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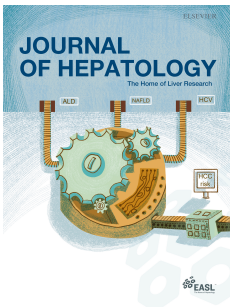

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PII: S0168-8278(22)02932-4

DOI: https://doi.org/10.1016/j.jhep.2022.06.033

Reference: JHEPAT 8797

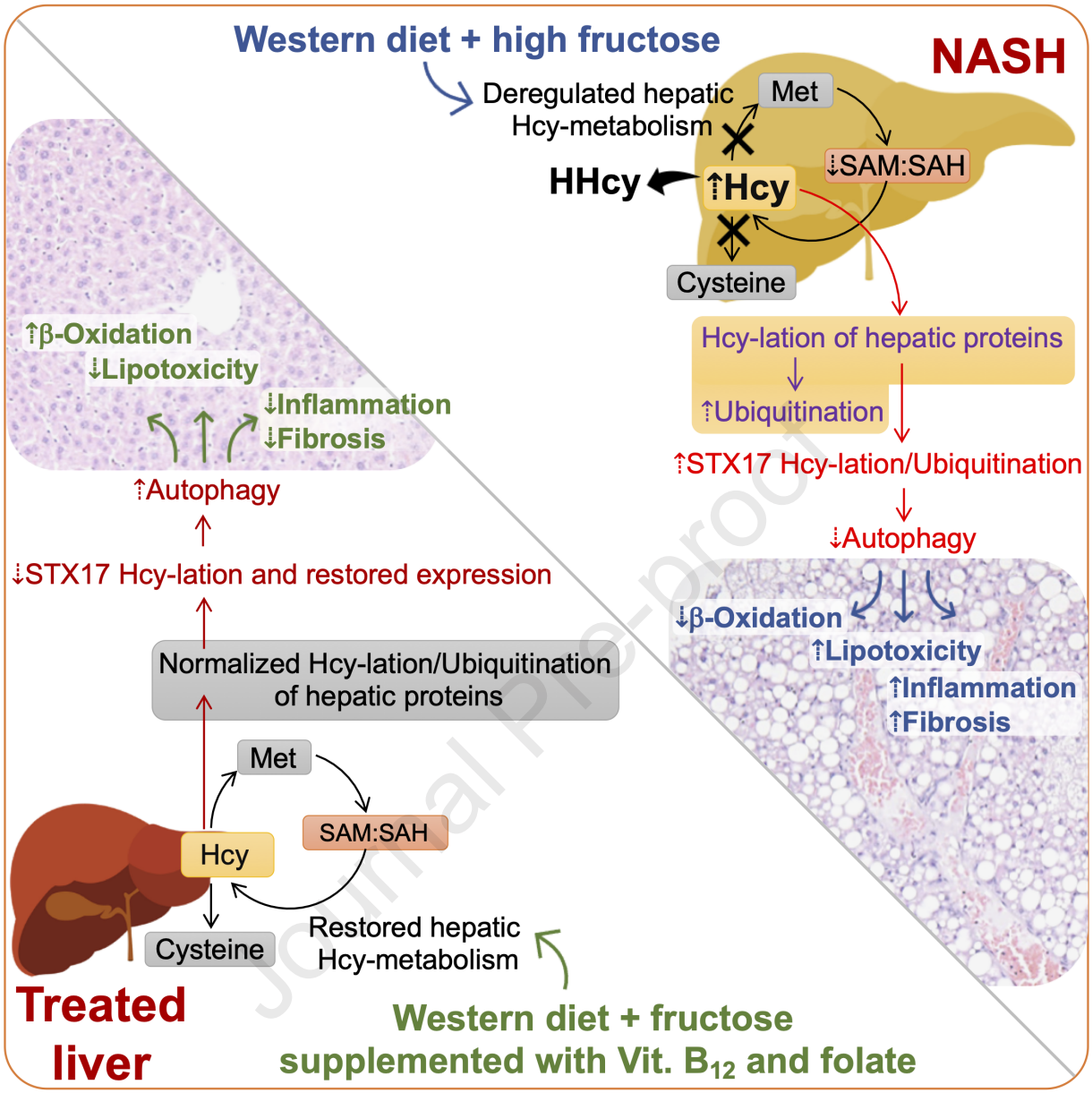
To appear in: *Journal of Hepatology*

Received Date: 4 December 2021

Revised Date: 22 June 2022

Accepted Date: 28 June 2022

Please cite this article as: Tripathi M, Singh BK, Zhou J, Tikno K, Widjaja A, Sandireddy R, Arul K, Ghani SABA, Bee GGB, Wong KA, Pei HJ, Shekeran SG, Sinha RA, Singh MK, Cook SA, Suzuki A, Lim TR, Cheah CC, Wang J, Xiao RP, Zhang X, Hoe Chow PK, Yen PM, Vitamin B_{12} and folate decrease inflammation and fibrosis in NASH by preventing Syntaxin 17 homocysteinylation, *Journal of Hepatology* (2022), doi: https://doi.org/10.1016/j.jhep.2022.06.033.



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

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

Abstract

Background and Aims: The relationship between hyperhomocysteinemia (HHcy) and non-alcoholic steatohepatitis (NASH) is poorly understood. **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 homocysteine (Hcy) correlated with hepatic inflammation and fibrosis in NASH. Elevated hepatic Hcy induced and exacerbated NASH. Gene expression of hepatic Hcy-metabolizing enzymes was down-regulated 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 autophagy block. 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 homocysteine 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 roles of high serum homocysteine levels (HHcy) and intrahepatic homocysteine on the development of non-alcoholic seatohepatitis (NASH) are not known. We found HHcy increased with NASH severity and showed that homocysteinylation of the key autophagy protein STX17 led to autophagy block during NASH and NASH progression. Vitmains B_{12} and folate supplementation restored autophagy and reduced NASH progression and could be a new first-line therapy for NASH.

Introduction

Hyperhomocysteinemia (HHcy) is a metabolic disorder mainly due to 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), leading to impaired protein structure/function and 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 NASH, and B_{12} and Fol levels were negatively correlated with NAFLD/NASH severity [5-8]. However, it is not known whether HHcy has a pathogenic role in NASH.

Here, we examined the the role of HHcy on NASH in mouse models, patients and primates. We found the HHcy correlated 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 weeks 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 NAFLD spectrum (from steatosis to mild NASH, and moderate NASH respectively) [9]. Customized WDs with either B_{12}+Fol (Vits), or Fol. were used (details are as 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 a FDA approved human equivalent dose.

HHcy dietary NASH model: 12 weeks 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) were generated by injecting AAV8-*Alb*-sh*Cbs* ($1X10^{12}$ gc/mice) via tail vein. Mice were then fed either control diet or WDF for 8 weeks.

All the diets were procured from Research Diets Inc.

For other materials and detailed methods please see Supplementary Methods.

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 two- tailed t-test was used to compute statistical differences between two 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 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 Western diet and 15% (w/v) fructose in drinking water (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 Supplementary Fig. 1a,b**). Liver histopathology showed mild steatosis and sinusoidal/perisinusoidal infiltration of inflammatory cells in mice fed WDF (8w); marked steatosis, mild focal, spotty hepatocyte ballooning and sinusoidal/perisinusoidal cell infiltrate were observed in mice fed WDF(16w), and diffuse distribution of hepatocyte ballooning and lobular infiltration of inflammatory cells in mice fed WDF (30w), and overall increased NAFLD activity score (NAS) (**Fig. 1g,h and Supplementary Fig. 1c,d**). Sirius red staining showed markedly increased collagen content in mice fed WDF for 30 weeks. (**Supplementary Fig. 1e**).

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 back 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 latter time points (**Supplementary Fig. 1f**). 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) (**Supplementary Fig. 2a,b**). . Additionally, a cohort of primates fed high fat diet (HFD) for 2.5 to 5 years developed NASH (**Supplementary Fig. 2j**) and had higher serum Hcy levels than their baselines or primates fed normal chow diet. Serum Hcy levels also were positively correlated with NAS (**Supplementary Fig. 2k-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 also was observed in hepatic Hcy metabolism genes (*MAT1A, MTHFR, CBS, MTR, PON1, PON2, PON3*) in the NAFLD

patients as their mRNA expression progressively decreased during steatosis and NASH (**Supplementary Fig. 2c**).

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 also were observed in the cohort of NAFLD patients (**Supplementary Fig. 2d,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 **(Supplementary Fig. 2f**). 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 Supplementary Fig. 2g**). 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 (**Supplementary Fig. 1g,h**).

We next analyzed the transcriptome data obtained from previously published studies of NASH patients available on the public GEO repository GSE48452 **(Supplementary Fig. 2h)** and Array Express (E-MEXP-3291) **(Supplementary Fig. 2i)** and found that expressions of Hcy metabolism genes also were 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]. 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 shRNA 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 Supplementary Fig. 3a,b**). Their liver index, serum ALT, and hepatic and serum TG and cholesterol levels further increased when *Cbs*-LKD mice were 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 Supplementary Fig. 3c and d**).

Increases in the serum Hcy were associated with concurrent decreases in hepatic SAM/SAH levels (**Fig. 2h,i**) and down-regulation 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 Supplementary Fig. 3e**). Serum Hcy levels also significantly correlated ($P<0.0001$) with hepatic hydroxyproline levels (**Fig. 2o**), whereas serum methionine did not correlated with serum Hcy (**Supplementary Fig. 3f,g**). Similar to mice chronically fed WDF to develop NASH (**Supplementary Fig. 1g,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 (**Supplementary Fig. 3h,i**)

We next provided excess dietary methionine to mice fed WDF to see whether HHcy itself could exacerbate or accelerate NASH. Mice were fed control diet for 8 weeks (8w), WDF for 8 and 16 weeks (8w and 16w) and WDF+Met for 8 weeks (8w) (**Supplementary Fig. 4a**). Mice fed WDF+Met (8w) had increased body weight, liver index serum ALT, liver and serum TG, cholesterol and histological changes consistent with NASH (**Supplementary Fig. 4b-j**). Mice fed WDF+Met (8w) had increased serum Hcy levels, and decreased SAM/SAH ratio (**Supplementary Fig. 4k-m**) and Hcy metabolism gene expression that were more significant than mice fed WDF (8w) and comparable to mice fed WDF (16w). Interestingly, inflammation

and fibrosis also were increased in mice fed WDF + Met (**Supplementary Fig. 4n-q).** Serum B_{12} and Fol levels also were significantly decreased (**Supplementary Fig. 4r,s)**.

Vits or Fol treatment reduces HHcy and improves NASH

To examine whether B_{12} and Fol (Vits) 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 score 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 Supplementary Fig. 5a,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 as mice fed NCD (**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-suplemented 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 (**Supplementary Fig. 5c-q**). In another model, $Lepr^{db/db}$ (db/db) mice were fed Western diet (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 (**Supplementary Fig. 6a-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 its 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 (8,16, and 30 w) 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 (**Supplementary Fig. 7a,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), 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 30w, *Cbs*-LKD mice and NASH patients. 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 Supplementary Fig. 7a,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

Homocysteinylation 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 25-75kDa 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**). There also was increased ubiquitination of hepatic proteins during HHcy and NASH in these models (**Supplementary Fig. 7c-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 NCD 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 NCD or WDF (**Fig. 4c,d**).

We next examined the effect of Hcy on autophagy when *Stx17* was knocked down (KD) or overexpressed (OE) 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 condition as evident by decreased accumulation of

Map1lc3b-ii after Bafilomycin A1 (Baf A1) treatment (**Supplementary Fig. 7g**). Additionally, both WT and *Stx17* KD cells treated with Hcy had increased expression of inflammation and fibrosis genes, with the latter having the higher induction (**Supplementary Fig. 7h,i**). Remarkably, overexpression of *Stx17* in AML12 cells at basal 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, Supplementary Fig. 7j**). Inflammation and fibrosis gene expressions also decreased to control cell levels in Stx17-overexpressing cells treated with Hcy (**Supplementary Fig. 7k,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 the elevated serum β-hydroxybutyrate and hepatic acylcarnitine levels (particularly C2, C3, and C4) by metabolomics analysis (**Fig. 5i,**

Supplementary Fig. 8a-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 TAG and DAG changes (**Supplementary Fig. 8e,f**) in our dietary model of pre-established NASH. These beneficial effects occurred, at least in partc due to decreased Hcy-lation of Stx17 to restore Stx17 expression and improve 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 systemic effects of HHcy. Accordingly, we examined whether intrahepatic Hcy had a pathogenic role in NASH by generating *Cbs*-LKD mice, to specifically increase intra-hepatic 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 decreased transmethylation rate of homocysteine to methionine in NASH [25]. An earlier report showed that mice fed Met- and choline-deficient (MCD) diet developed HHcy and NASH [27]. Surprisingly, Hcy supplementation improved 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 MCD diet and led to UPR-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, an autophagosome-lysosome fusion protein Stx17had increased Hcy-lation and ubiquitination, and reduced total protein expression during NASH. Stx17 Hcy-lation alsoled 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 methionine synthase (Fig. 1A). Together they play critical roles in the MHTFR cycle to convert homocysteine to methionine. 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 Food and Drug Administration (FDA), Vits or Fol could be used as potential a 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.

Acknowledgments

The authors would like to thank Prof. Christopher B. Newgard, Duke University School of Medicine, Durham, NC for his valuable discussions.

Author 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.

Funding

This research was funded by the Ministry of Health (MOH), and National Medical Research Council (NMRC), Singapore, grant number NMRC/OFYIRG/0002/2016 and MOH-000319 (MOH-OFIRG19may-0002) to BKS; NMRC/OFYIRG/077/2018 and 2019-AYOXXA-01 (AYOXXA Biosystems GmbH) to MT; CSA19may-0002 to PMY; Duke-NUS Medical School and Estate of Tan Sri Khoo Teck Puat Khoo Pilot Award (Collaborative) Duke-NUS-KP(Coll)/2018/0007A to JZ; NMRC/OFYIRG/0053/2017 to AW.

Conflicts of Interest

The authors declare no conflict of interest.

Data Availability

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

Figure 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 alanine transaminase (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.

Figure 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:100µm). **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.

Figure 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: 100µm). **i,** NAS score. **j,** Liver hydroxyproline content. **i,** Correlation analysis for hydroxyproline (x-axis) *vs.* serum Hcy (y-axis). **j,** Fold-changes (FC) 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-100µm). **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.

Figure 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 normalized densitometric values to GAPDH. **e and g,** Representative Western blot analyzing protein Hcy-lation (Hcy) in liver tissues (n=5 per group). **f and h,** their normalized densitometric values 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 in input (n=3 per group). **k and m,** Representative Western blots analyzing autophagy proteins in AML12 cells. **l and n,** their normalized densitometric values 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.

Figure 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-nation detection by Western blotting in the liver tissues 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 normalized densitometric values to GAPDH. **e,** Representative Western blot analyzing protein Ubiquitnation (Ubq) in liver tissues (n=5 per group). **f,** their normalized densitometric values to GAPDH. **g,** Representative Western blots analyzing autophagy proteins, SNARE proteins in liver tissue lysates (n=5 per group). **h,** their normalized densitometric values 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.

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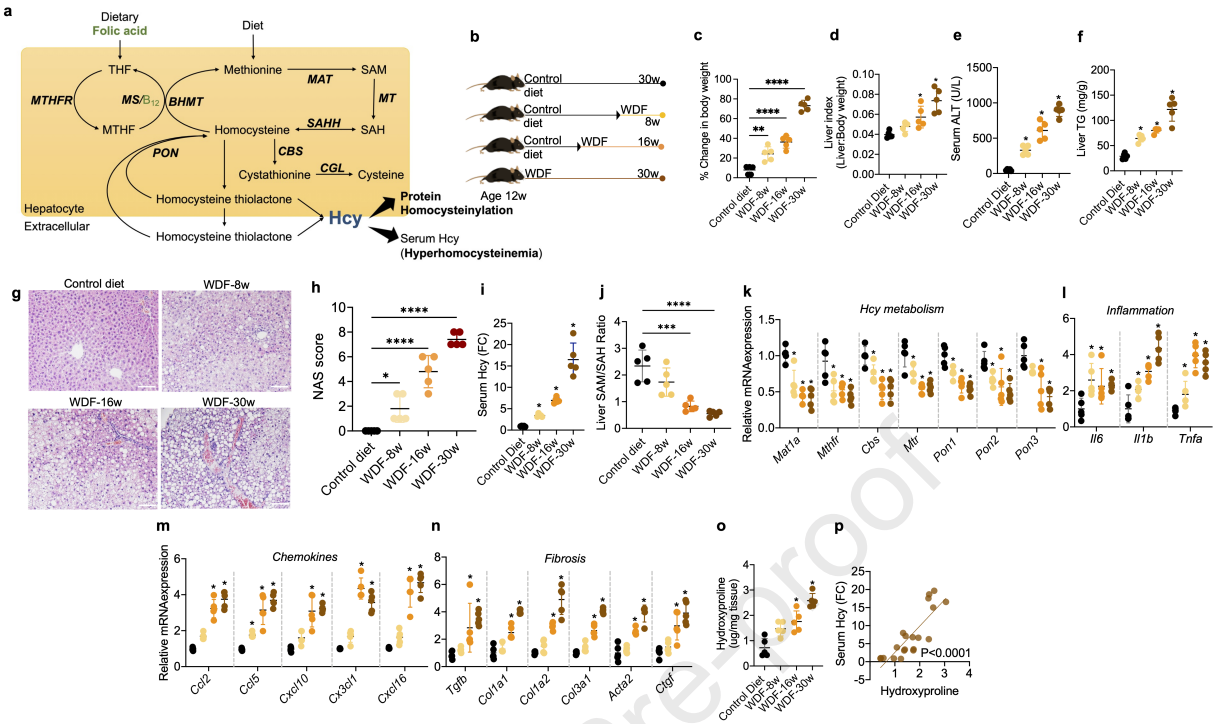
Figure 1:



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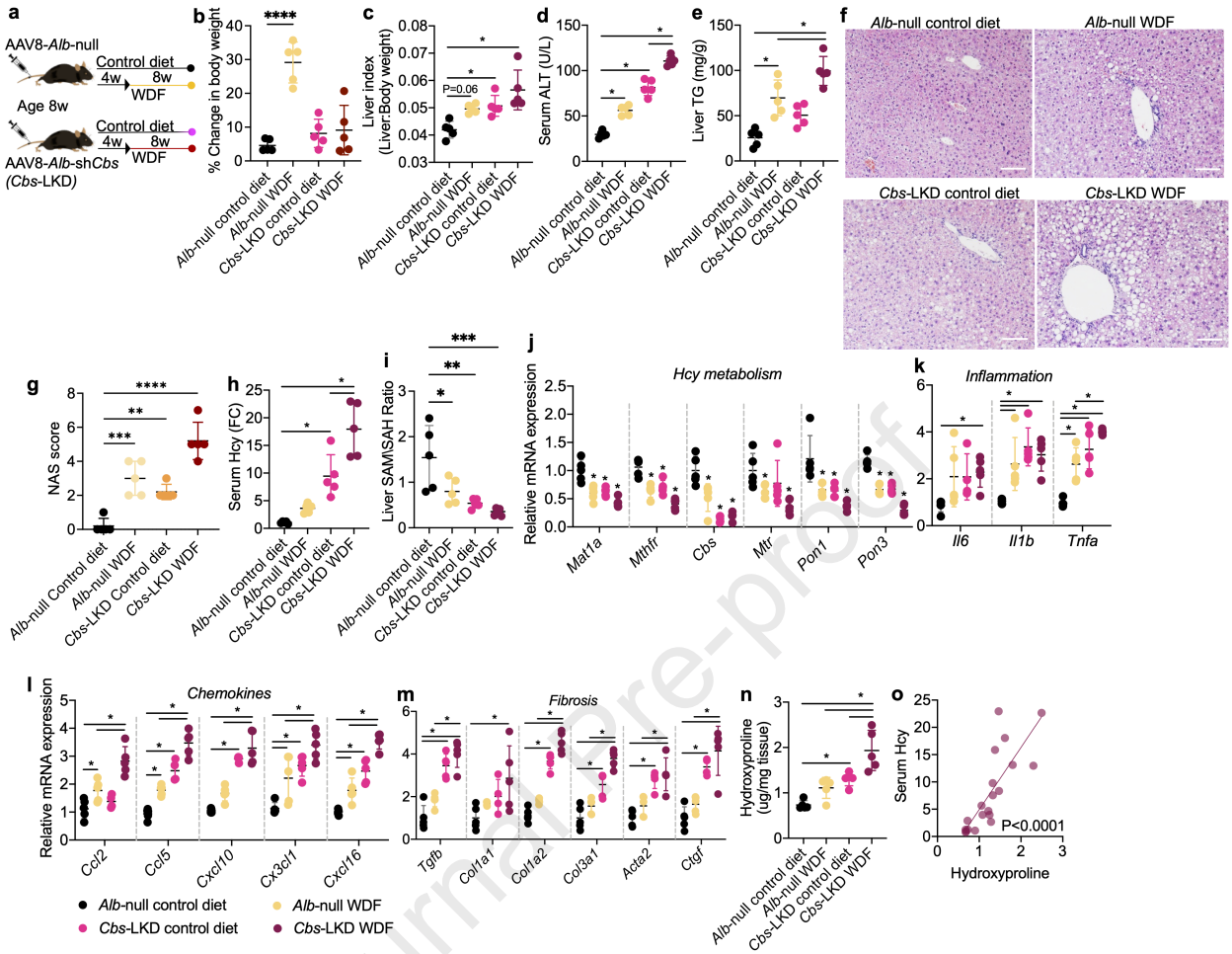

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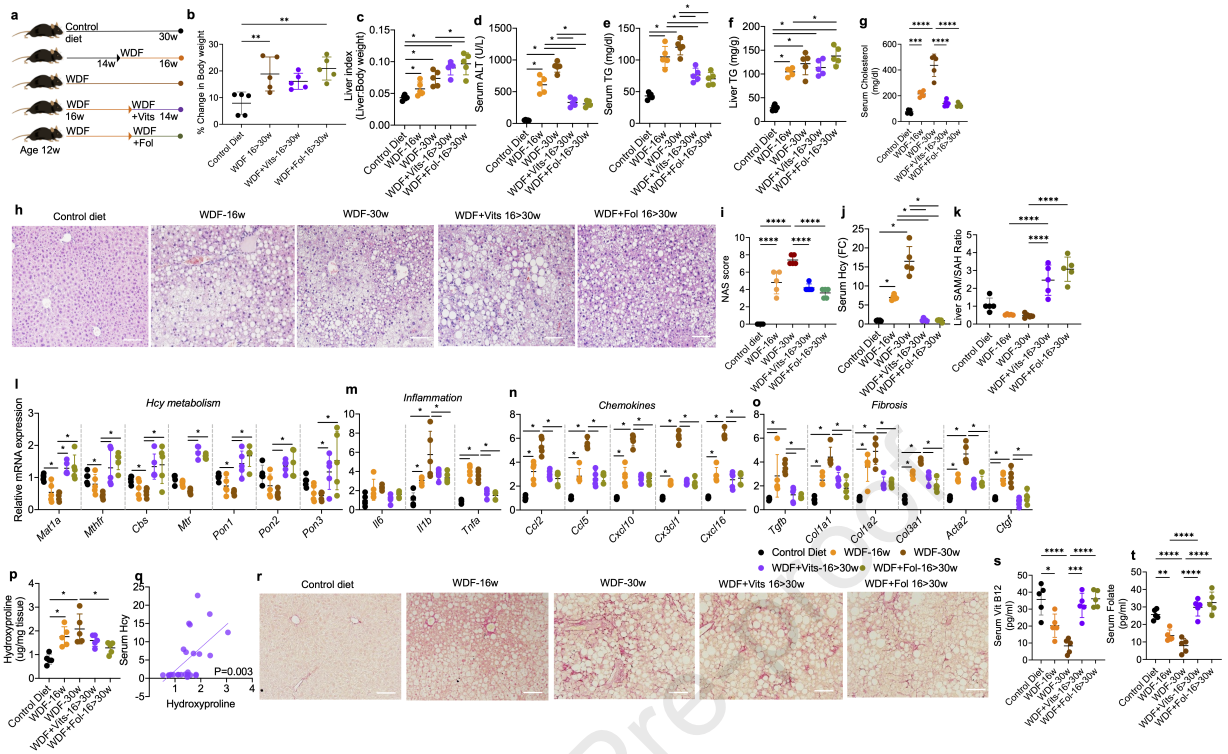

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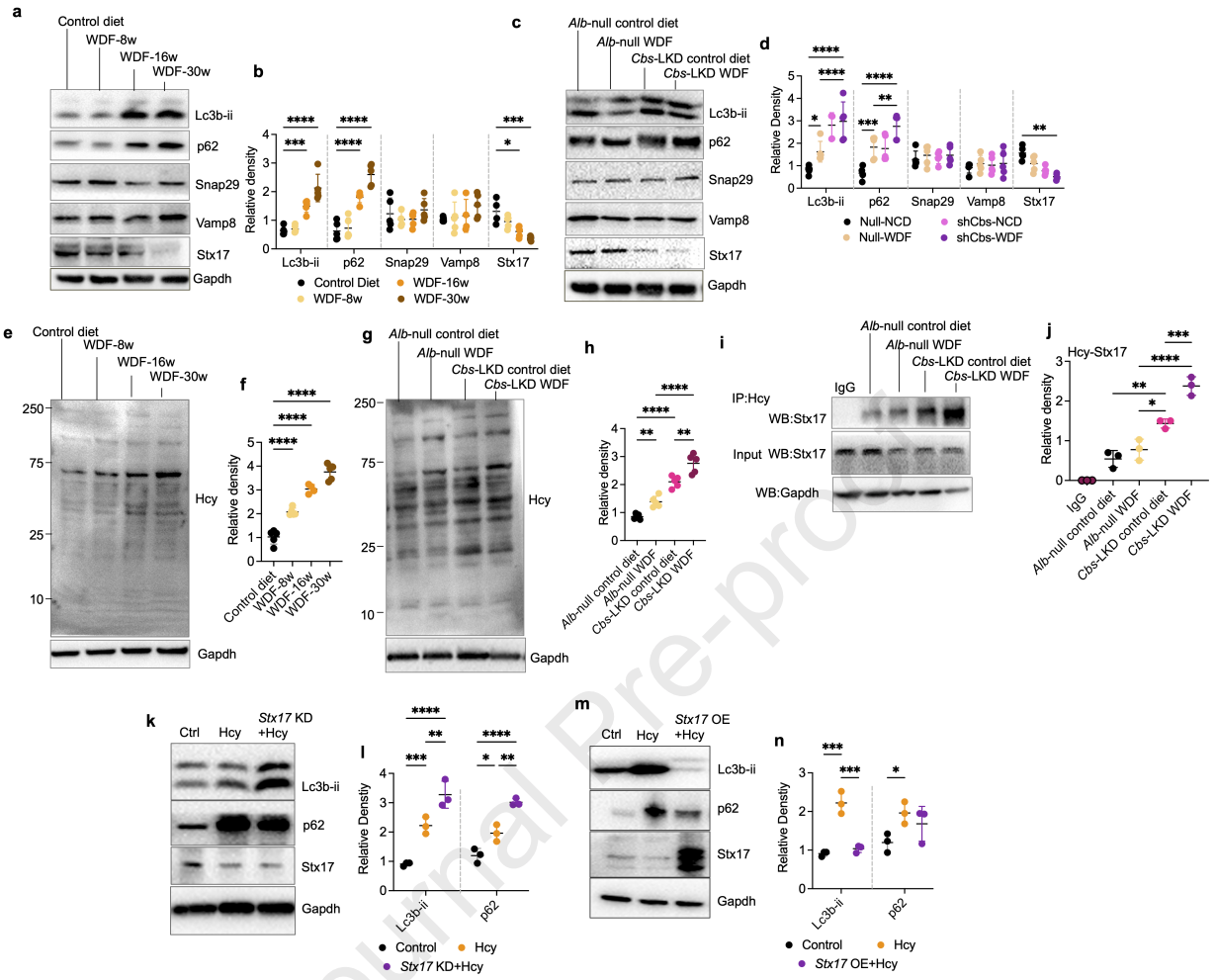

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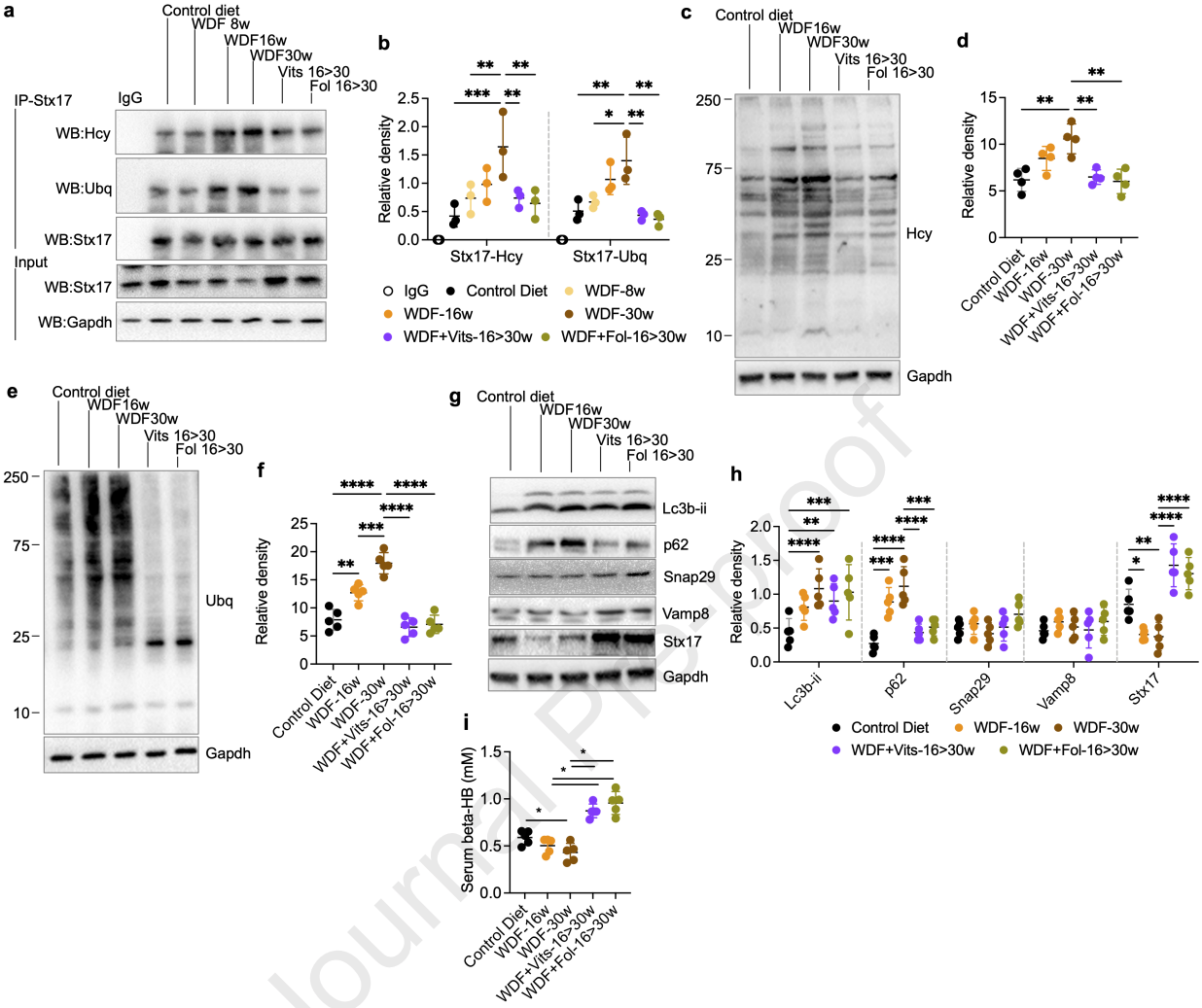

Highlights

- Hyperhomocysteinimia is positively associated with NASH progression.
- Increased intrahepatic homocysteine causes NASH.
- STX17 homocysteinylation and ubiquitination leads to autophagy block during NASH progression.
- Vitamin B12 and folate supplementation restores STX17 protein expression and autophagy to decrease inflammation and fibrosis in NASH.