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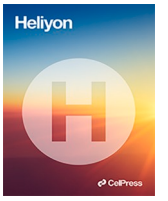

Research article

Modulation of liver cholesterol homeostasis by choline supplementation during fibrosis resolution

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ABSTRACT

Liver fibrosis is a critical global health challenge, often leading to severe liver diseases without timely intervention. Choline deficiency has been linked to metabolic dysfunction associated steatohepatitis (MASH) and liver fibrosis, suggesting choline supplementation as a potential therapeutic approach. This study aimed to explore the therapeutic potential of choline supplementation in liver fibrosis resolution and its effects on cholesterol homeostasis using a mouse model with induced liver fibrosis. Our findings reveal that choline supplementation significantly decreases blood lactate dehydrogenase (LDH) and non-high-density lipoprotein cholesterol (non-HDL-C) levels. Transcriptome analysis showed that choline supplementation primarily induces genes related to cholesterol homeostasis, suggesting a significant impact on liver cholesterol synthesis. However, choline supplementation did not significantly alter the expression of fibrosis-related, choline metabolism-related, or epigenetics-related genes. This study provides novel insights into the role of choline in liver health and cholesterol metabolism, potentially informing treatments for liver fibrosis and related conditions.

1. Introduction

Liver diseases, ranging from fibrosis and cirrhosis to metabolic dysfunction associated steatohepatitis (MASH) and liver cancer, pose significant health challenges worldwide. The global rise in obesity has escalated the prevalence of metabolic dysfunction associated steatotic liver disease (MASLD), which includes conditions such as MASH, highlighting the urgent need for effective therapeutic strategies. Disruption in cholesterol homeostasis is a key driver of MASH, making cholesterol metabolism a critical area of focus in liver disease research [1].

Choline, an essential nutrient often erroneously associated with the B vitamin family, plays pivotal roles in various physiological processes. It serves as a precursor to phospholipids, vital components of the cell membrane, and is involved in the synthesis of the neurotransmitter acetylcholine. As a methyl group donor, choline contributes to epigenetic DNA modification [2,3]. Beyond its function as a precursor to phospholipids and acetylcholine, choline's role in lipid transport and epigenetic regulation underscores its

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Abbreviations

MASLD	metabolic dysfunction associated steatotic liver disease
MASH	metabolic dysfunction associated steatohepatitis
LDL-C	Low Density Lipoprotein cholesterol
HDL-C	High Density Lipoprotein cholesterol
TAA	thioacetamide

potential in maintaining liver health and preventing steatosis [4–6].

In animal models subjected to a long-term choline-deficient diet, fatty liver and subsequent liver fibrosis are induced [7–9]. During pregnancy, acute fatty liver and MASLD may be associated with insufficient maternal choline intake [10]. Despite known consequences of choline deficiency, such as fatty liver and liver fibrosis in animal models, the therapeutic potential of choline supplementation in liver fibrosis resolution is inadequately explored. This study aims to bridge this gap by investigating the molecular effects of choline supplementation on liver fibrosis resolution, utilizing a liver fibrosis model to conduct a detailed analysis of the liver transcriptome during recovery. To achieve this, we established a liver fibrosis model and sequentially sampled the liver transcriptome during liver fibrosis resolution.

2. Materials and methods

2.1. Mice

All animal experiments adhered to the Guidelines for the Care and Use of Laboratory Animals of The University of Tokyo and received approval from the Institutional Animal Care and Use Committee of the Institute of Molecular and Cellular Biosciences, The University of Tokyo (approval number A2022IQB014-01). Male C57BL/6 mice, obtained from CLEA Japan (Tokyo, Japan), were maintained on a 12-h light/dark cycle with unrestricted access to food. To induce liver fibrosis, mice were given thioacetamide (TAA; 300 mg/L) in drinking water for 8 weeks. After fibrosis formation, choline chloride (3.5 g/L) was administered in drinking water for 1, 2, or 4 weeks. Mice received approximately 770 mg/kg/day of choline chloride, based on their daily water intake. All blood was collected from the retro-orbital plexus under anesthesia before euthanizing the mice via cervical dislocation. Immediately after euthanasia, the livers were excised and placed in ice-cold PBS. The largest lobe was then embedded in OCT compound (Sakura Finetek Japan), flash-frozen, and stored at −80 °C until sectioning.

2.2. Histochemistry

Liver cryosections (8 μm) were mounted on glass slides. Sirius red staining was performed as previously described [11]. In brief, sections were fixed overnight at 4 °C using Bouin's solution (Sigma-Aldrich, St. Louis, MO) and subsequently stained for collagen with Direct Red 80 (Sigma-Aldrich). Images were captured using BZ-X810 (Keyence, Osaka, Japan). Fibrotic areas detected by Sirius Red-stained collagen fibers were quantified in the liver sections at ×20 magnification. The mean value of 15 serial areas per sample was used to calculate the percentage area of fibrosis using Image J software (Version 1.54e).

2.3. Serum test

To prepare serum sample, blood was allowed to clot at room temperature for 30 min and centrifuged at 250×*g* for 10 min then the serum fraction was transferred to an empty vial. Serum was stored at −20 °C until use. Serum markers from choline-supplemented and control mice following were analyzed using SPOTCHEM II LIVER-1 (Cat# 77182, Arkray, Kyoto, Japan) and KENSHIN-2 (Cat# 77332, Arkray, Kyoto, Japan). non-high-density lipoprotein cholesterol (non-HDL-C) values were calculated by this formula: non-HDL-C = Total Cholesterol−HDL-C.

2.4. RNA-seq

Total RNA was isolated from the liver using TRIzol reagent (15596-026; Invitrogen) following the manufacturer's instructions. The isolated RNA was prepared for sequencing using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (E7420; NEB) and NEBNext rRNA Depletion Kit (E6310; NEB). We generated a set of 4 x 10^7 paired-end reads (36bp x 2, insert size 240 bp) from each cDNA library using a NextSeq 2000 (Illumina). Sequenced reads were mapped to the mouse genome (GRCm38) using STAR (v2.7.10a). Expression levels were estimated and compared using RSEM (v1.3.3) and DESeq2 (v1.36.0) programs. Differentially expressed genes (DEGs, false discovery rate [FDR] < 0.01) were visualized by a volcano plot and subjected to gene set enrichment analysis. Principal component analysis (PCA) was performed using R software (v4.0.5). All these programs are included in our recently developed RNA-seq analysis pipeline, RumBall (https://github.com/rnakato/RumBall). Expression profiles of cholesterol metabolism-related genes on the KEGG pathway were visualized with pathview (v1.38.0).

2.5. Gene set enrichment analysis

DEGs at each stage were subjected to gene set enrichment analysis using GSEA (v4.3.2) with 1000 permutations [12]. Selected gene sets constructed by Gene Ontology were downloaded from the Molecular Signature Database v2023.1.

2.6. Statistics

Data are presented as mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to assess the normality of data distribution. *P*-values >0.05 from the Shapiro-Wilk test indicated that the data followed a normal distribution. For normally distributed data, independent t-tests were employed for comparisons between groups. For data that did not follow a normal distribution (*P*-values ≤0.05), the non-parametric Mann-Whitney *U* test was utilized. Specific statistical tests used for each analysis are indicated in the corresponding figure legends. *P*-values <0.05 were considered statistically significant.

2.7. Data availability

The RNA-seq results published here are available on the GEO with the accession number: GSE261310. Reviewer token is mlafkgaafvwxzod.

3. Results

3.1. The impact of choline supplementation at the Individual level

Utilizing a thioacetamide (TAA)-induced fibrosis model in mice, we observed a significant increase in the liver-to-body weight ratio following 8 weeks of TAA treatment (Fig. 1B). This observation is consistent with the previous reports like the TAA-induced liver fibrosis model recapitulates key histological, molecular, and pathological features of human liver fibrosis [13–16]. After fibrosis establishment, we provided mice with either normal water or choline chloride. Briefly, choline chloride was administered in drinking water (0 or 25 mM), provided ad libitum, as described by Moon et al. [17] (Fig. 1A). Subsequent intervention with choline chloride did not result in any significant alterations in this ratio over a 4-week period, suggesting that choline supplementation does not affect liver size relative to body weight during recovery under current choline concentrations and period (Fig. 1B).

To further explore fibrosis establishment and the effect of choline supplementation, we assessed serum markers of liver injury. Compared to WT and TAA8w groups, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were significantly increased after 8 weeks of TAA administration (Fig. 1C). This elevation in AST and ALT levels confirms the successful establishment of liver injury and fibrosis. Serum analyses during the recovery phase revealed that indicators of liver function and injury, including serum total albumin (ALB), AST, and ALT, remained unchanged between control and choline-supplemented groups (Fig. 1C). This finding indicates that liver function and the degree of hepatic injury were not modified by choline supplementation.

Notably, choline supplementation was associated with a significant reduction in serum lactate dehydrogenase (LDH) levels (Fig. 1C). Furthermore, we observed a marked decrease in non-high-density lipoprotein cholesterol (non-HDL-C) levels in the choline-supplemented mice (Fig. 1C). Non-HDL-C, encompassing various lipoprotein particles such as VLDL-C, IDL-C, LDL-C, and Lp(a), serves as a pivotal marker for cardiovascular risk assessment. Serum triglyceride (TG) levels also reflected a positive lipoprotein status in the choline-supplemented mice (Fig. 1C).

To determine the direct effect of choline supplementation on liver fibrosis resolution, we assessed collagen accumulation in liver sections via Sirius red staining (Fig. 1D). Our analysis showed no significant change in the collagen-positive areas between choline-supplemented and control livers.

3.2. The impact of choline supplementation on the liver transcriptome

Given that choline deficiency exacerbates liver fibrosis, we sought to investigate whether choline supplementation conversely affects the whole transcriptomes in the liver. We extracted total RNA from the liver before TAA administration (WT), after 8 weeks of TAA administration (T8), and during fibrosis recovery with or without choline supplementation until 4 weeks. To elucidate the overarching patterns of gene expression variation and assess the impact of choline supplementation, we employed Principal Component Analysis (PCA). Our PCA demonstrated that gene expression patterns of WT and T8 were distinct from each other, and during recovery, the liver was positioned between WT and T8 (Fig. 2A). The distance between the control and choline supplementation groups increased along with the choline supplying time. Consistent with the PCA results, clustering analysis demonstrated that expression profiles of T8 were distinct from other liver samples (Fig. 2B). Interestingly, the number of DEGs in pairwise comparison of control and choline-supplemented liver significantly increased proportional to choline administration time (Fig. 2C and D). Thus, we decided to investigate these DEGs in more detail.

3.3. The expression levels of fibrosis-related genes and choline metabolism-related genes were not significantly altered by choline supplementation

Next, we focused on liver fibrosis-related genes. Initially, the expression levels of *Col1a1*, and *Acta2*, which are expressed by hepatic

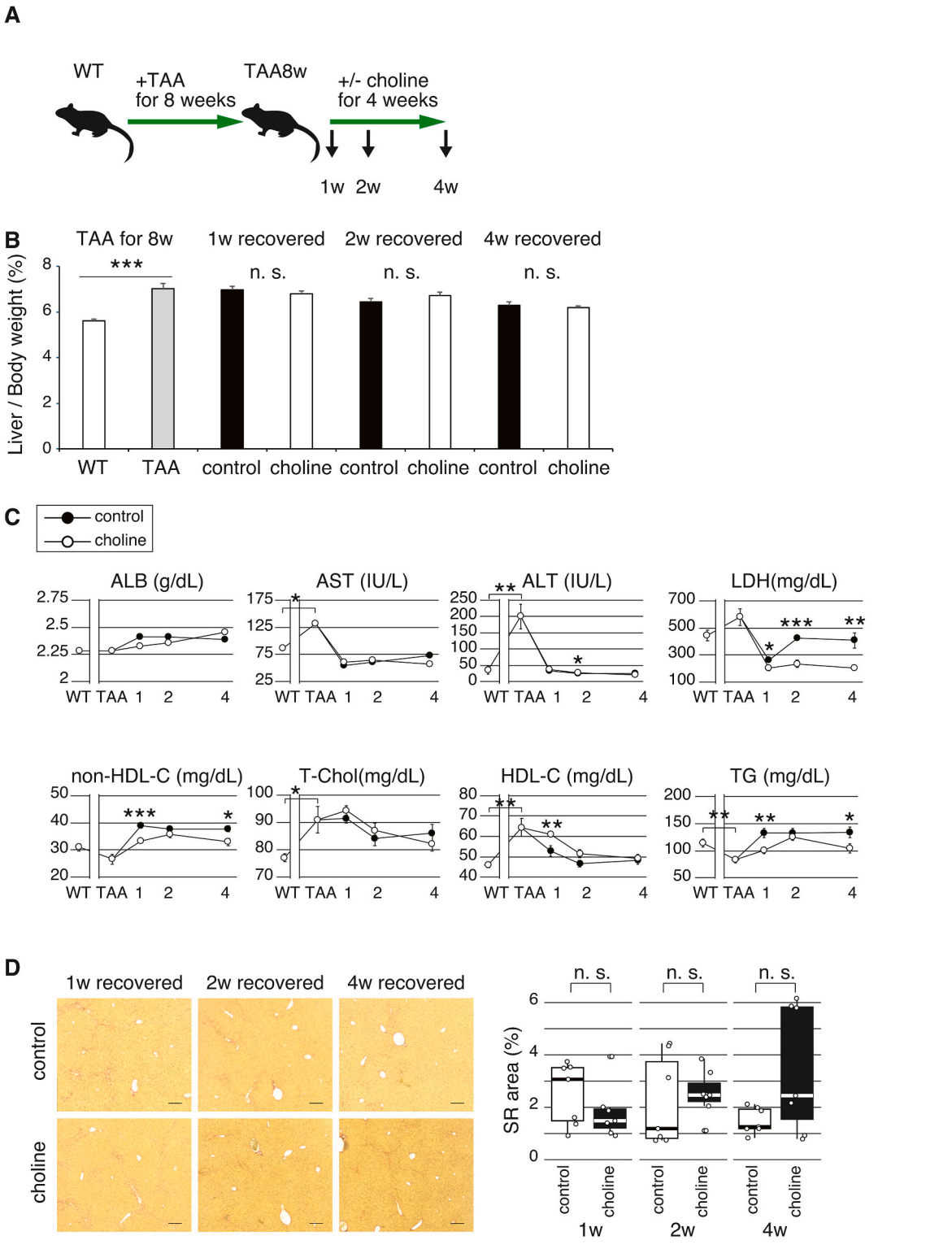

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Fig. 1. Effects of choline supplementation on liver parameters in thioacetamide (TAA)-Induced fibrosis mice.
(A) Experimental design depicting the timeline of TAA-induced liver fibrosis and choline chloride supplementation. (B) Liver weight to body weight ratio before (WT) and after TAA administration (TAA), with or without choline chloride supplementation for 1 week, 2 weeks, and 4 weeks post-TAA administration. Statistical analysis was performed using unpaired two-tailed Student's t-test. ***$p < 0.001$. N = 7. (C) Evaluation of serum markers including albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), non-high-density lipoprotein cholesterol (non-HDL-C), total cholesterol (T-Chol), high-density lipoprotein cholesterol (HDL-C), and triglycerides (TG) before and after choline chloride administration. Statistical analysis was performed using unpaired two-tailed Student's t-test. ALT at 4 weeks, LDH at 1 week, non-HDL-C at 2 week and TG at WT vs TAA 8 weeks were analyzed using the Mann-Whitney *U* test due to non-normal distribution. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. N = 7. (D) Histological assessment of liver fibrosis using Sirius red staining, with quantification of collagen-positive area. Scale bar: 200 μm. Statistical analysis was performed using unpaired two-tailed Student's t-test. N = 7.

stellate cells in response to chronic liver injury, were dramatically induced after 8 weeks of TAA administration. Similarly, the expression levels of *Timp1*, *Cd44*, *Vcam1* and *Lgals3* showed strong induction in TAA-administered livers compared to wild-type controls (Fig. 3A). However, there were no significant differences between the control and choline supplementation groups during fibrosis recovery (Fig. 3A). Choline is primarily metabolized by the choline cycle [18]. We plotted the levels of representative genes involved in choline metabolism (Fig. 3B). Interestingly, the expression of the Phosphatidylethanolamine N-methyltransferase (*Pemt*) gene was significantly induced by 4 weeks of choline administration. PEMT is an enzyme that catalyzes the methylation of phosphatidylethanolamine to phosphatidylcholine and replenishes cellular choline levels [18]. This intriguing finding is discussed in the Discussion section. Genome and histone methylation levels are directly affected by dietary methyl groups, including choline [19]. The relative expression levels of DNA methylation-related genes (Fig. 3C) and representative histone methylation genes (Fig. 3D) did not show any significant alteration between control and choline-supplemented mice. Contrary to expectations, choline supplementation has a subtle effect on liver fibrosis recovery, choline metabolism, and epigenetic regulation in the liver, while there are several differentially expressed genes (DEGs).

3.4. Gene set enrichment analysis shows that choline supplementation alters cholesterol homeostasis during liver fibrosis resolution

To elucidate the impact of choline supplementation on the expression patterns of crucial biological pathways during liver fibrosis recovery, we conducted gene set enrichment analysis (GSEA) [12]. Our analysis revealed a significant upregulation of genes involved in cholesterol homeostasis at 2- and 4-weeks post-choline supplementation, with no other gene set showing such changes under the normal settings (FDR cutoff <0.25) (Fig. 4A). Specifically, we observed a 1.96-fold upregulation in the expression of *Hmgcr*, the rate-limiting enzyme in LDL-C biosynthesis, alongside a tendency for decreased expression of *Cyp7a1*, a counteracting enzyme in this pathway [20] (Fig. 4B). This suggests a pronounced effect of choline supplementation on LDL-C metabolism during liver fibrosis recovery. Additionally, the steroid biosynthesis pathway, as visualized on the KEGG pathway (Fig. 4C). Interestingly, despite a significant decrease in blood non-HDL-C levels (Fig. 1D), we observed the upregulation of LDL-C synthesis pathways in choline-supplemented mice. These paradoxical findings highlight the complex interplay between dietary nutrients like choline and cholesterol metabolism, especially in the context of liver health and disease recovery.

4. Discussion

This study demonstrates that choline supplementation alters liver cholesterol homeostasis, significantly reducing blood non-HDL-C levels and inducing transcriptome changes related to cholesterol metabolisms. Choline has significant implications for liver health. A methionine/choline-deficient diet is a commonly used dietary model of metabolic dysfunction associated steatohepatitis (MASH) in rodents [8]. In contrast, the study of the effect of choline supplementation on liver disease is limited. While choline deficiency is known to exacerbate inflammatory processes and liver fibrosis, our findings indicate that choline supplementation exerts a limited effect on liver inflammation and fibrosis (Fig. 1D). Therefore, choline supplementation, when used as a therapeutic intervention following TAA administration, has a neutral effect on liver fibrosis recovery. We did not test choline for prophylactic purposes, but it may still hold potential benefits in that context. Furthermore, choline supplementation reduced serum lactate dehydrogenase (LDH) levels (Fig. 1C). Given that LDH is a ubiquitous enzyme released during tissue damage and is less liver-specific than AST or ALT, this reduction suggests a potential protective effect of choline on other organs. The study highlights that the cessation of TAA administration has a substantial impact on liver recovery, potentially overshadowing the effects of choline supplementation. As a result, the observed influence of choline supplementation may be less pronounced in comparison.

Choline supplementation increases the number of differentially expressed genes (DEGs) along the supplementation period (Fig. 2C and D). Unexpectedly, the DEGs induced by choline supplementation were less related to choline metabolisms, DNA methylation, and histone methylation (Fig. 3B, C, and 3D). Exception is the PEMT gene, which synthesizes choline. It is intriguing that the supplementation of choline increases the expression of the choline synthesis gene; this could be the result of some feedback regulation. Furthermore, the gene set related to cholesterol homeostasis was the only one enriched in the DEGs (Fig. 4A). The liver is the primary site of cholesterol biosynthesis and storage to maintain systemic cholesterol homeostasis under normal circumstances [21]. The cholesterol biosynthetic pathway is a complex biochemical process that involves more than 30 different reactions [22]. Notably, the rate-limiting enzyme *Hmgcr*, along with other key enzymes such as *Mvk*, *Pmvk*, and *Sc5d*, showed increased expression levels in response to choline supplementation. This suggests that choline has a significant impact on cholesterol metabolism, with *Hmgcr* acting as a central regulator and the other enzymes being influenced by its activity. Thus, the induction of cholesterol synthesis was

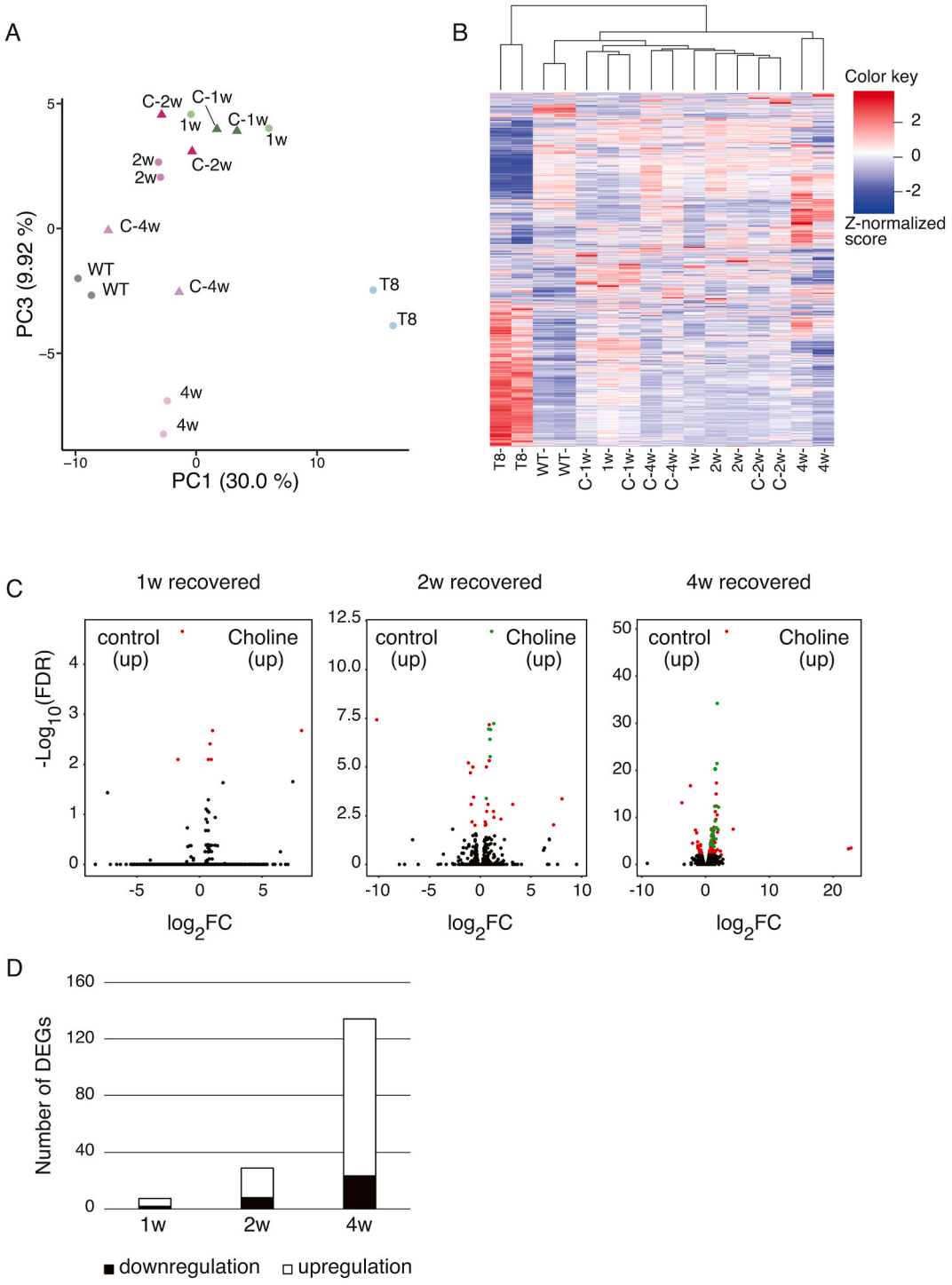

Fig. 2. Transcriptomic changes in liver recovery after TAA treatment with and without choline chloride supplementation.
Transcriptome analysis by RNA-seq reveals that (A) PCA of wildtype (WT), 8 weeks after TAA treatment (T8), 1 week, 2 weeks and 4 weeks recovered after 8 weeks after TAA treatment without (1w, 2w, and 4w) or with choline chloride (C-1w, C-2w, and C-4w) liver based on log (TPM). (B) Hierarchical clustering of RNA-seq samples based on Z scores of log (TPM) for wildtypes (WT), 8 weeks after TAA treatment (T8), 1 week (1w), 2 weeks (2w), and 4 weeks (4w) recovered after 8 weeks after TAA treatment without choline chloride or with choline chloride liver. The y axis indicates 2000 top variable genes. (C) Volcano plots showing changes in gene expression levels with or without choline chloride supplementation at the indicated time. The fold change of with versus without choline is presented in a log scale on the x-axis. The y-axis shows –log10(FDR). Significant differentially expressed genes (DEGs) from cholesterol homeostasis-related ones and the others are colored in green and red, respectively. (D) The number of differentially expressed genes.

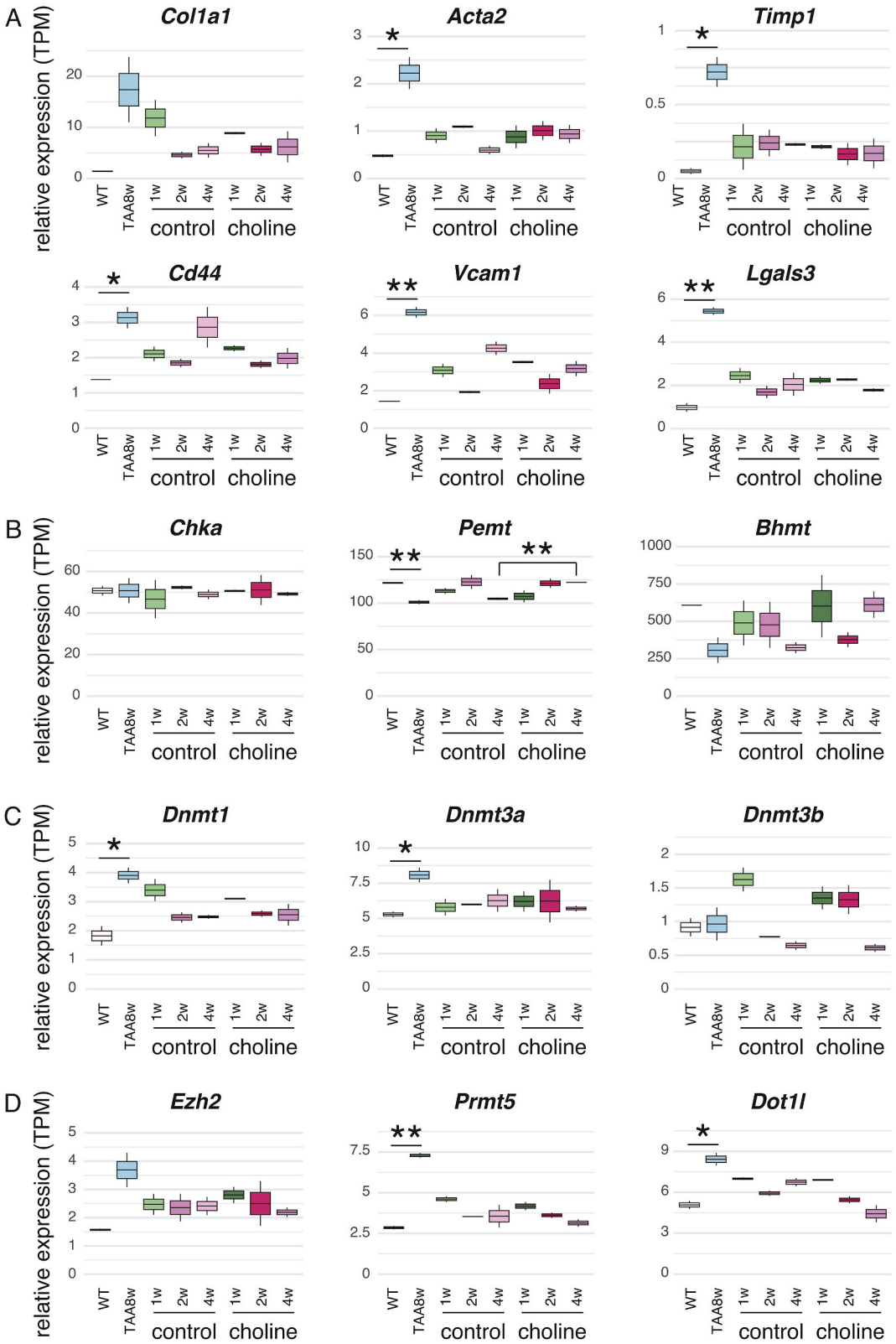

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Fig. 3. Expression levels of fibrosis-related, choline metabolism-related, and epigenetics-related genes in choline-supplemented liver.
(A, B, C, D) Box plots showing expression levels of fibrosis-related genes (A), choline metabolism-related genes (B), DNA methylation-related genes (C), and histone methylation-related genes (D) in choline-supplemented liver compared to controls. Fibrosis-related genes were significantly upregulated in fibrosis induced liver (A) whereas there are no significant differences between choline-supplemented liver and controls unless *p*-value is provided. Statistical analysis was performed using unpaired two-tailed Student's t-test. $^{**}p < 0.01$, $^{*}p < 0.05$.

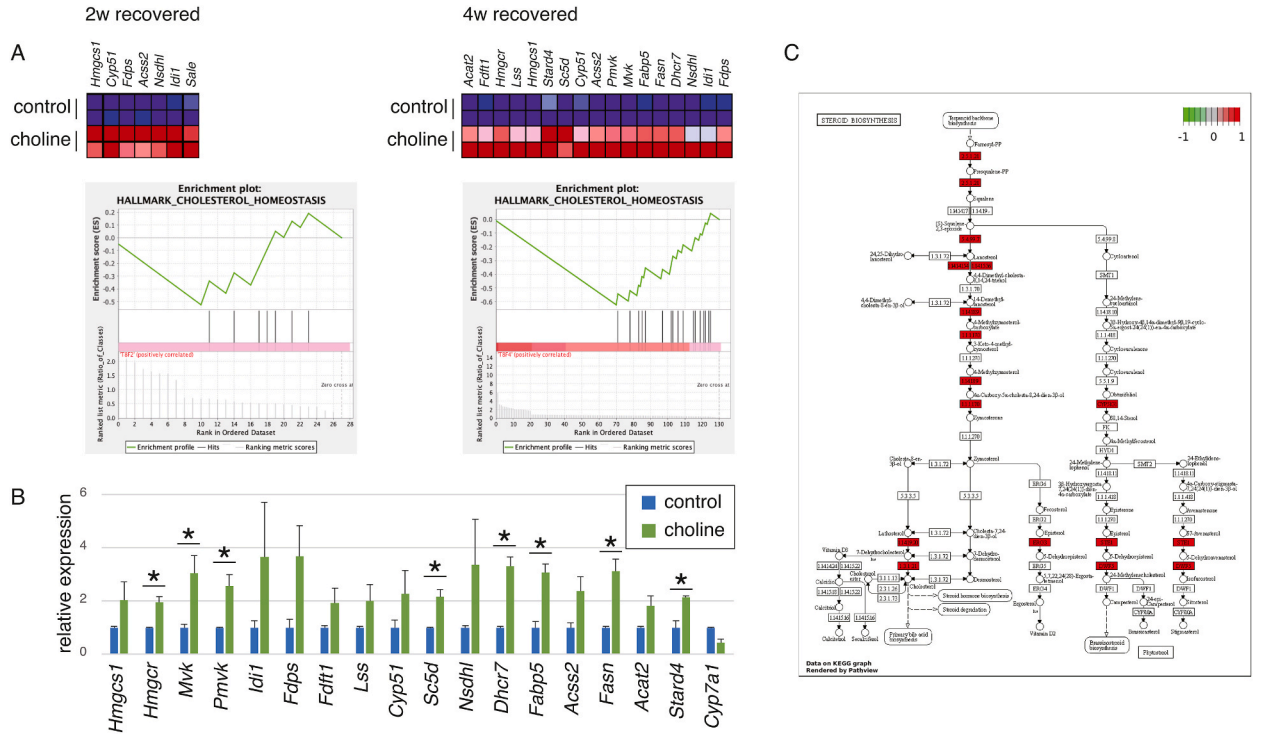

Fig. 4. Choline supplementation induces cholesterol homeostasis-related genes.
(A) Gene set enrichment analysis (GSEA) of liver samples with or without choline chloride supplementation, showing enrichment of cholesterol homeostasis-related genes. (B) Relative expression levels of cholesterol homeostasis-related genes. Statistical analysis was performed using unpaired two-tailed Student's t-test. $^{*}p < 0.05$. (C) Visualization of differentially expressed genes (DEGs) in the KEGG steroid biosynthesis pathway (fold-change (log2) cutoff >2).

considered the main action of choline supplementation specifically in the context of recovering injured livers.

In recent years, choline supplementation has attracted much attention as a potential regulator of plasma cholesterol. However, the effects of choline intake on blood "harmful" low-density lipoprotein-cholesterol (LDL-C) have remained controversial. Guo et al. showed that high choline intake significantly elevates serum LDL-C levels [23]. In contrast, Li et al. reported that choline supplementation in Helicobacter pylori-infected mice leads to liver injury and decreases blood LDL-C [24]. In *Ldlr* knockout metabolic disorder model mice [25] and in the vitamin B6 deficient hyperhomocysteinemia murine model [26], choline supplementation normalizes liver function and cholesterol metabolism. In the context of liver fibrosis recovery, choline supplementation seems to improve the increase of LDL-C levels in the body. While choline supplementation appears to activate the LDL-C biosynthesis pathway, it concurrently contributes to a systemic reduction in non-HDL-C levels, underscoring the potential of choline as a modulator of cholesterol homeostasis during liver fibrosis resolution. Reductions in non-HDL-C and TG suggest that choline supplementation may have a favorable impact on cholesterol metabolism and cardiovascular health.

Since choline supplementation in disease models improves cholesterol metabolisms so far, the molecular mechanisms of choline intake in this model may provide a novel insight into the development of an anti-metabolic syndrome cure.

5. Conclusion

In conclusion, our study reveals that choline supplementation during liver fibrosis resolution significantly decreases in blood LDH and non-HDL-C levels. Notably, choline primarily influences cholesterol homeostasis genes, suggesting a significant impact on cholesterol synthesis within the liver during fibrosis resolution. It is important to note that these observations pertain to therapeutic use, as prophylactic effects were not tested. These findings challenge traditional views on choline's role in liver health and open new avenues for potential therapeutic applications. Further research is needed to fully understand the complex interplay between dietary

nutrients like choline and liver health, particularly in the context of liver fibrosis and cholesterol metabolism.

Ethical approval statement

All animal experiments adhered to the Guidelines for the Care and Use of Laboratory Animals of The University of Tokyo and received approval from the Institutional Animal Care and Use Committee of the Institute of Molecular and Cellular Biosciences, The University of Tokyo (approval number A2022IQB014-01).

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Data availability statement

This study's RNA-seq datasets are stored in the GEO repository. The accession number is GSE261310.

CRediT authorship contribution statement

Eiko Saijou: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yoshiko Kamiya:** Visualization, Data curation. **Katsunori Fujiki:** Supervision, Funding acquisition. **Katsuhiko Shirahige:** Data curation. **Ryuichiro Nakato:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e36727.

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