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Lower hepatic *CBS* and *PEMT* expression in advanced NAFLD: inferencing strategies to lower homocysteine with a mathematical model

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Abstract

Aim: Hepatic homocysteine (Hcy) accumulation promotes inflammation and fibrosis in experimental nonalcoholic fatty liver disease (NAFLD), while vitamin B12 and folate reduce hepatic Hcy and protect animals from nonalcoholic steatohepatitis. This suggests clinical implications for preventing/treating patients with NAFLD. Given the known sex-specific regulation of one-carbon metabolism (OCM), the response to various OCM cofactors may vary by sex and reproductive status. We aimed to strategize an effective Hcy-lowering treatment in broader NAFLD patients while discerning disparities in treatment responses.

Methods: We analyzed existing hepatic microarray data relevant to Hcy metabolism with clinical and histologic data from patients with NAFLD (N = 82), while considering potential age/sex disparities. Additionally, we performed computer simulation analyses using a mathematical model of OCM to predict hepatic Hcy-lowering effects of OCM cofactors by sex.

Results: Of 82 patients with NAFLD, 98% had at least one metabolic feature [i.e., metabolic dysfunction-associated steatotic liver disease (MASLD)]. Lower hepatic gene expressions of cystathionine-beta synthase (*CBS*) and phosphatidyl- ethanolamine N-methyltransferase (*PEMT*) were associated with more severe fibrosis in NAFLD, while sub-analysis suggested possible variations by age and sex. The simulation analysis demonstrated sex differences in the Hcy-lowering effects of the OCM cofactors (vitamins B6 and B12, folate, and betaine), with the combination of these cofactors consistently showing the maximum Hcy-lowering effect in both sexes.

Conclusion: We theorize that the combination of OCM cofactors would maximize Hcy-lowering effects in the broader MASLD population. Our findings also underscore the importance of considering sex and age in designing future studies on homocysteine metabolism.

Keywords: Nonalcoholic fatty liver disease (NAFLD), nonalcoholic steatohepatitis (NASH), metabolic dysfunction-associated steatotic liver disease (MASLD), hepatic fibrosis, one-carbon metabolism, homocysteine

INTRODUCTION

Hepatic homocysteine (Hcy) accumulation promotes hepatic inflammation and fibrosis in experimental nonalcoholic fatty liver disease (NAFLD) by homocysteinylating cellular proteins essential to homeostasis[1]. Experimental NAFLD induced by a Western diet and high fructose demonstrated elevated serum Hcy, increased homocysteinylated hepatic proteins, including syntaxin 17 (STX17), an autophagy regulator, and inhibition of autophagy, and worsening lipotoxicity, hepatic inflammation, and fibrosis[1]. Interestingly, supplementing Vitamin B12 and folate, cofactors of enzymes in the one-carbon metabolism (OCM), significantly reduced homocysteinylated hepatic proteins, including STX17, restored autophagy, and ameliorated hepatic inflammation and fibrosis[1]. Furthermore, a recent population-based study demonstrated that baseline serum Hcy levels are directly correlated with an incidence of liver-related outcomes in NAFLD patients[2]. Serum Hcy levels were also positively correlated with the severity indexes of NAFLD[2]. These studies strongly implicate the therapeutic potential of Hcy-lowering therapies in NAFLD patients. Whether lowering Hcy improves hepatic inflammation and fibrosis and improves liver-related and overall outcomes in patients with NAFLD remains to be investigated.

Therapeutic response to Hcy-lowering supplements may vary depending on patients. Serum homocysteine levels are higher in older subjects, in men *vs.* women, and in postmenopausal *vs.* premenopausal women[3,4]. These differences are partly explained by sex hormone regulation of the enzymes involved in Hcy metabolism[5,6]. OCM and related pathways are complex and crossly interlinking; the activities of the involved enzymes are regulated via allostatic inhibition/activation, competing/non-competing inhibition, group substrate inhibitions, and transcription/translation of the enzyme proteins[5,7]. Thus, to formulate effective Hcy-lowering strategies in a heterogeneous NAFLD population, it is crucial to understand the biological complexity, disparities in the metabolic pathways, and population-specific aspects (e.g., race/ethnicity, comedications and diets). We sought to evaluate the associations between hepatic gene expressions relevant to Hcy metabolism and histologic severity in patients with NAFLD and to infer effective therapeutic strategies for lowering Hcy in NAFLD patients using an OCM mathematical model as a theory-generation tool.

MATERIALS AND METHODS

Data source and population

The existing hepatic microarray data (GSE49541; NIH RC2-AA019399, PI: AMD) were used[8]. The cohort comprised adult patients with a histologic diagnosis of NAFLD who did not have other liver diseases, a history of liver transplantation, and excess alcohol use (i.e., less than seven servings/week for women, less

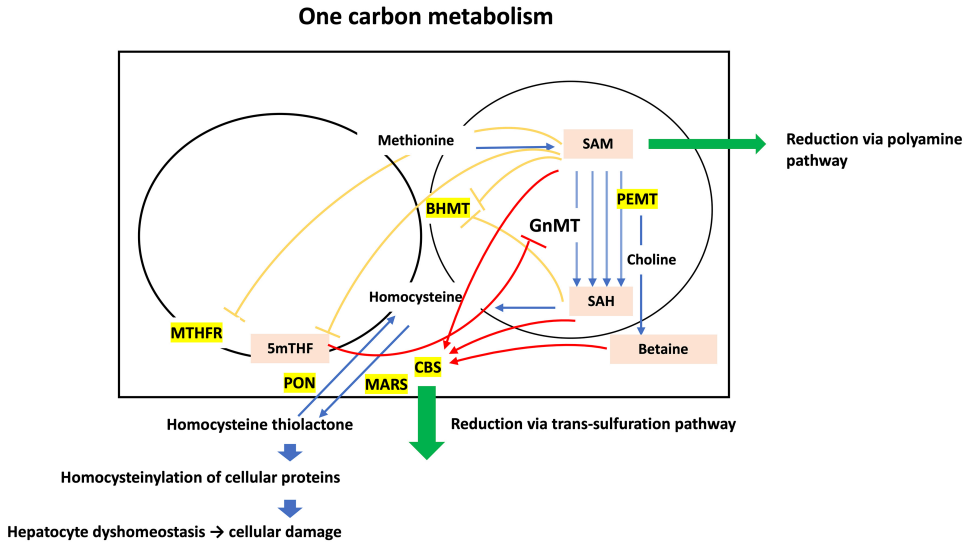

Figure 1. Simplified one-carbon metabolism pathway and homocysteine regulation. This figure illustrates key regulators of homocysteine and their interactions, as simulated by the mathematical model[5,7]. OCM and homocysteine are regulated intricately, not only through the transcription/translation of key enzymes but also through allosteric inhibition/activation, competing/non-competing inhibition, and group substrate inhibitions. OCM metabolites include all substrates and products of enzymes within the folate/methionine cycles. These metabolites undergo methylation and demethylation but remain within the cycles, maintaining a constant total molecular mass. In contrast, two exit pathways (indicated by red arrows), the transsulfuration and polyamine pathways, convert homocysteine and SAM to molecules that do not reenter the folate/methionine cycles, thereby reducing the total molecular mass. Enhanced transsulfuration, which converts homocysteine to cystathionine (a molecule that would not reenter the folate/methionine cycles), could effectively reduce tissue homocysteine. A similar theory can be applied to the polyamine pathway; converting SAM to polyamine and then further to hypusine (both of which would not reenter the folate/methionine cycles) would reduce tissue homocysteine, although experimental data for the polyamine pathway are currently limited. OCM: One-carbon metabolism; SAM: S-adenosylmethionine; BHMT: betaine-homocysteine methyltransferase; CBS: cystathionine-beta synthase; MARS: methionyl-tRNA synthetase; MTHFR: methylenetetrahydrofolate reductase; PEMT: phosphatidyl-ethanolamine N-methyltransferase; POS: paraoxonase. GNMT: glycine N-methyltransferase; 5mTHF: 5-methyltetrahydrofolate; SAH: S-adenosylhomocysteine.

than 14 servings/week for men) were analyzed in this study. We included both testing and validation cohorts while adjusting for batch effects (N = 82). Clinical data, such as age, sex, body mass index (BMI), current alcohol use, current smoking, and histologic data, were obtained from the linked Duke NAFLD Clinical Database and repository for the analysis[8]. The DUHS NAFLD Clinical Database was approved by the Duke University Institutional Review Board (IRB) (Pro: 00005368) and was performed in accordance with the Declaration of Helsinki ethical guidelines. The Duke University IRB reviewed this specific secondary analysis and determined that it met the criteria for category 4, exempting it from further IRB review (Pro: 00105083).

Study outcomes

The outcome variables were steatosis (grade 1 *vs.* 2 and 3), nonalcoholic steatohepatitis (NASH) (yes *vs.* no), and fibrosis stage (stages 0, 1, 3, 4). The histologic features were scored according to the NASH Clinical Research Network (CRN) scoring system[9], while the pathologist's diagnosis was used for the diagnosis of NASH.

Study predictors

We selected key enzymes from each pathway involved in remethylating or trans-sulfurating homocysteine [Figure 1], as well as several putative mechanisms related to homocysteine-induced hepatic inflammation and dyshomeostasis, based on our recent experimental findings[1]. We analyzed probes for ten selected genes: methylenetetrahydrofolate reductase (*MTHFR*), betaine-homocysteine methyltransferase (*BHMT*),

betaine-homocysteine methyltransferase 2 (*BHMT2*), cystathionine-beta synthase (*CBS*), phosphatidylethanolamine N-methyltransferase (*PEMT*), methionyl-tRNA synthetase (*MARS*), homocysteine inducible ER protein with ubiquitin-like domain 1(*HERPUD1*), and paraoxonase type 1 (*PON1*), paraoxonase type 2 (*PON2*), and paraoxanase type 3 (*PON3*).

HERPUD1 is induced in ER stress. Methionyl-tRNA synthetase, encoded by *MARS*, synthesizes homocysteine thiolactone, a highly reactive metabolite, that can form homocysteinylated proteins, contributing to dyshomeostasis, while paraoxonase (*PON*) reduces homocysteine thiolactone[10-12]. Gene expression analysis was done at a gene level, averaging individual probe data for each gene.

Analytic approach

Data are presented as mean ± SD for continuous variables and percentage for categorical/ranked variables. The associations between the histologic variables and the gene expression data were analyzed by multiple logistic (or ordinal logistic) regression models after adjusting for covariates [age, sex, BMI, DM type 2, current alcohol use, current smoking, and assay categories (testing *vs.* validation cohort)[8]]. Race and ethnicity were also considered as covariates in a secondary analysis. *P*-values were corrected for multiple testing via false discovery rates (Benjamini-Hochberg). All the statistical analyses were performed using R version 4.2.2. and JMP® Pro 17 software (version 2.0, Cary, NC).

Simulation analysis

The mathematical models simulating complex regulations of one-carbon metabolism and its peripheral pathways have been developed by Drs. Michael C. Reed and H. Frederik Nijhout and published incorporating variations by sex and sex hormone levels[5,7]. For this particular study, we focused on simulating sex differences in Hcy-lowering response to different OCM cofactors and the response of hepatic Hcy concentration to a wide range of folate levels, mimicking deficient, replete, and excess states using the existing models.

RESULTS

The clinical characteristics of the study population are summarized in Table 1. The mean age and BMI of the study population were 50.5 ± 10.8 years and 35.6 ± 7.6 kg/m^2. 67% were female. The majority were non-Hispanic White, 39% had type 2 diabetes, and 97.6% had at least one metabolic feature (BMI ≥ 30, diabetes mellitus, hypertension, or hyperlipidemia), meeting the criteria of the new nomenclature of metabolic-dysfunction associated steatotic liver disease (MASLD), 44% consumed up to a modest amount of alcohol, and 18% used cigarettes at the time of liver biopsy. 46% had advanced fibrosis (stage 3-4) per the original study design[8], 79% had histologic evidence of NASH, and 56% had moderate to severe steatosis (grade 2-3). For the exploratory analysis, the numbers of women of age ≤ 50, women of age > 50, men of age ≤ 50, and men of age > 50 were 20, 35, 17, and 10, respectively.

The results of multiple ordinal logistic (logistic) regression are summarized in Table 2. Overall, lower gene expressions of *CBS* and *PEMT* were associated with an increased odds ratio (OR) of hepatic fibrosis (adjusted *P*-values: 0.0017 for *CBS* and 0.0016 for *PEMT*), while lower gene expressions of *PON1* and *PON3* were associated with an increased OR of hepatic steatosis (adjusted *P*-values: 0.0042 for *PON1* and 0.0003 for *PON3*) [Table 2]. The results were consistent even after adjusting for race and ethnicity, with minor changes in OR and low statistical power [Table 2].

The results of the sub-analysis by age 50 and sex are summarized in the Supplementary Table 1. Due to the small sample size, the data were interpreted based on raw p-values, without considering multiple

Table 1. Clinical characteristics of the study population

	Summary statistics
Demographics	
Age, years	50.5 ± 10.8
Female sex, %	55 (67%)
White, *n* (%)	73 (89.0%)
Hispanic, *n* (%)	6 (7.3%)
Metabolic features	
BMI, kg/m^2	35.6 ± 7.6
Type 2 diabetes mellitus, *n* (%)	32 (39.0%)
Hypertension, *n* (%)	53 (64.6%)
Hyperlipidemia, *n* (%)	52 (63.4%)
Patients with any metabolic features, *n* (%)	80 (97.6%)
Other clinical variables	
Minimal to moderate alcohol use, *n* (%)*	36 (43.9%)
Current smoking, *n* (%)	14 (17.5%)
Histologic features	
Steatosis (0, 1, 2, 3), *n* (%)	2 (2.4%): 34 (41.5%): 28 (34.1%): 18 (22.0%)
NASH, *n* (%)	65 (79.3%)
Fibrosis (0, 1, 3, 4), *n* (%)	16 (19.5%): 28 (34.1%): 30 (36.6%): 8 (9.8%)

Continuous data are presented as mean ± SD. *Minimal to moderate alcohol use (i.e., non-excess alcohol use) was defined by less than seven servings/week for women and less than 14 servings/week for men. BMI: body mass index; NASH: nonalcoholic steatohepatitis.

companions. Lower gene expression of *CBS* was associated with more severe fibrosis among young women ($P = 0.0148$), and a similar tendency was observed in older subjects regardless of sex ($P = 0.075$ for older women and 0.09 for older men). In contrast, lower gene expression of *PEMT* was associated with more severe fibrosis in all subgroups ($P = 0.0014$ for older women, 0.023 for young men, and 0.027 for older men), except for young women. Lower gene expression of *PEMT* was also associated with an increased risk of NASH among older men (P-value = 0.048). Among young women, lower expression of *PON1*, which theoretically leads to the retention of highly reactive Hcy-thiolactone, was associated with more severe fibrosis ($P = 0.0004$). In addition, lower expression of *BHMT* was associated with more severe steatosis among older women ($P = 0.0071$), while lower *BHMT2* and *MARS* expression was associated with less fibrosis among older women ($P = 0.037$ and 0.018, respectively). Among young men, lower expression of most genes, except for *BHMT2*, was associated with more severe steatosis, which is probably attributed to overfitting and is not interpretable.

The mathematical model[5,7] simulated (1) the response of hepatic Hcy to OCM cofactors in men and women [Figure 2A and B]; and (2) the response of hepatic Hcy as a function of folate alone [Figure 2C]. Although men and women are predicted to respond differently to various OCM cofactors, both showed the best response to the combination of all cofactors. Folate is the most effective in lowering hepatic Hcy alone among the cofactors and is predicted to further lower Hcy even when folate is close to the reference.

DISCUSSION

We report that altered gene expression of enzymes, which result in increasing tissue Hcy or Hcy thiolactone, was associated with more severe fibrosis and steatosis in patients with NAFLD. This further supports lowering hepatic Hcy and reducing highly reactive Hcy thiolactone to prevent NASH progression. Based on the OCM mathematical model, several allosteric regulations are essential in lowering tissue Hcy,

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Table 2. The associations between the histologic severity of NAFLD and hepatic expression of genes involved in homocysteine metabolism in the overall population, without and with adjusting for race and ethnicity

Genes	Steatosis grade					NASH					Fibrosis stage				
	OR	LLC	ULC	Raw_*P*	FDR_*P*	OR	LLC	ULC	Raw_*P*	FDR_*P*	OR	LLC	ULC	Raw_*P*	FDR_*P*
Models without including race and ethnicity															
BHMT	1.30	1.99	0.85	0.1471	0.3677	1.04	1.30	0.83	0.7216	0.8620	0.99	1.20	0.81	0.8854	0.8854
BHMT2	0.99	1.36	0.72	0.8886	0.8886	1.08	1.28	0.92	0.3531	0.5885	0.93	1.08	0.81	0.3338	0.5563
CBS	1.14	1.49	0.87	0.4112	0.5960	1.11	1.27	0.96	0.1632	0.5441	**1.24**	**1.39**	**1.11**	**0.0003**	**0.0017**
MARS	0.91	1.14	0.72	0.4172	0.5960	0.93	1.05	0.83	0.2226	0.5566	0.90	1.00	0.82	0.0456	0.1521
MTHFR	0.97	1.13	0.83	0.7099	0.7888	1.01	1.09	0.93	0.8724	0.8724	1.04	1.11	0.97	0.3020	0.5563
PEMT	1.17	1.99	0.69	0.6737	0.7888	0.86	1.14	0.65	0.2793	0.5586	**1.56**	**1.94**	**1.25**	**0.0002**	**0.0016**
PON1	**1.31**	**1.74**	**0.99**	**0.0008**	**0.0042**	1.14	1.32	0.98	0.0876	0.4381	1.12	1.27	0.98	0.0889	0.2221
PON2	1.31	1.92	0.89	0.1207	0.3677	1.03	1.26	0.84	0.7758	0.8620	1.03	1.23	0.87	0.7195	0.8854
PON3	**1.77**	**2.68**	**1.17**	**0.0000**	**0.0003**	1.22	1.54	0.98	0.0779	0.4381	1.07	1.31	0.88	0.4921	0.7030
HERPUD1	1.11	1.38	0.90	0.2810	0.5620	0.97	1.08	0.86	0.5295	0.7564	0.99	1.09	0.90	0.8427	0.8854
Models including race and ethnicity															
BHMT	1.31	2.00	0.86	0.2024	0.5059	1.05	1.32	0.84	0.6475	0.7194	1.04	1.28	0.85	0.7099	0.7888
BHMT2	0.99	1.37	0.72	0.9696	0.9696	1.08	1.28	0.91	0.3832	0.6387	0.94	1.10	0.80	0.4172	0.5960
CBS	1.14	1.49	0.88	0.3151	0.5427	1.09	1.26	0.95	0.2223	0.6035	**1.30**	**1.46**	**1.16**	**0.0000**	**0.0003**
MARS	0.91	1.14	0.73	0.4201	0.6002	0.94	1.05	0.83	0.2715	0.6035	0.92	1.02	0.83	0.1207	0.3677
MTHFR	0.97	1.14	0.83	0.7177	0.7975	1.01	1.10	0.93	0.8189	0.8189	1.04	1.12	0.97	0.2810	0.5620
PEMT	1.15	1.97	0.68	0.5973	0.7467	0.86	1.14	0.65	0.3018	0.6035	**1.52**	**1.93**	**1.20**	**0.0008**	**0.0042**
PON1	1.30	1.72	0.98	0.0691	0.3455	1.16	1.35	0.99	0.0605	0.5934	1.11	1.27	0.96	0.1471	0.3677
PON2	1.28	1.87	0.88	0.1900	0.5059	1.06	1.29	0.87	0.5731	0.7164	0.96	1.15	0.80	0.6737	0.7888
PON3	1.78	2.70	1.18	0.0066	0.0657	1.20	1.51	0.95	0.1187	0.5934	1.09	1.35	0.88	0.4112	0.5960
HERPUD1	1.11	1.38	0.90	0.3256	0.5427	0.97	1.08	0.86	0.5541	0.7164	0.99	1.10	0.89	0.8886	0.8886

All the models were adjusted for age, sex, BMI, diabetes, alcohol, smoking, and assay categories (testing *vs.* validation cohort), without or with race and ethnicity. Statistically significant findings (after adjusting for multiple comparisons) are presented in bold. NAFLD: Nonalcoholic fatty liver disease; NASH: nonalcoholic steatohepatitis; OR: odds ratio; LLC: lower limit of 95% confidence interval; ULC: upper limit of 95% confidence interval; Raw_*P*: raw *P*-values, without adjusting for multiple comparisons; FDR_*P*: *P*-values after adjusting for multiple comparisons, FDR: false discovery rate; BHMT: betaine-homocysteine methyltransferase; BHMT2: betaine-homocysteine methyltransferase; CBS: cystathionine-beta synthase; MARS: methionyl-tRNA synthetase; MTHFR: methylenetetrahydrofolate reductase; PEMT: phosphatidyl-ethanolamine N-methyltransferase; PON1: paraoxonase type 1; PON2: paraoxonase type 2; PON3: paraoxanase type 3; HERPUD1: homocysteine inducible ER protein with ubiquitin-like domain 1; BMI: body mass index.

providing stable methylation capacity [S-adenosylmethionine (SAM)/S-adenosylhomocysteine (SAH) ratio], and sustaining the DNA methylation pathway to maintain homeostasis[5,7]. There are two primary exits to reduce "metabolic mass" in order to lower tissue Hcy: transsulfuration and polyamine pathways [Figure 1][5,7]. The model also indicated that re-methylation alone, without reducing the mass of OCM metabolites through the two exit pathways, would not effectively decrease Hcy concentration[5,7].

Taken together, we theorize that CBS activity is the most important in lowering Hcy through transsulfuration and that optimizing CBS activity, either allosterically or via upregulation of expression, is an effective approach for reducing Hcy. CBS activity is regulated through multiple mechanisms, but allosteric activation by SAM and betaine is essential[5,7,13]. Choline and betaine are derived both from foods and biosynthesis by PEMT, an estrogen-regulated enzyme. Thus, men and postmenopausal women are prone to develop choline/betaine deficiency due to the lower physiological estrogen level[14], which, in turn, reduces transsulfuration drainage and elevates hepatic Hcy, via reducing allosteric activation of CBS[5,7,13]. Among older women, lower expression of MARS, a gene coding methionyl-tRNA synthetase - an enzyme synthesizing homocysteine thiolactone, a highly reactive metabolite - was protective against hepatic fibrosis. *BHMT* showed inconsistent associations in some subgroups, depending on histologic features. Due to the limited sample size in the subgroup analyses, the significance of the *BHMT* finding cannot be determined in this study. While some of the observed disparities by age and sex have biological plausibility, the findings from the subgroup analyses should be interpreted with caution, given the limited sample size. Nevertheless, a strategic approach considering these possible biological disparities is crucial in future research to formulate effective Hcy-lowering supplements for a broader population with NAFLD.

The OCM mathematical model provided simulation data demonstrating the response of hepatic Hcy to OCM cofactor supplements and the combination in men and women [Figure 2A and B][5]. The simulation data suggest that folate may exert the strongest Hcy-lowering effect, in both men and women, likely by increasing SAM and then SAH and allosterically activating CBS[5,7]. Vitamin B6 shows a sex-specific lowering effect, greater in males *vs.* females. Twelve-week Vitamin B6 supplementation has been shown to significantly reduce hepatic fat accumulation, with insignificant male dominance among responders (61.5% *vs.* 55.6%)[15]. Thus, folate (or combination with B6) might be a good approach to lowering Hcy. It is important to note that orally administered SAM has a poor bioavailability, compared to intravenously administered SAM[16], thus SAM supplementation may not be an effective approach to lower Hcy. The homocysteine-lowering effects of vitamin B12 and betaine are rather insignificant based on the simulation data [Figure 2A and B]. However, a few factors must be considered. Most patients with NAFLD are obese or overweight, suffer gastroesophageal reflux disease, and have type 2 diabetes or impaired glucose tolerance. Thus, a significant portion of patients are on PPI and/or metformin, both of which have been associated with Vitamin B12 deficiency[17,18], a cofactor of methionine synthetase. Thus, Vitamin B12 supplementation may be required in this particular population. Furthermore, PEMT activity and betaine levels are influenced by estrogen levels. Thus, to maximize betaine-driven allosteric activation of CBS[5,7,13] in estrogen-depleted subjects, betaine should be supplemented. The simulation, in fact, demonstrated the strongest Hcy-lowering effect in the combination for men and women, even above normal levels [Figure 2A-C].

Genetic variants regulating key enzymes in homocysteine metabolism may also impact NAFLD progression, which was not evaluated in this study. Studies that assessed genetic polymorphisms related to homocysteine and NAFLD have not demonstrated significant associations between such polymorphisms and NAFLD severity[19,20]. Given the sex-specific regulation of one-carbon metabolism and sexually dimorphic NAFLD pathobiology[5,7,21], further studies are warranted, considering genetic variants of enzymes in homocysteine metabolism and their interplay with sex and reproductive status. The results of our main analysis were

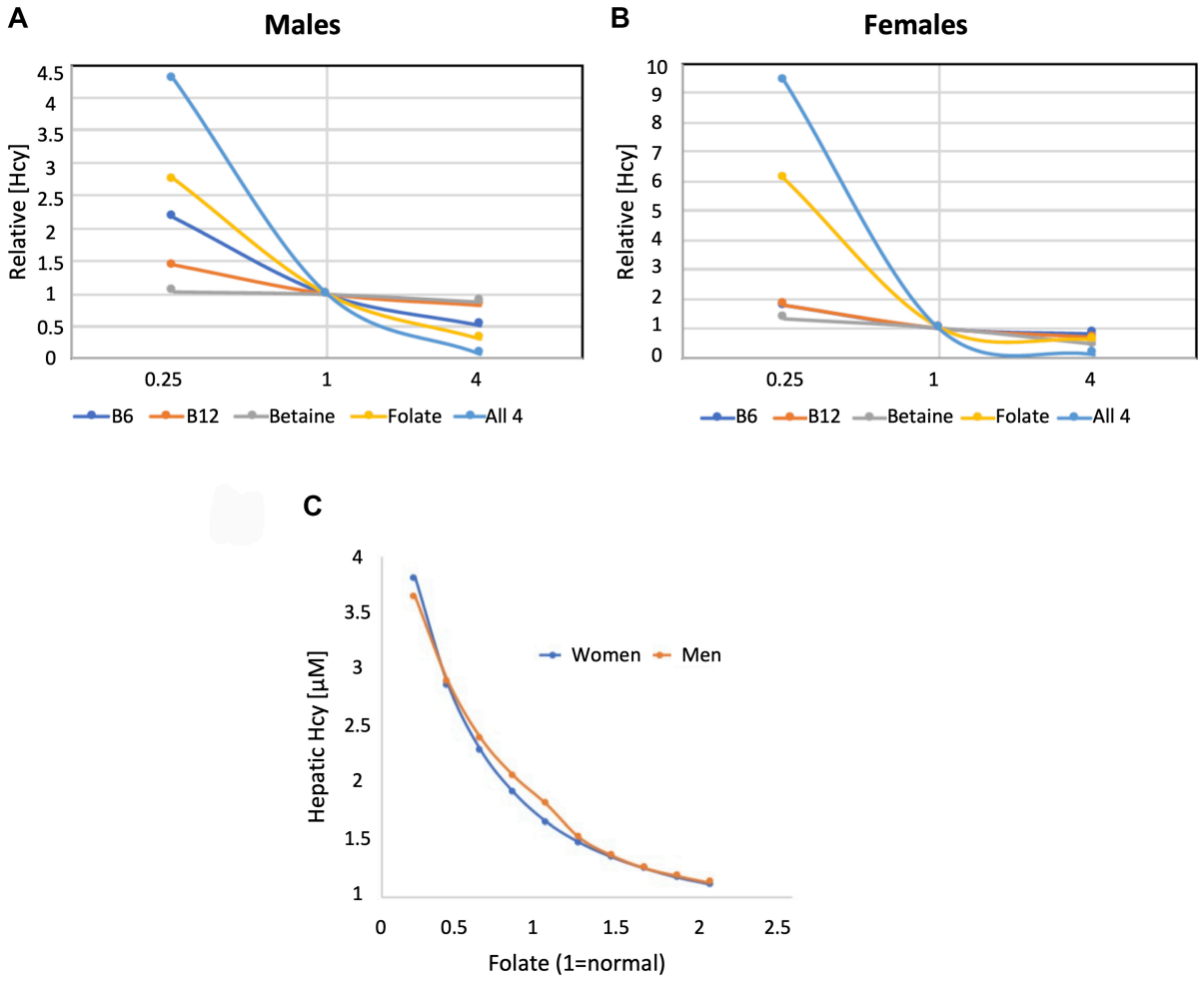

Figure 2. Homocysteine-lowering effects of OCM cofactor supplements in males and females (A-C). Figures depict the simulated effects of OCM cofactors (Vitamins B6 and B12, folate, and betaine) and their combination on hepatic homocysteine under different nutritional statuses, ranging from 0.2 or 0.25 (i.e., deficient) to 2 or 4 (i.e., high intake), where 1 is the reference state. OCM: One-carbon metabolism.

consistent even after adjusting for race and ethnicity in this racially homogeneous population. Our cohort used in this analysis was enrolled at the liver clinic and bariatric surgery clinic at the tertiary center. Thus, the results may not be generalizable to a broader NAFLD population (e.g., lean NAFLD). In light of the recent change in nomenclature and associated definitions from NAFLD and metabolic dysfunction-associated fatty liver disease (MAFLD) to MASLD, it is important to note that 98% of our NAFLD cohort met the criteria for MASLD, with about 48% having three or more metabolic features. Insulin resistance syndrome (metabolic syndrome) has been associated with elevated serum homocysteine compared to those without this syndrome[22]. Thus, individuals with MASLD accompanied by metabolic syndrome may benefit more from lowering Hcy compared to those without metabolic syndrome. Future studies should be designed to discern potential disparities in response to Hcy-lowering supplements based on the level of metabolic dysfunction. Lastly, our existing data did not include the parameters necessary for analyzing sarcopenia, a crucial factor in the pathobiology of NAFLD/MASLD. Future trials should evaluate the effects of homocysteine-lowering supplements on sarcopenia.

In summary, our analysis and inference using the OMC mathematical model generated hypotheses to strategize a broadly effective homocysteine-lowering therapy in patients with NAFLD using the combination of folate, Vitamin B6 (PIP), Vitamin B12, and betaine. A feasibility/pilot trial for the combination therapy is underway (clinicaltrials.gov # NCT05720702) to design and power a future clinical trial. If proven effective, this approach of lowering homocysteine in patients with NAFLD is particularly appealing since it is inexpensive and safe.

DECLARATIONS

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Authors' contributions

Conceptualization: Suzuki A, Tripathi M, Singh BK, Yen PM, Reed MC, Nijhout HF
Data curation: Diehl AM, Abdelmalek MF, Suzuki A
Methodology and analysis: Suzuki A, Henao R, Reed MC, Nijhout HF
Development of original draft: Suzuki A
Critical review and edits: Henao R, Reed MC, Nijhout HF, Tripathi M, Singh BK, Yen PM, Diehl AM, Abdelmalek MF

Availability of data and materials

Not applicable.

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Conflicts of interest

Suzuki A is an Associate Editor of the journal *Metabolism and Target Organ Damage*, while the other authors have declared that they have no conflicts of interest.

Ethical approval and consent to participate

The DUHS NAFLD Clinical Database was approved by the Duke University Institutional Review Board (IRB) (Pro: 00005368) and was performed in accordance with the Declaration of Helsinki ethical guidelines. The Duke University IRB reviewed this specific secondary analysis and determined it met the criteria for category 4, exempting it from further IRB review (Pro: 00105083). All patients provided verbal informed consent prior to participation.

Consent for publication

Not applicable.

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