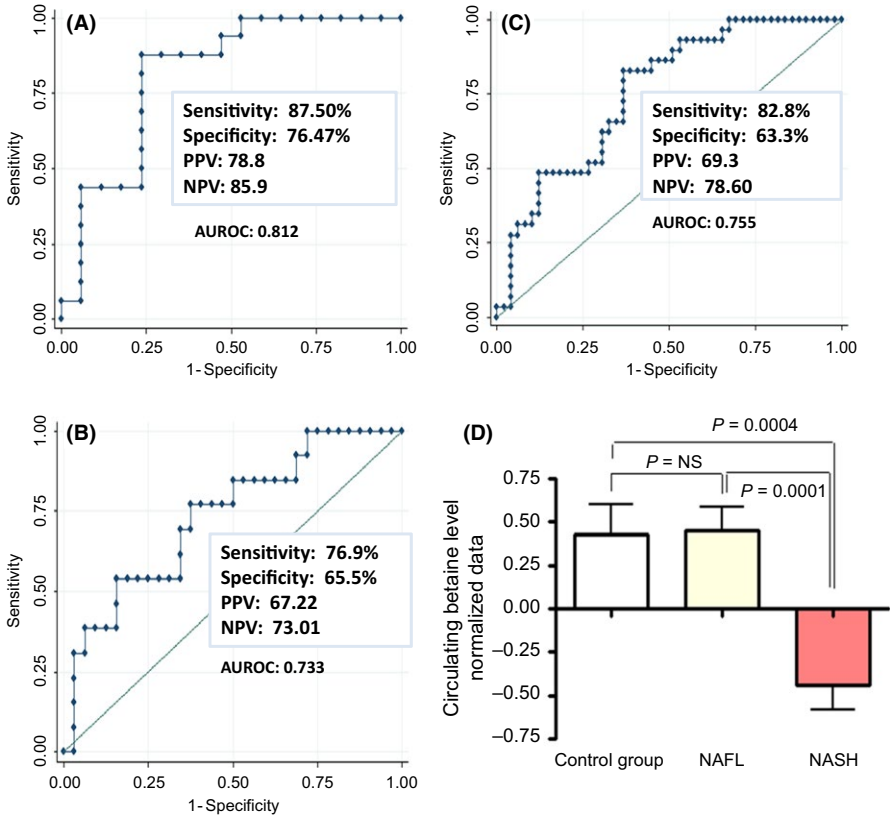

FIGURE 2 NAFLD severity is associated with a state of "betaine-insufficiency". Figure displays receiver operating characteristic (ROC) plot for circulating betaine levels in differentiating nonalcoholic steatohepatitis (NASH) from nonalcoholic fatty liver (NAFL) in the discovery (panel A) and replication study (panel B), and the joined dataset (C). The cut-off for normalized betaine values to rule-out NASH are −0.1589, −0.087 and −0.158 in the discovery, replication and joined dataset respectively. PPV, positive predictive value; NPP, negative predictive value. AUROC, area under the receiver-operator curve. Panel D shows that betaine levels (Z scores) in circulation are significantly reduced in patients with NASH in comparison with controls and NAFL; graph displays results of the joined dataset

folate as cofactors. Moreover, DMGDH is highly expressed in the liver http://www.proteinatlas.org/ENSG00000132837-DMGDH/tissue. Therefore, it is plausible to suggest that a variant that modulates dimethylglycine levels indirectly modifies the susceptibility to advanced disease by influencing the methyl-group metabolism. In agreement with our observations, the results from a recent experimental study showed that diseased animals had decreased levels of dimethylglycine that resulted in a decrease in the ratio between dimethylglycine and betaine.[12]

While the specific mechanisms by which the rs1805074 or any other variant in linkage disequilibrium (LD) regulates the levels of dimethylglycine are not entirely known, it is plausible to hypothesize that the variant in *DMGDH* might be associated with changes in the enzymatic activity. To test this hypothesis, we explored in silico the protein domain(s) that could be possible affected by the missense rs1805074 variant. Interestingly, we found two protein domains in DMGDH that are predicted to have significant functional impact on the protein function (pfam01571 *P* value=8.2e-79 and COG0404 *P* value=3.8e-117) (http://bioinf.umbc.edu/dmdm/). The pfam01571 domain is indeed an "aminomethyltransferase folate-binding domain" known as GcvT domain or "glycine cleavage system T protein" (T protein is an aminomethyl transferase); COG0404 also belongs to a glycine cleavage system T protein. Moreover, the explored rs1805074 and the missense rs1805073 (p.Ala530Gly), which is in strong LD with the rs1805074 (R^2=0.957), both share the same predicted domains. Hence, as dimethylglycine is the product of betaine demethylation,[14] it is plausible to suggest that the potential protective effect of the variant on NASH could indeed be explained by avoiding the development of a "methyl-deficient state".

Furthermore, as dimethylglycine acts as a feed-back regulator of betaine-homocysteine methyltransferase,[14] it is reasonable to suggest that either homocysteine levels or the ratio between betaine/homocysteine could be affected by the rs1805074. To answer this question, we searched in the Metabolomics GWAS Server for this putative association; unfortunately, there was no evidence for the rs1805074 or any other variant in LD to be possible associated with circulating homocysteine levels (the search was done on all the variants with a R^2=1 with the rs1805074). Surprisingly, significant associations were found with betaine/amino acid ratios, including, betaine/glutamine (*P* value=9.474e-19) or tyrosine/betaine (*P* value=4.048e-18).[22] Hence, the potential protective effect of the rs1805074 on NASH could be additionally explained by the effect/s on other metabolites or metabolic parameters not explored in our work.

Likewise, gene variants in *BHMT* might also account for changes in serum levels of betaine or dimethylglycine. Accordingly, we searched into the Metabolomics GWAS Server and we found 10 intronic SNPs located in *BHMT* associated with betaine levels (Table S1). Remarkably, two of 10 variants in *BHMT*, including rs6860801 and rs6881725, are in strong linkage disequilibrium (R^2: 0.957) with the missense *DMGDH* rs1805074 explored in our research and then the signal may not be assigned to one of these two genes.

Potential limitations of our study should be highlighted, including the cross-sectional design, which cannot demonstrate causality. Moreover, it would have been valuable to have measured SAM concentrations, SAM/S-adenosylhomocysteine (SAH) ratio, and PEMT and/or BHMT enzymatic activities to fully understand the role of

TABLE 3 Clinical and biochemical evaluation of patients with NAFLD and healthy controls enrolled in the candidate-gene association study

Variable (mean ± SD)	Controls	NAFL	NASH	*P* value* *P*<	*P* value** *P*<	*P* value# *P*<
Number of subjects	138	107	145	–	–	–
Female/male, %	87/51	60/47	99/46	NS	NS	NS
Age, years	47 ± 16	52 ± 10	50 ± 14	.03	NS	.04
BMI, kg/m^2	24 ± 4	32 ± 5	33.5 ± 5	1.0×10^{-8}	.004	1.0×10^{-8}
Waist circumference, cm	85.5 ± 15.5	102 ± 16	108 ± 13	1.0×10^{-8}	.005	1.0×10^{-8}
Fasting plasma glucose, mg/dL	84 ± 14	98 ± 20	129 ± 122	1.0×10^{-8}	1.0×10^{-8}	1.0×10^{-8}
Fasting plasma insulin, mg/dL	7 ± 5	13 ± 9	16.3 ± 11	1.0×10^{-8}	.002	1.0×10^{-8}
HOMA-IR index	1.4 ± 1.03	3.1 ± 3	5.2 ± 7	1.0×10^{-8}	.00005	1.0×10^{-8}
SABP, mm Hg	116 ± 15	126 ± 13	130 ± 16	1.0×10^{-3}	NS	1.0×10^{-8}
DABP, mm Hg	71 ± 9	77 ± 10	79 ± 12	.00034	NS	1.0×10^{-8}
Total cholesterol, mg/dL	205 ± 41	206 ± 48	210 ± 44	NS	NS	NS
HDL-cholesterol, mg/dL	55 ± 15	52 ± 23	50 ± 12	.01	NS	.0003
LDL-cholesterol, mg/dL	123 ± 37	124 ± 46	124 ± 42	NS	NS	NS
Triglycerides, mg/dL	120 ± 78	151 ± 75	191 ± 120	.0006	.003	1.0×10^{-8}
ALT, U/L	26 ± 10	56 ± 72	75 ± 55	.000001	.0003	1.0×10^{-8}
AST, U/L	28 ± 11	35 ± 17	51 ± 33	.00001	1.0×10^{-2}	1.0×10^{-8}
GGT, U/L	31 ± 14	68 ± 59	82.5 ± 75	.00007	NS	1.0×10^{-8}
AP, U/L	160 ± 36	233 ± 100	227 ± 112	1.0×10^{-3}	NS	.00007
Histological features						
Degree of steatosis, %	–	48 ± 26	60 ± 20	–	.0001	–
Lobular inflammation (0-3)	–	0.6 ± 0.4	1.5 ± 0.65	–	1.0×10^{-8}	–
Hepatocellular ballooning (0-2)	–	–	0.9 ± 0.6	–	1.0×10^{-8}	
Fibrosis Stage	–		1.4 ± 1.27	–	1.0×10^{-8}	
p.Ser646Pro (rs1805074)						
AA	74	58	90			
AG	44	40	48			
GG	20	48	7			

ALT and AST, serum alanine and aspartate aminotransferase, respectively; AP, alkaline phosphatase; BMI, body mass index; GGT, gamma-glutamyl-transferase; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment-insulin resistance; LDL-C, low-density lipoprotein cholesterol; NAFL, nonalcoholic fatty liver or simple steatosis; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.
Results are expressed as mean ± SD. *Indicates comparisons between NAFL and controls, **denotes NAFL vs. NASH comparisons, and #pertains to the comparisons between NASH and controls. The *P* value reflects the statistical significance calculated using the Mann–Whitney *U*-test, with the exception of the female/male ratio, where the *P* value reflects statistical significance calculated via a Chi-squared test. NS: nonsignificant.

betaine in human NASH development. Finally, genetic variation in the entire betaine/methionine transmetilation pathway is worth to be further examined, including variants in *PMET*.[39]

In conclusion, the role of betaine in the modulation of the methylome is particularly relevant as the process of methylogenesis, including SAM as the major methyl donor, is specifically active in the liver; moreover, the liver is one of the largest reservoirs of betaine in the body.[40] Whether betaine depletion is the cause or the consequence of the progression of NAFLD cannot be confirmed by this study; nevertheless, the present observation supports the notion that NAFLD disease severity is associated with changes in the levels of metabolites that modulate the liver methylome, which can be modified by therapeutic intervention.

CONFLICT OF INTEREST

The authors do not have any disclosures to report.

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