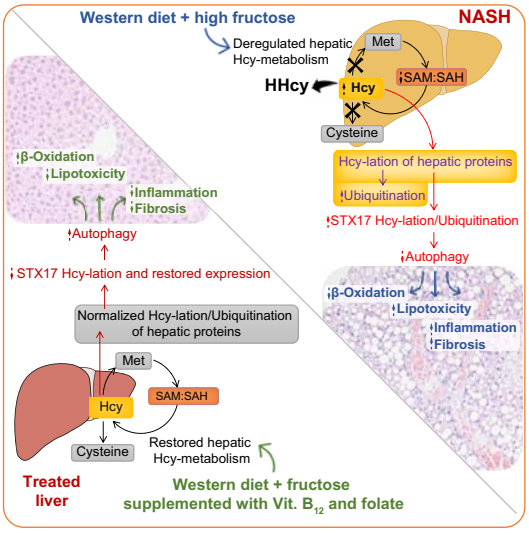
Research Article
NAFLD and Alcohol-Related Liver Diseases

JOURNAL OF HEPATOLOGY

Vitamin B_{12} and folate decrease inflammation and fibrosis in NASH by preventing syntaxin 17 homocysteinylation

Graphical abstract



Highlights

- Hyperhomocysteinemia is positively associated with NASH progression.
- Increased intrahepatic homocysteine causes NASH.
- STX17 homocysteinylation and ubiquitination leads to a block in autophagy during NASH progression.
- Supplementary vitamin B12 and folate restore STX17 expression and autophagy to decrease inflammation and fibrosis in NASH.

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Lay summary

The incidence of non-alcoholic steatohepatitis, for which there are no approved pharmacological therapies, is increasing, posing a significant healthcare challenge. Herein, based on studies in mice, primates and humans, we found that dietary supplementation with vitamin B_{12} and folate could have therapeutic potential for the prevention or treatment of non-alcoholic steatohepatitis.

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Vitamin B_{12} and folate decrease inflammation and fibrosis in NASH by preventing syntaxin 17 homocysteinylation

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Background & Aims: Several recent clinical studies have shown that serum homocysteine (Hcy) levels are positively correlated, while vitamin B_{12} (B_{12}) and folate levels are negative correlated, with non-alcoholic steatohepatitis (NASH) severity. However, it is not known whether hyperhomocysteinemia (HHcy) plays a pathogenic role in NASH.

Methods: We examined the effects of HHcy on NASH progression, metabolism, and autophagy in dietary and genetic mouse models, patients, and primates. We employed vitamin B_{12} (B_{12}) and folate (Fol) to reverse NASH features in mice and cell culture.

Results: Serum Hcy correlated with hepatic inflammation and fibrosis in NASH. Elevated hepatic Hcy induced and exacerbated NASH. Gene expression of hepatic Hcy-metabolizing enzymes was downregulated in NASH. Surprisingly, we found increased homocysteinylation (Hcy-lation) and ubiquitination of multiple hepatic proteins in NASH including the key autophagosome/lysosome fusion protein, Syntaxin 17 (Stx17). This protein was Hcy-lated and ubiquitinated, and its degradation led to a block in autophagy. Genetic manipulation of *Stx17* revealed its critical role in regulating autophagy, inflammation and fibrosis during HHcy. Remarkably, dietary B_{12}/Fol, which promotes enzymatic conversion of Hcy to methionine, decreased HHcy and hepatic Hcy-lated protein levels, restored Stx17 expression and autophagy, stimulated β -oxidation of fatty acids, and improved hepatic histology in mice with pre-established NASH.

Conclusions: HHcy plays a key role in the pathogenesis of NASH via Stx17 homocysteinylation. B_{12}/folate also may represent a novel first-line therapy for NASH.

Lay summary: The incidence of non-alcoholic steatohepatitis, for which there are no approved pharmacological therapies, is increasing, posing a significant healthcare challenge. Herein, based on studies in mice, primates and humans, we found that dietary supplementation with vitamin B_{12} and folate could have therapeutic potential for the prevention or treatment of non-alcoholic steatohepatitis.

Introduction

Hyperhomocysteinemia (HHcy) is a metabolic disorder caused by improper removal and/or accumulation of homocysteine (Hcy) most commonly arising from low dietary intake of Folate (Fol) or Vitamin B_{12} (B_{12}), or mutations in *MTHFR* and *CBS* genes (Fig 1A).[2] Hcy can be covalently linked to proteins via an isopeptide bond to lysine (Lys) residues, and this unique post-translational modification is termed "homocysteinylation" (Hcy-lation), which leads to impaired protein structure/function and is associated with cytotoxic, proinflammatory and proatherogenic effects linked to cardiovascular disease, diabetes, etc.[3,4] Several recent clinical studies showed that serum Hcy levels were positively associated with non-alcoholic steatohepatitis (NASH), and B_{12} and Fol levels were negatively correlated with non-alcoholic fatty liver disease (NAFLD)/NASH severity.[5–8] However, it is not known whether HHcy plays a pathogenic role in NASH.

Herein, we examined the role of HHcy on NASH in mouse models, patients and primates. We found the HHcy correlated

Keywords: Homocysteine; Vitamin therapy; Syntaxin-17; Autophagy; Protein homocysteinylation; Non-alcoholic steatohepatitis (NASH); Fibrosis; B_{12}; Folate.
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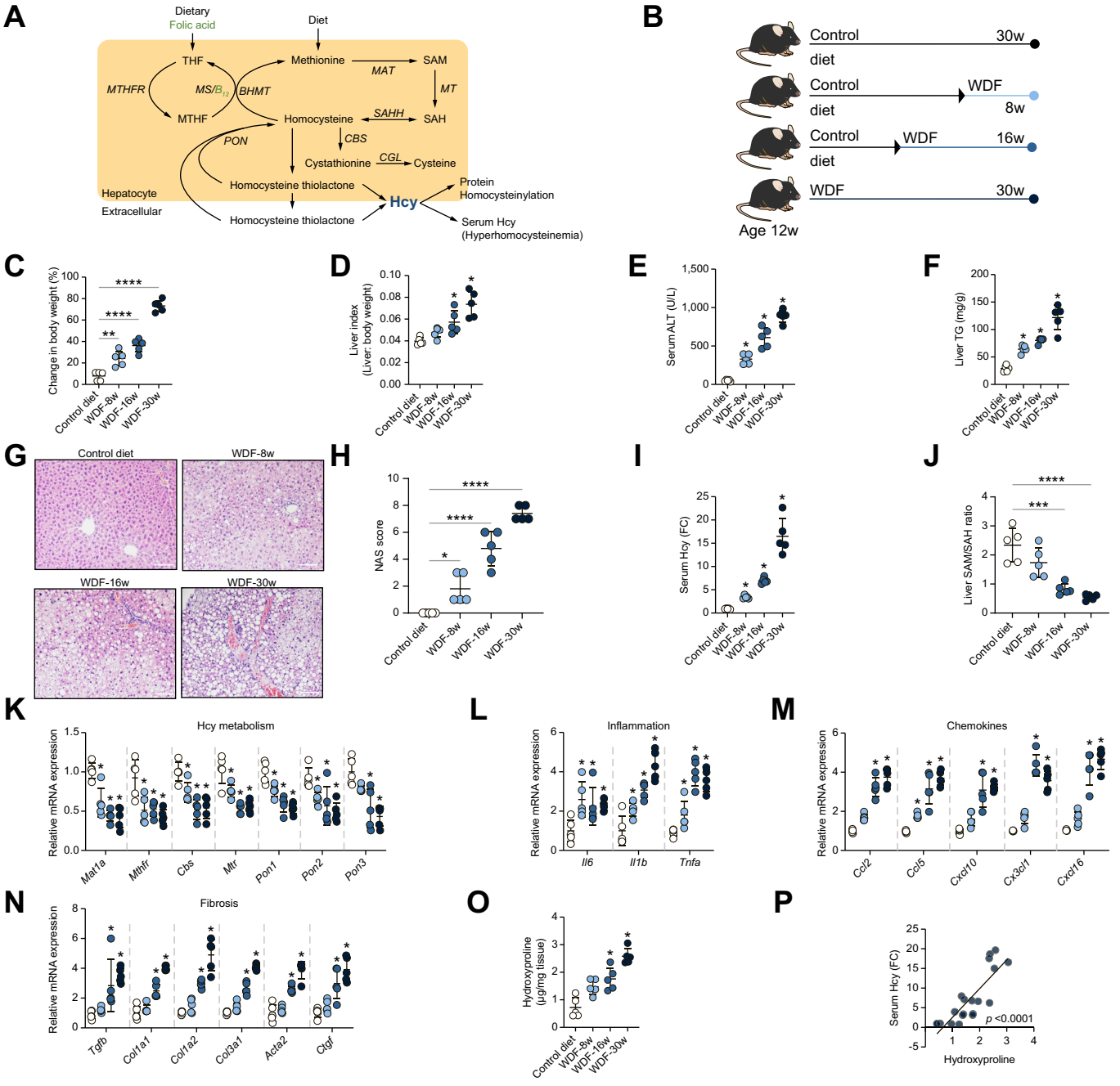

Fig. 1. Dietary mouse model of progressive NASH had concomitant increases in serum Hcy levels. (A) Schematic diagram of Hcy metabolism and protein Hcy-lation. (B) Experimental design for the induction of steatosis, mild, and moderate NASH (WDF-8w, WDF-16w, and WDF-30w, respectively) (n = 5 animals/group). (C) % Change in body weight. (D) liver index (liver weight: body weight). (E) Serum ALT. (F) Liver TG. (G) H&E-stained images of liver sections (scale bar-100μm). (H) NAS scores. (I) Serum Hcy. (J) Liver SAM:SAH. (K-N) Relative mRNA expression of genes by RT-qPCR. (O) Hepatic hydroxyproline content. (P) Correlation analysis for hepatic hydroxyproline levels (x-axis) *vs.* serum Hcy levels (y-axis). Results are expressed as mean ± SD. The statistical significance of differences (*p <0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test. ALT, alanine aminotransferase; Hcy, homocysteine; NASH, non-alcoholic steatohepatitis; NAS, NAFLD activity score; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethione; TG, triglyceride; WDF, Western diet+fructose. (This figure appears in color on the web.)

with severity of hepatic inflammation and fibrosis, and increased intrahepatic Hcy induced NASH. We identified Stx17, a protein involved in autophagosome/lysosome fusion, as homocysteinylated (Hcy-lated), ubiquitinated, and down-regulated in NASH. Remarkably, dietary B_{12}/Fol supplementation increased Stx17 expression, restored autophagy, slowed NASH

progression, and reversed inflammation and fibrosis in mice with pre-established NASH.

Material and methods

Mouse models

NASH-inducing dietary model

12-week-old male C57BL/6J mice fed *ad libitum* with Western diet (WD) (D12079B) and 15% (w/v) fructose in drinking water (WDF) for 8, 16 and, 30 weeks to progressively generate the spectrum of NAFLD (from steatosis to mild NASH, and moderate NASH, respectively).[9] Customized WDs with either B_{12}+Fol (Vits), or Fol were used (details are provided in the supplementary methods). All mice were maintained according to the Guide for the care and use of laboratory animals (NIH), and the experiments performed were approved by the IACUC's at SingHealth (2015/SHS/1104 and 2020/SHS/1549). The dosage was an FDA-approved human equivalent dose.

HHcy dietary NASH model

12-week-old male C57BL/6J mice were fed control diet or WD supplemented with 3X methionine (Met) (D18012301) and 15% (w/v) fructose in drinking water (WDF+Met) for 8 weeks.

HHcy genetic model

Liver-specific *Cbs* knockdown (*Cbs*-LKD) mice were generated by injecting AAV8-*Alb*-sh*Cbs* ($1X10^{12}$ gc/mice) into the tail vein. Mice were then fed either control diet or WDF for 8 weeks.

All the diets were procured from Research Diets Inc. For further details regarding the materials and methods used, please refer to the CTAT table and supplementary information.

Quantitative and statistical analyses

Results are expressed as mean ± SD. The statistical significance of differences (*p <0.05, **p <0.01, ***p <0.001, and ****p <0.0001) was assessed by a one-way or two-way ANOVA for multiple group comparisons wherever applicable, followed by Tukey's multiple-comparisons test. An unpaired 2-tailed t test was used to compute statistical differences between 2 groups. All statistical tests were performed using Prism 9 for Mac OS X (GraphPad Software).

Results

HHcy is associated with NASH progression in a dietary mouse model of NASH as well in patients and primates with NASH

To determine whether HHcy was associated with NASH, we examined serum Hcy and hepatic steatosis, inflammation, and fibrosis in a dietary mouse model of progressive NASH. Mice were fed WDF for 8, 16 and, 30 weeks to mimic human NAFLD progression by inducing steatosis, and mild to moderate/severe NASH, respectively (Fig. 1B).[9] HHcy was associated with progressive increases in the body weight and liver index, serum alanine aminotransferase (ALT), and hepatic and serum triglyceride (TG) and cholesterol levels in mice fed WDF for 8, 16, and 30 weeks (WDF 8, 16, and 30w) (Fig. 1C-F and Fig. S1A,B). Liver histopathology showed mild steatosis and sinusoidal/perisinusoidal infiltration of inflammatory cells in mice fed WDF for 8 weeks; marked steatosis, mild focal, spotty hepatocyte ballooning and sinusoidal/perisinusoidal cell infiltrate were observed in mice fed WDF for 16 weeks, and diffuse distribution of hepatocyte ballooning and lobular infiltration of inflammatory cells in mice fed WDF for 30 weeks, and overall increased NAFLD activity score (NAS) (Fig. 1G,H and Fig. S1C,D). Sirius red staining showed markedly increased collagen content in mice fed WDF for 30 weeks (Fig. S1E).

Importantly, we observed that progressive increases in serum Hcy levels occurred concurrently with significant decreases in hepatic s-adenosylmethionine (SAM)/s-adenosylhomocysteine (SAH) ratio in mice fed WDF (Fig. 1I,J), reflecting HHcy and hepatic Hcy accumulation with disease progression. Serum levels of Met returned to normal in mice fed WDF for 16 and 30 weeks, suggesting that increased serum HHcy, and not serum Met, was associated with the hepatic changes that occurred at the later time points (Fig. S1F). A similar correlation between HHcy and NAFLD progression was observed in the sera of a cohort of patients with steatosis and NASH from Singapore General Hospital (Control, n = 6; Steatosis, n = 6; NASH, n = 24) (Fig. S2A,B). Additionally, a cohort of primates fed a high-fat diet for 2.5 to 5 years developed NASH (Fig. S2J) and had higher serum Hcy levels than their baselines or primates fed a normal chow diet. Serum Hcy levels were also positively correlated with NAS (Fig. S2K-M). Interestingly, the mRNA expression of key genes involved in Hcy metabolism (*Mat1a, Mthfr, Cbs, Mtr, Pon1, Pon2, Pon3*) were temporally downregulated in mice fed WDF for 8 to 30 weeks (Fig. 1K). A similar pattern was also observed in hepatic Hcy metabolism genes (*MAT1A, MTHFR, CBS, MTR, PON1, PON2, PON3*) in patients with NAFLD, as their mRNA expression progressively decreased during steatosis and NASH (Fig. S2C).

Hepatic inflammation (*Il6, Il1b, Tnf-a*) and chemokine (*Ccl2, Ccl5, Cxcl10, Cx3cl1, Cxcl16*) gene expression increased progressively in mice fed WDF (Fig. 1L,M). Similar results were also observed in the cohort of patients with NAFLD (Fig. S2D,E). Hepatic fibrosis (*Tgfb, Col1a1, Col1a2, Col3a1, Acta2, Ctgf*) gene expression and hydroxyproline levels (to measure collagen content) increased in parallel with NAFLD progression in mice fed WDF (Fig. 1N,O) and the cohort of patients with NAFLD (Fig. S2F). Serum Hcy and hydroxyproline levels also positively correlated with each other (p <0.0001) in both mice fed WDF and patients with NAFLD (Fig. 1P and Fig. S2G). Previously, Mahamid *et al.* 2018[7] showed low serum levels of Fol and B_{12} were associated with the histological severity of NASH. Interestingly, we also observed a significant decrease in the levels of serum Fol and B_{12} in our progressive model of NASH (Fig. S1G,H).

We next analyzed the transcriptome data obtained from previously published studies of patients with NASH available on the public GEO repository GSE48452 (Fig. S2H) and Array Express (E-MEXP-3291) (Fig. S2I) and found that expressions of Hcy metabolism genes were also decreased in NASH. Taken together, these findings showed that HHcy was associated with NASH progression and correlated with changes in hepatic steatosis, inflammation, and fibrosis.

HHcy induces NASH in genetic (Cbs-LKD) and dietary models

Patients with *CBS* deficiency have HHcy and develop hepatic steatosis and fibrosis, and the global *Cbs* knockout mouse model recapitulates the human disease phenotype.[13,14,23] To determine whether elevated hepatic Hcy itself could induce NASH, we generated liver-specific *Cbs* knockdown mice (*Cbs*-LKD) via tail vein injection with AAV8-mediated gene delivery of short-hairpin RNA against the *Cbs* gene (Fig. 2A). Interestingly, these mice had increased body weight and liver index, serum ALT, hepatic and serum TG and cholesterol when fed control diet (Fig. 2B-E and Fig. S3A,B). Their liver index, serum ALT, and hepatic

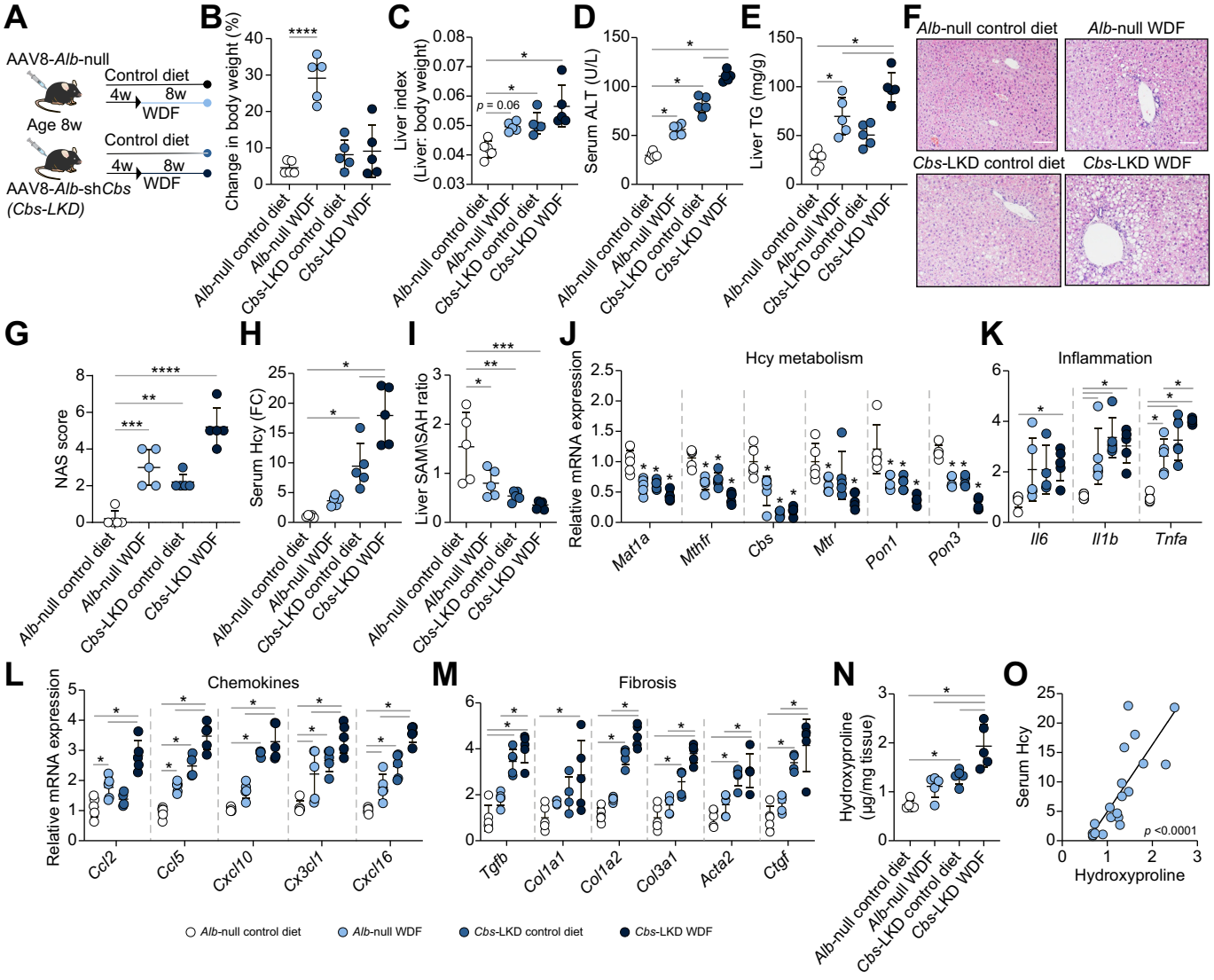

Fig. 2. *Cbs*-LKD mice had increased serum Hcy, hepatic inflammation, and fibrosis when fed either control diet or WDF. (A) Experimental design for the generation of *Cbs*-LKD mice and NASH. (B) % change in body weight. (C) Liver index. (D) Serum ALT. (E) Liver TG. (F) H&E staining of liver sections (Scale bar: 100 μm). (G) NAS score. (H) Serum Hcy. (I) Liver SAM:SAH. (J-M) Relative mRNA expression of hepatic genes by RT-qPCR. (N) Hepatic hydroxyproline content. (O) Correlation analysis for hydroxyproline (x-axis) *vs.* serum Hcy (y-axis). Results are expressed as mean ± SD. The statistical significance of differences (*p <0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test. ALT, alanine transaminase; Cbs-LKD, cystathinine beta synthase liver-specific knock down; Hcy, homocysteine; NASH, non-alcoholic steatohepatitis; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethione; TG, triglyceride; WDF, Western diet+fructose. (This figure appears in color on the web.)

and serum TG and cholesterol levels further increased when fed WDF. H&E staining of liver tissues from *Cbs*-LKD mice fed WDF or control diet showed increases in micro- and macro-vesicular perivenular steatosis, pericentral infiltration of inflammatory cells, mild ballooning and increased NAS (Fig. 2F,G and Fig. S3C,D).

Increases in serum Hcy were associated with concurrent decreases in hepatic SAM/SAH levels (Fig. 2H,I) and downregulation of Hcy metabolism genes in *Cbs*-LKD mice fed either control diet or WDF diet compared to *Alb*-null mice fed control diet (Fig. 2J). Hepatic expression of inflammation and chemokine genes was increased in *Cbs*-LKD mice fed WDF compared to *Alb*-null mice fed WDF for 8 weeks and were comparable to *Alb*-null mice fed WDF for 16 weeks (Fig. 2K,L). Hepatic fibrosis gene expression, Sirius Red staining and hydroxyproline levels in *Cbs*-LKD mice were modestly increased when fed control diet and further increased when fed WDF (Fig. 2M,N and Fig. S3E). Serum Hcy levels also significantly correlated (p <0.0001) with hepatic hydroxyproline levels (Fig. 2O), whereas serum Met did not correlate with serum Hcy (Fig. S3F,G). Similar to mice chronically fed WDF to develop NASH (Fig. S1G,H), serum B_{12} and Fol levels decreased in *Cbs*-LKD mice fed control or WDF diets compared to *Alb*-null mice fed control diet (Fig. S3H,I).

We next provided excess dietary Met to mice fed WDF to see whether HHcy itself could exacerbate or accelerate NASH. Mice were fed control diet for 8 weeks, WDF for 8 and 16 weeks and WDF+Met for 8 weeks (Fig. S4A). Mice fed WDF+Met for 8 weeks had increased body weight, liver index serum ALT, liver and

serum TG, cholesterol and histological changes consistent with NASH (Fig. S4B-J). Mice fed WDF+Met for 8 weeks had increased serum Hcy levels, and decreased SAM/SAH ratio (Fig. S4K-M) and Hcy metabolism gene expression that were more significant than mice fed WDF for 8 weeks and comparable to mice fed WDF for 16 weeks. Interestingly, inflammation and fibrosis also were increased in mice fed WDF+Met (Fig. S4N-Q). Serum B_{12} and Fol levels also were significantly decreased (Fig. S4R,S).

Vits or Fol treatment reduces HHcy and improves NASH

To examine whether Vits (B_{12} and Fol) or Fol could reverse NASH, mice were fed WDF for 16 weeks to establish NASH, and then given Vits or Fol supplementation of WDF for an additional 14 weeks (WDF+Vits-16>30w, WDF+Fol-16>30w) (Fig. 3A). There were no significant changes in body weight and liver index in mice fed WDF supplemented with Vits or Fol compared to mice only fed WDF (Fig. 3B,C). Interestingly, serum ALT, TG, and cholesterol levels markedly improved in mice fed WDF supplemented with Vits or Fol (Fig. 3D,E,G). In contrast, hepatic TG levels were not significantly changed in mice fed WDF supplemented with Vits or Fol (Fig. 3F). Likewise, H&E and NAS of liver samples from mice fed WDF supplemented with Vits of Fol exhibited histological improvements (except steatosis) in inflammatory cell infiltration and fibrosis (Fig. 3H,I and Fig. S5A,B).

Mice fed WDF supplemented with Vits or Fol exhibited decreased serum Hcy levels and increased hepatic SAM/SAH ratios (Fig. 3J,K). Remarkably, Vits or Fol restored the expression of Hcy metabolism genes and markedly reduced expression of inflammation and chemokine genes to levels similar to mice fed control diet (Fig. 3L-N). Interestingly, Vits and Fol also reduced fibrosis gene expression and hepatic hydroxyproline in these mice (Fig. 3O,P). Hepatic hydroxyproline and serum Hcy levels were positively correlated in both supplemented and non-supplemented mice (Fig. 3Q). Sirius red staining decreased although hepatosteatosis persisted in vitamin-treated mice (Fig. 3R). Serum B_{12} and Fol levels in mice fed WDF also improved after supplementation with Vits or Fol (Fig. 3S,T).

Vits and Fol also prevented NASH development when supplemented to WDF diet from 0-16 weeks (Fig. S5C-Q). In another model, *Lepr*$^{db/db}$ (db/db) mice were fed WD for 8 weeks to induce NASH with and without Vits or Fol supplementation. Vits or Fol supplementation reduced serum HHcy, inflammation, and fibrosis in Lepr$^{db/db}$ (*db/db*) mice fed WD and prevented development of NASH (Fig. S6A-O).[15]

Decreased autophagy and reduced Stx17 occurs in NASH models

We and others previously showed that decreased autophagy led to reduced lipophagy, mitophagy, and β-oxidation of fatty acids that contributed to hepatosteatosis in NAFLD.[16–18] This reduced autophagy and the subsequent changes in cellular metabolism increased lipotoxicity and oxidative stress.[19–21] To examine the effects of HHcy and NASH on autophagy, we examined the hepatic expression of autophagy proteins, Map1lc3b-ii and Sqstm1/p62, in mice fed WDF for 8, 16, and 30 weeks and found progressively increased levels of both Map1lc3b-ii and Sqstm1/p62 as NASH advanced (Fig. 4A,B). We also saw increased Map1lc3b-ii and Sqstm1/p62 protein levels in *Cbs*-LKD mice fed control diet or WDF (Fig. 4C,D), suggesting that HHcy likely contributed to the block in autophagy. Furthermore, we saw a similar pattern in patients with steatosis and NASH (Fig. S7A,B); This profile of increased Map1lc3b-ii and Sqstm1/p62 suggested that there was a late block in autophagy in both NASH and HHcy mouse models and patients.

Since we observed a potential late block in autophagy in mice with HHcy and NASH, we examined the expression of SNARE proteins: syntaxin 17 (Stx17), synaptosomal-associated protein 29 (Snap29) and vesicle-associated membrane protein 8 (Vamp8), which are involved in SNARE-mediated autophagosome-lysosome fusion. Interestingly, we found a selective decrease in Stx17 protein expression during NASH in mice fed WDF for 8, 16, and 30 weeks, *Cbs*-LKD mice and patients with NASH. However, there were no changes in Vamp8 and Snap29 protein expression in hepatic tissues from the three mouse models of NASH and patients with NASH (Fig. 4A-D and Fig. S7A,B). Thus, our findings strongly suggested that decreased Stx17 protein expression could contribute to the impaired autophagy found in NASH and HHcy.

Stx17 Hcy-lation and ubiquitination occurs during HHcy and NASH

Hcy-lation is a rare post-translational protein modification. It previously was shown that Hcy-lated proteins were ubiquitinated and degraded via the proteasomal pathway.[22] Remarkably, we found hepatic protein Hcy-lation using anti-Hcy antibodies on Western blots. Several specific bands appearing between 25-75 kDa on Western blots increased in intensity in parallel with the severity of HHcy and NASH in mice chronically fed WDF and *Cbs*-LKD mice fed WDF (Fig. 4E-H). We also observed increased ubiquitination of hepatic proteins during HHcy and NASH in these models (Fig. S7C-F), although we could not determine the size of specific ubiquitinylated proteins due to the diffuse pattern observed in the ubiquitination blots.

Stx17 is Hcy-lated and undergoes proteasomal degradation during NASH

Upon closer inspection, we observed that there was a Hcy-lated protein expressed during NASH that was 33 kD in size, which coincidentally corresponded to the molecular weight of Stx17 on Western blot. This observation raised the interesting possibility that Stx17 might be Hcy-lated and undergo increased proteasomal degradation. Accordingly, we performed immunoprecipitation of Hcy-lated proteins in *Cbs*-LKD and control mice, followed by Western blotting with anti-Stx17 antibody (Fig. 4I,J). We observed increased Hcy-lated Stx17 in liver tissue samples from *Cbs*-LKD mice fed control diet or WDF even though total Stx17 protein expression was decreased. The decrease in Stx17 expression corresponded with the reduction in autophagy reflected by the increase in p62 protein expression in *Cbs*-LKD mice fed control diet or WDF (Fig. 4C,D).

We next examined the effect of Hcy on autophagy following *Stx17* knockdown (KD) or overexpression in mouse hepatic AML12 cells. We found increased Map1lc3b-ii and Sqstm1/p62 in both *Stx17* KD cells and WT cells treated with Hcy, suggesting there was a late block in autophagy in both cases (Fig. 4K,L). Significantly, Hcy reduced *Stx17* expression in WT cells to the same level as *Stx17* KD cells treated with Hcy. We also observed that Stx17 KD decreased autophagy flux at basal conditions, as demonstrated by decreased accumulation of Map1lc3b-ii after Bafilomycin A1 treatment (Fig. S7G). Additionally, both WT and *Stx17* KD cells treated with Hcy had increased expression of inflammation and fibrosis genes, with the latter having the

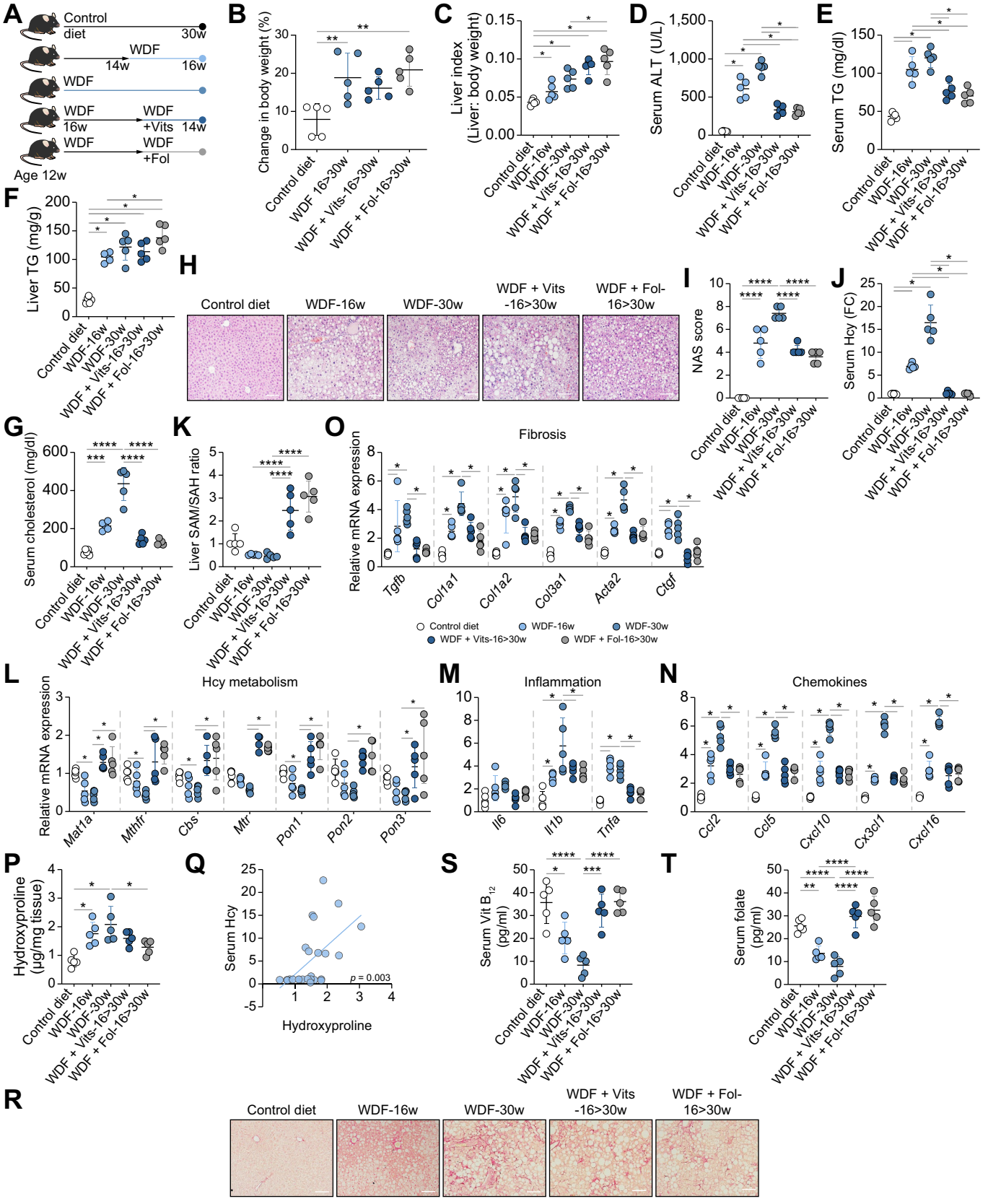

Fig. 3. Vits or Fol reduced hepatic inflammation and fibrosis in mice with pre-established NASH. (A) Experimental design for reversal study. Mice were fed WDF supplemented with vitamin B_{12} + Fol (WDF+Vits) or Fol (WDF+Fol) for 14 weeks after NASH induction (WDF 16 weeks). (B) % change in body weight. (C) Liver index. (D) Serum ALT. (E) Serum TG. (F) Liver TG. (G) Serum cholesterol. (H) H&E staining of liver sections (scale bar: 100 μm). (I) NAS score. (J) Liver

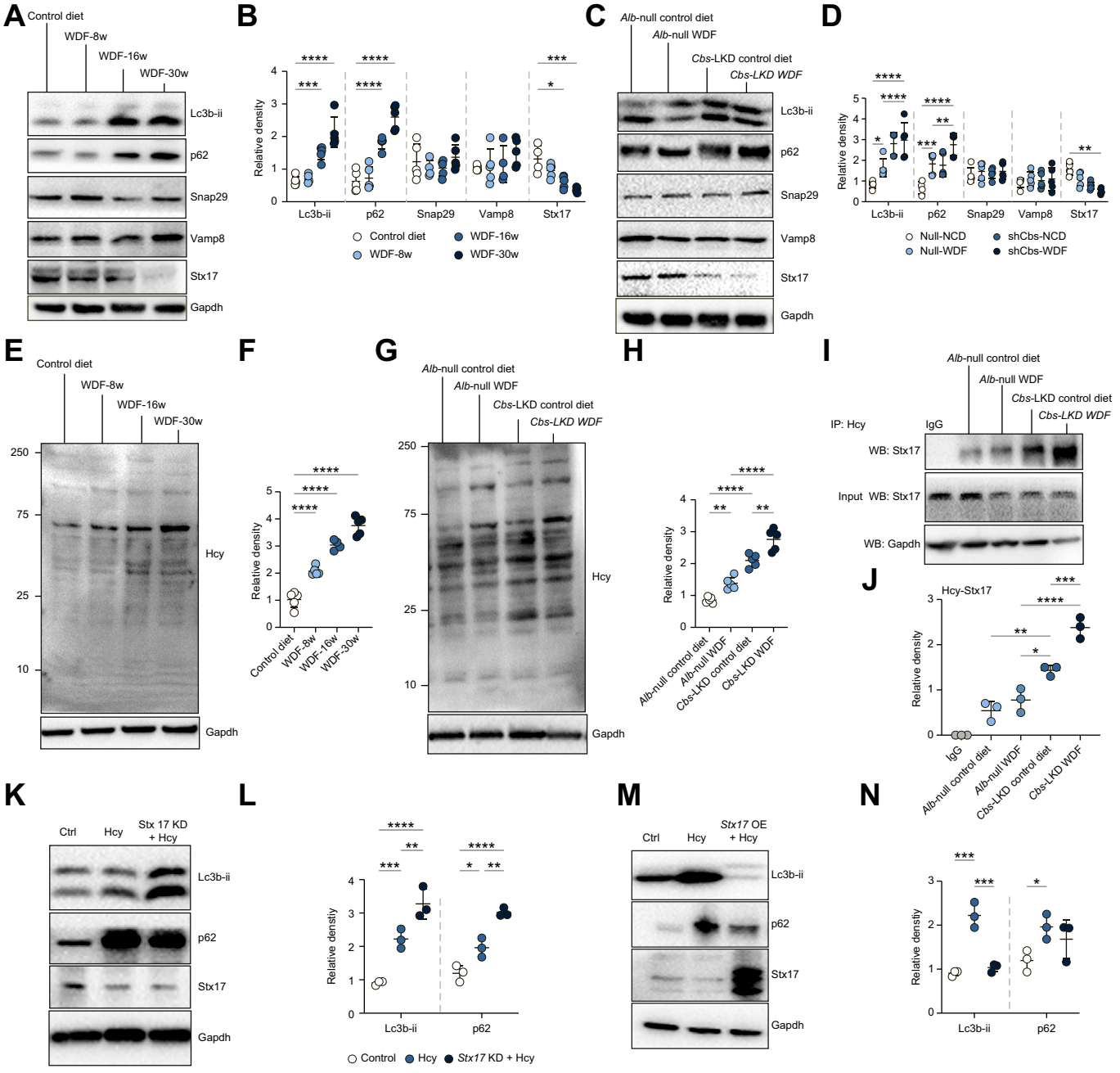

Fig. 4. Autophagy inhibition, protein Hcy-lation, and Stx17 Hcy-lation occurred in mice with NASH. (A and C) Representative Western blots analyzing autophagy proteins (Lc3b-ii/Map1lc3b-ii and p62/Sqstm1), SNARE proteins (Snap29, Vamp8 and Stx17) in liver samples (n = 5 per group). (B and D) Their densitometric values normalized to GAPDH. (E and G) Representative Western blot analyzing protein Hcy-lation (Hcy) in liver tissues (n = 5 per group). (F and H) Their densitometric values normalized to GAPDH. (I) Immunoprecipitation of Hcy-lated proteins and detection of Stx17 and Lc3b-ii protein levels by Western blotting in the liver tissues (n = 3 per group). Representative Western blots are presented. (J) Their densitometric values normalized to Stx17 protein level in input (n = 3 per group). (K and M) Representative Western blots analyzing autophagy proteins in AML12 cells. (L and N) Their densitometric values normalized to GAPDH. Results are expressed as mean ± SD. The statistical significance of differences (*p <0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test. Hcy, homocysteine; NASH: non-alcoholic steatohepatitis; WDF, Western diet+fructose.

hydroxyproline content. (I) Correlation analysis for hydroxyproline (x-axis) *vs.* serum Hcy (y-axis). (J) Fold-changes in serum Hcy. (K) Liver SAM:SAH. (L-O) Relative mRNA expression of hepatic genes by RT-qPCR. (P) Liver hydroxyproline content. (Q) Correlation analysis for hydroxyproline levels (x-axis) *vs.* serum Hcy levels (y-axis). (R) Sirius Red stained images of liver sections (scale bar: 100 μm). (S-T) Serum B_{12} (pg/ml), and Fol (pg/ml) concentrations. Results are expressed as mean ± SD. The statistical significance of differences (*p <0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test. ALT, alanine transaminase; Hcy, homocysteine; NASH, non-alcoholic steatohepatitis; SAHc, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TG, triglyceride; WDF, Western diet+fructose. (This figure appears in color on the web.)

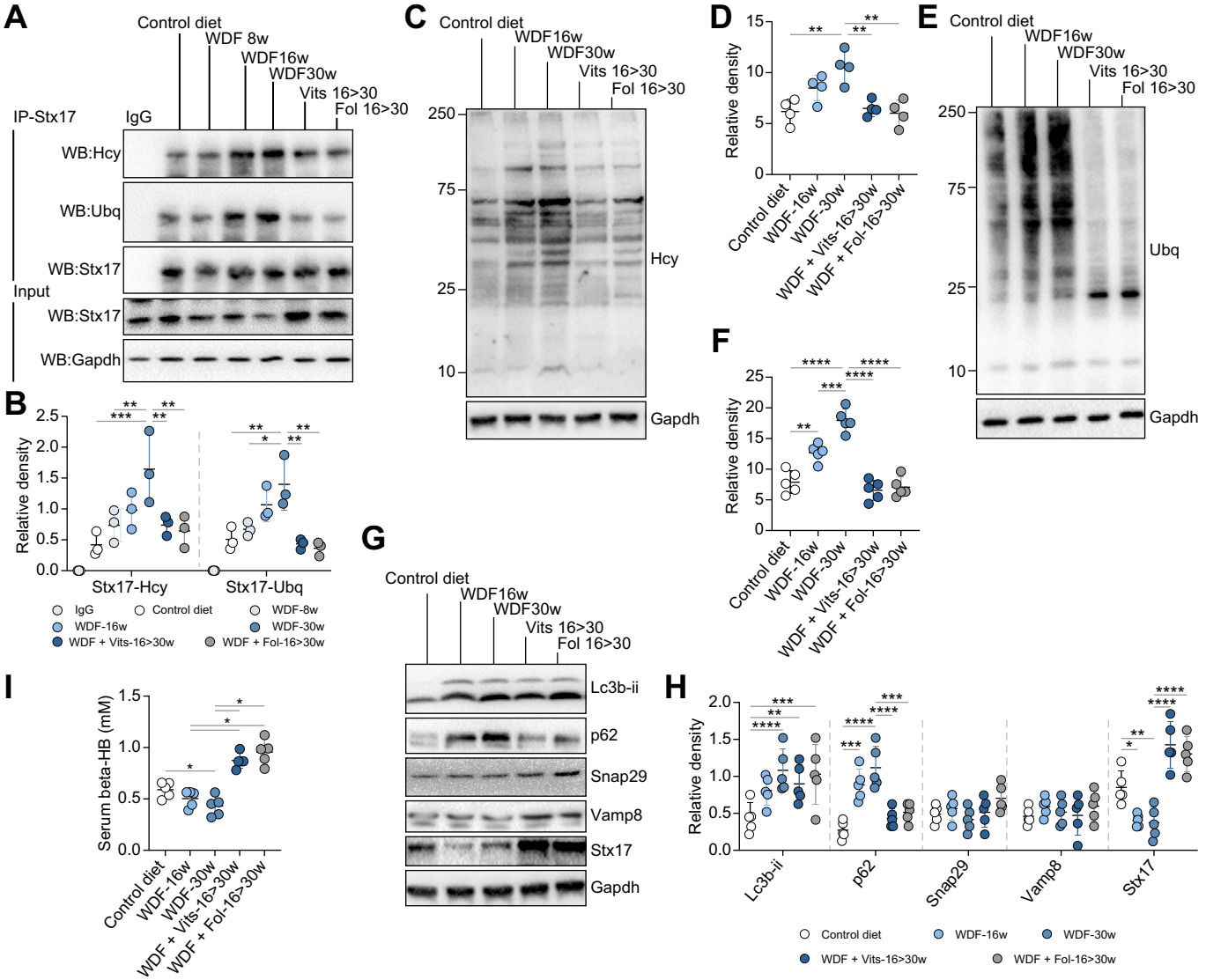

Fig. 5. Vits and Fol increased autophagy, reduced Hcy-lation and ubiquitination of Stx17, and increased β-oxidation of fatty acids in mice with pre-established NASH. (A) Immunoprecipitation of Stx17 and Hcy-lation and Ubq detection by Western blotting using liver tissue lysates as inputs (n = 3 per group). Representative Western blots are presented. (B) Hcy-lated (Stx17-Hcy) and ubiquitinated Stx17 (Stx17-Ubq) densitometric values normalized to Stx17 protein level in input. (C) Representative Western blot analyzing Hcy-lated (Hcy) proteins in the liver tissues (n = 5 per group). (D) Their densitometric values normalized to GAPDH. (E) Representative Western blot analyzing protein Ubq in liver tissues (n = 5 per group). (F) Their densitometric values normalized to GAPDH. (G) Representative Western blots analyzing autophagy proteins, SNARE proteins in liver tissue lysates (n = 5 per group). (H) Their densitometric values normalized to GAPDH. (I) Serum β-hydroxubutyrate (mM). Results are expressed as mean ± SD. The statistical significance of differences (*p <0.05) was assessed by a one-way or two-way ANOVA wherever applicable, followed by Tukey's multiple-comparisons test. Hcy, homocysteine; Ubq, ubiquitination; WDF, Western diet+fructose.

higher induction (Fig. S7H,I). Remarkably, overexpression of *Stx17* in AML12 cells increased autophagy flux and rescued the late block in autophagy, as Map1lc3b-ii and Sqstm1/p62 expression levels were restored in Hcy-treated cells (Fig. 4M,N, Fig. S7J). Inflammation and fibrosis gene expression also decreased to control cell levels in Stx17-overexpressing cells treated with Hcy (Fig. S7K,L). These findings suggested that Stx17 had a critical role in autophagy, and its decreased expression by Hcy led to decreased autophagy and increased expression of inflammation and fibrosis genes. On the other hand, *Stx17* overexpression reversed the autophagy inhibition, and decreased the expression of inflammation and fibrosis genes that were induced by Hcy.

Stx17 Hcy-lation and ubiquitination is reversed by Vits or Fol

To study the effects of vitamin therapy on Stx17 during NASH, we immunoprecipitated Stx17 in liver tissues collected from mice fed WDF for 8, 16 and 30 weeks or Vits or Fol supplemented WDF for 16>30 weeks. We observed that hepatic Stx17 was progressively Hcy-lated and ubiquitinated in a time-dependent manner in mice fed WDF from 8 to 30 weeks (Fig. 5A,B). The increases in these post-translational modifications of Stx17 occurred in parallel with the decreases in total Stx17 expression in the input (whole tissue lysate) (Fig. 5A).

Remarkably, Vits or Fol decreased Stx17 Hcy-lation and ubiquitination and global hepatic Hcy-lation and ubiquitination in mice fed WDF for 16 and 30 weeks (Fig. 5A-F). The decreases

in Hcy-lated Stx17 protein expression by Vits and Fol were associated with increases in total Stx17 expression, and reversal of the autophagy defect as evident from the significantly reduced Sqstm1 levels (Fig. 5A,B,G,H). This improvement in autophagy also increased β-oxidation of fatty acids, reflected by elevated serum β-hydroxybutyrate and hepatic acylcarnitine levels (particularly C2, C3, and C4) on metabolomics analysis (Fig. 5I, Fig. S8A-D). Thus, our findings strongly suggested that Vits or Fol supplementation increased β-oxidation of fatty acids led to decreased inflammation and fibrosis in tandem with the reversal of hepatic TG and diacylglycerol changes (Fig. S8E,F) in our dietary model of pre-established NASH. These beneficial effects occurred, at least in part due to decreased Hcy-lation of Stx17, which restored Stx17 expression and hepatic autophagy.

Discussion

Earlier studies showed that serum Hcy levels were positively associated with NAFLD, whereas serum B_{12} and Fol levels were negatively correlated with NAFLD/NASH severity.[5–8] Here, we showed conclusively that serum Hcy was positively correlated with NASH severity in patients, primates and mice. However, it was not known whether this association was due to intrahepatic Hcy or the systemic effects of HHcy. Accordingly, we examined whether intrahepatic Hcy had a pathogenic role in NASH by generating *Cbs*-LKD mice, to specifically increase intrahepatic Hcy. *Cbs*-LKD mice fed control diet for 8 weeks developed early signs of inflammation and fibrosis that were not evident in null mice fed WDF. *Cbs*-LKD mice fed WDF for 8 weeks developed accelerated NASH comparable to null mice fed WDF for 16 weeks. In another experiment, mice fed WDF+Met to induce HHcy also had more severe NASH than mice fed WDF alone. These findings were consistent with previous studies that showed a decreased rate of transmethylation of Hcy into Met in NASH.[25] An earlier report showed that mice fed Met- and choline-deficient diet developed HHcy and NASH.[27] Surprisingly, Hcy supplementation improved the NASH phenotype in these mice. The reason(s) for the differences between these findings and ours is not known; however, it is possible that Hcy supplementation helped replenish intrahepatic Met and SAM, which were depleted by the Met- and choline-deficient diet and led to unfolded protein response-related dysfunction.[27]

We examined the mechanism(s) by which intrahepatic Hcy induces NASH. One strong contributor to NASH development and progression may be protein Hcy-lation that regulates protein activity, function, and stability through ubiquitin-mediated degradation.[3,4] Here, we found progressive increases in hepatic protein Hcy-lation and ubiquitination during NASH progression that were associated with an autophagic block. Remarkably, we found that the Hcy-lation and ubiquitination of an autophagosome-lysosome fusion protein (Stx17) were increased, while the total protein expression of Stx17 was reduced during NASH. Stx17 Hcy-lation also led to the decrease in autophagy observed during NASH progression,[16] and impaired autophagy's critical roles in fatty acid β-oxidation, mitochondrial turnover and quality control, and inflammation that together prevented lipotoxicity in the liver.[28,29]

Fol is a substrate for THFR and B_{12} is a co-factor for Met synthase (Fig. 1A). Together they play critical roles in the MHTFR cycle to convert Hcy to Met. Accordingly, we investigated whether they restored hepatic autophagy and decreased NASH progression. Interestingly, Vits or Fol prevented and reversed the rises in serum Hcy and hepatic SAH levels and increased autophagy in mice fed WDF. This led to increased β-oxidation of fatty acids, decreased inflammation and fibrosis, and less NASH progression. These effects were mediated by decreased Hcy-lation of hepatic proteins in general, and Stx17 in particular. This reduction in Hcy-lation of Stx17 led to increased *Stx17* protein expression and reversed the late block in autophagy. Additionally, we observed several other proteins that were Hcy-lated besides Stx17 during NASH, so it is likely that other hepatic cell functions are dysregulated through Hcy-lation of these proteins. Currently, we are identifying these Hcy-lated proteins and characterizing them. Interestingly, Vits of Fol also improved serum B_{12} and Fol levels. Since B_{12} is not synthesized endogenously in mice and humans and most folate is obtained by diet, it is possible that there could be increased absorption of B_{12} and Fol with the reversal of NASH.

Since HHcy correlated with the progression of liver fibrosis in our dietary model of NASH, it is a potential biomarker for NASH severity, perhaps in combination with other serum biomarkers such as ALT, TG, and serum inflammatory cytokines and chemokines. Currently, its specificity for NASH is not known. Nevertheless, our findings suggest that HHcy, particularly in patients with diabetes, obesity, or other features of metabolic syndrome, should warrant further investigation for the diagnosis of NASH. Presently, there are no pharmacological therapies for the prevention and treatment of NASH. Given their high safety profiles and their designation as dietary supplements by the FDA, Vits or Fol could be used as potential first-line therapies for the prevention and treatment of NASH either by themselves, or in combination with other drugs, particularly since B_{12} and Fol absorption decrease with age and certain types of diets. The low cost of therapy is attractive since it would represent tremendous cost savings and health burden reductions for NASH in both developed and undeveloped countries. We believe that further clinical studies to examine the effectiveness of Vits and Fol to prevent and treat NASH are warranted.

Abbreviations

ALT, alanine aminotransferase; BafA1, bafilomycin A1; B_{12}, vitamin B12; Cbs, cystathionine beta-synthase; Cbs-LKD, cystathionine beta-synthase liver specific knockdown; Fol, folate; Hcy, homocysteine; Hcy-lation, homocysteinylation; HHcy, hyperhomocysteinemia; KD, knockdown; LKD, liver-specific knockdown; Lys, lysine; Mat1a, methionine adenosyltransferase I alpha; Met, methionine; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity score; NASH, non-alcoholic steatohepatitis; NCD, normal chow diet; PON1, paraoxonase 1; PON 2, paraoxonase 2; PON 3, paraoxonase 3; SAH, s-adenosylhomocysteine; SAM, s-adenosylmethionine; Snap29, synaptosomal-associated protein 29; Stx17, syntaxin 17; TG, triglyceride; Vamp8, vesicle-associated membrane protein 8; Vits, vitamin B12 + folate; WD, Western diet; WDF, Western diet + fructose.

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Conflicts of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

M.T., B.K.S. and, P.M.Y. conceived and designed the study; M.T., B.K.S., K.T., R.S., S.A.B.A.G., A.W., Z.J., K.A.W., S.G.S and, J.P., performed experiments; P.K.H.C provided control, NAFLD and NASH patients serum and liver tissues, R.X.,J.W. and X.Z. for proving data on High-fat diet fed primates; G.G.B.B., A.S., comments and proofread the draft; M.T., B.K.S., M.K.S, S.A.C., and P.M.Y. finalized the manuscript.

Data availability statement

The data sets analysed for this study were publically availbales as GSE48452 and E-MEXP-3291.

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Supplementary data

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Author names in bold designate shared co-first authorship

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