Title

Wilson's disease: changes in methionine metabolism and inflammation affect global DNA methylation in early liver disease.

Journal

Hepatology (Baltimore, Md.), 57(2)

ISSN

0270-9139

Authors

Medici, Valentina
Shibata, Noreene M
Kharbanda, Kusum K
et al.

Publication Date

2013-02-01

DOI

10.1002/hep.26047

Peer reviewed

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Wilson's Disease: Changes in Methionine Metabolism and Inflammation Affect Global DNA Methylation in Early Liver Disease

Valentina Medici,[1] Noreene M. Shibata,[1] Kusum K. Kharbanda,[2] Janine M. LaSalle,[3] Rima Woods,[3] Sarah Liu,[1] Jesse A. Engelberg,[4] Sridevi Devaraj,[5] Natalie J. Török,[1] Joy X. Jiang,[1] Peter J. Havel,[6,7] Bo Lönnerdal,[7] Kyoungmi Kim,[8] and Charles H. Halsted[1]

Hepatic methionine metabolism may play an essential role in regulating methylation status and liver injury in Wilson's disease (WD) through the inhibition of S-adenosylhomocysteine hydrolase (SAHH) by copper (Cu) and the consequent accumulation of S-adenosylhomocysteine (SAH). We studied the transcript levels of selected genes related to liver injury, levels of SAHH, SAH, DNA methyltransferases genes (*Dnmt1, Dnmt3a, Dnmt3b*), and global DNA methylation in the tx-j mouse (tx-j), an animal model of WD. Findings were compared to those in control C3H mice, and in response to Cu chelation by penicillamine (PCA) and dietary supplementation of the methyl donor betaine to modulate inflammatory and methylation status. Transcript levels of selected genes related to endoplasmic reticulum stress, lipid synthesis, and fatty acid oxidation were down-regulated at baseline in tx-j mice, further down-regulated in response to PCA, and showed little to no response to betaine. Hepatic *Sahh* transcript and protein levels were reduced in tx-j mice with consequent increase of SAH levels. Hepatic Cu accumulation was associated with inflammation, as indicated by histopathology and elevated serum alanine aminotransferase (ALT) and liver tumor necrosis factor alpha (*Tnf-α*) levels. *Dnmt3b* was down-regulated in tx-j mice together with global DNA hypomethylation. PCA treatment of tx-j mice reduced *Tnf-α* and ALT levels, betaine treatment increased S-adenosylmethionine and up-regulated *Dnmt3b* levels, and both treatments restored global DNA methylation levels. *Conclusion*: Reduced hepatic *Sahh* expression was associated with increased liver SAH levels in the tx-j model of WD, with consequent global DNA hypomethylation. Increased global DNA methylation was achieved by reducing inflammation by Cu chelation or by providing methyl groups. We propose that increased SAH levels and inflammation affect widespread epigenetic regulation of gene expression in WD. (HEPATOLOGY 2013;57:555-565)

Abbreviations: CPT1A, carnitine palmitoyltransferase 1A; Cu, copper; DNMT, DNA methyltransferase; ER, endoplasmic reticulum; GRP78, glucose-regulated protein 78; PCA, penicillamine; PPARα, peroxisome proliferator-activated receptor alpha; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; SREBP1c, sterol regulatory element-binding protein 1c; TNF-α, tumor necrosis factor alpha; WD, Wilson's disease.

From the [1]Department of Internal Medicine, Division of Gastroenterology and Hepatology, University of California Davis, Davis, CA; [2]Research Service, Veterans Affairs Nebraska-Western Iowa Health Care System, Omaha, NE; [3]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA; [4]Center for Comparative Medicine, University of California Davis, Davis, CA; [5]Texas Children Hospital, Houston, TX; [6]Department of Molecular Biosciences, University of California Davis, Davis, CA; [7]Department of Nutrition, University of California Davis, Davis, CA; [8]Department of Public Health Sciences, Division of Biostatistics, University of California Davis, Davis, CA.

Received April 20, 2012; accepted August 14, 2012.

Supported by grant number K08DK084111 from the National Institute of Diabetes and Digestive and Kidney Diseases, by Department and Divisional funds, by University of California Davis Center for Health and Nutrition Research (CHNR), by the National Center for Research Resources (NIH) through grant #UL1 RR024146 (to V.M.), by grant number R03AA020577-01 from the National Institute on Alcohol Abuse and Alcoholism (to C.H.H.), by 2R01HD041462 from the National Institute of Child Health and Human Development (to J.M.L.), T32ES002321 from the National Institute of Environmental Health Sciences (to R.W.), and by a Veterans Health Administration Biomedical Laboratory Research and Development National Merit Review grant (to K.K.K.). V.M. is a full member of the University of California San Francisco Liver Center (Liver Center grant number P30 DK026743). The content is the sole responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

The earliest phases of hepatic involvement in Wilson's disease (WD) include portal inflammation that may present as lymphocyte and neutrophil infiltrations,[1] and microvesicular and macrovesicular steatosis,[2] which is exhibited both clinically[2] and in animal models of WD.[3,4] Previous studies on the pathogenesis of WD explored the possibilities of genetic polymorphisms in the ATP7B copper (Cu) transporter,[5] alternative ATP7B gene splice variants,[6] alterations in the RNA processing machinery,[7] and the presence of gene modifiers.[8] The mechanisms connecting Cu accumulation to hepatocyte damage are poorly understood and may include oxidative damage,[9] apoptosis,[10] and mitochondrial membrane cross-linking.[11]

Abnormal methionine metabolism occurs in animal models of hepatic Cu overload,[12,13] is connected to epigenetic regulation of gene expression,[14] and could represent a link between Cu accumulation and the variety of hepatic manifestations in WD. Methionine metabolism is central to the regulation of S-adenosylhomocysteine (SAH), which inhibits methylation reactions, and is known to sensitize hepatocytes to the presence of tumor necrosis factor alpha (TNF-α).[15] Similarly, by promoting increased cell division and DNA repair, inflammation could increase the demand for methylation.[16,17]

SAH is the substrate for the bidirectional enzyme SAH hydrolase (SAHH) (Supporting Material Fig.). Cu may regulate methionine metabolism through its known inhibitory effect on SAHH with consequent increase in SAH, the principal inhibitor of transmethylation reactions.[12,13,18] Cu binds noncompetitively to the SAHH enzyme and reduces its activity by releasing NAD^+ cofactors.[19] The regulatory role of increased Cu in down-regulation of SAHH activity, with consequent elevation of its substrate SAH and its potential secondary epigenetic effects on gene expression, suggest that methionine metabolism could be the missing link between Cu accumulation and hepatocyte damage in WD. Of note, there has been a growing interest in SAHH due to its relationship with SAH levels and gene expression in hepatic steatosis[20] and human SAHH deficiency.[21]

We hypothesized that by regulating *Sahh* expression, Cu and its associated hepatic inflammation initiate alterations in methionine metabolism that affect DNA methylation status and potentially the expression of selected genes central to endoplasmic reticulum (ER) stress and lipid metabolism in WD. To test this hypothesis, we modulated Cu levels and inflammation by administering the Cu chelator penicillamine (PCA) and hepatic methylation status by administering the methyl donor betaine in the tx-j mouse model of WD.

Materials and Methods

Animal Models and Experimental Protocols

We used the C3HeB/FeJ-Atp7b$^{tx\text{-}J}$/J mouse (tx-j) model of WD with its background strain C3HeB/FeJ (C3H) as a control. The tx-j mouse model has a G712D missense mutation predicted to be in the second transmembrane region of the Atp7B gene, which results in a phenotypic disorder similar to WD.[22] Mice in the baseline and PCA experiments were obtained from the Jackson Laboratory (Bar Harbor, ME), whereas mice in the betaine experiments were obtained from our in-house UC Davis colony that was developed from C3H breeder pairs and homozygous-affected tx-j breeder pairs purchased from the Jackson Laboratory. At 24 weeks of age, seven males from each strain were taken for harvest of blood and tissues and served as control groups for mice in PCA and betaine studies.

PCA Study. From age 12 to 24 weeks, a subgroup of seven male tx-j mice received treatment with oral PCA (Sigma Aldrich, St. Louis, MO) that was dissolved in deionized water at 100 mg/kg bodyweight/day, a dose shown to reduce hepatic Cu concentration in a rat model of WD.[23] PCA was not administered to control mice since Cu deficiency could independently modify lipid[24] and methionine metabolism.[25]

Betaine Study. From age 20 to 24 weeks, six male C3H and seven tx-j mice were treated with betaine (Sigma Aldrich) supplemented at 1.5% in the drinking water.[26]

All other methods are described in the Supporting Material.

Results

Baseline Characteristics and Responses to PCA and Betaine Treatments

Twenty-four-week-old tx-j mice had lower body weights and higher liver/bodyweight ratios than

Address reprint requests to: Valentina Medici, M.D., University of California, Davis, Division of Gastroenterology and Hepatology, Department of Internal Medicine, 4150 V St., Suite 3500, Sacramento, CA 95817. E-mail: valentina.medici@ucdmc.ucdavis.edu; fax: 916-734-7908.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.26047

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Table 1. Effects of Copper Accumulation, Penicillamine and Betaine Administration

	Control (C3H) (7)	Tx-j (7)	Tx-j + PCA (7)	Control (C3H) + betaine (6)	Tx-j + betaine (7)
Body weight (g)	39.5±2.4[a]	29.3±1.2[a]	27.8±1.8	40.5±1.41[d]	29.3±1.1[d]
Liver/body weight	0.046±0.002[a]	0.058±0.001[a,b]	0.054±0.003[b]	0.049±0.002[d]	0.06±0.001[d]
Copper (liver) (μg/g d.w.)	7.2±1.5[a,c]	259±104[a,b]	125±26[b]	2.8±0.4[c,d]	181±13[d]
Iron (liver) (μg/g d.w.)	94.6±10.8	100±11	98.8±8.6	79.5±4.6	101±13
ALT (plasma)(U/L)	36.2±14.5[a]	238±49[a,b]	104±39.5[b]	34.8±9.1[d]	234±111[d]
Bilirubin (mg/dL)	0.38±0.21	0.4±0.11[b]	0.3±0.17	0.1±0	0.12±0.04[b]
Tnf-α liver transcripts (fold changes)	-0.56±1.4[a]	2.91±1.26 [a,b]	0.28±1.8 [b]	-1.34±1.4[d]	2.85±1.1 [d]
Lobular inflammation (0-3)	0±0[a]	1.08±0.5[a,b]	0.4±0.41[a,b]	0±0[d]	0.37±0.25[b,d]
Portal inflammation (0-3)	0.12±0.2[a]	1.66±0.51[a,b]	1.1±0.22[b]	0±0[d]	1.75±0.5[d]
Hepatocyte nuclear diameter (N) (μm)	5.6±0.2[a]	16±2[a]	14.7±0.19	5.6±0.3[d]	15±0.8[d]
Hepatocyte cell diameter (C) (μm)	16.9±0.9[a]	41±4.8[a]	40±1.8	17.4±1[d]	38±2.9[d]
N/C ratio	0.3±0.01[a]	0.4±0.01[a]	0.4±0.01	0.3±0.007[d]	0.4±0.01[d]
Hepatocyte nuclear area (μm^2)	46±2.9[a]	99±7.7[a]	114±14	47±0.62[d]	101±19[d]

Values are expressed as mean ± SD. In parenthesis, the number of mice in each group.

Values with same letter symbol are significantly different ($P < 0.05$) from each other. Hepatocyte nuclear and cell diameter were calculated on 4-6 mice/group. The relative expression of *Tnf*-α (and other target genes in subsequent figures) was calculated using the equation $2-\Delta\Delta Cq$ where $\Delta\Delta Cq = \Delta Cq(sample) - \Delta Cq(calibrator)$. Gene transcription is expressed as an n-fold difference relative to the calibrator (mean of control groups). The chosen reference gene is HPRT1 (coefficient of variation= 3.3%). See Supporting Material for additional methods. PCA: penicillamine.

control C3H mice (Table 1). Mean hepatic Cu concentration was more than 30 times increased in the tx-j mice and was associated with marked lobular and portal inflammation, with ∼6-fold increase in serum alanine aminotransferase (ALT) levels and increased liver *Tnf*-α transcript levels. There were no differences in hepatic iron levels between the groups. Liver histopathology of tx-j mice at 24 weeks of age showed lymphocytic lobular and portal infiltrates and perisinusoidal fibrosis (Fig. 1). The oral provision of PCA to tx-j mice from age 12 to 24 weeks resulted in 50% reduction of hepatic Cu and serum ALT levels, 90% reduction in liver *Tnf*-α expression, and concomitant improvements of both lobular and portal infiltration, whereas betaine treatment had no effect on *Tnf*-α transcripts or serum ALT in tx-j mice, but significantly lowered mean hepatic Cu levels in control mice by 61% and nonsignificantly lowered mean hepatic Cu levels by 30% while reducing lobular inflammation (Table 1). Hepatocyte and nuclear diameters and their ratios and hepatocyte nuclear areas were increased in tx-j mice, but were unchanged by either PCA or betaine.

Selected Gene Expressions for ER Stress and Steatosis. The transcript levels of selected genes related to ER stress (glucose-regulated protein 78 [*Grp78*]), lipogenesis (sterol regulatory element-binding protein [*Srebp1c*]), and fatty acid β oxidation (peroxisome proliferator-activated receptor α [*Pparα*] and carnitine palmitoyl transferase 1A [*Cpt1A*]), and protein levels of SREBP1c and PPARα were each lower in untreated tx-j than in C3H mice (Fig. 2). PCA further down-regulated *Srebp1c* and *Pparα* transcript levels and protein levels of GRP78 and CPT1A in tx-j mice. Betaine down-regulated the transcript levels of *Grp78, Pparα*, and *Cpt1A* in the control mice and *Cpt1A* in tx-j mice, whereas both transcript and protein levels of SREBP1c, PPARα, and CPT1A were each lower in betaine-treated tx-j mice than in betaine-treated control mice.

Methionine Metabolism, DNA Methyltransferases, and Global DNA Methylation in Untreated tx-j Mice

Liver S-adenosylmethionine (SAM) levels were similar in the untreated groups, whereas SAH levels were increased and SAM to SAH ratios were lower in the tx-j mice versus control mice (Fig. 3A-C). Although SAHH activity was similar in both untreated groups (Fig. 3D), both SAHH gene and protein expressions were decreased in the tx-j mice (Fig. 3F). DNA methyltransferase 1 (*Dnmt1*) transcripts were up-regulated, *Dnmt3a* transcripts were similar, and *Dnmt3b* transcripts were down-regulated in tx-j mice (Fig. 4A-C). According to dot blot analysis, global DNA methylation was lower in tx-j than in C3H mice (Fig. 5).

Effects of PCA on SAHH Activity, Tnf-α *and Dnmts Transcripts, and Global DNA Methylation*

PCA treatment reduced *Tnf*-α transcripts by 10-fold (Table 1) and increased SAHH activity in tx-j mice (Fig. 3D), whereas *Sahh* gene expression remained low and protein levels were further decreased (Fig. 3F), although SAM and SAH levels and SAM/SAH ratios were unchanged (Fig. 3A-C). PCA treatment restored global DNA methylation to control levels (Fig. 5), despite unchanged *Dnmt1* and reduction of *Dnmt3a* and *Dnmt3b* transcripts (Fig. 4B,C).

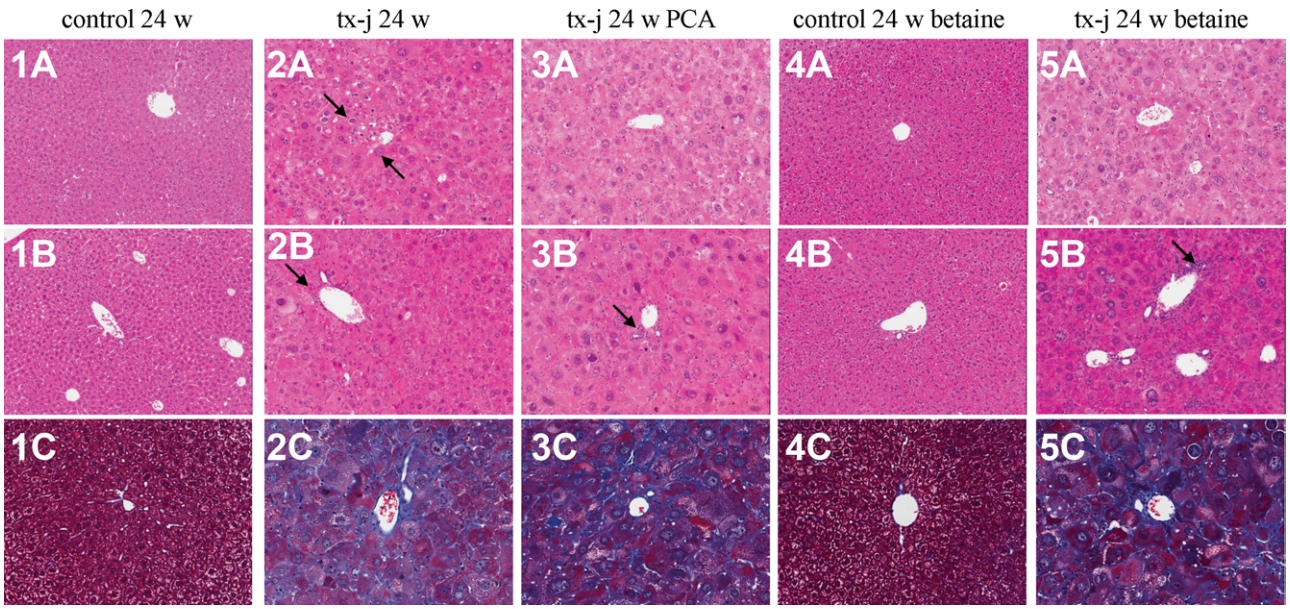


Fig. 1. Liver histology from control and tx-j mice at 24 weeks of age (A,B, hematoxylin and eosin, 10×; A, central vein; B, portal space; C, trichrome, 20×). Control (C3H) mice histology was normal (panels 1 and 4). Untreated tx-j mice presented enlarged and glycogenated hepatocyte nuclei (arrows) (2A), with marked lymphocytic lobular (2A) and portal infiltrate (arrow) (2B) and perisinusoidal fibrosis (2C). Minimal steatosis was present and was unaffected by either treatment. Lobular inflammatory infiltrate partially improved after penicillamine (arrow) (3A) and betaine treatment (arrow) (5A), with no change in portal inflammatory infiltrate after betaine treatment (arrow) (5B).

Effects of Betaine Treatment on SAM/SAH Ratio, Transcript Levels of Dnmt3b, and Global DNA Methylation

Betaine treatment from 20 to 24 weeks increased hepatic SAM levels in both groups, SAH levels in control mice, and the SAM/SAH ratio in tx-j mice (Fig. 3A-C). Although betaine treatment did not affect SAHH activity (Fig. 3E), it down-regulated *Sahh* transcripts in tx-j mice (Fig. 3F). *Dnmt1* and *Dnmt3a* transcripts were unchanged by betaine in both groups (Fig. 4A,B), whereas *Dnmt3b* transcript levels were up-regulated in tx-j mice (Fig. 4C). Global DNA methylation was increased by betaine treatment in both groups (Fig. 5).

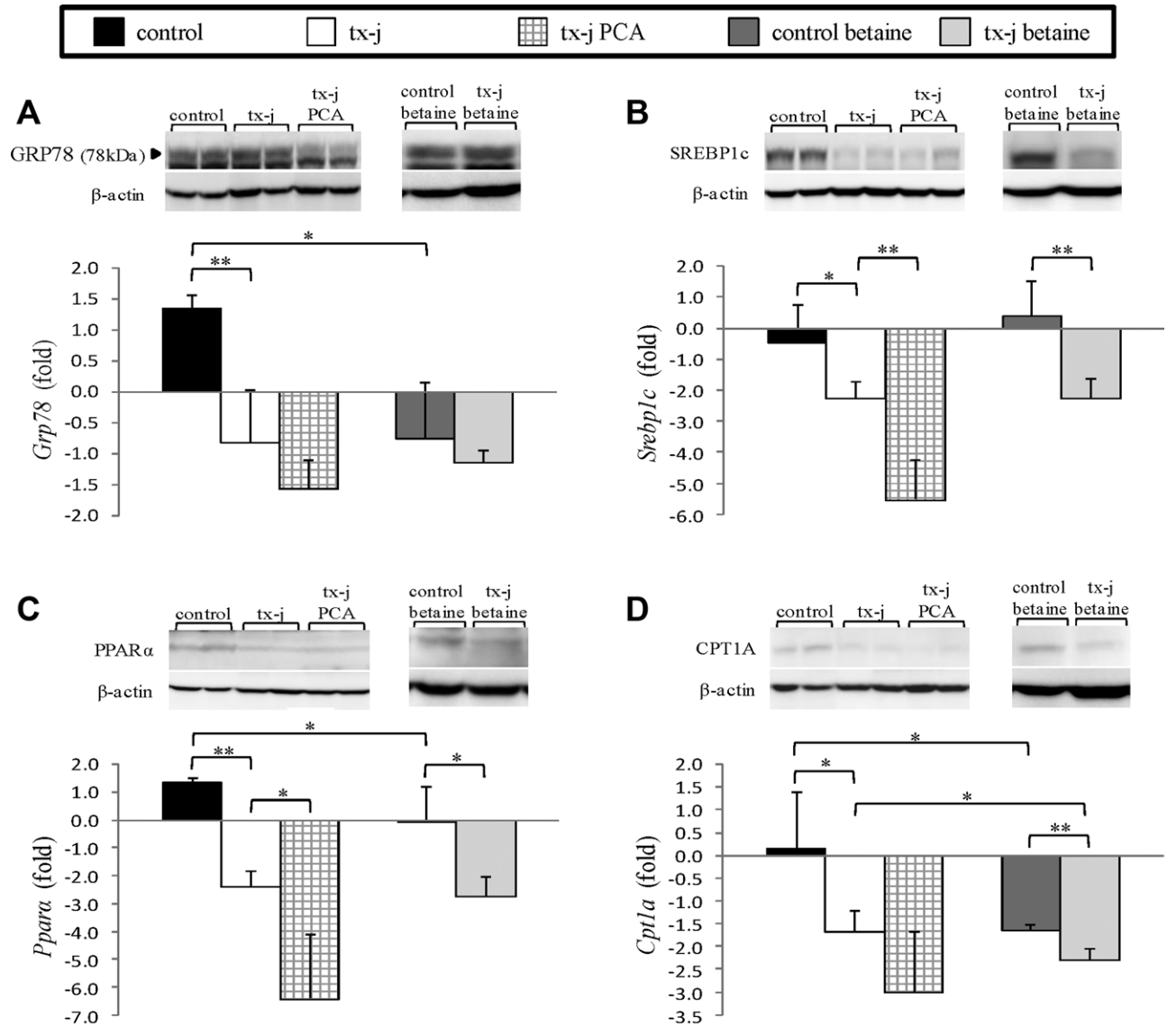
Correlations of Dnmts Transcript Levels with Global DNA Methylation and Selected Gene Expressions. Using data from all groups, *Dnmt3b* expression correlated positively with global DNA methylation and with transcript levels of *Sahh, Grp78, Srebp1c, Pparα*, and *Cpt1A* (Table 2). In addition (not shown), global DNA methylation values correlated with *Srebp1c* and *Pparα* transcript levels ($r = 0.39$, $P = 0.02$ and $r = 0.41$, $P = 0.02$, respectively).

Immunostaining for 5-Methylcytosine.

The immunostaining pattern for 5-methylcytosine showed diffuse and less intense signals in hepatocyte nuclei from tx-j mice than in C3H mice (Fig. 61A,2A). Normalized signal intensity was significantly higher in C3H mice than in tx-j mice, but not significantly different in betaine treated tx-j and control mice. In addition, when comparing betaine versus PCA treated tx-j mice, betaine treatment was associated with stronger nuclear intensity peaks (Fig. 62B,2C), indicating that provision of methyl groups is associated with a different pattern of DNA methylation.

Discussion

This study investigated the potential role of Cu-induced abnormal methionine metabolism in the tx-j mouse model of WD and found several novel results relevant to treatment. First, elevated levels of SAH and reduced levels of the SAM to SAH methylation ratio were observed in untreated tx-j mice in association with reduced levels of SAHH and global DNA methylation. DNA hypomethylation in tx-j mice was correlated with reduced expression of *Dnmt3b*, but was paradoxically associated with increased expression of *Dnmt1*. Second, Cu chelation by PCA improved inflammation in the tx-j mice, reduced the expression of *Tnf-α* and selected genes related to ER stress and lipid metabolism, and normalized global DNA methylation levels while reducing transcript levels of *Dnmt3a* and *Dnmt3b*. Lastly, the methyl donor betaine also normalized global DNA methylation while enhancing SAM levels and SAM-to-SAH ratio and reducing transcript levels of *Cpt1A* in the tx-j mice.



E

	control (7)	tx-j (7)	tx-j PCA (7)	control betaine (7)	tx-j betaine (7)
GRP78/β-actin ratio	0.30 ± 0.12	0.30 ± 0.08[b]	0.11 ± 0.04[b]	1.25 ± 0.42	1.27 ± 0.25
SREBP1c/β-actin ratio	2.00 ± 0.29[a]	0.46 ± 0.20[a]	0.68 ± 0.49	4.54 ± 1.52[c]	2.23 ± 1.75[c]
PPARα/β-actin ratio	1.37 ± 0.36[a]	0.50 ± 0.11[a]	0.43 ± 0.14	0.59 ± 0.18[c]	0.20 ± 0.03[c]
CPT1A/β-actin ratio	0.74 ± 0.32	0.53 ± 0.17[b,†]	0.31 ± 0.10[b]	1.65 ± 0.35[c]	0.70 ± 0.16[c]

Fig. 2. Western blot analysis and transcripts levels of genes representative of ER stress, lipogenesis, and fatty acid oxidation. "Control" refers to untreated C3H mice in all panels. (A-D) GRP78; SREBP1c; PPARa; CPT1A, each showing protein and transcript expression data normalized to β-actin (6-7 mice/group); (E) Numerical mean ± SD of protein expressions according to Western blots, in parenthesis the number of mice used for the quantification (seven in all groups, except CPT1A (†) in six tx-j mice) in this and subsequent figures. *$P < 0.05$; **$P < 0.001$. See Supporting Information for methods. Values with the same letter symbol are significantly different ($P < 0.05$) from each other.

We propose that interplay between inflammation and methionine metabolism is related to Cu-mediated inhibition of *Sahh* resulting in elevated SAH levels, which dysregulates methylation status and gene expression in WD. We demonstrated that *Sahh* transcript and protein levels were down-regulated in the tx-j mouse model of WD, whereas SAHH activity was increased by Cu chelation with PCA, although liver SAH levels remained increased and the SAM/SAH level decreased. A previous study on the natural course

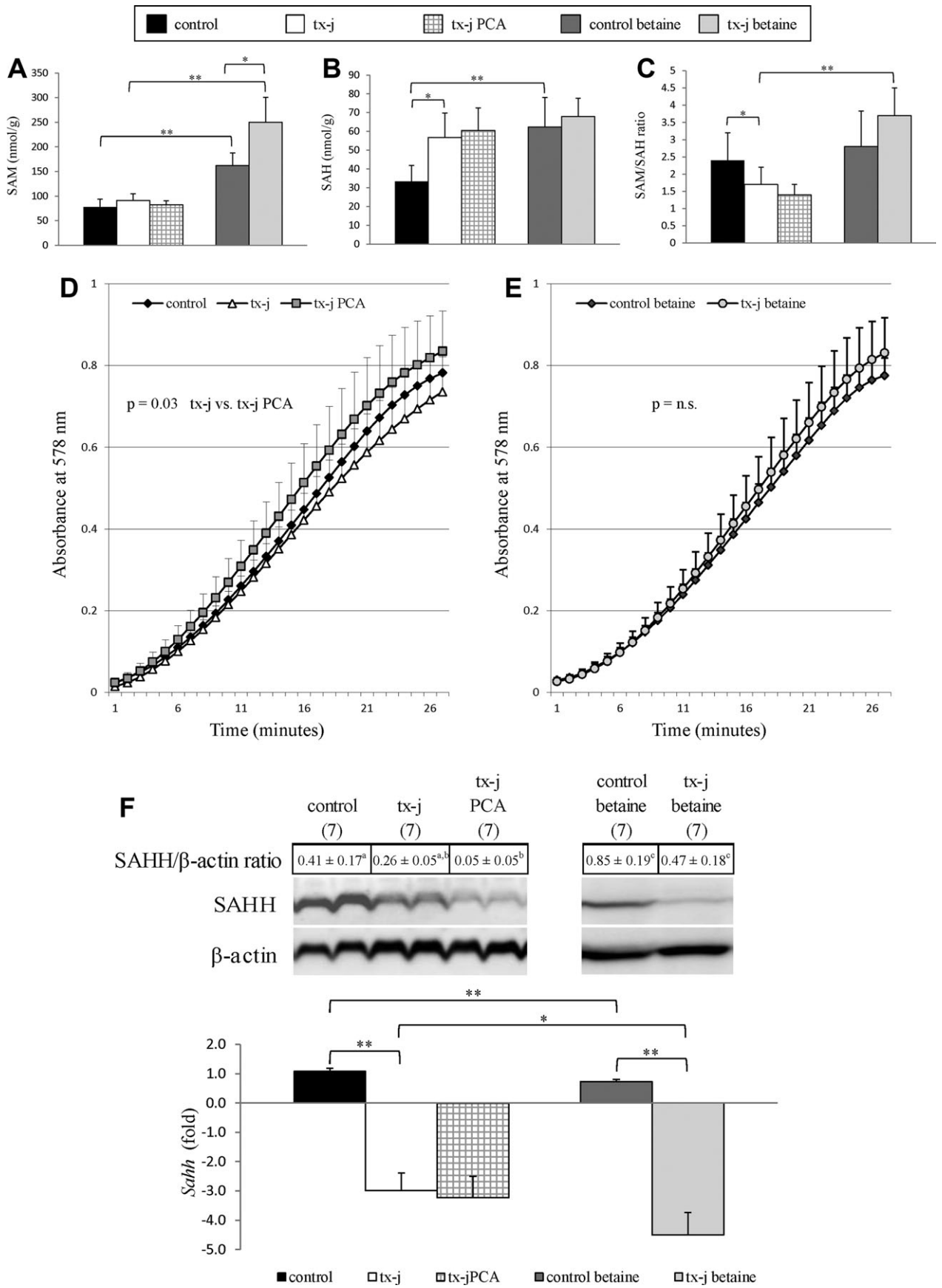


Fig. 3. (A) Hepatic S-adenosylmethionine (SAM); (B) S-adenosylhomocysteine (SAH); (C) SAM/SAH ratio; (D,E) S-adenosylhomocysteine hydrolase (SAHH) activity; (F) Western blot analyses and mRNA transcript levels. The curves for SAHH activities in C3H and tx-j mice represent the average of absorbance measurements of 6-7 mice from each group of 24-week-old mice, untreated and in response to penicillamine (PCA) (D) and betaine (E). Protein and gene transcript levels of SAHH were significantly lower in tx-j mice compared to control CH3 mice (F). Values with the same letter symbol are significantly different ($P < 0.05$) from each other. *$P < 0.05$; **$P < 0.001$.

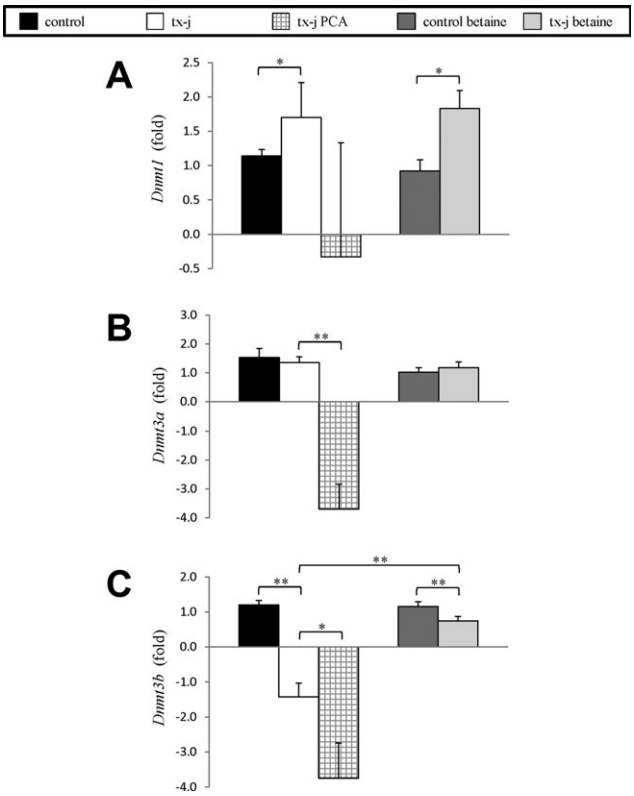


Fig. 4. DNA methyltransferases transcript levels. *Dnmt1 levels* were up-regulated (A), whereas *Dnmt3b* (C) levels were down-regulated in untreated tx-j mice and further down-regulated by PCA treatment in both *Dnmt3a* and *Dnmt3b*. Betaine treatment had opposite effects on *Dnmt1* and *Dnmt3b* expression in control and tx-j mice, while increasing the expression of *Dnmt3b* in the treated tx-j mice. $*P < 0.05$; $**P < 0.001$.

of WD in 24-week-old LEC rats showed reduced SAHH protein levels only in advanced stages of liver disease,[27] in contrast to findings in the present study of the same age tx-j mice.

SAH is a potent inhibitor of DNMTs activities and reduced levels of *Dnmts* transcripts were described in response to elevated SAH levels in a recent clinical study.[28] Notably, we observed that *Dnmt1*, which encodes the principal DNMT in mammalian cells and is mainly implicated in the maintenance of methylation status, was up-regulated, whereas *Dnmt3b*, encoding a *de novo* methyltransferase, was down-regulated in untreated tx-j mice, and both *Dnmt3a* and *Dnmt3b* transcripts were down-regulated by PCA. Transcript levels of *Dnmt* genes show compensatory increased levels in response to reduced DNA methylation in liver or brain.[29] Others described up-regulation of *Dnmt1* expression without changes in *Dnmt3a* in livers of choline-deficient rat embryos with global DNA hypomethylation,[30] whereas *Dnmts* levels are increased in chronic hepatitis, cirrhosis, and human hepatocellular carcinoma.[31] The finding that *Dnmt3b* was down-regulated in untreated tx-j mice with increased SAH levels and correlated with global DNA methylation and expressions of each of the studied genes suggests that this enzyme plays a major role in controlling global DNA methylation in WD. There were some discrepancies in the *Dnmts* expression in response to betaine treatment. We did not observe any change in *Dnmts* levels in control mice nor in *Dnmt1* and *Dnmt3a* in tx-j mice, whereas *Dnmt3b* was markedly up-regulated in tx-j mice in response to betaine.

Tx-j mice demonstrated reduced levels of global DNA methylation, which was restored either by PCA-induced Cu chelation or by provision of the methyl donor betaine. The positive effect of PCA on increasing global DNA methylation may be due to relatively reduced inflammation and demand for methyl groups.[17] At the same time, methyl group supplementation by betaine could increase global DNA methylation in both control and tx-j mice, regardless of inflammation. We propose that global DNA hypomethylation in WD was influenced by inflammation which was resolved by reducing Cu hepatic levels by PCA treatment, or by the increasing availability of methyl groups by betaine. The major difference between PCA and betaine treatment in tx-j mice was that PCA was exclusively associated with a significant improvement of inflammation, whereas only betaine induced significant increased SAM and *Dnmt3b* levels. These observations suggest a potential positive additive effect of including betaine in the PCA treatment of patients with WD.

Methyl donors such as betaine and/or SAM have been used effectively in treatment of other liver diseases, including adjuvant therapy for hepatitis C in which either agent restored methylation of STAT1 which regulates the response to interferon treatment,[32] whereas SAM improved viral kinetics by increasing the methylation of STAT1 and the expressions of its representative genes.[33] A study of experimental acetaminophen liver toxicity found that SAM administration restored its mitochondrial and nuclear levels, possibly due to increased expression and activity of methionine adenosyltransferase.[34] These results support our findings that the administration of the methyl donor betaine modified SAM levels and consequently the expressions of selected genes.

The observed significant 61% reduction of the mean liver Cu level by betaine treatment in control mice and nonsignificant 30% reduction of mean hepatic Cu level in the tx-j mice (Table 1) are original findings, which could have been influenced by uneven hepatic Cu distribution in the liver.[35] Potentially,

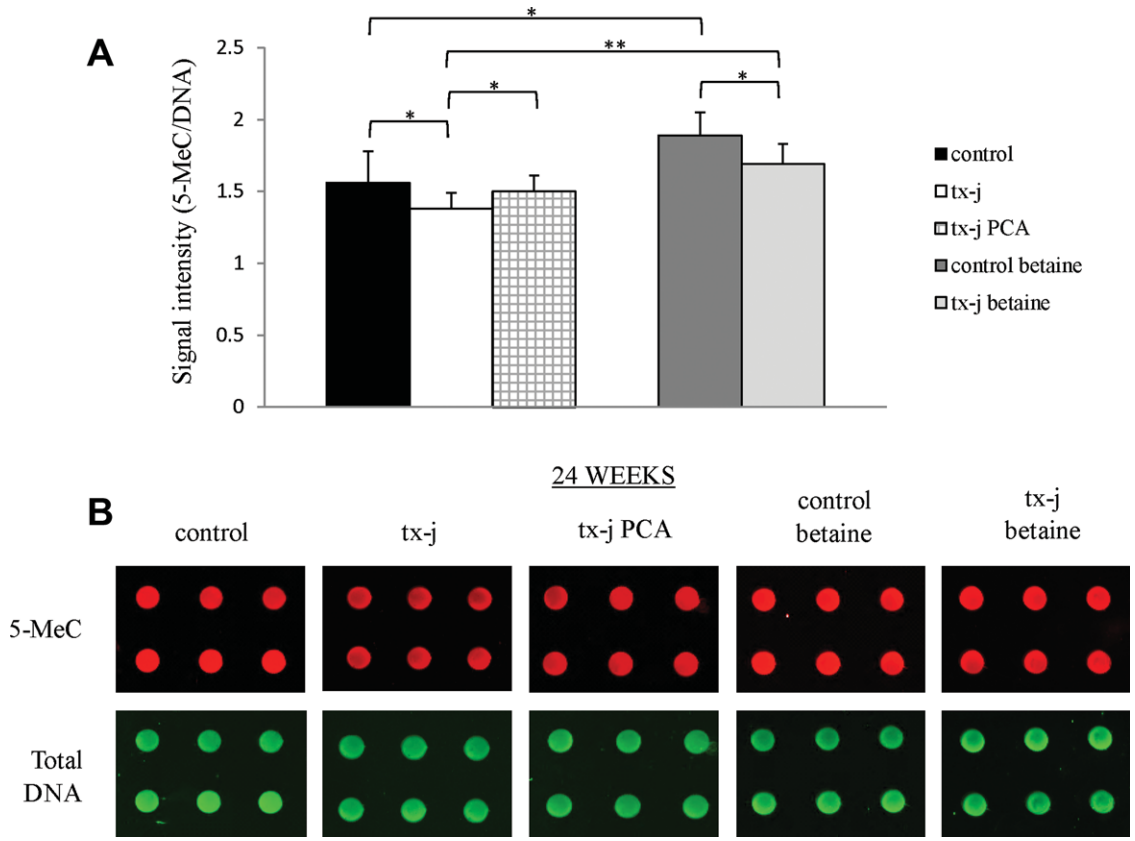


Fig. 5. Global DNA methylation. (A) Global DNA methylation was lower in the tx-j mice and improved to control levels after Cu chelation by PCA. Betaine treatment resulted in increased methylation in both untreated groups. (B) The red blots represent 5-methylcytosine (5-MeC) and the green blots represent total DNA signal. Each timepoint and genotype is represented by two mice (upper and lower row in each black square), each of them in triplicate. Each blot represents methylated DNA from one mouse. $*P < 0.05$; $**P < 0.001$.

betaine could reduce Cu levels by modification of the expressions of genes for Cu chaperones or cellular transport. Ultimately, only long-term studies on betaine treatment for tx-j mice may clarify this issue.

Currently, the molecular mechanisms linking Cu and gene expression are unknown but it seems likely that methylation plays an important role. Reduced global methylation in the untreated tx-j mice could relate to increased inflammation, which others have associated with increased demand for gene methylation resulting from increased cell division and DNA.[16,17] Consequently, WD may be associated with increased demand for methyl groups as result of both an increase of the methylation inhibitor SAH and increased inflammation. Although the associations of Cu-mediated SAHH inhibition with SAH accumulation and reduced *Dnmt3b* transcripts were consistent with global DNA hypomethylation in tx-j mouse livers, the relationship between DNA hypomethylation and down-regulation of the expressions of genes representative of ER stress, lipogenesis, and fatty acid oxidation in untreated tx-j mice is less clear. Similar to our data, others described down-regulation of gene transcripts representative of lipid metabolism in the Atp7b−/−

Table 2. Correlation Analysis of the Relationship Between *Dnmts* Transcript Levels with Global DNA Methylation and the Expressions of the Studied Genes

	Global DNA methylation		*Sahh*		*Grp78*		*Srebp1c*		*Pparα*		*Cpt1A*	
	r	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value	*r*	*P* value
Dnmt1	-0.19	0.30	-0.29	0.13	-0.01	0.97	0.30	0.10	0.05	0.77	0.04	0.82
Dnmt3a	0.07	0.70	0.18	0.35	0.39	**0.04**	0.71	**<0.0001**	0.67	**<0.0001**	0.54	**0.002**
Dnmt3b	0.48	**0.006**	0.46	**0.01**	0.44	**0.02**	0.78	**<0.0001**	0.79	**<0.0001**	0.47	**0.01**

Sahh, S-adenosylhomocysteine hydrolase; *Grp78*, glucose-related protein 78; *Srebp1c*, sterol regulatory element-binding protein 1c; *Pparα*, peroxisome proliferator-activated receptor α; *Cpt1A*, carnitine palmitoyltransferase 1A; *Dnmt*, DNA methyltransferase.

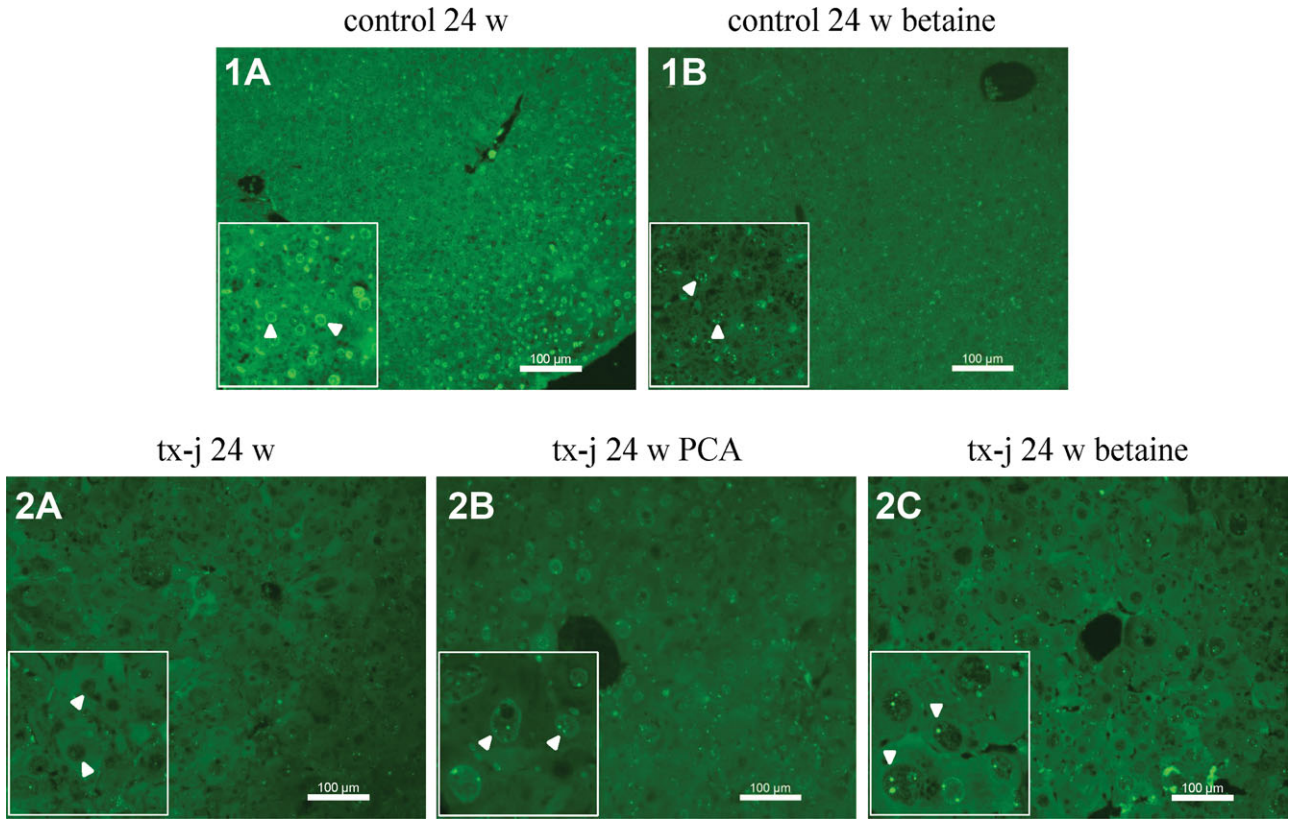


Fig. 6. Immunostaining for 5-methylcytosine. Compared to C3H mice (1A), untreated tx-j mice presented diffuse and less intense nuclear staining (2A) ($P < 0.01$). Betaine-treated control (1B) and tx-j mice (2C) had similar immunostaining patterns. Betaine-treated tx-j (2C) presented a higher percentage of nuclear peaks than PCA-treated tx-j mice (2B) ($P = 0.01$). Arrowheads in the insets indicate representative nuclei.

knockout mouse at 6 weeks of age, prior to development of histological damage.[36] However, this study did not find any changes in *Sahh* and *Dnmt1* expressions, whereas *Dnmt3a, Dnmt3b*, and potential changes in lipid metabolism in response to Cu chelation were not measured. In the present study, the hypothesis that the observed changes in gene transcript levels with PCA treatment are due to changes in methylation is supported by observations that both PCA and betaine treatments were associated with significant increases in global DNA methylation and by the positive correlations between *Dnmt3b* transcripts and global DNA methylation and transcripts of genes representative of ER stress, lipogenesis, and fatty acid oxidation.

There are potentially other mechanisms involved in the gene expression response to Cu accumulation and PCA. As discussed by others, various proteins could mediate crosstalk between Cu status and lipid metabolism,[37] and others showed that Cu enters nuclei where it can bind to specific targets.[38]

The observed changes in hepatocellular morphology with increased hepatocyte and nuclear diameter, and nuclear to hepatocyte diameter ratios that persisted with treatment, are novel, and differ from previously reported data in the LEC rat where they responded to Cu chelation therapy.[23] Nuclear and cellular hepatocyte enlargement, which in general are associated with chronic hepatitis with hepatocyte enzymatic activation,[39] is well known in humans with WD[1,40] and in animal models of this condition[3,23] but the response to Cu chelation treatment has not been consistently described.[41]

Prior studies on the effects of betaine included its down-regulation of selected ethanol-induced ER stress-related genes *Grp78* and *Srebp1c* in mice,[42] that *Ppara* promoter methylation in offspring was affected by maternal dietary betaine in rats,[43] and that the *Cpt1A* promoter is hypermethylated in human embryonic stem cells.[44] Although both PCA and betaine treatments were associated with increased global DNA methylation in our study, betaine treatment achieved significant down-regulation of selected ER stress and fatty acid oxidation related transcripts only in control mice, i.e., specifically in the absence of inflammation and Cu accumulation. Increased methylation of promoter CpG islands has been correlated with gene silencing, and may be occurring in our control mice with normally saturated levels

of DNA methylation following betaine treatment. In contrast, gene body and intergenic methylation were unsaturated in the global hypomethylated state that we observed in the tx-j mouse livers, so increasing the availability of methyl groups can be predicted to target nonpromoter methylation sites associated with increased gene expression levels.[45] Of note, the directions and magnitude of changes in proteins levels for the studied genes were generally similar to those in transcript levels. It is possible that the observed differences between messenger RNA (mRNA) and protein levels of several of the studied genes could be attributed to posttranslational modifications.

In summary, WD appears to be a condition associated with increased demand for methyl groups due to both increased levels of the methylation inhibitor SAH, which is based on the inhibitory effect of Cu on SAHH as shown by its activation after PCA chelation, and also due to chronic inflammatory status (Supporting Fig.). Our results demonstrate reduced SAHH levels in a mouse model of WD that is characterized by Cu accumulation and hepatic inflammation in the early phases of liver damage. The consequent elevation of liver SAH as a result of SAHH inhibition, and the concomitant inflammation, were associated with down-regulation of *Dnmt3b* and global DNA hypomethylation. Our study supports the hypothesis that gene expression and possibly phenotypic presentations of WD are affected by interactions between Cu accumulation and methionine metabolism.

Acknowledgment: We thank Dr. Robert D. Cardiff for expert assistance with the histopathology analysis.

References

1. Stromeyer FW, Ishak KG. Histology of the liver in Wilson's disease: a study of 34 cases. Am J Clin Pathol 1980;73:12-24.
2. Medici V, Trevisan CP, D'Incà R, Barollo M, Zancan L, Fagiuoli S, et al. Diagnosis and management of Wilson's disease: results of a single center experience. J Clin Gastroenterol 2006;40:936-941.
3. Biempica L, Rauch H, Quintana N, Sternlieb I. Morphologic and chemical studies on a murine mutation (Toxic Milk Mice) resulting in hepatic copper toxicosis. Lab Invest 1988;59:500-508.
4. Roberts EA, Robinson BH, Yang S. Mitochondrial structure and function in the untreated Jackson toxic milk (tx-j) mouse, a model for Wilson disease. Mol Genet Metab 2008;93:54-65.
5. Huster D, Kühne A, Bhattacharjee A, Raines L, Jantsch V, Noe J, et al. Diverse functional properties of Wilson disease ATP7B variants. Gastroenterology 2012;142:947-956.
6. Wan L, Tsai CH, Hsu CM, Huang CC, Yang CC, Liao CC, et al. Mutation analysis and characterization of alternative splice variants of the Wilson disease gene ATP7B. Hepatology 2010;52:1662-1670.
7. Burkhead JL, Ralle M, Wilmarth P, David L, Lutsenko S. Elevated copper remodels hepatic RNA processing machinery in the mouse model of Wilson's disease. J Mol Biol 2011;406:44-58.
8. Weiss KH, Runz H, Noe B, Gotthardt DN, Merle U, Ferenci P, et al. Genetic analysis of BIRC4/XIAP as a putative modifier gene of Wilson disease. J Inherit Metab Dis 2010 [Epub ahead of print].
9. Medici V, Santon A, Sturniolo GC, D'Incà R, Giannetto S, Albergoni V, et al. Metallothionein and antioxidant enzymes in Long-Evans Cinnamon rats treated with zinc. Arch Toxicol 2002;76:509-516.
10. Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, et al. Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. Nat Med 2007;13:164-170.
11. Zischka H, Lichtmannegger J, Schmitt S, Jägemann N, Schulz S, Wartini D, et al. Liver mitochondrial membrane crosslinking and destruction in a rat model of Wilson disease. J Clin Invest 2011;1;121:1508-1518.
12. Bethin KE, Cimato TR, Ettinger MJ. Copper binding to mouse liver S-adenosylhomocysteine hydrolase and the effects of copper on its levels. J Biol Chem 1995;270:20703-20711.
13. Delgado M, Perez-Miguelsanz J, Garrido F, Rodriguez-Tarduchy G, Perez-Sala D, Pajares MA. Early effects of copper accumulation on methionine metabolism. Cell Mol Life Sci 2008;65:2080-2090.
14. Esfandiari F, Medici V, Wong DH, Jose S, Dolatshahi M, Quinlivan E, et al. Epigenetic regulation of hepatic endoplasmic reticulum stress in the ethanol-fed cystathionine beta synthase-deficient mouse. Hepatology 2010;51:932-941.
15. Song Z, Zhou Z, Song M, Uriarte S, Chen T, Deaciuc I, et al. Alcohol-induced S-adenosylhomocysteine accumulation in the liver sensitizes to TNF hepatotoxicity: possible involvement of mitochondrial S-adenosylmethionine transport. Biochem Pharmacol 2007;1;74:521-531.
16. LaSalle JM. A genomic point-of-view on environmental factors influencing the human brain methylome. Epigenetics 2011;6:862-869.
17. Gonda TA, Kim YI, Salas MC, Gamble MV, Shibata W, Muthupalani S, et al. Folic acid increases global DNA methylation and reduces inflammation to prevent Helicobacter-associated gastric cancer in mice. Gastroenterology 2012;142:824-833.
18. Bethin KE, Petrovic N, Ettinger MJ. Identification of a major hepatic copper binding protein as S-adenosylhomocysteine hydrolase. J Biol Chem 1995;270:20698-206702.
19. Li M, Li Y, Chen J, Wei W, Pan X, Liu J, et al. Copper ions inhibit S-adenosylhomocysteine hydrolase by causing dissociation of NAD+ cofactor. Biochemistry 2007;46:11451-11458.
20. Matthews RP, Lorent K, Mañoral-Mobias R, Huang Y, Gong W, Murray IV, et al. TNF alpha-dependent hepatic steatosis and liver degeneration caused by mutation of zebrafish S-adenosylhomocysteine hydrolase. Development 2009;136:865-875.
21. Buist NR, Glenn B, Vugrek O, Wagner C, Stabler S, Allen RH, Pogribny I, et al. S-adenosylhomocysteine hydrolase deficiency in a 26-year-old man. J Inherit Metab Dis 2006;29:538-545.
22. Coronado V, Nanji M, Cox DW. The Jackson toxic milk mouse as a model for copper loading. Mamm Genome 2001;12:793-795.
23. Togashi Y, Li Y, Kang JH, Takeichi N, Fujioka Y, Nagashima K, et al. D-penicillamine prevents the development of hepatitis in Long-Evans Cinnamon rats with abnormal copper metabolism. Hepatology 1992;15:82-87.
24. Lei KY. Alterations in plasma lipid, lipoprotein and apolipoprotein concentrations in copper-deficient rats. J Nutr 1983;113:2178-2183.
25. Koupparis AJ, Jeremy J, Angelini G, Persad R, Shukla N. Penicillamine administration reverses the inhibitory effect of hyperhomocysteinaemia on endothelium-dependent relaxation in the corpus cavernosum in the rabbit. BJU Int 2006;98:440-444.
26. Barak AJ, Beckenhauer HC, Mailliard ME, Kharbanda KK, Tuma DJ. Betaine lowers elevated s-adenosylhomocysteine levels in hepatocytes from ethanol-fed rats. J Nutr 2003;133:2845-2848.
27. Lee BH, Kim JM, Heo SH, Mun JH, Kim J, Kim JH, et al. Proteomic analysis of the hepatic tissue of Long-Evans Cinnamon (LEC) rats according to the natural course of Wilson disease. Proteomics 2011;11:3698-3705.
28. Tao J, Yang M, Chen Z, Huang Y, Zhao Q, Xu J, et al. Decreased DNA methyltransferase 3A and 3B mRNA expression in peripheral

blood mononuclear cells and increased plasma SAH concentration in adult patients with idiopathic thrombocytopenic purpura. J Clin Immunol 2008;28:432-439.
29. Ghoshal K, Li X, Datta J, Bai S, Pogribny I, Pogribny M, et al. A folate- and methyl-deficient diet alters the expression of DNA methyltransferases and methyl CpG binding proteins involved in epigenetic gene silencing in livers of F344 rats. J Nutr 2006;136: 1522-1527.
30. Kovacheva VP, Mellott TJ, Davison JM, Wagner N, Lopez-Coviella I, Schnitzler AC, et al. Gestational choline deficiency causes global and Igf2 gene DNA hypermethylation by up-regulation of Dnmt1 expression. J Biol Chem 2007;282:31777-31788.
31. Oh BK, Kim H, Park HJ, Shim YH, Choi J, Park C, et al. DNA methyltransferase expression and DNA methylation in human hepatocellular carcinoma and their clinicopathological correlation. Int J Mol Med 2007;20:65-73.
32. Duong FH, Christen V, Filipowicz M, Heim MH. S-Adenosylmethionine and betaine correct hepatitis C virus induced inhibition of interferon signaling in vitro. HEPATOLOGY 2006;43:796-806.
33. Feld JJ, Modi AA, El-Diwany R, Rotman Y, Thomas E, Ahlenstiel G, et al. S-adenosyl methionine improves early viral responses and interferon-stimulated gene induction in hepatitis C nonresponders. Gastroenterology 2011;140:830-839.
34. Brown JM, Ball JG, Hogsett A, Williams T, Valentovic M. Temporal study of acetaminophen (APAP) and S-adenosyl-L-methionine (SAMe) effects on subcellular hepatic SAMe levels and methionine adenosyltransferase (MAT) expression and activity. Toxicol Appl Pharmacol 2010;247:1-9.
35. Faa G, Nurchi V, Demelia L, Ambu R, Parodo G, Congiu T, et al. Uneven hepatic copper distribution in Wilson's disease. J Hepatol 1995;22:303-308.
36. Huster D, Purnat TD, Burkhead JL, Ralle M, Fiehn O, Stuckert F, et al. High copper selectively alters lipid metabolism and cell cycle machinery in the mouse model of Wilson disease. J Biol Chem 2007;282: 8343-8355.
37. Huster D, Lutsenko S. Wilson disease: not just a copper disorder. Analysis of a Wilson disease model demonstrates the link between copper and lipid metabolism. Mol Biosyst 2007;3:816-824.
38. Ralle M, Huster D, Vogt S, Schirrmeister W, Burkhead JL, Capps TR, et al. Wilson disease at a single cell level: intracellular copper trafficking activates compartment-specific responses in hepatocytes. J Biol Chem 2010;285:30875-30883.
39. Maronpot RR, Yoshizawa K, Nyska A, Harada T, Flake G, Mueller G, et al. Hepatic enzyme induction: histopathology. Toxicol Pathol 2010; 38:776-795.
40. Müller T, Langner C, Fuchsbichler A, Heinz-Erian P, Ellemunter H, Schlenck B, et al. Immunohistochemical analysis of Mallory bodies in Wilsonian and non-Wilsonian hepatic copper toxicosis. HEPATOLOGY 2004;39:963-969.
41. Cope-Yokoyama S, Finegold MJ, Sturniolo GC, Kim K, Mescoli C, Rugge M, Medici V. Wilson disease: histopathological correlations with treatment on follow-up liver biopsies. World J Gastroenterol 2010;16: 1487-1494.
42. Ji C, Kaplowitz N. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. Gastroenterology 2003;124:1488-1499.
43. Lillycrop KA, Phillips ES, Jackson AA, Hanson MA, Burdge GC. Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. J Nutr 2005;135:1382-1386.
44. Shen Y, Chow J, Wang Z, Fan G. Abnormal CpG island methylation occurs during in vitro differentiation of human embryonic stem cells. Hum Mol Genet 2006;15:2623-2635.
45. Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, et al. Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 2009;462:315-322.